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Nonselective Effects of the Putative Phospholipase C Inhibitor, U73122, on Adenosine A_1 Receptor-mediated Signal Transduction Events in Chinese Hamster Ovary Cells

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ABSTRACT. Adenosine A_1 receptors can signal, through $G_{i/o}$ proteins, to inhibit adenylyl cyclase activity and also to stimulate phosphoinositide hydrolysis and the subsequent release of intracellular Ca^{2+} stores. The aminosteroid U73122 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione) has been widely used as an inhibitor of phospholipase C, the enzyme mediating phosphoinositide hydrolysis. Using U73122, we sought to selectively block signalling through the phospholipase C pathway, in Chinese hamster ovary (CHO-K1) cells heterologously expressing human adenosine A_1 receptors. U73122 inhibited A_1 receptor-mediated phosphoinositide hydrolysis, as measured by total inositol phosphate accumulation, over the concentration range 1–15 μM. However, over the same concentration range, it also appeared to inhibit A_1 receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation, A_1 receptor agonist-promoted [^{35}S]GTPγS binding, and at the higher concentrations (10–15 μM) produced marked morphological changes, leading to cytolysis. The structural analogue of U73122, U73343 (1-[6-[[17β-3-methoxyestra-1,3,5(10-trien-17-yl]amino]hexyl]-2,5-pyrrolidone-dione), typically used as an inactive control compound, had little effect on these events. The data suggest that U73122 is not a selective inhibitor of phospholipase C activity, interfering with adenosine A_1 receptor signalling generally, either at the pre-effector level involving $G_{i/o}$ proteins, or as a consequence of the morphological changes it induces. BIOCHEM PHARMACOL **56**;11:1455–1462, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. U73122; U73343; CHO-K1 cells; adenosine A_1 receptors; phospholipase C; adenylyl cyclase

Many extracellular signals regulate cellular processes by stimulating the hydrolysis of PIP_2,† yielding the two secondary messengers IP_3 and 1,2-diacylglycerol, whose primary effects are to release Ca^{2+} ions from intracellular stores and activate protein kinase C isoenzymes, respectively [1, 2]. In mammalian cells, this metabolism of phosphoinositides is mediated by three families of inositol lipid-specific PLC enzymes, classified as PLC-β, PLC-γ and PLC-δ [3]. The physiological role and regulation of members of the PKC-δ family are not clearly defined. The PLC-γ isoenzymes are typically activated by a combination of SH2-domain-dependent complex formation with phosphorylated growth hormone receptor tyrosine kinases and its subsequent phosphorylation on tyrosine residues [3]. The four identified PLC-β isoforms are principally activated by members of the heptahelical class of receptors through the intermediary of heterotrimeric G-proteins. A range of biogenic amines and peptides stimulate cell surface receptors (e.g. α_1 adrenoceptors, m1 muscarinic receptors, angiotensin, and bradykinin receptors [4–6]) which couple to the G_q family of heterotrimeric G-proteins, releasing $G_q\alpha$ subunits. These pertussis toxin-insensitive subunits then interact with the C-terminal region of PLC-β [7], thereby activating this enzyme. PLC-β isoforms can also be activated by G-protein βγ subunits [8]. These subunits are thought to interact with the N-terminus and possibly the inter X-Y region of PLC-β to promote PI metabolism [9, 10], and receptors which couple with the pertussis toxin-sensitive $G_{i/o}$ family of G-proteins, e.g. m2 muscarinic receptors, α_2 adrenoceptors, and receptors for fMLP, may signal in part through this $G_{\beta\gamma}$/PLC-β pathway [11, 12].

