α_{2A}-Adrenergic Receptor Stimulated Calcium Release Is Transduced by G_i-Associated $G_{\beta\gamma}$-Mediated Activation of Phospholipase C†

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ABSTRACT: Proposed mechanisms by which α_2-adrenergic receptors (α_2AR) regulate intracellular calcium ($[Ca^{2+}]_i$) include stimulation and inhibition of cell surface calcium channels, stimulation of calcium release via receptor coupling to G_q with subsequent activation of phospholipase C and release of IP_3, or stimulation of calcium release via coupling to G_i in an IP_3-independent manner. These potential mechanisms were explored in cells that expressed α_{2A}AR endogenously (HEL cells), permanently transfected CHO cells, and transiently transfected COS-7 cells. Each cell type displayed agonist (UK14304)-dependent increases in $[Ca^{2+}]_i$ that were blocked by yohimbine, ablated by pertussis toxin, and largely unaffected by chelation of extracellular calcium. Furthermore, calcium release was associated with IP_3 accumulation and was blocked by an inhibitor of phospholipase C (PLC). When expressed in CHO cells, a mutated α_{2A}AR which has the amino and carboxyl termini of the third intracellular loop substituted with β_2AR sequence poorly coupled to G_i in adenylyl cyclase assays, and likewise displayed virtually no coupling to increased $[Ca^{2+}]_i$. These results all point toward a G_i- versus a G_q-mediated coupling pathway triggering release of intracellular calcium stores. The possibility that $G_{\beta\gamma}$ subunits released from α_{2A}AR−G_i coupling is the mechanism of PLC activation was explored in COS-7 cells by coexpressing α_{2A}AR with the $G_{\beta\gamma}$ inhibitors transducin or a carboxy-terminal portion of the βAR kinase. Both $\beta\gamma$ inhibitors markedly inhibited α_{2A}AR modulation of $[Ca^{2+}]_i$ while not affecting thromboxane A_2 receptor mediated stimulation of $[Ca^{2+}]_i$ via G_q coupling. Thus, α_{2A}AR couple to calcium release via G_i-associated $G_{\beta\gamma}$ subunits. This coupling is present in multiple cell types and should be considered a major signal transduction pathway of this receptor.

While the cell signaling pathways for G_q-coupled receptor-mediated calcium signaling have been well established, confusion remains regarding calcium signaling transduced by non-G_q-coupled receptors such as α_2-adrenergic receptors (α_2AR). These receptors are ubiquitously coupled to inhibition of adenylyl cyclase, yet this cannot explain receptor-stimulated increases in intracellular calcium. Conflicting reports in the literature suggest that α_{2A}(C10)AR stimulate calcium release via nonspecified G_i-mediated effects which are independent of phospholipase C activation (Michel et al., 1989), or by direct activation of G_q and phospholipase C (PLC) representing promiscuous coupling of these receptors to multiple G-proteins (Conklin et al., 1992; Chabre et al., 1994; Gesek, 1996). Motulsky and colleagues, in a pharmacologic analysis of α_{2A}AR signaling in human erythroleukemia (HEL) cells (Michel et al., 1989), found that the α_2AR agonists epinephrine and UK14304 stimulated calcium release which was pertussis toxin sensitive, was not associated with a significant increase in IP_3, and which preceded any measurable increase in inositol phosphate. They concluded that the α_2AR of these cells couple to calcium mobilization by a mechanism which is independent of inhibition of adenylyl cyclase and is not due to activation of phospholipase C. In apparent contrast, others (Conklin et al., 1992) have observed that cotransfection of $G_{\alpha q}$ with α_{2A}AR into human embryonal kidney (HEK) 293 cells resulted in UK14304-stimulated increases in phospholipase C activity of large magnitude, suggesting that α_2AR may couple to PLC (and calcium release) via G_q interactions. Controversy also exists regarding the source of α_2AR-stimulated calcium ions in different cell types with evidence for receptor-coupled ion channels which mediate calcium entry in some cells (Graham et al., 1996), but no calcium influx in other cell lines (Michel et al., 1989; Erdbrugger et al., 1993).

Recent studies indicating that G-protein-mediated signal transduction is not the exclusive domain of the Gα subunit, but can also be accomplished via $G_{\beta\gamma}$ effects (Wu et al., 1993; Smrcka & Sternweis, 1993; Lee et al., 1993; Boyer et al., 1994), together with the aforementioned observations, prompted us to reexamine α_{2A}AR-mediated calcium signaling. Our goal was to define α_{2A}AR receptor signaling effectors which transduce calcium mobilization. Our approach was to characterize signaling in HEL cells which express a homogeneous population of α_{2A}AR receptors, and also to study receptor signaling in transiently or stably transfected cells which do not naturally express α_{2A}AR so that the effects of specific cell signaling perturbations could be assessed. We sought to address three questions: What receptor−G-protein interaction transduces the physiologically relevant α_{2A}AR-mediated calcium release reaction? Is this

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calcium release a G_α- or $G_{\beta\gamma}$-mediated event? Is α_{2A}AR-mediated calcium signaling mediated via phospholipase C activation?

MATERIALS AND METHODS

Materials. Tissue culture supplies were purchased from Life Technologies, Inc. [^{3}H]Myoinositol and [^{3}H]yohimbine were purchased from Dupont NEN. HEL, COS-7, and CHO cells, and the cDNA for $G_{\alpha t}$ (transducin) (Van Dop et al., 1989) were obtained from ATCC, Rockville, MD. The eukaryotic expression vector pcDNA3 was from Invitrogen. Expression plasmids encoding human α_{2A}AR (Jewell-Motz & Liggett, 1996), the α_{2A}AR mutant denoted $\alpha_2(\beta_2$NT+CT) (Eason & Liggett, 1996), and TP_α thromboxane receptors (D'Angelo et al., 1994) have been previously described. A plasmid (denoted as βARK minigene) encoding a carboxyl-terminal polypeptide of the βAR kinase (Zhang et al., 1996) was a generous gift of Robert Lefkowitz. Fura-2 AM, human α thrombin, the thromboxane mimetic U46619, and other reagents and buffers were purchased from Sigma. U73122 and U73343 were from BioMol. The selective α_2AR agonist UK14304 was obtained from Tocris-Cookson.

