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Coupling of Expressed α_{1B}- and α_{1D}-Adrenergic Receptors to Multiple Signaling Pathways Is Both G Protein and Cell Type Specific

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SUMMARY

α_1-Adrenergic receptors (ARs) are members of the G protein-coupled receptor superfamily. α_1-AR subtypes mediate the effects of the sympathetic nervous system, especially those involved in cardiac homeostasis. To investigate signal transduction by a novel subtype (α_{1D}), which we recently cloned, and to compare it with that by the previously characterized α_{1B}-AR, we assessed the ability of each subtype to activate polyphosphoinositide (PI) metabolism, cAMP accumulation, and arachidonic acid release in Chinese hamster ovary (CHO) and COS-1 cells expressing these subtypes after stable or transient transfection, respectively. In COS-1 and CHO cells, both the α_{1D}- and α_{1B}-AR were found to couple to PI hydrolysis through a pertussis toxin-insensitive G protein. Both α_1-AR subtypes also increased intracellular cAMP by an indirect mechanism, although this effect was observed only in COS-1 cells and not in CHO cells. Interestingly, α_1-AR-stimulated arachidonic acid release was also demonstrated for both subtypes in COS-1 cells. This release was mediated through phospholipase A_2 activation and involved a pertussis toxin-sensitive G protein. α_1-AR-stimulated arachidonic acid release was dependent upon extracellular calcium and was inhibited by 1 μM nifedipine. Inhibitors of protein kinase C, phospholipase C, and diacylglycerol lipase did not alter α_1-AR-stimulated release of arachidonic acid. These findings indicate that in COS-1 cells α_1-AR-stimulated arachidonic acid release is most likely coupled to dihydropyridine-sensitive L-type calcium channels via a pertussis toxin-sensitive G protein. The influx of extracellular calcium then stimulates phospholipase A_2 to release arachidonic acid. α_1-AR-stimulated arachidonic acid release could also be demonstrated in CHO cells and was pertussis toxin sensitive but nifedipine insensitive. These cells were also unresponsive to Bay K8644, indicating a lack of voltage-sensitive calcium channels in CHO cells. Nevertheless, α_1-AR activation increased intracellular Ca^{2+} levels, as assessed by fura-2 fluorescence studies. Neomycin blocked both α_1-AR-stimulated PI hydrolysis and increases in intracellular Ca^{2+} levels but did not inhibit the increase in arachidonic acid release. Taken together, these data indicate that in CHO cells α_1-ARs can couple directly to phospholipase A_2 activation via a pertussis toxin-sensitive pathway. Thus, in these model systems we demonstrate for the first time that a single α_1-AR subtype can activate multiple distinct signal transduction pathways, in which receptor-effector coupling is modulated by distinct G proteins.

Since the initial observation by Morrow *et al.* (1) that α_1-AR-mediated responses are differentially sensitive to various agonists and antagonists, many studies have been reported that support the notion of α_1-AR heterogeneity. These studies, which evaluated α_1-ARs in various tissues and cell lines, provided evidence for the existence of at least two (α_{1A} and α_{1B}) α_1-AR subtypes (2–6). The α_{1A} subtype mediates responses that are sensitive to agonists such as methoxamine and antagonists such as 5-methylurapidil, (+)-niguldipine, and WB4101, and this α_1 subtype is insensitive to alkylation by CEC. Additionally, stimulation of the α_{1A}-AR subtype leads to extracellular calcium entry and, thus, α_{1A}-AR-mediated responses can be inhibited by voltage-sensitive L-type calcium channel blockers, such as nifedipine (6). In contrast, α_{1B}-AR-mediated responses are less sensitive to methoxamine and the aforementioned antagonists, and α_{1B}-ARs can be totally inactivated by CEC (2–6). Also, α_{1B}-ARs mediate PLC activation by a mechanism that is not dependent on calcium influx and is nifedipine insensitive (7). Thus, both subtypes lead to increases in intracellular calcium levels, although with the α_{1A}-AR this response involves calcium influx, whereas with the α_{1B}-AR calcium is mobilized as a consequence of PLC-mediated increases in IP_3 (7).

More recently, it has been demonstrated that the α_{1A}-AR can

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ABBREVIATIONS: AR, adrenergic receptor; CEC, chlorethylclonidine; [^{125}I]HEAT, 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]tetralone; KHB, Krebs Henseleit buffer; PI, polyphosphoinositide; MDCK, Madin-Darby canine kidney; IP_3, inositol-1,4,5-trisphosphate; PLC, phospholipase C; CHO, Chinese hamster ovary; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester; SDS, sodium dodecyl sulfate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

also activate PLC and that α_1-ARs can activate a variety of other effectors such as phospholipase D, phospholipase A_2, cAMP metabolism, and various ion channels (8–12). In some cases, e.g., PLC activation, receptor signaling involves coupling via a pertussis toxin-insensitive G protein, whereas in others, e.g., phospholipase A_2 activation, a pertussis toxin-sensitive pathway is implicated (13).

These pharmacological and biochemical studies have also led to the notion that a single α_1-AR subtype can couple to several effectors via different G proteins, because some of the tissues and cell lines evaluated in these studies appear to express only one receptor subtype and yet receptor stimulation activates multiple effector pathways. However, the ability to resolve multiple subtypes is still limited by the lack of highly selective subtype-specific ligands, particularly if several subtypes are expressed at different levels in a single population of cells. This contention is further underscored by the findings of molecular cloning studies, which have confirmed the existence of multiple α_1-AR subtypes but have also identified several previously unrecognized receptor subtypes (14–17). Also, the transcripts for the various α_1-AR subtypes are often extremely low in abundance (17). Thus, it remains unclear whether, *in vivo*, a single α_1-AR subtype can couple to one or more than one effector pathway.

cDNA clones have been isolated that encode three distinct subtypes (α_{1B}, α_{1C}, and α_{1D}) (14, 15, 17). The pharmacological, biochemical, and signaling properties of the expressed α_{1B}-AR are identical to those observed for the α_{1B}-AR expressed endogenously in tissues and cell lines (14). The α_{1C} and α_{1D} clones encode distinct α_1-ARs (15, 17). An additional cDNA clone, which is identical to the α_{1D} clone, has also been described, and it has been suggested that the receptor encoded by this cDNA clone is the α_{1A}-AR (16). However, the properties of the expressed receptor encoded by this cDNA clone differ from those observed for the α_{1A}-AR characterized previously in various tissues and cells (2–6, 17, 18). Signaling by the expressed receptors that are encoded by the α_{1B} and α_{1C} cDNAs has been investigated (7). However, the signal transduction mechanisms of the α_{1D}-AR remain unknown.

In the present study we investigated and compared signaling by the expressed α_{1D}- and α_{1B}-ARs. The findings of this study indicate that the expressed α_{1D}-AR, like the α_{1B}-AR, can activate multiple effector responses, whether the receptor is expressed transiently in COS-1 cells or stably in CHO cells. Both subtypes activate phospholipase A_2 and generate arachidonic acid. This activation proceeds through a pertussis toxin-sensitive G protein. We also demonstrate coupling of both subtypes to inositol phosphate generation through a pertussis toxin-insensitive G protein. Thus, in these model systems involving the expression of a single α_1-AR subtype, it is evident that α_1-AR activation can generate several second messengers via coupling to distinct G proteins.

Materials and Methods

Transient and stable transfections. cDNAs encoding the α_{1D}- and α_{1B}-ARs were subcloned into the mammalian expression vector pMT2′, as described previously (19). Transient expression in COS-1 cells was accomplished by the DEAE-dextran method (20). For stable transfection of α_1AR cDNAs, CHO cells were co-transfected with either pMT2′α_{1D} or pMT2′α_{1B} and pSV2-HIS (21), using the DEAE/polybrene method (22). Clones were selected for their ability to survive and grow in histidinol (5–10 mM)-supplemented medium (21) and were tested for their ability to bind the selective α_1-AR antagonist [^{125}I] HEAT (23).

Inositol phosphate measurement. COS or CHO cells expressing α_1-AR subtypes, grown in 100-mm dishes (6×10^6 cells/dish), were labeled for 16–24 hr with [^{3}H]inositol (DuPont-New England Nuclear) at 1–2 μCi/ml. After labeling, cells were washed and incubated in HBSS (no added calcium) for 45 min to 1 hr, followed by a 30-min incubation with 20 mM LiCl. Various agents were then added and the reaction was stopped by the addition of ice-cold 0.4 M perchloric acid (24). The culture dishes were then frozen by placing them on dry ice. One-half volume of 0.72 N KOH/0.6 M $KHCO_3$ was added and the sample was centrifuged to settle the precipitate. The supernatant was applied to 1-ml packed AG1-X8 columns (100–200 mesh, formate form) and total inositol phosphates were eluted with 1 M ammonium formate/0.1 M formic acid, after the column was washed with 16 ml of 0.1 M formic acid. In some experiments, IP_3 was eluted after the column was washed sequentially with 0.2 M and then 0.4 M ammonium formate/0.1 M formic acid to remove inositol monophosphate and inositol bisphosphate. IP_3 was then eluted in the 1 M ammonium formate/0.1 M formic acid fraction. Thin layer chromatographic analysis of IP_3 was performed according to the method of Martin (24).