The adenosine A_1 subtype of P_1 purinoceptor has been shown to couple with G_{i1}, G_{i2}, and G_{i3} [13, 14], and accordingly to inhibit adenylyl cyclase activity [15]. However, these receptors can also stimulate PI hydrolysis and

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† *Abbreviations:* CHO-A1 cells, CHO-K1 cells transfected with the human brain adenosine A_1 receptor cDNA; CHO-H1 cells, cells transfected with the bovine histamine H_1 receptor cDNA; CHO-K1 cells, Chinese hamster ovary cells; CPA, cyclopentyladenosine; fMLP, formyl-methionoyl-leucylphenylalanine; [^{3}H]DPCPX, cyclopentyl-1,3-[^{3}H]dipropylxanthine; IP, inositol phosphates; IP_3, inositol 1,4,5-triphosphate; PI, phosphoinositide; PIP_2, phosphatidylinositol 4,5-bisphosphate; and PLC, phospholipase C.

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release intracellular Ca^{2+} stores by a pertussis toxin-sensitive mechanism [16, 17], suggesting that they also couple to the $G_{\beta\gamma}$/PLC-β pathway through G_i proteins. In this paper, using A_1 receptors heterologously expressed in CHO-K1 cells, we sought to disassociate the G_i-mediated coupling of the A_1 receptors to adenylyl cyclase from the G_i-mediated $G_{\beta\gamma}$/PLC-β pathway using the putative PLC inhibitor U73122.

U73122 was originally described as a potent inhibitor of platelet aggregation induced by a number of agonists [18]. As U73122 was also found to inhibit agonist-stimulated increases in cellular IP_3 content and $[Ca^{2+}]_i$ in intact platelets and block the activity of platelet-soluble PLC, it was concluded that this aminosteroid might act by inhibiting PLC. Since its discovery, U73122 has been used successfully in a number of studies examining the role of PLC in Ca^{2+} signalling and other intracellular signalling mechanisms [19–22]. Here, as anticipated we have shown that U73122 had the ability to block A_1 receptor-mediated PI hydrolysis, but at concentrations which also compromised signalling through the adenylyl cyclase system, G-protein activation, and cell viability. The close structural analogue of U73122, U73343, which is believed to have negligible activity as a PLC inhibitor [18], was relatively inactive in these regards.

MATERIALS AND METHODS

Cell Culture

CHO-A1 or CHO-H1 cells [23, 24] were grown at 37° in a humidified air/CO_2 atmosphere (95:5) in 75 cm^2 flasks. The cells were grown in Dulbecco's modified Eagle's medium/nutrient mix F12 HAM (1:1) supplemented with 2 mM L-glutamine and 10% (v/v) foetal bovine serum. Cells for assay of cAMP accumulation, PI hydrolysis, and [^{3}H]DPCPX binding were grown in 24-well cluster dishes. Experiments were performed on confluent monolayers. Cells for [^{35}S]GTPγS binding were grown in 162 cm^2 flasks.

Phosphoinositide Hydrolysis

Confluent monolayer cultures were loaded for 24 hr with *myo*-[2-^{3}H(N)]inositol (37 kBq/well) in 24-well cluster dishes in inositol-free Dulbecco's modified Eagle's medium containing 2 mM of glutamine and 0.5% foetal bovine serum. After being washed twice with 1 mL per well Hanks'/20 mM HEPES buffer (pH 7.4), the cells were incubated at 37° for 30 min in Hanks'/HEPES (290 μL/well) containing 20 mM of LiCl and the required concentration of U73122, U73343 or dimethylsulfoxide, as the vehicle control. Agonists were added in 10 μL of medium and the incubation continued for 40 min. Incubations were terminated by aspiration of the incubation medium and addition of 900 μL cold (−20°) methanol/0.12 M HCl (1:1, v/v). The cells were left at −20° for at least 2 hr before isolating total IP as described previously [16]. [^{3}H]IP levels were determined by liquid scintillation counting.