Cell Transfection. COS-7 were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and were transfected using calcium phosphate precipitation as previously described (Jewell-Motz & Liggett, 1996; Eason & Liggett, 1996). CHO cells were permanently transfected with cDNAs encoding for the human wild-type or mutated α_{2A}AR in the expression vector pBC12BI as described (Eason & Liggett, 1996) and grown in Ham's F12 plus 10% fetal calf serum, 100 units/mL penicillin, 100 μg/uL streptomycin, and 80 ug/mL G418 to maintain selection pressure. α_{2A}AR expression was monitored by radioligand binding with the α_2AR antagonist [^{3}H]yohimbine as described (Eason et al., 1995). HEL cells were cultured in RPMI medium plus 10% fetal calf serum in 1 L magnetic stirring flasks. Antibiotic/antimycotic solution from Life Technologies, Inc (1%) was included in the culture media for HEL cells.

Calcium Measurements. Agonist-stimulated increases in intracellular free calcium ($[Ca^{2+}]_i$) were quantitated by monitoring the fluorescence of Fura-2-loaded cells as previously described (Dorn, 1992). Fluorescence ratios were converted to $[Ca^{2+}]_i$ using Photon Technologies Felix software and assuming a K_d for calcium binding to Fura-2 of 224 nM (Grynkiewicz et al., 1985). Calcium entry was discriminated from calcium release in studies where EGTA (17 mM final concentration) was added to the cells 2 min prior to agonist stimulation. In some experiments, cells were cultured in the presence of 500 ng/mL pertussis toxin or 1 μg/mL cholera toxin for 18 h prior to study. In these experiments, thrombin (0.3 unit/mL), which increases $[Ca^{2+}]_i$ in HEL, COS-7, and CHO cells via a pertussis toxin insensitive mechanism (Schwaner et al., 1992; vide infra), was used to control for cell viability and integrity of calcium signaling. The effects of PLC inhibition on agonist-stimulated calcium signaling were assessed by adding (4 μM each) the PLC inhibitor U73122 [or, as a control, its inactive analog U73343 (Smith et al., 1990; Dorn & Becker, 1993)] 5 min prior to agonist stimulation. Inhibition of $G_{\beta\gamma}$ activity was studied using transient coexpression of α_{2A}AR and βARK minigene or transducin in COS-7 cells. In these experiments, cotransfection of α_2AR with empty vector (pcDNA3) controlled for the effects of cotransfection. Coexpression of thromboxane TP_α receptor (D'Angelo et al., 1994), which is not naturally expressed by COS-7 cells and which increases $[Ca^{2+}]_i$ via $G_{\alpha q}$ effects (Shenker et al., 1991), with βARK minigene or transducin controlled for any potential non-$G_{\beta\gamma}$-mediated effects of these agents.

Measurement of Inositol Phosphates. HEL cells were loaded in serum- and inositol-free RPMI with [^{3}H]myoinositol (10 μCi/mL) for 24 h and stimulated for the indicated periods of time with UK14304 (10 μM), α thrombin (1 unit/mL), or vehicle. All studies were performed 10 min after the addition of 10 mM LiCl so that hydrolysis of inositol phosphate was inhibited. The reaction was stopped with trichloroacetic acid, and water soluble inositol phosphates were extracted and resolved by anion-exchange HPLC as previously described (Dorn et al., 1992; Dorn & Becker, 1993).

Statistical Methods. The half-maximal effective concentration (EC_{50}) of agonist-stimulated calcium signaling was calculated using nonlinear iterative least-squares techniques and software from Graph Pad. Maximal increases in $[Ca^{2+}]_i$ were defined as those in response to 10 μM UK14304 or 3 units/mL thrombin, respectively. Multiple comparison between cell lines or different treatment groups was by one-way analysis of variance followed by Bonferroni's test for means comparisons. The effects of transducin and the βARK minigene on calcium signaling were compared to empty vector transfected controls by Student's *t* test for paired studies. Unless otherwise noted, all data are expressed as mean $\pm$ SEM with $p < 0.05$ determining significance.

RESULTS

α_{2A}AR Signaling in HEL Cells. Human erythroleukemia cells are a model system for α_2AR mediated adenylyl cyclase inhibition and calcium signaling (McKernan et al., 1987; Michel et al., 1989). The receptor expressed in these cells is the α_{2A} subtype (Michel, 1994) and was found to be expressed at levels of $\simeq$400 fmol/mg. When HEL cells were loaded with Fura-2 and exposed to various concentrations of the α_2AR agonist UK14304, there was a transient, concentration-dependent, increase in $[Ca^{2+}]_i$ (Figure 1a). UK14304-stimulated calcium signaling was prevented by preincubation of HEL cells with the α_2AR antagonist yohimbine (10 μM), but not by the βAR antagonist propranolol (10 μM) (data not shown). The EC_{50} for UK14304-stimulated increases in calcium in HEL cells (eight experiments) was 59 $\pm$ 10 nM (Figure 1b).

α_{2A}AR-Mediated Calcium Signaling in CHO Cells. CHO cells were chosen for stable expression of α_{2A}AR as they have intact cell signaling properties and have been employed for prior studies of this receptor and its coupling to inhibition of adenylyl cyclase (Eason & Liggett, 1992, 1996; Eason et al., 1994a,b, 1995). Binding studies using [^{3}H]yohimbine as the radioligand demonstrated that the clonal α_{2A}AR receptor expressing CHO cell line studied herein expressed $\simeq$1700 fmol of receptor/mg of membrane protein. As with HEL cells, transfected CHO cells exhibited concentration-dependent increases in intracellular free calcium when exposed to UK14304 (Figure 1c,d), but the EC_{50} for this relationship was lower than in HEL cells, being 6 $\pm$ 2 nM ($n = 4$, $p = 0.001$). Intracellular calcium levels were unaffected by UK14304 in nontransfected CHO cells (not