[^{3}H]Arachidonic acid release. Cells in 100-mm dishes (7×10^6 cells/dish) were incubated overnight with [^{3}H]arachidonic acid (1 μCi/ml). The medium was aspirated and the cells were washed three times at 10-min intervals with HBSS containing 1.6 mM $CaCl_2$, 0.8 mM $MgSO_4$, 10 mM HEPES, pH 7.4, and 2 mg/ml fatty acid-free bovine serum albumin (Sigma). After the final wash the experimental agents were added in the same modified HBSS (2 ml) and the cells were incubated for various times. AR agonists were then added and, after 5 min, the medium was removed, acidified with 100 μl of 1 N formic acid, and extracted with 3 ml of chloroform. The extracts were evaporated to dryness, resuspended in 50 μl of chloroform, and applied to silica gel thin layer chromatography plates (LK5D; Whatman). The plates were developed in a heptane/diethyl ether/acetic acid (75:25:4, v/v/v) solvent system. Nonradioactive standards (2 μg) were run in each lane as carriers. Carriers were visualized with iodine vapor and radioactivity was quantified by scraping the plates and adding 10 ml of scintillation cocktail to the resin. Samples were dark-adapted overnight before being counted.

Assay of phospholipase A_2 activity. Cells growing in T-175 tissue culture flasks were rinsed with 10 ml of Ca^{2+}- and Mg^{2+}-free HBSS. Five milliliters of ice-cold 10 mM Tris·HCl, pH 8.0, were added and the cells were scraped from the flask. Cells were placed into a Dounce homogenizer and homogenized by hand with six strokes. The homogenate was centrifuged at 30,000 × g for 15 min. The pellet was resuspended in 10 ml of ice-cold 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, and rehomogenized. The suspension was centrifuged at 1000 × g for 15 min and the supernatant was centrifuged at 30,000 × g for 20 min. The pellet was resuspended in 100 mM Tris·HCl, pH 8.5. Assays were conducted in 15 mM HEPES, pH 7.4, containing 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM Ca^{2+}, 100 μM GTP, 0.125 μCi of 1-acyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine (Amersham), and 100 μg of membrane protein. After 5 min at 37°, the reaction was stopped by the addition of 300 μl of ethanol. The released [^{3}H]arachidonic acid in the extract was separated from the unreacted substrate by thin layer chromatography on Whatman LK5D plates using chloroform/methanol/water (63:27:4) (the R_F was 0.69 for the [^{3}H]phosphatidylcholine substrate and 0.92 for the [^{3}H]arachidonic acid product). Plates were spotted with 2 μl of arachidonic acid as a standard and were visualized with iodine vapor after running. The band was scraped from the plate, eluted into 20 ml of scintillation liquid, and counted.

cAMP assay. COS-1 or CHO cells expressing either the α_{1D}- or α_{1B}-AR were grown in 100-mm dishes (7×10^6 cells/dish), washed twice, and then incubated for 30 min in serum-free Dulbecco's minimum essential medium with 20 mM HEPES, pH 7.5, and 5 mM theophylline. Alprenolol (10^{-5} M) was also added to block endogenous β-ARs, if any, in these cells. After a 10-min incubation with different drugs, the supernatant was aspirated and 100 mM HCl (200 μl) was added to lyse

the cells. Aliquots were taken, diluted to 1/2 to 1/4, and assayed directly for cAMP by radioimmunoassay according to the manufacturer's instructions (Amersham cAMP kit).

Intracellular calcium measurements. CHO cells expressing either the α_{1D}- or α_{1B}-AR were grown in suspension culture in Dulbecco's minimum essential medium supplemented with 5% fetal calf serum. Cells were harvested, washed, and resuspended in KHB containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 25 mM HEPES, 16.5 mM dextrose, and 7.5 mM pyruvate and supplemented with 0.68 mM glutamine and 1× basal minimum Eagle's medium amino acids and vitamin solutions, pH 7.35. CHO cells were loaded in the same KHB with 1 μM fura-2/AM (Molecular Probes, Eugene, OR) at 37°, in a shaking waterbath, for 30 min. After loading, the cells were sedimented at 100 × *g* and washed with KHB. Sedimented CHO cells were resuspended in fresh KHB at a concentration of approximately 2×10^7 cells/ml. Fluorescence measurements were carried out using 2-ml cell suspensions (~0.2×10^6 cells/cuvette), with constant stirring and under constant temperature, in a custom-built fluorimeter specifically designed for single-excitation wavelength measurements of cells in suspension (University of Pennsylvania Biomedical Instrumentation Group, Philadelphia, PA) (25). A 75-W xenon lamp (Ultraviolet Products, Inc.) was the excitation source. Emission was monitored through a 510-nm interference filter (20-nm half-bandwidth; Omega Optical Company, Brattleboro, VT) 90° from the excitation beam, filtered to 340 nm with a 5-nm half-bandwidth filter.

Ligand binding. COS-1 and CHO cell membranes were prepared as described previously (17). The ligand-binding characteristics of the expressed receptors were determined in a series of radioligand binding studies using the α_1 antagonist radioligand [^{125}I]HEAT. Competition reactions (total volume, 0.25 ml) contained 20 mM HEPES, pH 7.5, 1.4 mM EGTA, 12.5 mM $MgCl_2$, 200 pM [^{125}I]HEAT, COS-1 or CHO cell membranes, and increasing amounts of unlabeled ligands known to interact with ARs. Nonspecific binding was determined in the presence of 10^{-4} M phentolamine. Reactions were allowed to proceed for 1 hr at room temperature. Reactions were stopped by the addition of ice-cold HEPES buffer and were filtered onto Whatman GF/C glass fiber filters with a Brandel cell harvester. Filters were washed five times with HEPES buffer, and bound radioactivity was determined using a Packard Auto-gamma 500 counter. Binding data were analyzed by the iterative curve-fitting program LIGAND. For saturation binding studies, [^{125}I]HEAT concentrations ranging from 25 to 800 pM were used.

ADP-ribosylation. Pertussis toxin was preactivated for 1 hr at room temperature in 100 mM Tris, pH 8.0, containing 50 mM dithiothreitol. The activated toxin (2.9 μg) was then added to a mixture of 50 μg of COS-1 membranes, 100 mM Tris, pH 8.0, 25 mM dithiothreitol, 2 mM ATP, and 50 nM [^{32}P]NAD (1 μCi/tube), in a final volume of 100 μl. The reaction mixture was incubated for 1 hr at 37°, terminated by the addition of Laemmli sample buffer (26), and then heated for 5 min at 95°. SDS-polyacrylamide gel electrophoresis was performed on the sample and the wet gel was subjected to autoradiography.

Materials. Drugs were obtained from the following manufacturers: WB4101, Research Biochemicals Inc. (Natick, MA); (−)-epinephrine, (−)-norepinephrine, (−)-alprenolol, oxymetazoline, (−)-isoproterenol, mepacrine, dibucaine, BAPTA, and neomycin, Sigma; phentolamine, CIBA-Geigy; prazosin, Pfizer; and [^{125}I]HEAT, [^{3}H]inositol, and [^{3}H] arachidonic acid, New England Nuclear (Boston, MA). AG1-X8 resin was from Bio-Rad. Staurosporine and BAPTA/AM were from CalBiochem, and RHC-80267 was from Biomol.

Results

Expression of α_1-AR subtypes in COS-1 and CHO cells. To compare the functional characteristics of the α_{1D}-AR with those of the previously cloned and characterized hamster α_{1B}-AR, the signal transduction pathways of both α_1-AR subtypes were analyzed in transiently transfected COS-1 cells and in stably transfected CHO cells. COS-1 cells are useful for receptor characterization because they generally allow rapid, convenient, high level expression of mammalian genes and they are virtually devoid of endogenous α_1-ARs. However, because of the high level of receptor expression in COS-1 cells, coupling of receptor to the proteins involved in signal transduction that are expressed endogenously in COS-1 cells may be promiscuous and nonphysiological. For this reason, we elected to also characterize α_{1D}- and α_{1B}-AR-coupled signal transduction pathways in CHO cells, which express receptor proteins at levels approaching those found *in vivo*. These cells also allow the expression of transfected genes in a stable manner, and they lack endogenous α_1-ARs.

Radioligand binding studies performed as described previously (17), using the α_1-AR-selective radioligand [^{125}I]HEAT, indeed confirmed high level receptor expression in COS-1 cells transfected with either the α_{1D}-AR (B_{max}, 1 pmol of receptor/mg of protein; K_d, 0.2 nM) or α_{1B}-AR (B_{max}, 2 pmol of receptor/mg of protein; K_d, 0.24 nM). Similar studies with transfected CHO cells revealed receptor densities (B_{max}) of 0.219 and 0.247 pmol/mg of protein and equilibrium dissociation constants (K_d) of 174 and 100 pM, respectively. Additionally, competition binding studies with both the transfected COS-1 and CHO cells, using a variety of AR ligands, revealed rank orders of potency for agonists and antagonists similar to those observed previously (17) for the expressed α_{1D}- and α_{1B}-ARs (data not shown).

Characteristics of PI hydrolysis. Incubation of either COS-1 or CHO cells expressing the α_{1D}- or α_{1B}-AR with (−)-epinephrine (100 μM) resulted in a 2–3-fold accumulation of inositol phosphates (Fig. 1). These agonist-stimulated responses could be blocked by pretreatment with the α_1-selective antagonist prazosin (1 μM) or with neomycin (100 μM), indicating an α_1-specific pathway and activation of PLC, respectively (Fig. 1). The α_1-selective agonist phenylephrine (10 μM) was as effective as (−)-epinephrine in stimulating PI hydrolysis, as was (−)-norepinephrine (100 μM). Dose-response studies performed with CHO cells gave EC_{50} values for (−)-norepinephrine of 4.8 and 0.8 μM for the α_{1D}- and α_{1B}-ARs, respectively. However, EC_{50} values were the same for both receptors (0.7 μM versus 0.69 μM) when (−)-epinephrine was used.