Accumulation of cyclic[^{3}H]AMP

Confluent cell monolayers, in 24-well cluster dishes, were incubated for 2 hr at 37° with 0.3 mL Hanks'/20 mM HEPES buffer (pH 7.4) containing [2,8-^{3}H]adenine (74 kBq/well). The cells were washed once and then incubated for 30 min at 37°, in 0.3 mL Hanks'/HEPES buffer containing the cAMP phosphodiesterase inhibitor rolipram (10 μM) and the required concentration of U73122, U73343 or dimethylsulfoxide, as the vehicle control. Agonists were added in 3 μL of medium and the incubation continued for a further 10 min. Incubations were terminated by the addition of 50 μL of concentrated HCl and an additional 0.7 mL Hanks'/HEPES was added to each well. Cyclic [^{3}H]AMP was then isolated by sequential Dowex-alumina chromatography [25]. To allow for percentage recovery correction, the samples were spiked with cyclic [8-^{14}C]AMP before being applied to the columns. After elution the levels of cyclic [^{3}H]AMP and cyclic [^{14}C]AMP were determined by liquid scintillation counting.

[^{35}S]GTPγS Binding

Cells from confluent 162 cm^2 flasks were initially washed twice with PBS and then detached in Tris–HCl buffer (50 mM, pH 7.4) using a cell scraper. Following centrifugation (200 *g* for 5 min), the pelleted cells were resuspended in Tris–HCl buffer and homogenised using a hand-held glass homogeniser (20 strokes), over ice. The cell homogenates were centrifuged at 20,000 *g* for 10 min, resuspended in Tris–HCl buffer, and assayed for protein content using BSA as a standard [26], before storage at −20°, or immediate use.

CHO-A1 cell homogenates (0.8–1.2 mg/mL) were pretreated with adenosine deaminase 1 unit/mL, for 30 min at 25° immediately prior to use. [^{35}S]GTPγS binding was then determined by incubating these homogenates (20–30 μg protein per assay tube) in 1 mL of assay buffer (50 mM of Tris–HCl, 100 mM of NaCl, 10 mM of $MgCl_2$, 10 μM of GDP, and 0.1 nM [^{35}S]GTP γS, pH 7.4), for 30 min at 25°. CHO-H1 cell homogenates (≈100 μg protein/tube) were assayed for 60 min at 25° in the same assay buffer, but containing only 1 μM of GDP. The assay was terminated by rapid filtration (using a Brandel cell harvester) through Whatman GF/B filters, presoaked in ice-cold water. The filters were then washed twice with 4 mL of ice-cold water and then subjected to liquid scintillation counting (75% counting efficiency).

Whole Cell [^{3}H]DPCPX Binding

Ligand binding to the A_1 receptors was measured using the A_1 receptor antagonist [^{3}H]DPCPX. After initially washing confluent CHO-A1 cells in 24-well cluster dishes with 0.5 mL per well Hanks'/20 mM HEPES buffer (pH 7.4), the cells were incubated in 0.265 mL/well Hanks'/HEPES containing adenosine deaminase (1 unit/mL), for 30 min at 37°. The cells were then incubated for a further 1 hr at 37°,

in a total volume of 0.3 mL Hanks'/HEPES buffer, in the presence (nonspecific binding) or absence (total binding) of 5 mM of theophylline, and in the presence of 2 nM [^{3}H]DPCPX and 0.0005% Triton X-100. The assay was then terminated by aspirating the incubation medium, washing each well with 1 mL of Hanks'/HEPES buffer and then adding 0.5 mL/well trypsin/EDTA (trypsin, 0.5 g; EDTA, 0.2 g; and NaCl, 0.85 g per litre) to detach the cells from the culture dish. The amount of [^{3}H]DPCPX bound to the detached cells was then determined by liquid scintillation counting.

Assessment of Cell Viability

Cell viability was assessed by trypan blue exclusion. Confluent monolayer cultures in 6-well cluster dishes were incubated at 37°, for 30 min, in Hanks'/HEPES buffer (3 mL/well) containing U73122, U73343 or dimethylsulfoxide, as the vehicle control. At the end of this period, incubation media were then replaced with 1 mL of Hanks'/HEPES buffer/0.4% trypan blue solution (1:1), and the cells were examined immediately by phase contrast microscopy (Nikon Diaphot).

Data Analysis

Responses obtained in the presence of dimethylsulfoxide (vehicle control), U73343, and U73122 were compared by analysing the data from at least three experiments by two-way analysis of variance and then post hoc, using Newman–Keuls tests.