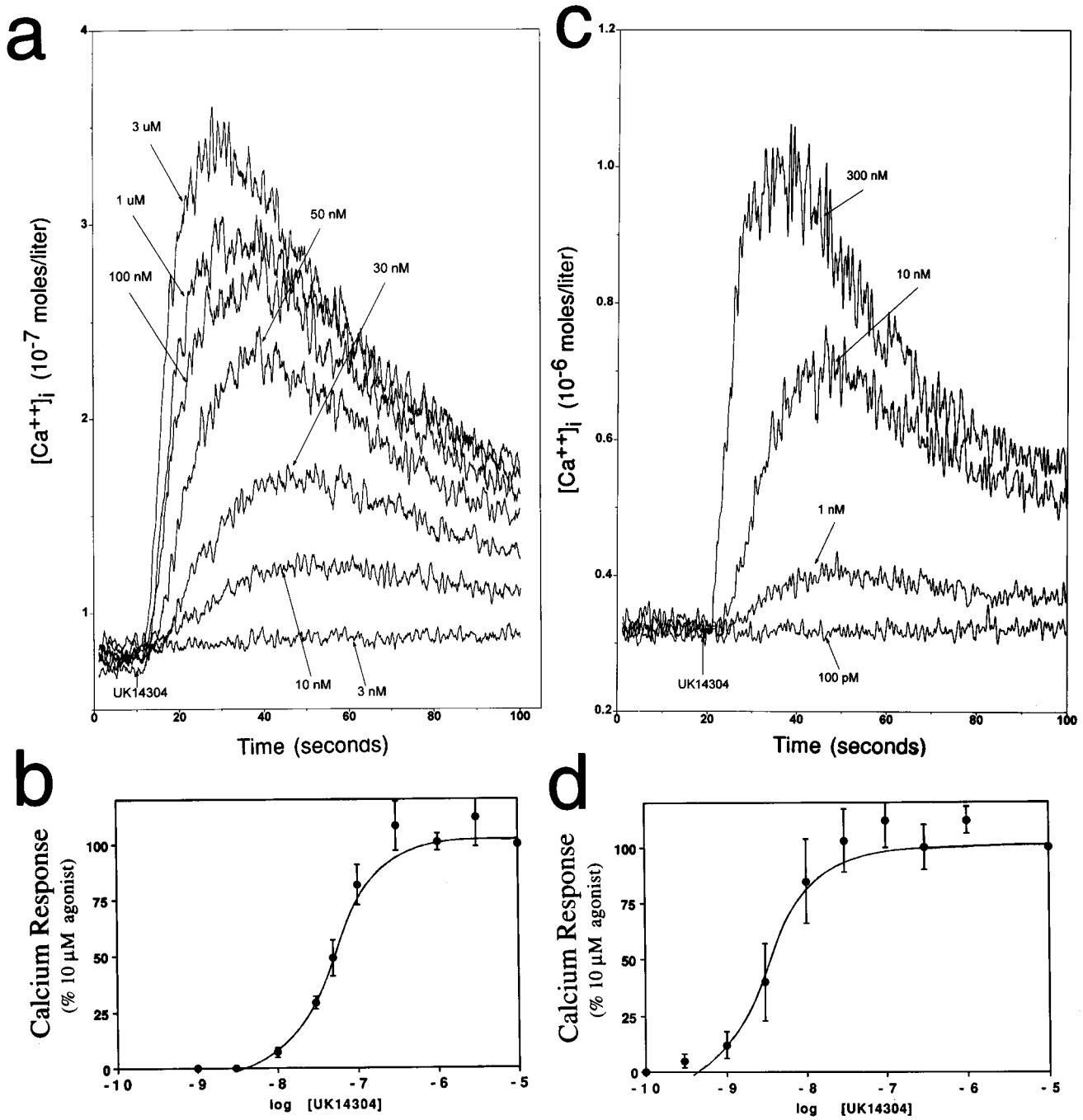

FIGURE 1: UK14304 increases HEL and α_{2A}AR expressing CHO cell $[Ca^{2+}]_i$ in a concentration-dependent manner. (a) Representative tracings of calcium transients in UK14304-stimulated, Fura-2 loaded HEL cells. UK14304 was added in the indicated concentrations at the 10 s time point. Vertical axis is $[Ca^{2+}]_i$ in moles $\times$ 10^{-7}/liter. (b) Cumulative concentration response curve ($n = 8$ experiments) for UK14304−stimulated HEL cell $[Ca^{2+}]_i$. (c and d) Representative calcium signals and concentration response curve ($n = 4$ experiments) for clonal α_{2A}AR receptor expressing CHO cell line (vertical axis for panel is $[Ca^{2+}]_i$ in moles $\times$ 10^{-6}/liter). There was no measurable calcium response to UK14304 in nontransfected CHO cells. EC_{50} values are given in the text and in Table 1.

shown). CHO cells were found to naturally express a thrombin receptor with similar calcium signaling characteristics as in HEL cells (Table 1). These studies in HEL and CHO cells demonstrated the feasibility of using these cultured lines as models of α_2AR calcium signaling and provided an experimental framework for subsequent studies which defined the components of receptor-stimulated calcium signaling.

Characteristics of α_{2A}AR-Stimulated Calcium Transients. HEL or CHO cells stably expressing α_{2A}AR were studied to determine the source of the UK14304-stimulated calcium signal and identify the responsible calcium signal transducers. In each of these experiments, the response to UK14304 was contrasted to that of α thrombin which increases intracellular calcium through G_q-mediated mobilization of intracellular stores and influx of extracellular calcium (Schwaner et al., 1992). The relative contribution of calcium release and calcium influx was assessed by chelating extracellular calcium with EGTA prior to agonist stimulation. The maximal UK14304-induced calcium response in CHO cells was unaffected by chelation of extracellular calcium (Figure 2a). In addition, this calcium response in CHO cells was unaffected by blockade of L- and N-type calcium channels by nitrendipine and conotoxin, respectively (data not shown).

Table 1: Summary of Results for Pharmacologic Inhibitors of Calcium Signaling in UK14304- or Thrombin-Stimulated HEL or Stably Transfected CHO Cells[a]

cell type	HEL	CHO
	α_{2A}AR Signaling	
UK14304 EC_{50} (nM)	59 ± 10 (8)	6 ± 2 (4)
10 μM UK calcium amplitude (nM)	160 ± 15 (17)	576 ± 78 (4)
% PTX inhib	*100 ± 0 (3)	*100 ± 0 (3)
% EGTA inhib	*33 ± 6 (6)	10 ± 5 (3)
% U73122 inhib	*100 ± 0 (3)	*100 ± 0 (3)
% U73343 inhib	2 ± 4 (3)	7 ± 4 (2)
	Thrombin Signaling	
thrombin EC_{50} (units/mL)	0.34 ± .03 (4)	0.23 ± 0.05 (4)
1 unit/mL thr calcium amplitude (nM)	1342 ± 103 (7)	862 ± 149 (4)
% PTX inhib	3 ± 2 (3)	4 ± 3 (3)
% EGTA inhib	*50 ± 6 (6)	*57 ± 8 (3)
% U73122 inhib	*100 ± 0 (3)	*100 ± 0 (3)
% U73343 inhib	10 ± 5 (3)	9 ± 5 (3)

[a] Values are means ± SEM (n). PTX = pertussis toxin. An asterisk denotes that the p value is less than 0.05 as compared to control.