To characterize the G protein involved in α_1-AR-mediated PI hydrolysis, both CHO and COS-1 cells were pretreated with pertussis toxin (1 μg/ml) for 24 hr. This results in ADP-ribosylation of susceptible G proteins endogenously expressed in these cells and, thus, uncouples receptor interactions with these pertussis toxin-sensitive substrates. To ensure the completeness of the pertussis toxin-catalyzed reaction, control studies were performed in which membranes were prepared from cells that had been pretreated with or without pertussis toxin. The membranes were then subjected to pertussis toxin-catalyzed ADP-ribosylation in the presence of [^{32}P]NAD, and covalent incorporation of the radiolabel was monitored by SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 1 (*inset*), pretreatment of cells with pertussis toxin blocked the incorporation of [^{32}P]NAD into a 41-kDa protein, which is most likely a "G_i-like" G protein. However, pertussis toxin pretreatment did not inhibit PI hydrolysis with agonist activation of either the α_{1D}- or α_{1B}-AR.

To investigate the inositol phosphate released with α_{1D}-AR activation, CHO cells expressing the α_{1D}-AR were stimulated with (−)-epinephrine (100 μM) for various times, and the inositol phosphates generated were separated sequentially by ion exchange chromatography using increasing concentrations of

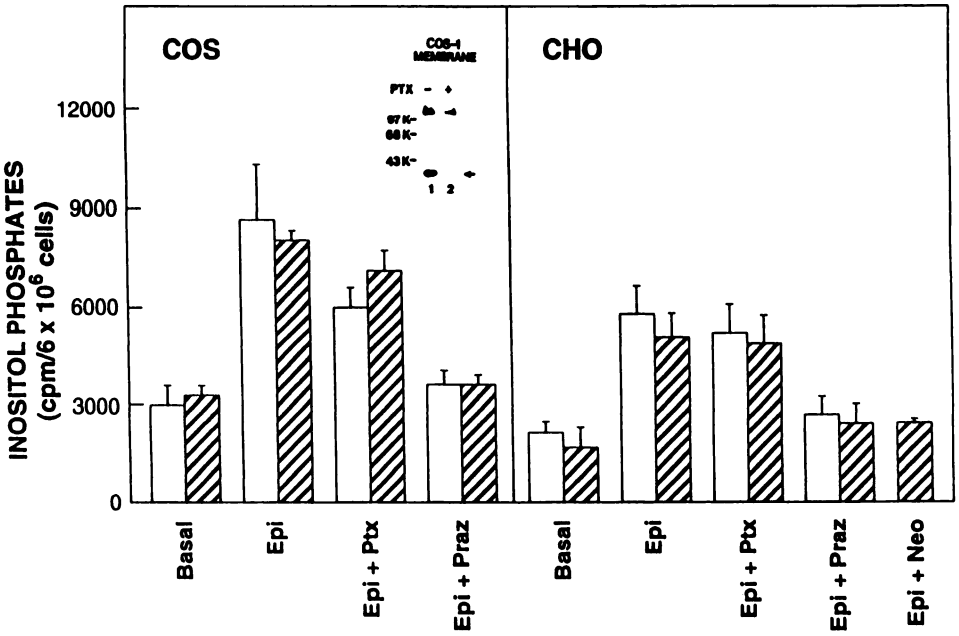

Fig. 1. Inositol phosphate release in COS-1 and CHO cells expressing the α_{1B}-AR (□) or α_{1D}-AR (▨). Cells were labeled with [^{3}H]inositol (1–2 μCi/ml) as described in the text and total inositol phosphates were measured after a 30-min incubation in the presence of vehicle (*Basal*), 100 μM (−)-epinephrine (*Epi*), or both (−)-epinephrine and 1 μM prazosin (*Epi + Praz*). Sensitivity of receptor-activated inositol phosphate release to pertussis toxin (*Ptx*) was determined exactly as described above except the cells were pretreated with 1 μg/ml pertussis toxin for 24 hr. The effect of neomycin on α_{1D}-AR-stimulated inositol phosphate generation in CHO cells was determined after pretreatment of cells for 15 min with 100 μM neomycin before addition of agonist. Results are means ± standard errors of two to four independent experiments per treatment. Receptor expression was approximately 2 pmol/mg for the α_{1B}-AR and 1 pmol/mg for the α_{1D}-AR in COS-1 cells. CHO cells expressed 247 and 219 fmol/mg levels of the α_{1B}-AR and α_{1D}-AR, respectively. Responses to epinephrine and epinephrine plus pertussis toxin were significantly greater than basal levels ($p < 0.05$ for each). *Inset*, ADP-ribosylation of COS-1 membranes. Pertussis toxin-catalyzed ADP-ribosylation of COS-1 membranes was carried out as described in the text. Products were run on a 12% SDS-polyacrylamide gel and then subjected to autoradiography. *Lane 1*, [^{32}P]NAD/ADP-ribosylation of native COS-1 membranes. A radiolabeled 41-kDa protein is seen (*arrow*) that is not apparent in *lane 2*. *Lane 2*, [^{32}P]NAD/ADP-ribosylation of COS-1 membranes that had been pretreated with pertussis toxin (1 μg/ml) for 24 hr.

ammonium formate. Within 5 sec, the inositol phosphate release was maximal, suggesting PLC activation and IP_3 production (data not shown). IP_3 production was also verified by thin layer chromatography using [^{32}P]IP_3 as a standard.

The effect of extracellular calcium chelation, which also results in a reduction of intracellular calcium levels, on α_1-AR-induced PI hydrolysis was investigated in CHO cells expressing either the α_{1D}- or α_{1B}-AR. Incubation of cells for 30 min with 2 mM EGTA did not affect epinephrine-stimulated release of inositol phosphates (Fig. 2). When CHO cells expressing the α_1-AR subtypes were incubated in the presence of 2 mM $CaCl_2$, stimulation with 100 μM epinephrine resulted in a greater release of total inositol phosphates, compared with the responses in the absence of extracellular calcium. This effect of calcium on PI hydrolysis was not sensitive to 1 μM nifedipine, indicating that dihydropyridine-sensitive calcium channels are not involved. These findings are consistent with a PLC-mediated mechanism that does not require calcium influx, although activation of this pathway can be enhanced by Ca^{2+} loading, as demonstrated for α_{1B}- and α_{1C}-AR-mediated PI hydrolysis (7). Taken together, these data are consistent with both the α_{1B}-AR and the recently cloned α_{1D}-AR being coupled to PLC. Also, activation of PI hydrolysis by these α_1-ARs is not dependent on calcium influx but involves receptor-PLC coupling via a pertussis toxin-insensitive G protein, such as G_q (27) or G_h (28).

cAMP metabolism of α_1-AR subtypes. It has recently been demonstrated that the α_{1B}- and α_{1C}-ARs can increase cAMP levels in COS-7 cells transiently expressing these α_1-AR subtypes (7). This effect does not involve a direct activation of adenylyl cyclase but, rather, appears to require protein kinase C activation, because the increases in cAMP can be partially blocked by sphingosine (29). To investigate whether the α_{1D}-AR also mediates this effect, cAMP levels were measured in COS-1 and CHO cells expressing either α_{1D}- or α_{1B}-ARs. Stimulation of COS-1 cells with (−)-epinephrine (100 μM) caused a 2-fold increase in cAMP levels, compared with basal levels (Fig. 3). In contrast, (−)-epinephrine stimulation of CHO cells, or of nontransfected COS-1 cells, did not result in cAMP increases, although forskolin stimulation of cAMP levels was readily apparent in nontransfected COS-1 cells (Fig. 3). In the transfected COS-1 cells, both the α_{1D}- and α_{1B}-AR-mediated responses were blocked by prazosin or by the protein kinase C inhibitor staurosporine (10 nM), indicating an α_1-AR-specific pathway involving activation of protein kinase C.

Characterization of α_1-AR-mediated arachidonic acid release. Because α_1-AR-mediated arachidonic acid release has been described in a number of cell types (12, 30), we evaluated this receptor-coupled effect for both α_1-AR subtypes in the transfected COS-1 and CHO cell lines. In preliminary studies we examined the ability of bradykinin receptors, expressed endogenously in COS-1 cells, to elicit arachidonic acid release. We reasoned that this monkey kidney epithelial (COS-1) cell line, like the MDCK cell line (30), may also contain bradykinin (B_2) receptors coupled to PLC and arachidonic acid release. Indeed, as shown in Fig. 4, bradykinin (0.5 μM) caused a 3-fold