Chemicals

Cell culture flasks and 24-well cluster dishes were from Costar. Adenosine deaminase, N^6-CPA, dimethylsulfoxide, Dulbecco's modified Eagle's medium/nutrient mix F12 HAM, foetal bovine serum, forskolin, GDP, L-glutamine, Hanks' balanced salt solution, HEPES, histamine dihydrochloride, theophylline, Tris, Triton X-100, and 0.4% trypan blue solution were supplied by Sigma. The radiochemicals [2,8-^{3}H]adenine (991.6 GBq/mmol), cyclic [8-^{14}C]AMP (1.9 GBq/mmol), *myo*-[2-^{3}H(N)]inositol (569.8 GBq/mmol), [^{35}S]guanosine 5′[γ-thio] triphosphate (37 TBq/mmol), and [^{3}H]DPCPX (4440 GBq/mmol) were obtained from New England Nuclear. U73122 and U73343 were from Calbiochem, inositol-free Dulbecco's modified Eagle's medium from Flow Laboratories, pertussis toxin from Porton Products Ltd. and rolipram from Schering A.G. All other chemicals were of analytical grade.

RESULTS

Effect of U73122 on Agonist-stimulated Phosphoinositide Hydrolysis

The ability of U73122 to inhibit agonist-stimulated PI hydrolysis was first examined in CHO-H1 cells expressing the histamine H_1 receptors. Stimulation of these $G_{q/11}$-coupled receptors with histamine (300 nM) elicited a strong PI response of 962 ± 81% of basal levels. U73122 reduced this response in a concentration-dependent manner, consistent with its proposed action as a PLC inhibitor (Fig. 1).

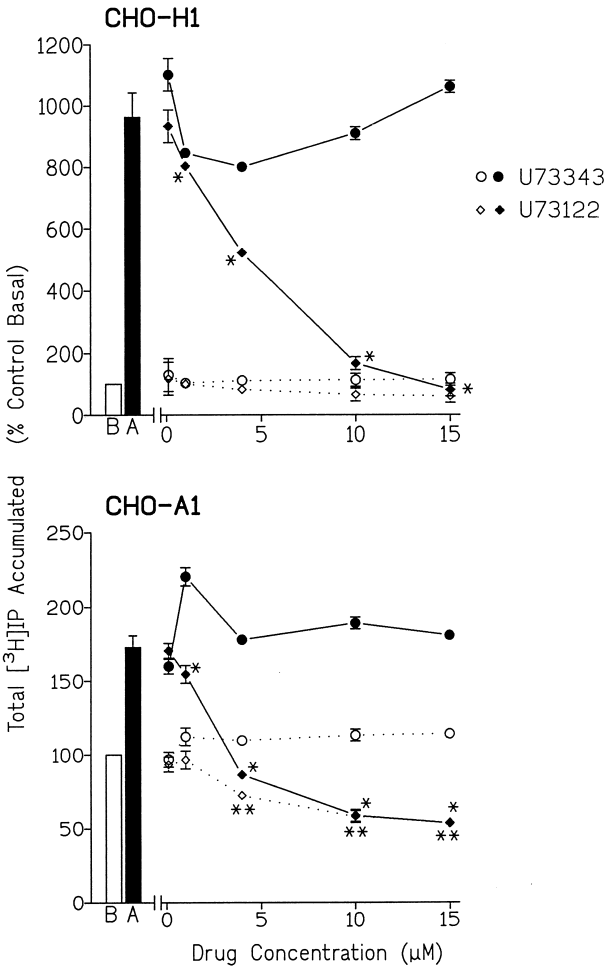

FIG. 1. The effect of U73122, and its inactive analogue U73343, on the PI responses to HA (300 nM) in CHO-H1 cells or CPA (1 μM) in CHO-A1 cells. The bars represent responses measured under control conditions (B, basal; A, agonist). Basal and agonist-stimulated responses, measured in the presence of U73343 (circles) or U73122 (diamonds), are represented by open and closed symbols, respectively. Data are the means ± SEM for observations from three independent experiments. Values significantly lower than **control basal or *control agonist-stimulated responses ($P < 0.05$).