The amplitude of the UK14304-stimulated calcium transient was, however, decreased by 33 ± 6% ($n = 6$, $p = 0.002$) in calcium-free (EGTA) buffer in HEL cells (Figure 2b). Thus, it appears that in CHO and HEL, the majority of the α_{2A}AR-stimulated increase in intracellular free calcium is due to mobilization of intracellular stores. In contrast, the thrombin-stimulated calcium signal was significantly attenuated in calcium-free buffer (Table 1), indicating that calcium influx accounts for fully half of the calcium signal stimulated by thrombin in HEL and CHO cells.

The nature of the G-protein which transduced UK14304-stimulated calcium signals was determined by pretreating the cells with pertussis toxin, an inhibitor of G-proteins of the G_i and G_o subtypes. Pertussis toxin treated CHO and HEL cells exhibited no calcium signaling to UK14304 compared to non-pertussis toxin treated cells studied in parallel (Figure 2) whereas pertussis toxin treatment did not significantly affect thrombin-stimulated calcium signaling (Table 1). Because we (Eason et al., 1992) and others (Conklin et al., 1992) have previously shown that α_{2A}AR also weakly couple to G_s, we also examined the effects of cholera toxin exposure (1 ug/mL for 24 h) on α_2AR−calcium signaling. Such treatment had no effect on the basal or agonist-stimulated intracellular calcium concentration (data not shown). Given the results of the pertussis toxin studies, an α_2AR−G_i/G_o pathway was strongly suggested. To confirm this, we studied calcium signaling of a mutated α_{2A}AR which has a β_2AR sequence substituted at the amino- and carboxyl-terminal portions of the third intracellular loop of the receptor. This mutated receptor, $\alpha_2(\beta_2$NT+CT), binds agonists with wild-type affinities, but minimally couples to G_i as assessed in adenylyl cyclase assays (Eason & Liggett, 1996). We considered then, that agonist-mediated stimulation of intracellular calcium accumulation would be substantially impaired in CHO cells expressing this receptor. As shown in Figure 3, this turned out to be the case with the mutant receptor calcium signaling amplitude averaging 9 ± 3% ($n = 4$) of wild-type α_2AR, strongly implicating a receptor−G_i coupling pathway in α_2AR−calcium signaling.

Role of Phospholipase C in α_{2A}AR-Mediated Calcium Signaling. The above experiments suggested that the major component of α_{2A}AR-mediated calcium signaling resulted from G_i-stimulated mobilization of calcium stores. These findings are in general agreement with those of Michel (1989), who further suggested that these events were independent of both adenylate cyclase inhibition and PLC activation. We examined the role of PLC using two methodologies. First, we directly assayed PLC activation measured as formation of inositol phosphates after stimulation of HEL cells with UK14304. The anion-exchange HPLC tracings in Figure 4a,b demonstrate that exposure for 1 min to a maximally effective concentration of UK14304 increased IP, IP_2, and IP_3 formation in HEL cells. The time-dependent accumulation of inositol phosphate after UK14304 stimulation is illustrated in Figure 4c. These results confirm the presence of rapid PI hydrolysis after α_{2A}AR stimulation of HEL cells with UK14304.

If α_2AR-stimulated calcium signaling were mediated by inositol trisphosphate, then UK14304-stimulated inositol phosphate accumulation, like calcium release, should be inhibited in cells exposed to pertussis toxin treatment. This was indeed the case as there was no significant increase in inositol phosphates 10 min after stimulation of pertussis toxin treated HEL cells with 10 μM UK14304 (not shown). In contrast, inositol phosphate accumulation in response to thrombin stimulation was not affected by pertussis toxin (not shown). To demonstrate that α_2AR-stimulated activation of PLC was necessary for calcium signaling, we pretreated HEL- and α_{2A}AR-expressing CHO cells with the PLC inhibitor compound U73122 (or its inactive analog U73343 as a control) and repeated the inositol phosphate and calcium determinations after stimulation with 10 μM UK14304. As shown in Figure 5a−c, U73122 inhibited UK14304-stimulated inositol phosphate accumulation and calcium signaling in HEL cells whereas the inactive compound had no effect. We have previously shown that U73122 also inhibits thrombin-stimulated calcium signaling (Dorn & Davis, 1992). The results in HEL cells and α_{2A}AR transfected CHO cells were again similar (see Table).

Role of $G_{\beta\gamma}$ Subunits in α_{2A}AR Stimulated Calcium Signaling. As $G_{\alpha i}$ subunits are not known to activate PLC, the relative roles of $G_{\alpha i}$ and associated $G_{\beta\gamma}$ subunits in agonist-stimulated calcium signaling were determined in studies where α_{2A}AR were cotransfected either with the pleckstrin homology domain of βARK (βARK minigene) or with $G_{\alpha t}$ (transducin), both of which bind to and inactivate $G_{\beta\gamma}$ subunits of G-proteins (Federman et al., 1992; Koch et al., 1993). Cotransfection of 5−20 μg of α_{2A}AR and βARK minigene expression plasmids decreased the amplitude of 1 μM UK14304-stimulated calcium signals by 46% from 65 ± 9 nM (empty vector control) to 35 ± 5 nM ($n = 6$, $p = 0.009$). A 65% decrease in UK14304-stimulated calcium amplitude was observed in experiments in which α_{2A}AR and 20 μg transducin expression plasmids were cotransfected (Figure 6). To establish that the βARK peptide and transducin did not have nonspecific effects on intracellular calcium signaling, we cotransfected these plasmids with one that expresses the thromboxane A_2 (TP_α subtype) in COS-7 cells. This receptor increases $[Ca^{2+}]_i$ via the G_q-PLC pathway (Shenker et al., 1991; D'Angelo et al., 1994). In these experiments, there was no inhibition of 1 μM thromboxane agonist U46619-stimulated calcium signaling relative to cotransfection with empty vector (Figure 6). Taken together, these results provide strong evidence that α_{2A}AR