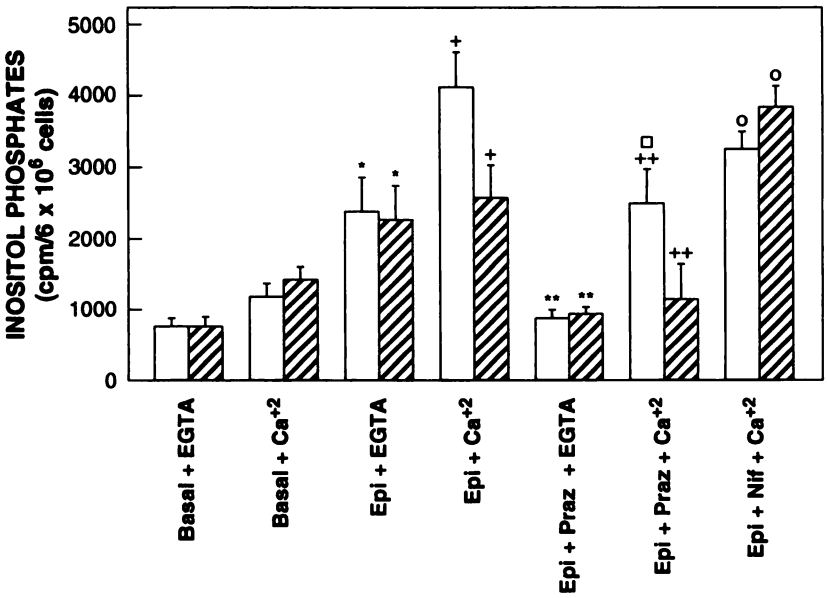

Fig. 2. Effect of Ca^{2+} and EGTA on inositol phosphate metabolism. CHO cells expressing either the α_{1B}-AR (□) or α_{1D}-AR (▨) were labeled with [^{3}H]inositol as described and, after washing, were incubated in HBSS containing either 2 mM EGTA or 2 mM $CaCl_2$. Cells were then stimulated for 30 min with 100 μM (−)-epinephrine in the absence (*Epi*) or presence of either prazosin (1 μM) (*Epi + Praz*) or nifedipine (1 μM) (*Epi + Nif*). Total inositol phosphates were measured as described in the text. Results are means ± standard errors for three independent experiments per treatment. CHO cells expressed 247 and 219 fmol/mg levels of the α_{1B}- and α_{1D}-AR, respectively. *, $p < 0.05$, epinephrine plus EGTA versus basal plus EGTA; +, $p < 0.05$, epinephrine plus Ca^{2+} versus basal plus Ca^{2+}; **, $p < 0.05$, epinephrine plus prazosin plus EGTA versus epinephrine plus EGTA; ++, $p < 0.05$, epinephrine plus prazosin plus Ca^{2+} versus epinephrine plus Ca^{2+}; O, p = not significant, epinephrine plus nifedipine plus Ca^{2+} versus epinephrine plus Ca^{2+}. □, This value is abnormally high because one experiment of three was aberrant.

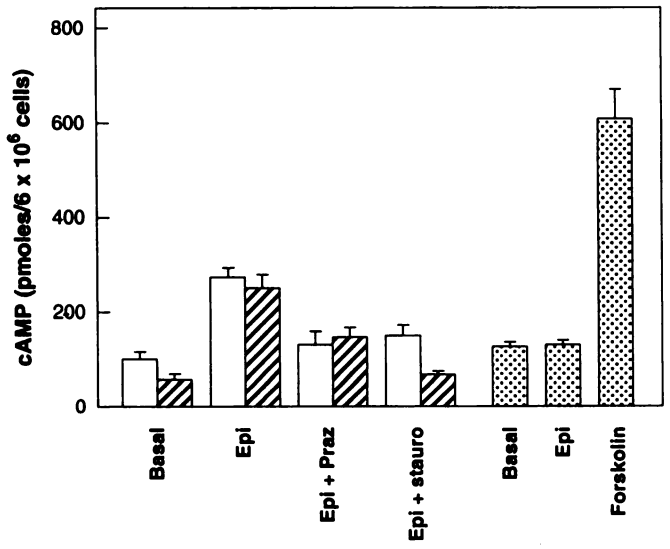

Fig. 3. α_1-AR-mediated cAMP metabolism in COS-1 cells. COS-1 cells expressing either the α_{1B}-AR (□) or α_{1D}-AR (▨) were incubated with vehicle (*Basal*) or (−)-epinephrine (100 μM) without (*Epi*) or with pretreatment with prazosin (1 μM) (*Epi + Praz*) or staurosporine (10 nM) (*Epi + stauro*) for 10 min at 37°, and cAMP levels were measured as described in the text. Responses to epinephrine were significantly greater than basal for epinephrine plus prazosin and epinephrine plus staurosporine ($p < 0.05$). Untransfected COS-1 cells (□) were similarly evaluated for cAMP generation after incubation with either vehicle (*Basal*), (−)-epinephrine, or forskolin (10 μM). Results are means ± standard errors of four independent experiments per treatment. COS-1 cells expressed approximately 2 and 1 pmol/mg levels of the α_{1B}- and α_{1D}-AR, respectively.

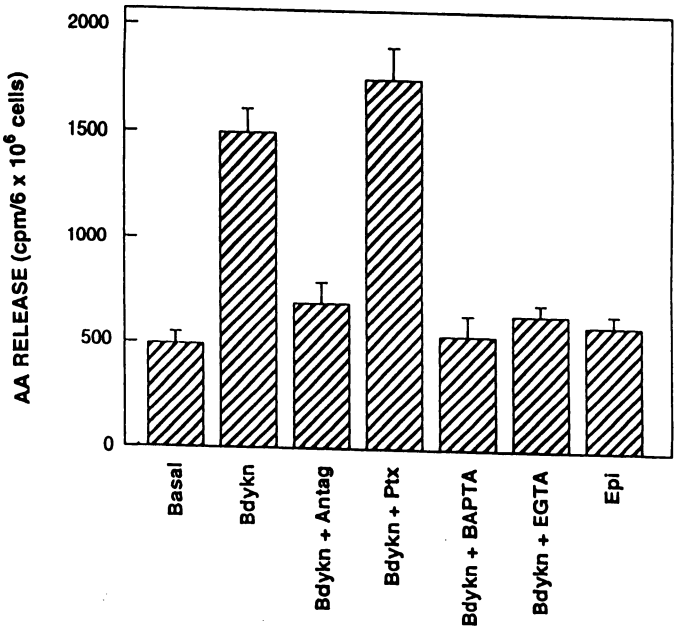

Fig. 4. Characterization of the bradykinin response in nontransfected COS-1 cells. Nontransfected COS-1 cells were labeled overnight with [^{3}H]arachidonic acid and, after thorough washing, arachidonic acid (*AA*) release was measured as described in the text, after a 5-min incubation with vehicle (*Basal*) or with 0.5 μM bradykinin without (*Bdykn*) or with pretreatment with 50 μM D-Arg-[Hyp^3,Thi^{5,8}-D-Phe^7]bradykinin (*Bdykn + Antag*) for 10 min, 1 μg/ml pertussis toxin (*Bdykn + Ptx*) for 24 hr, 100 μM BAPTA (*Bdykn + BAPTA*) for 1 hr, or 2 mM EGTA (*Bdykn + EGTA*) for 30 min. Nontransfected COS-1 cells were incubated with 100 μM (−)-epinephrine (*Epi*) to determine the presence of endogenous α-ARs. Results are means ± standard errors of three independent experiments per treatment.

stimulation of arachidonic acid release, which could be blocked by the B_2 receptor-specific antagonist D-Arg-[Hyp^3,Thi^{5,8}-D-Phe^7]bradykinin (50 μM). This bradykinin response requires calcium influx, because arachidonic acid release was blocked by chelation of extracellular calcium with either EGTA (2 mM) or BAPTA (100 μM). Additionally, bradykinin-stimulated arachidonic acid release involves coupling via a pertussis toxin-insensitive G protein, because it was resistant to pretreatment with pertussis toxin (1 μg/ml). These findings indicate that, like the MDCK cell line, COS-1 cells express bradykinin receptors and activation of these receptors elicits arachidonic acid release.

To determine whether the α_{1D}-AR or the α_{1B}-AR also can mediate arachidonic acid release, the effects of various α_1 agonists and antagonists were evaluated. In COS-1 cells transfected with either the α_{1D}- or α_{1B}-AR, the α_1 agonist (−)-epinephrine (100 μM) caused a 4-fold increase in arachidonic acid release over basal levels (Fig. 5). The specificity of this

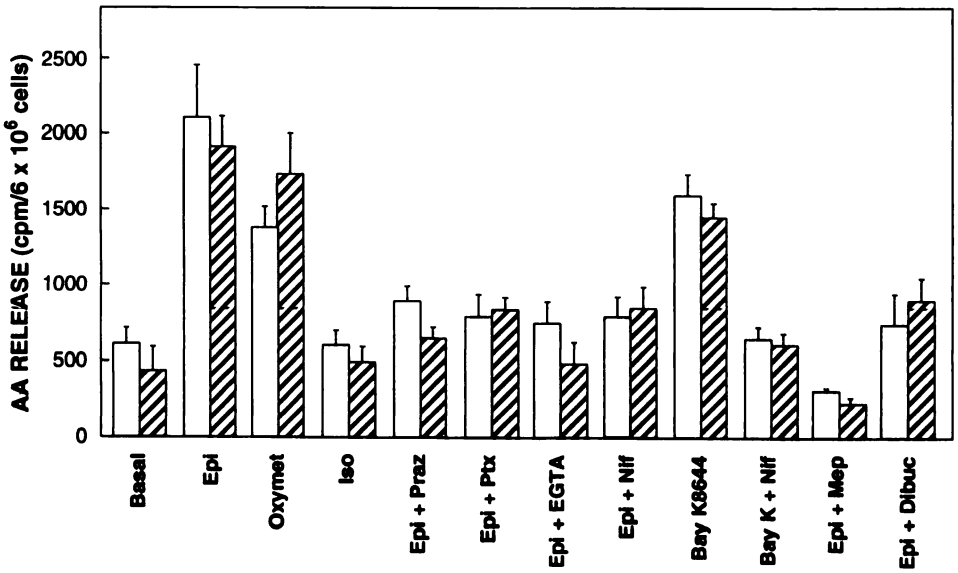

Fig. 5. Characterization of α_1-AR-mediated arachidonic acid release in COS-1 cells. COS-1 cells expressing either the α_{1B}-AR (□) or α_{1D}-AR (■) were labeled overnight with [^{3}H]arachidonic acid, and arachidonic acid (*AA*) release was measured as described in the text, after a 5-min incubation with vehicle (*Basal*) or 100 μM (−)-epinephrine without (*Epi*) or with pretreatment with 1 μM prazosin (*Epi + Praz*), 1 μM nifedipine (*Epi + Nif*), 200 μM mepacrine (*Epi + Mep*), or 200 μM dibucaine (*Epi + Dibuc*) for 15 min, 1 μg/ml pertussis toxin (*Epi + Ptx*) for 24 hr, or 2 mM EGTA (*Epi + EGTA*) for 30 min. COS-1 cells expressing the α_1 subtypes were also incubated with 100 μM oxymetazoline (*Oxymet*), 10 μM isoproterenol (*Iso*), or 1 μM Bay K8644 for 5 min. Results are means ± standard errors of four or five independent experiments per treatment. COS-1 cells expressed approximately 2 and 1 pmol/mg levels of the α_{1B}- and α_{1D}-AR, respectively. Responses to epinephrine, oxymetazoline, and Bay K8644 were significantly greater than basal ($p < 0.05$ for each). All inhibitory responses, i.e., epinephrine plus prazosin, epinephrine plus pertussis toxin, epinephrine plus EGTA, epinephrine plus nifedipine, epinephrine plus mepacrine, and epinephrine plus dibucaine, were significantly less than that to epinephrine ($p < 0.05$ for each). The Bay K8644 responses in the presence of nifedipine (*Bay K + Nif*) were also less than the responses to Bay K8644 alone ($p < 0.01$).

response for α_1-ARs is evident from the finding that arachidonic acid release could also be stimulated by the α_1 agonist oxymetazoline (100 μM) but not by the β agonist isoproterenol (Fig. 5). Moreover, α_1 agonist-stimulated release could be blocked by the α_1-selective antagonist prazosin (1 μM), and no increase in arachidonic acid release was observed in nontransfected COS-1 cells treated with (−)-epinephrine (100 μM) (Fig. 4). Another α_1-selective antagonist, WB4101 (10 μM), also blocked (−)-epinephrine-stimulated arachidonic acid release.

In COS-1 cells, further characterization of α_1-AR-mediated arachidonic acid release indicated that it involves calcium influx via activation of voltage-gated, L-type calcium channels, coupling via a pertussis toxin-sensitive G protein, and activation of phospholipase A_2 (Table 1). However, it appears to be independent of diacylglycerol lipase activation and, unlike bradykinin-mediated arachidonic acid release in MDCK cells (30), does not involve activation of PLC or protein kinase C.

Thus, chelation of extracellular calcium with EGTA (2 mM), buffering of intracellular calcium transients with BAPTA/AM, pretreatment with the L-type calcium channel blocker nifedipine (1 μM), or pretreatment with pertussis toxin (1 μg/ml) all inhibited (−)-epinephrine-stimulated arachidonic acid release (Fig. 5; Table 1). Evidence for the involvement of phospholipase A_2 is the finding that mepacrine or dibucaine, when used in doses (200 μM each) that are reasonably specific for producing phospholipase A_2 inhibition without causing nonspecific membrane-perturbant effects, inhibited (−)-epinephrine-stimulated arachidonic acid release. Also, arachidonic acid release was not blocked by inhibitors of PLC, protein kinase C, or diacylglycerol lipase, i.e., neomycin (100 μM), staurosporine (10 nM), or RHC-

TABLE 1

Effects of various agents on α_1-AR-stimulated arachidonic acid release in COS-1 cells transfected with either the α_{1D}- or α_{1B}-AR cDNA

Results are means ± standard errors for three or four individual experiments per agent.

Agent	Time of preincubation	Stimulated/basal	
		α_{1D}	α_{1B}
Basal		1.0 ± 0.2	1.0 ± 0.3
Epinephrine (100 μM)		3.8 ± 0.4[a]	3.9 ± 0.7[a]
Epinephrine + BAPTA/AM (100 μM)	1 hr	1.9 ± 0.2[b]	1.9 ± 0.3[b]
Epinephrine + staurosporine (10 nM)	15 min	4.1 ± 0.8	3.1 ± 0.3
Epinephrine + neomycin (100 μM)	15 min	5.0 ± 0.1	4.6 ± 0.8
Epinephrine + RHC-80267 (10 μM)	15 min	4.9 ± 1.0	3.4 ± 0.6
Epinephrine + EGTA (2 mM)	30 min	1.0 ± 0.3[c]	1.4 ± 0.3[b]
Veratridine only (40 μM)		2.9 ± 0.1[d]	2.6 ± 0.1[d]
KCl (25 mM)		2.0 ± 0.2[d]	1.6 ± 0.1[a]

[a] $p < 0.005$, versus basal.
[b] $p < 0.05$, versus epinephrine alone.
[c] $p < 0.005$ versus epinephrine alone.
[d] $p < 0.001$ versus basal.

80236 (10 μM), respectively. The dose of RHC-80236 used in these studies has previously been shown to markedly reduce monoacylglycerol levels without inhibiting phospholipase A_2 (31). Similarly, the doses of staurosporine and neomycin were the minimal effective concentrations that inhibited (−)-epinephrine-stimulated cAMP levels (Fig. 3) or increases in intracellular calcium levels (Fig. 6C), respectively. Thus, this α_1-AR-coupled phospholipase A_2 response is most likely due, in large part, to activation of phospholipase A_2 by the increases in intracellular calcium levels associated with receptor stimulation of calcium channels.

Involvement of voltage-gated, L-type calcium channels in this pathway was further investigated in additional studies. As shown in Fig. 5, the calcium channel agonist Bay K8644 (1 μM) increased arachidonic acid release, a response that was blocked by nifedipine (1 μM). Additionally, depolarization of COS-1 cells with KCl (25 mM) or with the sodium channel activator veratridine (40 μM), both of which activate voltage-sensitive calcium channels, also increased arachidonic acid release (Table 1).

In contrast to the responses in COS-1 cells, activation of the α_{1D}-AR in transfected CHO cells (Fig. 7) increased arachidonic acid release through a nifedipine-insensitive pathway, although arachidonic acid release in these cells was also sensitive to inhibition by pertussis toxin. This finding suggests that CHO cells lack L-type calcium channels and implicates either direct receptor coupling to phospholipase A_2 via a pertussis toxin-sensitive G protein or the involvement of some other dihydropyridine-insensitive calcium channel. As with COS-1 cells, various inhibitors of signaling were used to characterize the release of arachidonic acid in CHO cells (Table 2). The results of these studies were similar to those observed with COS-1 cells and ruled out the involvement of PLC, protein kinase C, diacylglycerol lipase, and intracellular calcium stores in the activation of phospholipase A_2. (−)-Epinephrine-stimulated arachidonic acid release was also evaluated in CHO cells expressing the α_{1B}-AR subtype but reached levels only about 40% above basal levels (data not shown). However, the α_{1B}-AR displayed the same responsiveness to the various agonists, blockers, and inhibitors in its activation of arachidonic acid release as did the α_{1D}-AR.

To further establish that the phospholipase responsible for arachidonic acid release in CHO cells is phospholipase A_2 and that the mechanism of coupling to α_1-ARs is direct, experiments were performed with cell-free membrane preparations. When exogenous 1-acyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine was used to assay membrane-bound phospholipase A_2 activity, (−)-epinephrine (100 μM) was found to stimulate [^{3}H] arachidonic acid release (Table 3). This response required both calcium and GTP and could be blocked by phentolamine (100 μM). GTPγS but not GDPβS also activated phospholipase A_2 in this membrane preparation, and an additional increase in [^{3}H]arachidonic acid release was observed when GTPγS and (−)-epinephrine were combined (Table 3).

In additional studies we investigated the temporal changes in α_1-AR-stimulated arachidonic acid release. We reasoned that this release may be faster in CHO cells, where receptor coupling to phospholipase A_2 appears to be direct, than in COS-1 cells,

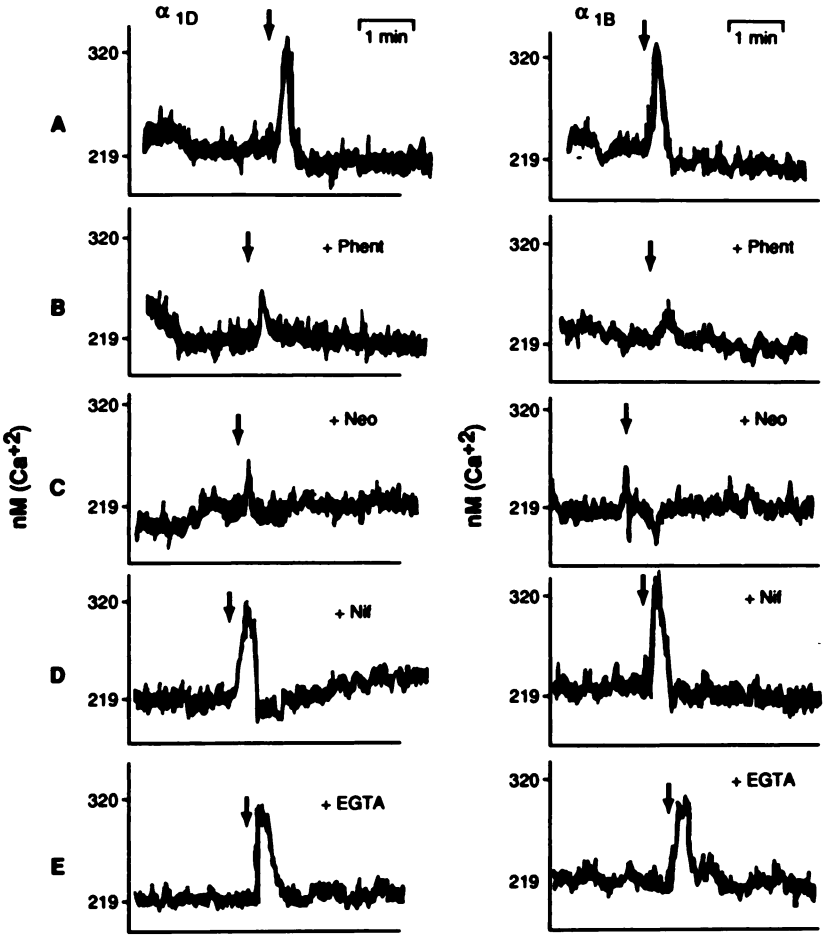

Fig. 6. Intracellular calcium responses to (−)-epinephrine in CHO cells expressing either the α_{1B}- or α_{1D}-AR. Cells were loaded with 1 μM fura-2/AM as described in the text. Calcium transients in CHO cells expressing the α_{1D}-AR (*left*) or α_{1B}-AR (*right*) are shown. In A-E, (−)-epinephrine (100 μM) was added at the times indicated by the *arrows*. A, Control; B-E, pretreatment with either phentolamine (100 μM) for 10 min (*+Phent*) (B), neomycin (100 μM) for 30 min (*+Neo*) (C), nifedipine (1 μM) for 30 min (*+Nif*) (D), or EGTA (5 mM) for 1 min (*+EGTA*) (E), before the addition of (−)-epinephrine. CHO cells expressed 247 and 219 fmol/mg levels of the α_{1B}- and α_{1D}-AR, respectively.