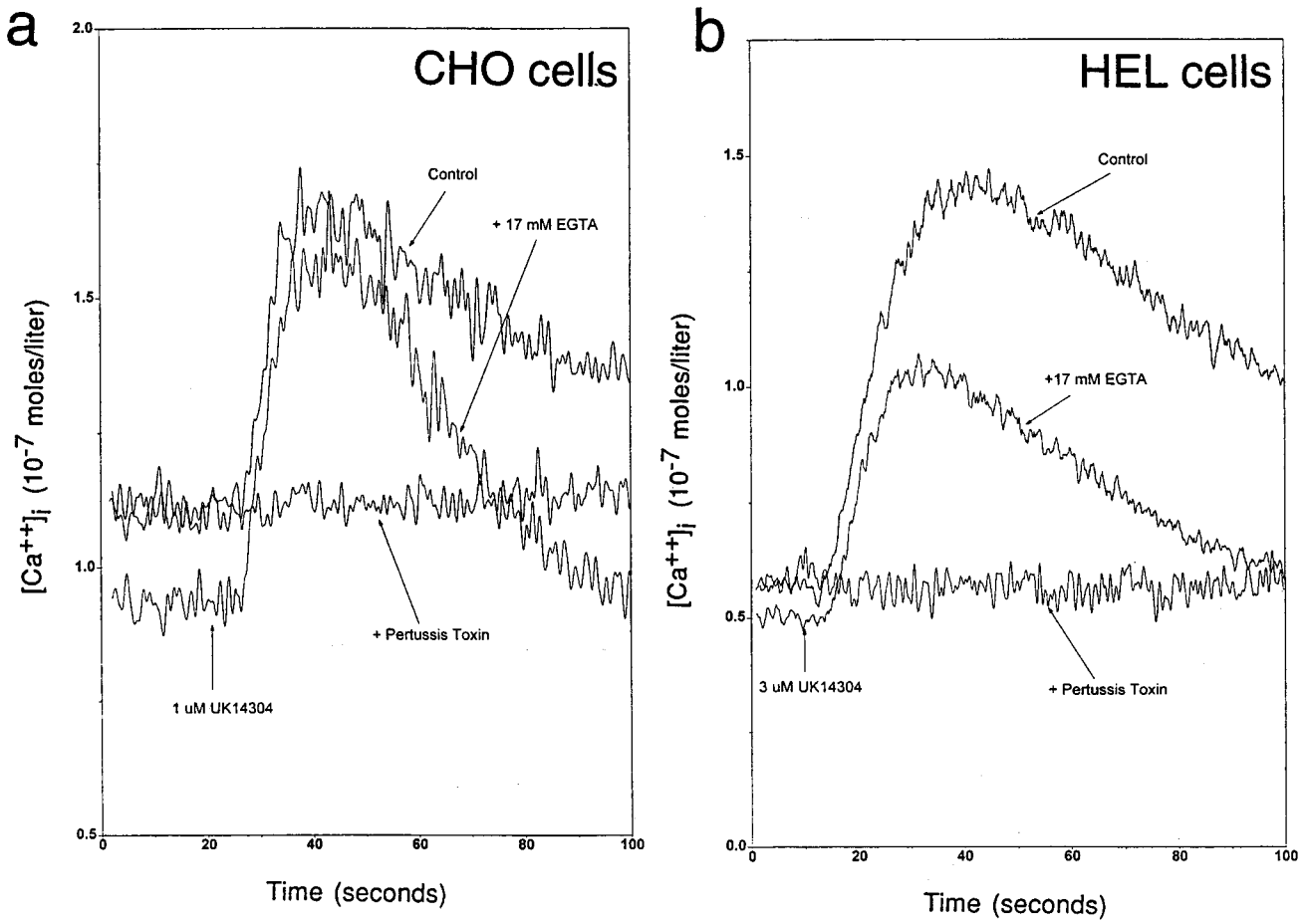

FIGURE 2: Effects of EGTA and pertussis toxin on UK14304-stimulated calcium signaling in CHO and HEL cells. Representative tracings demonstrating that chelation of extracellular calcium with EGTA had no effect on the amplitude of the UK14304-stimulated calcium transients in CHO cells (a) and decreased by approximately one third the response in HEL cells (b). Pertussis toxin abolished UK14304-stimulated increases in $[Ca^{2+}]_i$ in both cell lines.

calcium signaling is transduced by $G_{\beta\gamma}$ subunit-mediated activation of phospholipase C.

To further assess the generality of α_2AR-mediated increases in intracellular calcium release, we investigated additional cell lines that endogenously express the α_2AR or were transfected with the cDNA for the human receptor. Transfected human embryonic kidney cells (HEK-293) and Chinese hamster fibroblasts (CHW1102), and a natively expressing colonic adenocarcinoma cell line (HT29), each exhibited pertussis toxin sensitive increases in intracellular calcium that were unaffected by extracellular calcium chelation in response to α_2AR agonist (not shown). Thus, the α_2AR of these cells transduces a calcium signal qualitatively similar to that of the more extensively studied HEL, CHO, and COS-7 cells.

DISCUSSION

A primary coupling pathway for the α_2AR is inhibition of cAMP production via coupling to G_i and inhibition of adenylyl cyclase. This pathway alone, however, is insufficient to account for all of the actions of α_2AR in various organs and cell types (Limbird, 1988; Liggett & Raymond, 1993). Additional pathways have also been described which include opening of potassium channels and modulation of intracellular calcium (Limbird, 1988). In cells where cAMP levels are increased, protein kinase A phosphorylation of voltage-gated calcium channels results in their opening; thus, inhibition of cAMP production by α_2AR might serve to inhibit channel activation under these circumstances. An additional non-cAMP-dependent inhibition of voltage-gated calcium channels by α_2AR has also been described (Surprenant et al., 1992). However, observations in a variety of cells have revealed a robust α_2AR-mediated *stimulation* of $[Ca^{2+}]_i$. The mechanism by which these receptors transduce this signal is not well understood. Some studies, particularly in transfected cells overexpressing the receptor and/or various G-proteins, suggest that α_2AR can couple to G_q directly, thereby activating PLC (Conklin et al., 1992; Chabre et al., 1994; Gesek, 1996). This pathway requires high receptor or G_q expression and high agonist concentrations (Conklin et al., 1992; Chabre et al., 1994), and is unlikely to explain receptor-mediated increases in $[Ca^{2+}]_i$ in cells natively expressing the receptor. Likewise, α_2AR have been shown to couple to G_s and to stimulate cAMP (Eason et al., 1992, 1994a,b; Eason & Liggett, 1995). This could potentially result in voltage-gated calcium channel activation, although it is doubtful that this pathway is of major significance given its inefficiency and the observation that in many cell types the source of calcium ions appears to be from intracellular sources. In HEL cells, α_2AR-mediated increases in $[Ca^{2+}]_i$ have been well documented (Michel et al., 1989; Michel, 1994). However, the mechanism is not clear since the signal has been reported to be inhibited by pertussis toxin and was not temporally associated with IP_3 accumulation. In other studies, investigators have concluded that α_2AR couple to a pertussis toxin sensitive pathway resulting in activation of