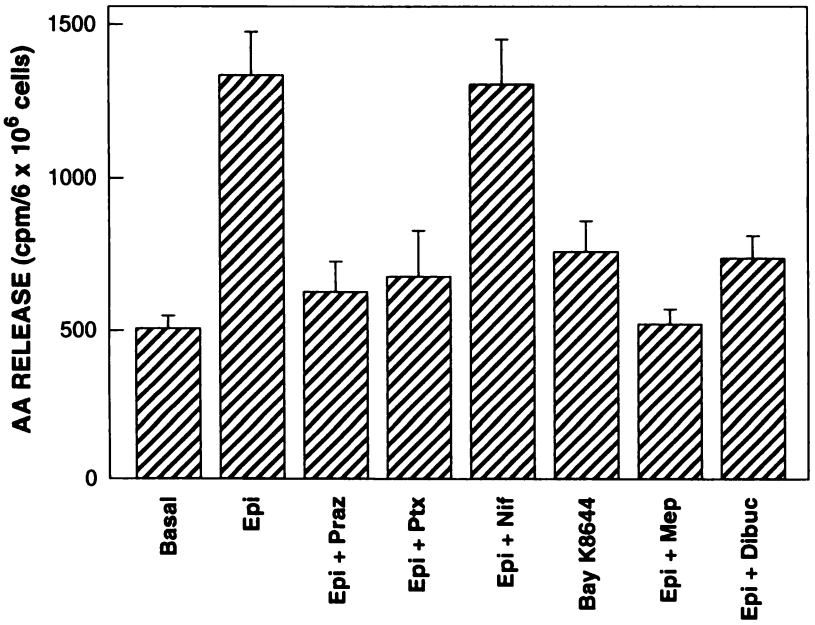

Fig. 7. Characterization of α_{1D}-AR-mediated arachidonic acid release in CHO cells. CHO cells expressing the α_{1D}-AR subtype were labeled overnight with [^{3}H]arachidonic acid, and arachidonic acid (*AA*) release was measured as described in the text, after a 5-min incubation with vehicle (*Basal*) or 100 μM (−)-epinephrine without (*Epi*) or with pretreatment with 1 μM prazosin (*Epi + Praz*), 1 μM nifedipine (*Epi + Nif*), 200 μM mepacrine (*Epi + Mep*), or 200 μM dibucaine (*Epi + Dibuc*) for 15 min or 1 μg/ml pertussis toxin (*Epi + Ptx*) for 24 hr. CHO cells expressing the α_{1D}-AR subtype were also incubated with 1 μM Bay K8644 for 5 min. Results are means ± standard errors of four or five independent experiments per treatment. CHO cells expressed 247 and 219 fmol/mg levels of the α_{1B}- and α_{1D}-AR, respectively.

TABLE 2

Effects of various agents on α_1-AR-stimulated arachidonic acid release in CHO cells transfected with the α_{1D}-AR cDNA

Results are means ± standard errors for four to six individual experiments per agent.

Agent	Time of preincubation	Stimulated/basal
Basal		1.0 ± 0.2
Epinephrine (100 μM)		2.7 ± 0.3[a]
Epinephrine + BAPTA/AM (100 μM)	1 hr	2.0 ± 0.5
Epinephrine + staurosporine (10 nM)	15 min	2.5 ± 0.3
Epinephrine + neomycin (100 μM)	15 min	3.0 ± 0.2
Epinephrine + RHC-80267 (10 μM)	15 min	2.4 ± 0.3
Epinephrine + EGTA (2 mM)	30 min	1.3 ± 0.1[b]
Veratridine only (40 μM)		3.6 ± 0.4[a]

[a] $p < 0.005$, versus basal.
[b] $p < 0.05$, versus epinephrine alone.

TABLE 3

Activation of phospholipase A_2 in CHO cell membranes

Membranes were prepared as described in the text, from CHO cells stably transfected with either the α_{1D}- or α_{1B}-AR cDNAs. Assays were conducted in 15 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM $CaCl_2$, with 0.125 μCi of 1-acyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine and 100 μg of membrane protein. All assays contained 1 mM Ca^{2+} unless otherwise indicated. All nucleotide concentrations were 10^{-4} M (final). After 5 min at 37°, the reaction was stopped by the addition of 300 μl of ethanol, followed by thin layer chromatography as described in the text. Values shown are means ± standard errors for three independent experiments for each treatment. Values in parentheses are the fold increases above the release of arachidonic acid in the presence of nucleotide alone.

Treatment[a]	[^{3}H]Arachidonic acid release	
	α_{1D}-AR	α_{1B}-AR
	cpm	
−GTP	1,530 ± 74	1,315 ± 62
+Epi, −GTP	1,551 ± 69 (1.0)	1,269 ± 75 (1.0)
+GTP	1,377 ± 136 (1.0)	1,209 ± 104 (1.0)
+Epi, +GTP	3,292 ± 326 (2.4[b])	2,083 ± 88 (1.7[b])
+Epi, +Phent, +GTP	1,161 ± 61 (0.8)	1,024 ± 129 (0.8)
+GTP, $-Ca^{2+}$	1,235 ± 101	1,268 ± 72
+Epi, +GTP, $-Ca^{2+}$	1,181 ± 75 (1.0)	1,308 ± 99 (1.0)
+GTPγS	18,214 ± 268	20,272 ± 950
+Epi, +GTPγS	23,946 ± 2,692 (1.3[b])	31,735 ± 1,552 (1.6[b])
+GDPβS	1,121 ± 12	1,301 ± 30
+Epi, +GDPβS	1,124 ± 44 (1.0)	1,314 ± 76 (1.0)

[a] Epi, (−)-epinephrine (100 μM); Phent, phentolamine (100 μM).
[b] $p < 0.05$.

where initial activation of calcium channels is required. However, as shown in Fig. 8, the time course of arachidonic acid release with activation of the α_{1D}-AR was similar in transfected COS-1 and CHO cells. In COS-1 cells, arachidonic acid release increased 3–4-fold over basal levels, whereas in CHO cells the increase was only 2–3-fold. This difference may be related to the differing levels of α_{1D}-AR expression in the two cell lines. The time course of α_{1B}-AR-mediated arachidonic acid release was similar to that of the α_{1D}-AR-mediated response. The magnitude of the response was also similar in COS-1 cells but was only 1.5-fold over basal levels in CHO cells (data not shown).

Measurement of cytosolic calcium levels. As shown in Fig. 6A, stimulation of CHO cells expressing either the α_{1D}- or α_{1B}-AR resulted in a rapid increase in intracellular calcium levels. This increase in cytosolic calcium concentration was approximately 100 nM above basal levels. Pretreatment of CHO cells with phentolamine (100 μM) or neomycin (100 μM) inhibited the calcium transient (Fig. 6, B and C), indicating that calcium release from intracellular stores is mediated by the IP_3 generated by α_1-AR-stimulated activation of PLC. Also, pretreatment with nifedipine (1 μM) did not affect the calcium transient, and addition of EGTA (5 mM) to CHO cells did not change the height or shape of the calcium transient. These findings indicate that calcium influx is not required for α_1-AR-mediated increases in intracellular calcium levels in CHO cells and rule out the possibility of receptor coupling to a dihydropyridine-insensitive calcium channel.