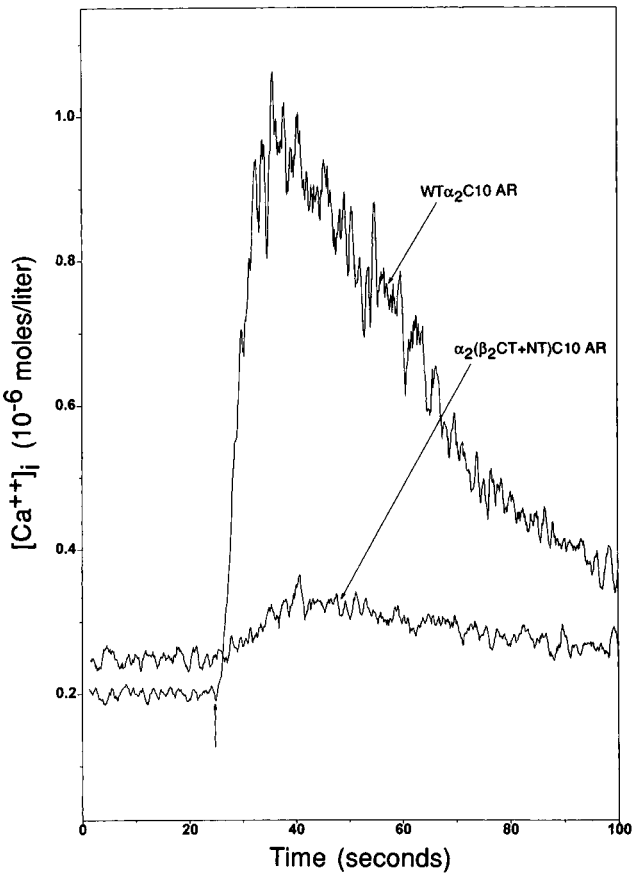

FIGURE 3: Mutant $\alpha_2(\beta_2$NT+CT) defective in coupling to G_i does not efficiently transduce a UK14304-stimulated calcium signal. Calcium studies were performed as described under Materials and Methods using a clonal CHO cell line stably expressing wild-type (WT) α_{2A}AR or the $\alpha_2(\beta_2$NT+CT) mutant. Both cells responded equally to thrombin (not shown). In this representative experiment (of four), the UK14304-stimulated calcium signal in the mutant receptor expressing cell lines was 12% of the amplitude in the wild-type receptor expressing cells.

protein kinase C which opens cell surface calcium channels (Lepretre et al., 1994).

To clarify the role of α_2AR in regulating $[Ca^{2+}]_i$, we utilized three cell types. HEL cells were used since they endogenously express the α_{2A} subtype as well as a thrombin receptor which couples to calcium release via a well-characterized G_q pathway (Schwaner et al., 1992). Permanently transfected CHO cells were also studied since we have previously characterized α_{2A}AR signaling to adenylyl cyclase in these cells, and we have stable lines expressing a number of mutated receptors which lack domains essential for G-protein coupling (Eason & Liggett, 1995, 1996). Finally, we utilized transient expression of α_{2A}AR in COS-7 cells because it was possible to obtain high-level transient expression of receptors plus transducin or the βARK minigene, which were important in assessing the role of $G_{\beta\gamma}$ subunits in the signal transduction pathway.

Our studies indicated efficient α_2AR-mediated increases in $[Ca^{2+}]_i$ with EC_{50} values for the α_2AR-specific agonist UK14304 of ~60 nM in HEL cells. In CHO and COS-7 cells, these values were somewhat lower (~6 and ~17 nM, respectively), most likely due to the higher expression of the receptor. The UK14304 EC_{50}s for α_2AR-mediated increases in $[Ca^{2+}]_i$ in CHO cells reported here are similar to those we and others have previously observed for inhibition of cAMP (Eason et al., 1994a,b; Eason & Liggett, 1995; Kurose & Lefkowitz, 1994) with this parental cell. In all three cell types, the UK14304 calcium response was blocked by yohimbine and phentolamine but not propranolol and was ablated by overnight pertussis toxin exposure. Exposure to a concentration of cholera toxin that we have previously shown eliminates α_2AR−G_s coupling (Eason et al., 1992; Eason & Liggett, 1995) had no effect on resting or agonist stimulated calcium transients in CHO cells. Finally, α_2AR calcium signaling in transfected CHO and COS-7 cells was unaffected by chelation of extracellular calcium with EGTA whereas in HEL cells a measurable (~33%) decrease in the maximal signal was noted with extracellular calcium chelation, suggesting that an additional pathway (potentially involving a cell surface channel) is present in this cell. Taken together, the above results indicate that α_2AR can increase $[Ca^{2+}]_i$ via activation of G_i/G_o which results in release of intracellular calcium. We also explored this putative signaling pathway using a mutated α_{2A}AR that we have previously shown is defective in coupling to G_i but not to G_s (Eason & Liggett, 1996). This receptor is processed normally, displays wild-type affinity for agonist, and was expressed at the same level as wild-type in the current study. If receptor−G_i coupling was the initial step in calcium signaling, we expected that this mutated receptor would exhibit impaired signaling, as was the case.

Since α_2AR calcium signaling appeared to be mediated by receptor−G_i interactions, we explored whether PLC was activated under these conditions. If so, the well-recognized pathway whereby PLC activation leads to IP_3 generation and in turn promotes the release of intracellular calcium via IP_3 receptors seemed likely. Previous studies by others reported no accumulation of IP_3 in HEL cells during agonist treatment, except after prolonged (e.g., 10 min) exposure to agonist (Michel et al., 1989). We approached this by using an HPLC-based assay which has previously been shown to be highly sensitive (Dorn et al., 1992; Dorn & Davis, 1992; Dorn & Becker, 1993). As indicated, we detected rapid IP_3 accumulation after agonist exposure, and confirmed that a time-dependent increase in total IPs occurs after stimulation with UK14304, thus demonstrating an intact α_2AR−PLC pathway. The relevance of PLC activation to calcium release was ascertained by examining the effects of the PLC inhibitor U73122 on α_2AR calcium signaling in CHO and HEL cells. Agonist-mediated calcium transients were completely eliminated by PLC inhibition in both cell types.