Because veratridine caused release of arachidonic acid in CHO cells, we also investigated the effect of veratridine on intracellular calcium changes, using fura-2/AM. Veratridine (40 μM) caused a slow but steady increase in intracellular calcium levels, from 210 nM to 483 nM, in 12 min. Therefore, it appears that veratridine, a Na^+ channel activator, increases intracellular calcium levels in CHO cells by enhancing Na^+/Ca^{2+} exchange and/or by depolarizing the cell and activating voltage-gated calcium channels. The effect of EGTA on intracellular calcium levels was also investigated. EGTA (2 mM) decreased intracellular calcium levels from 210 nM to 101 nM

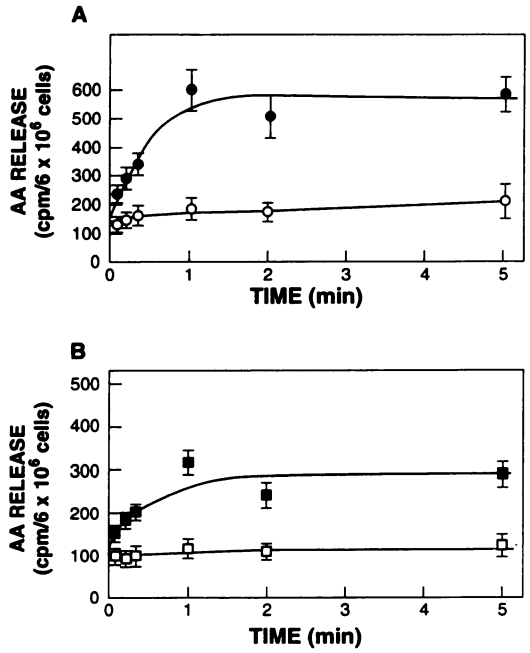

Fig. 8. Time course of arachidonic acid (*AA*) release in COS-1 (A) or CHO (B) cells expressing the α_{1D}-AR. COS-1 cells (●) and CHO (■) cells expressing the α_{1D}-AR were labeled with [^{3}H]arachidonic acid as described in the text and then were incubated with (−)-epinephrine (100 μM). At various times, samples of the medium were removed and measured for arachidonic acid release as described in the text. As controls, nontransfected COS-1 (○) and CHO (□) cells were also evaluated for (−)-epinephrine-stimulated arachidonic acid release. Results are means ± standard errors for two independent experiments. COS-1 cells expressed approximately 2 and 1 pmol/mg levels of the α_{1B}- and α_{1D}-AR, respectively, whereas CHO cells expressed 247 and 219 fmol/mg.

in 15 min. Thus, EGTA not only chelates extracellular calcium but also can eventually decrease intracellular calcium levels with longer periods of incubation.

Characterization of receptor density versus signaling. To determine the relationship between receptor density (B_{max}) and the subtype response to (−)-epinephrine for either receptor (Fig. 9, *inset*), COS-1 cells were transfected with various amounts of the expression vector pMT2′α_{1B} or pMT2′α_{1D} and arachidonic acid release was measured with whole cells after a 5-min exposure to 100 μM (−)-epinephrine. As shown in Fig. 9, (−)-epinephrine-stimulated arachidonic acid release was directly related both to the amount of vector transfected and to the number of α_1-ARs expressed. Additionally, the relationship between receptor-mediated signaling and receptor expression was similar for both the α_{1B}- and α_{1D}-AR subtypes (Fig. 9, *inset*).

Discussion

Recently, we reported the cloning of a cDNA for the α_{1D}-AR, a novel subtype that differs from the previously characterized α_{1B}- and α_{1C}-ARs (17). Interestingly, the properties of this α_{1D}-AR also differ from those of the typical, endogenously expressed, α_{1A}-AR characterized pharmacologically in various tissues and cultured cell lines (2–6, 17, 18). Here, we present an analysis of the signal transduction properties of the α_{1D}-AR. These properties were compared with those of the α_{1B}-AR after the transient or stable transfection in COS-1 or CHO cells, respectively, of cDNAs encoding either the α_{1D}- or α_{1B}-AR. Receptor expression and analysis of receptor-coupled signaling pathways in eukaryotic cell lines that lack endogenous α_1-ARs may differ from those observed *in vivo*, because these cells may contain a different complement of G proteins and effectors. Nevertheless, use of these model systems allows evaluation of the potential for each subtype, whether expressed in high copy number, as in COS-1 cells, or in levels approximating those observed *in vivo*, as observed for the receptors expressed in CHO cells, to independently couple to one or more effector pathways.

In agreement with the findings of Schwinn *et al.* (7), activation of the α_{1B}-AR, whether expressed stably or transiently, results in PI turnover, a response that involves coupling via a pertussis toxin-insensitive G protein. Comparable receptor-mediated, pertussis toxin-insensitive activation of PLC is also observed with the expressed α_{1D}-AR, although dose-response studies suggest that the potency of the neurotransmitter (−)-norepinephrine is less for the α_{1D}-AR- than for the α_{1B}-AR-mediated response (EC_{50}, 4.8 and 0.8 μM, respectively). In contrast, the efficacy of the neurohormone (−)-epinephrine is similar for both α_1-AR subtypes (EC_{50}, 0.7 and 0.69 μM, respectively). Calcium entry is not required for either α_{1D}- or α_{1B}-AR-mediated PLC activation, because receptor-stimulated increases in inositol phosphates are resistant to calcium channel blockade by nifedipine and to chelation of extracellular calcium with EGTA. However, activation of both receptor subtypes results in intracellular calcium mobilization, which is presumably mediated by PLC-catalyzed IP_3 generation, because it can be inhibited by neomycin.

Like the α_{1C}-AR (7), when transiently expressed in COS-1 cells the α_{1D}-AR, as well as the α_{1B}-AR, can mediate increases in cAMP levels. This α_1-AR-specific effect appears to involve protein kinase C activation, because it is sensitive to inhibition by staurosporine. Activation of protein kinase C is presumably a consequence of α_1-AR-stimulated increases in diacylglycerol and calcium mobilization that are associated with PLC-catalyzed phosphatidylinositol-4,5-bisphosphate hydrolysis (29). Moreover, protein kinase C-mediated activation of adenylyl cyclase has been reported for α_1-ARs in rat pinealocytes (32), as well as for other PLC-coupled receptor systems (33). The finding that agonist stimulation of α_1-ARs expressed in CHO cells does not increase cAMP suggests that these cells contain either an isoform of protein kinase C that is resistant to receptor activation or an isoform of adenylyl cyclase that is resistant to protein kinase C activation. Alternatively, the amounts of diacylglycerol generated by the lower levels of receptor expressed in CHO cells may not be sufficient to effectively activate protein kinase C.

In both transfected COS-1 and CHO cells activation of either the α_{1D}- or α_{1B}-AR results in arachidonic acid release, and in both cell types this response is mediated by a pertussis toxin-sensitive G protein. This α_1-AR-mediated response is due, most likely, to activation of phospholipase A_2, because it can be blocked by either mepacrine or dibucaine. Additionally, arachidonic acid release is resistant to inhibitors of PLC, protein kinase C, or diacylglycerol lipase. However, the signal transduction pathways involved in this α_1-AR-coupled response differ in COS-1 and CHO cells. In COS-1 cells, phospholipase A_2 activation is primarily indirect and involves receptor-mediated gating of voltage-sensitive L-type calcium channels. Activation of the effector enzyme is, thus, mainly a consequence of the resultant increases in cytosolic calcium. A direct coupling between α_1-AR and phospholipase A_2 may also exist but can account for only a minor component of the total arachidonic

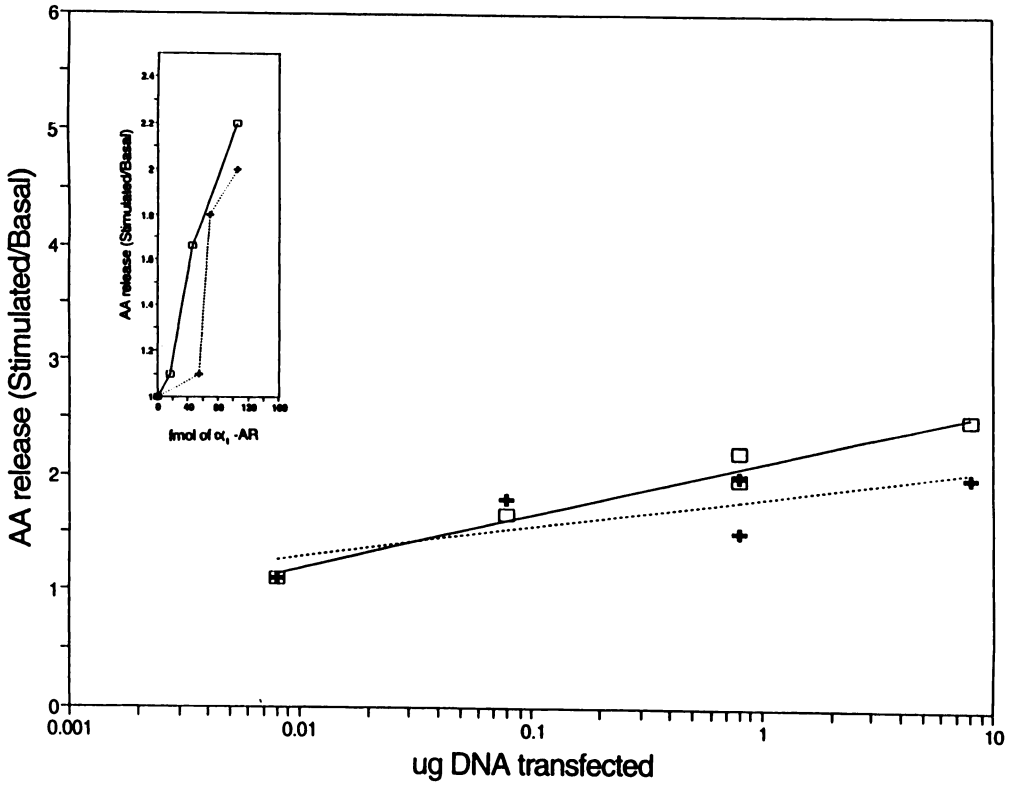

Fig. 9. Characterization of receptor density versus signaling. COS-1 cells (6×10^6) were transfected with various amounts of either pMT2′α_{1B} (+) or pMT2′α_{1D} (□) and after 2 days were labeled with [^{3}H]arachidonic acid, and arachidonic acid (*AA*) release was measured as described in the text, after a 5-min incubation with vehicle (*Basal*) or 100 μM (−)-epinephrine (*Stimulated*). *Inset*, after the transfection described above, the total fmol of receptor were calculated, based upon ligand binding studies using ^{125}I-HEAT as the radioligand, and were plotted versus the corresponding signal response. Results are the means of two independent experiments for each α_1 subtype.