Since the $G_{i\alpha}$ subunit is not known to couple directly to PLC, we considered that $G_{\beta\gamma}$ released from the heterotrimer during agonist activation could be the relevant G-protein transducer in α_2AR-mediated stimulation of $[Ca^{2+}]_i$ release. Indeed, $G_{\beta\gamma}$ is known to stimulate PLCβ isoforms 2 and 3 (Wu et al., 1993; Smrcka & Sternweis, 1993; Lee et al., 1993; Boyer et al., 1994; Stehno-Bittel et al., 1995). To address this, we coexpressed transducin or the pleckstrin homology domain (a carboxy-terminal portion) of the βAR kinase into COS-7 cells along with the α_{2A}AR. Both transducin (Federman et al., 1992) and this βARK minigene (Zhang et al., 1996) have been found to be capable of binding and thus effectively removing $G_{\beta\gamma}$ subunits from signal transduction events. Expression of either construct substantially inhibited α_2AR calcium signaling. (Of note, these data were always compared to those obtained with transfection of empty

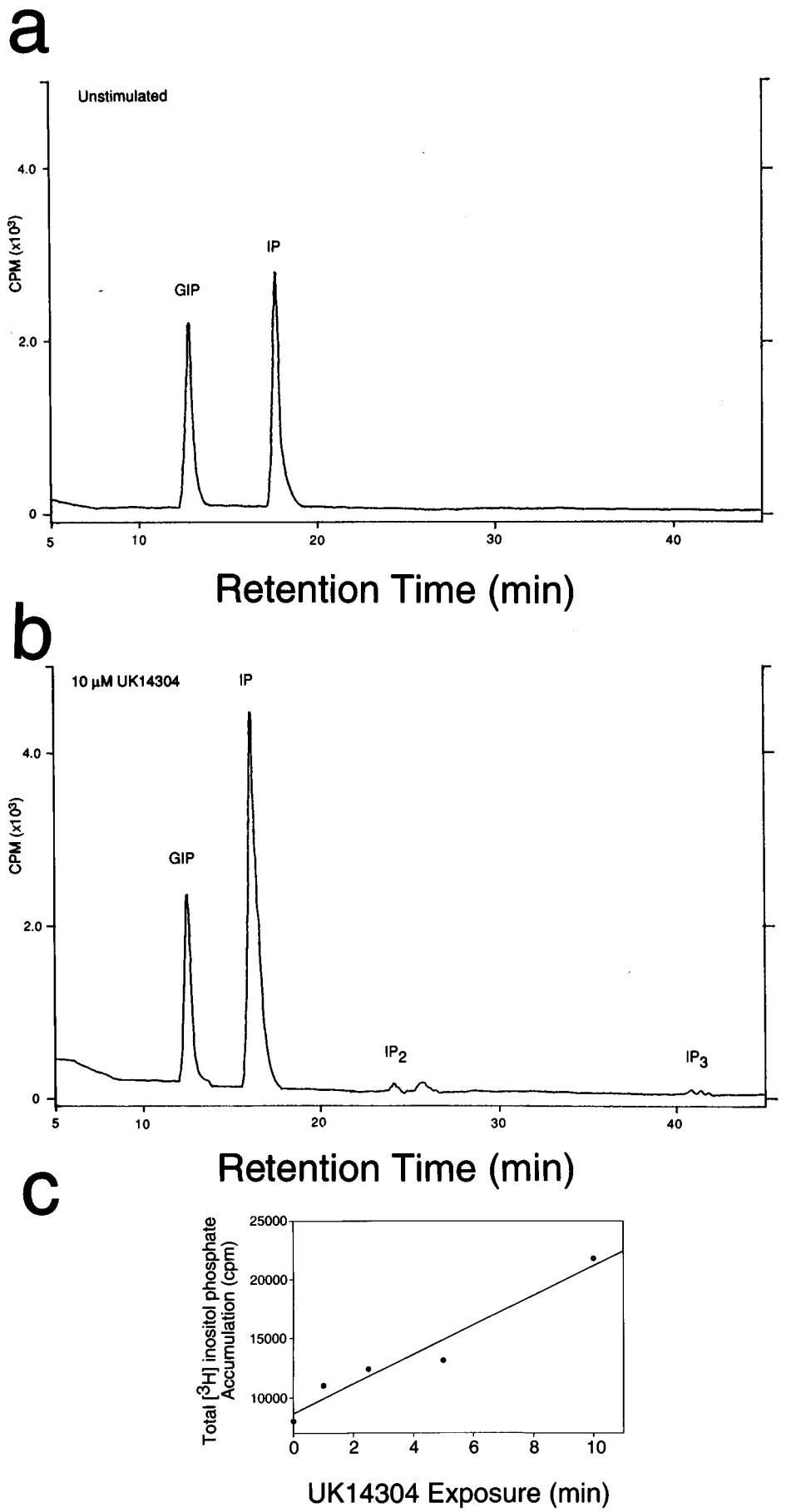

FIGURE 4: Stimulation of α_{2A}AR rapidly increases levels of inositol phosphates. Representative anion-exchange HPLC chromatograms of vehicle (a) and 10 μM × 1 min UK14304-stimulated (b) [^{3}H]myoinositol loaded HEL cells. In vehicle-treated cells, IP levels are low, and no IP_2 or IP_3 is evident. After 1 min of UK14304 treatment, IP levels have increased, and IP_2 and IP_3 are measurable. (c) Time course of IP accumulation in HEL cells stimulated with 10 μM UK14304 (shown is the mean of two experiments).