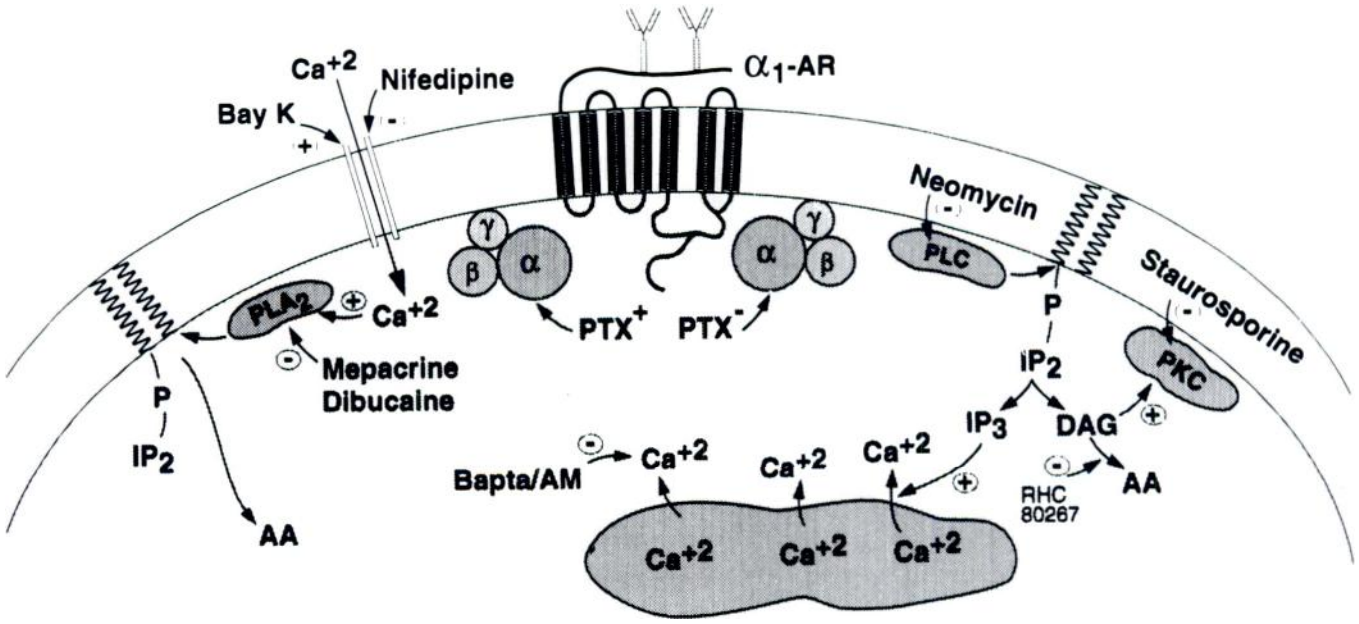

Fig. 10. Biochemical pathways activated by α_1-AR stimulation. The α_1-AR (α_{1B}- or α_{1D}-AR) can couple via a heterotrimeric ($\alpha\beta\gamma$) pertussis toxin-sensitive (*PTX*$^+$) G protein, either directly or through activation of voltage-sensitive, L-type calcium channels and, thus, increased intracellular calcium, to phospholipase A_2 (*PLA*$_2$) and via a pertussis toxin-insensitive (*PTX*$^-$) G protein to PLC. Calcium channels can be activated by Bay K8644 (*Bay K*) or inhibited by nifedipine. Phospholipase A_2 hydrolyzes membrane phospholipids at the *sn*-2 position to yield arachidonic acid (*AA*) and can be inhibited by mepacrine or dibucaine. Activation of PLC leads to the generation of the second messengers IP_3 and diacylglycerol (*DAG*), which mobilize calcium from intracellular stores and activate protein kinase C (*PKC*), respectively. The PLC reaction can be inhibited by neomycin, and protein kinase C activation can be inhibited by staurosporine. Arachidonic acid can also be generated by the hydrolysis of diacylglycerol by diacylglycerol lipase, which can be inhibited by RHC-80267. Intracellular calcium can be chelated by BAPTA/AM.

acid response. In contrast, in CHO cells, which lack L-type calcium channels, phospholipase A_2 activation appears to be coupled exclusively to receptor activation by a direct mechanism. Thus, this response is independent of either calcium channel activation or calcium mobilization, although changes in basal intracellular calcium concentrations can modulate phospholipase A_2 activity. In addition, this activation involves a G_i- or G_o-like protein that is sensitive to pertussis toxin.

Evidence for a direct mechanism in CHO cells comes from the fura-2/AM studies, where inhibition of the PLC pathway with neomycin completely blocked all changes in intracellular calcium levels. This indicates that the increase in intracellular calcium levels associated with α_1-AR stimulation results entirely from PLC activation and mobilization of intracellular calcium stores, rather than enhancement of calcium entry. In addition, cell-free assays of phospholipase A_2 activity, using phosphatidylcholine specifically labeled at the *sn*-2 position with [^{3}H]arachidonic acid, further indicate a direct mechanism that is independent of second messengers, because (−)-epinephrine also stimulates [^{3}H]arachidonic acid release in this membrane preparation. This response is dependent upon GTP and can be mediated directly by G protein activation with the nonhydrolyzable GTP analog GTPγS but not with the inactive analog GDPβS. Arachidonic acid release in this cell-free system is also dependent upon calcium, indicating that calcium is necessary but not sufficient for (−)-epinephrine-stimulated arachidonic acid release.

Taken together, the findings of these studies provide support for the notion that a single α_1-AR subtype can activate multiple effector pathways via coupling to distinct G proteins. Thus, activation of multiple effector responses is observed for both the α_{1D}- and α_{1B}-ARs, whether overexpressed in COS-1 cells or expressed at physiological levels in CHO cells. Similar findings of multiple effector pathways being coupled to a single receptor subtype have been reported for the cloned α_2-AR and muscarinic receptors (34, 35). Studies of α_1-AR-mediated responses in spinal cord neurons (36) and in the MDCK-D1 (37) and FRTL-5 (12) cell lines have also implicated receptor coupling to multiple effector pathways. However, definitive evidence that these cells express only a single α_1-AR subtype is lacking. In spinal cord neurons and in MDCK-D1 cells, α_1-ARs mediate both arachidonic acid release and PI turnover. Arachidonic acid release in these cells is dependent upon extracellular calcium and in spinal cord neurons is sensitive to inhibition by nifedipine. In MDCK-D1 cells, which express a non-α_{1A}, CEC-sensitive α_1-AR, arachidonic acid release is almost completely dependent on the activation of protein kinase C (37, 38). This contrasts with the arachidonic acid responses observed in this study for both the α_{1D}- and α_{1B}-ARs, which are protein kinase C independent. In FRTL-5 cells, an α_{1B}-AR couples to both arachidonic acid release and PI turnover, through pertussis toxin-sensitive and -insensitive G proteins, respectively. Like the α_{1D}-AR- and α_{1B}-AR-mediated responses in COS-1 cells, and like the α_1-AR-mediated response in MDCK-D1 cells, phospholipase A_2 activation in FRTL-5 cells is calcium dependent. However, a role for protein kinase C in this FRTL-5 cell response has not been examined.

The finding that calcium entry or increases in intracellular calcium levels are not required for the α_{1D}- or α_{1B}-AR-mediated arachidonic acid response in CHO cells suggests either that CHO cells express a calcium-independent phospholipase A_2 (39), which can couple directly to the α_1-ARs, or that, like PLC, receptor activation in CHO cells enhances the sensitivity of a calcium-dependent phospholipase A_2, so that it is activated by the basal concentrations of calcium in the cell. Evidence that CHO cells, indeed, contain a calcium-sensitive phospholipase A_2 involves the finding that veratridine activates arachidonic acid release (Table 2). This sodium channel activator depolarizes the cell and increases intracellular calcium levels by, thus, activating voltage-sensitive (but dihydropyridine-insensitive) calcium channels and/or stimulating Na^+/Ca^{2+} exchange. Alternatively, CHO cells may express more than one isoform of phospholipase A_2, one calcium activated and one receptor coupled.

In COS-1 cells arachidonic acid release mediated by the bradykinin B_2 receptor is coupled through a pertussis toxin-insensitive G protein, whereas that mediated by the α_{1D}- and α_{1B}-ARs is coupled through a pertussis toxin-sensitive G protein. This is of interest because it indicates that, despite overexpression of α_1-ARs in these cells, selectivity of their coupling to G proteins is maintained. Involvement of either a pertussis toxin-sensitive or -insensitive G protein in receptor-mediated arachidonic acid release has also been observed for α_1-ARs in vascular smooth muscle cells (40) and thrombin receptors in platelets (41), respectively. In vascular smooth muscle cells, this α_1-AR-mediated response is calcium influx dependent, whereas in platelets arachidonic acid release is calcium influx independent.

Based on the data presented here, a model of the biochemical events and signal transduction pathways coupled to activation of either the transiently or stably expressed α_{1D}- or α_{1B}-ARs is shown in Fig. 10. It is interesting to note that the α_{1D}- and α_{1B}-ARs exhibit almost identical signaling responses with either effector pathway in both cell types. As seen in Fig. 9, with any amount of DNA transfected and at any level of receptor expression (Fig. 9, *inset*), the α_{1B}-AR releases essentially the same amount of arachidonic acid as does the α_{1D}-AR. Thus, it appears that signaling by the two α_1 subtypes is similar. The physiological significance of multiple α_1 subtypes demonstrating identical signaling is not known. However, it is clear that the coupling of α_1-AR subtypes to multiple effectors in these model eukaryotic expression systems is both G protein and cell type specific.

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