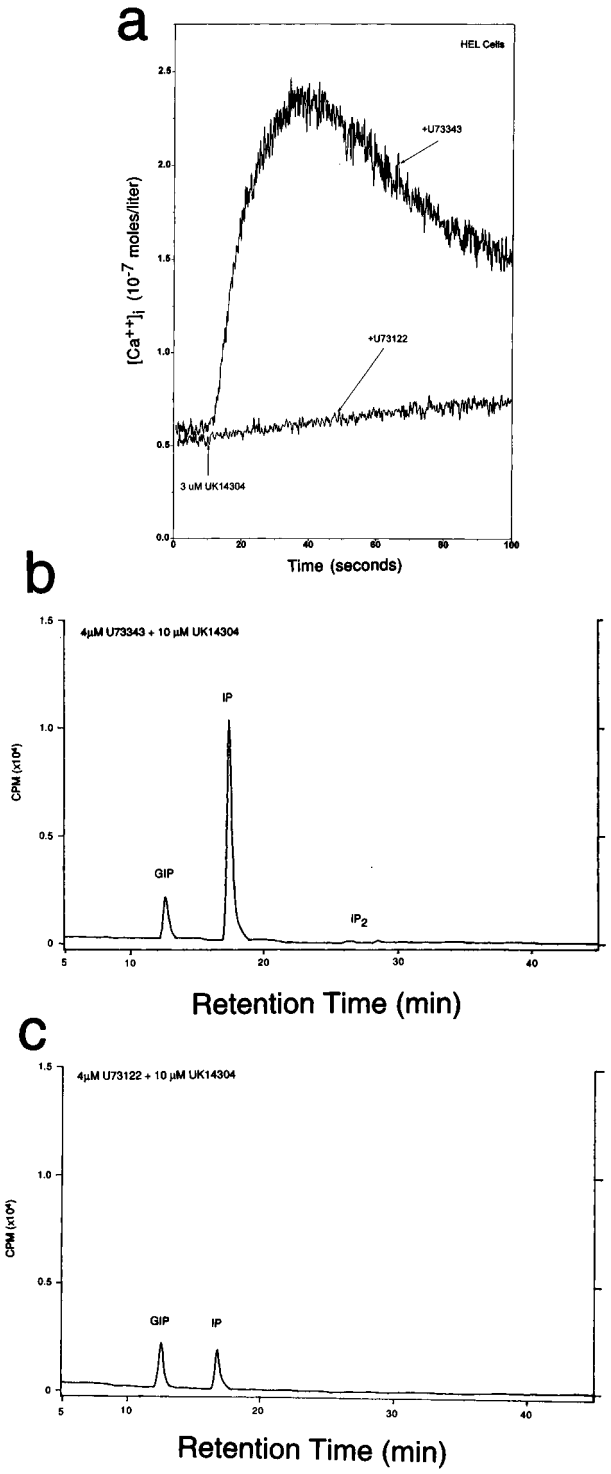

FIGURE 5: Effects of phospholipase C inhibition on UK14304-stimulated increases in $[Ca^{2+}]_i$ and inositol phosphate accumulation. HEL cells were prepared for Fura-2 (a) and [^{3}H]myoinositol studies (b, c) as described under Materials and Methods, pretreated for 5 min with the PLC inhibitor U73122 or inactive analog U73343, and stimulated with 3 μM UK14304 for calcium and 10 μM UK14304 for inositol studies. PLC inhibition prevented α_2AR-mediated calcium signaling and IP accumulation. Shown are representative results similar to three experiments performed.

vector.) The extent of inhibition of calcium signaling observed with cotransfection of βARK minigene or transducin is comparable to that observed by others assessing $G_{\beta\gamma}$ signaling in other pathways (Inglese et al., 1994; Koch et al., 1994). Consistent with the specific effects of such cotransfections, we found no effects on calcium signaling

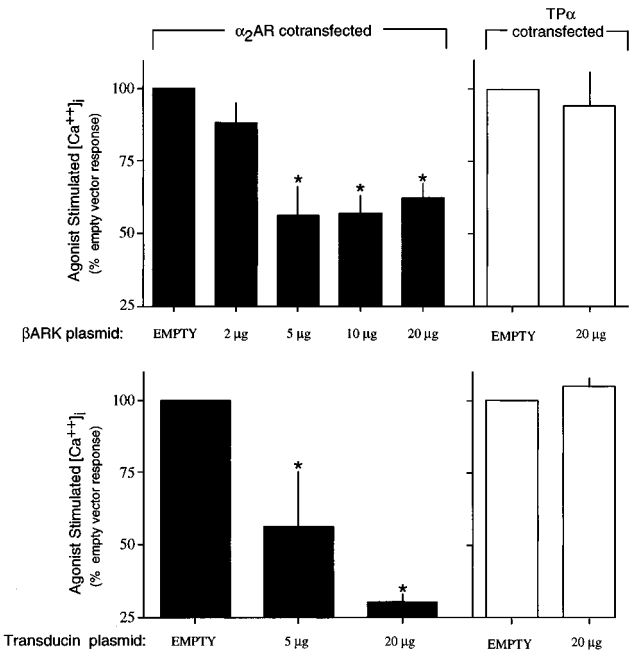

FIGURE 6: Effects of $G_{\beta\gamma}$ inhibition on agonist-mediated calcium signaling via α_{2A}AR and thromboxane A_2 receptors. COS-7 cells were cotransfected with equal amounts of α_{2A}AR (left panels) or TP_α (right panels) expression plasmids together with expression plasmids encoding βARK minigene (upper panels) or transducin (lower panels). UK14304 or U46619 calcium signaling of Fura-2-loaded cells was assessed 48 h after transfection. βARK minigene coexpression inhibited UK14304-stimulated calcium signaling by approximately 40% at plasmid concentrations of 5 μg or greater. Transducin coexpression resulted in ~65% reduction in agonist-mediated increases in $[Ca^{2+}]_i$. Neither βARK minigene nor transducin inhibited thromboxane-stimulated calcium signaling when cells were co-transfected with TP_α receptors (see text). Shown are results from 4 experiments with α_{2A}AR and 2−7 experiments with TP_α. $* = p < 0.05$ compared to empty plasmid.

via another receptor, the TP_α thromboxane receptor which couples to G_q (Shenker et al., 1991; D'Angelo et al., 1994).

In conclusion, we have elucidated a mechanism by which the α_{2A}AR couples to increases in intracellular calcium. This signal transduction involves activation of PLC by $G_{\beta\gamma}$ subunits released from the agonist-promoted dissociation of the G_i heterotrimer. Given the nearly identical characteristics of α_2AR-mediated increases in $[Ca^{2+}]_i$ observed in six different cell lines (two endogenously expressing and four transfected), this pathway does not appear to be restricted to only a few cell types, suggesting that this mechanism of α_2AR-mediated increases in $[Ca^{2+}]_i$ may be applicable to multiple organs and physiologic responses.

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