In CHO-A1 cells, the adenosine A_1 agonist, N^6-CPA (1 μM), stimulated a PI response of 173 ± 8% of basal levels. This PI signal was unaffected by submicromolar concentrations of U73122 (e.g. 0.1 μM, see Fig. 1). A small but significant inhibition (25%, $P < 0.01$) was observed at 1 μM of U73122. However, this compound only reduced the CPA response substantially at concentrations which also diminished basal levels of IP accumulation (Fig. 1). At the highest concentrations examined (10 and 15 μM), the CPA PI response equalled basal levels of IP accumulation, and this common signal size was only 54% of control basal levels. The inactive analogue U73343 showed no consistent effect on basal or CPA PI responses in CHO-A1 cells.

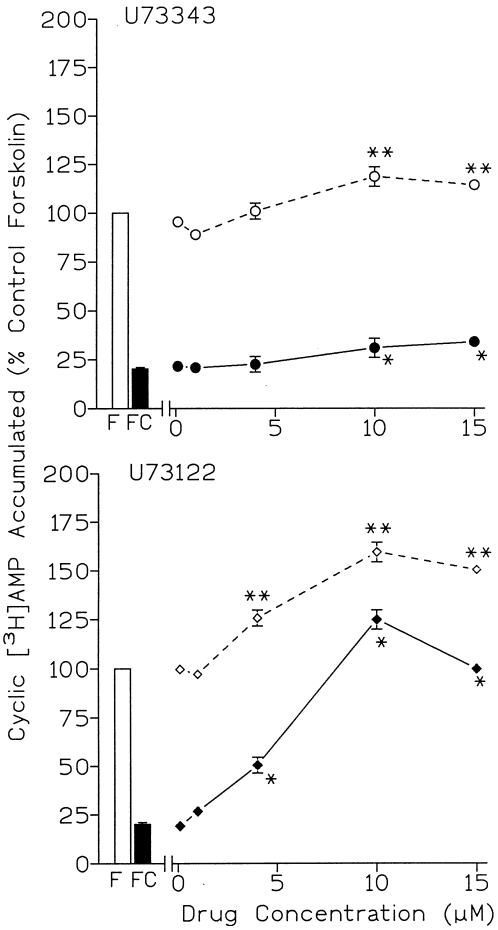

FIG. 2. The effect of U73122, and its inactive analogue U73343, on the cyclic AMP response to CPA in CHO-A1 cells. The bars represent responses measured under control conditions (F, 3 μM of forskolin; FC, 3 μM of forskolin + 1 μM of CPA). Responses to forskolin alone or costimulated with CPA, measured in the presence of U73343 (circles) or U73122 (diamonds), are shown by open and closed symbols, respectively. Data are the means ± SEM for observations from four independent experiments. Values significantly higher than control cyclic [^{3}H]AMP levels stimulated with **forskolin or costimulated with *forskolin and CPA ($P < 0.05$).

Effect of U73122 on Adenosine A_1 Receptor-mediated Inhibition of Forskolin-stimulated Cyclic AMP Accumulation

Under control conditions, forskolin, a direct activator of adenylyl cyclase, produced a sizeable cAMP response (≈6000 dpm above a basal response of ≈1500 dpm) in CHO-A1 cells. Neither U73343 nor U73122 stimulated cAMP accumulation on its own (data not shown). U73343, at the highest concentrations (10–15 μM), caused a slight increase in the response to forskolin, by 14–18% (Fig. 2). However, U73122 was much more potent in this effect, augmenting the forskolin response at a lower concentration, and to a higher degree (up to 59%, Fig. 2). As expected for a G_i-coupled receptor, the control response to stimulation by CPA (1 μM) was to reduce the cAMP response to forskolin (3 μM), in this case by 80 ± 1% (Fig. 2; $P < 0.01$). U73122 appeared to significantly reduce the ability of CPA to inhibit forskolin-stimulated cAMP response (Fig. 2), although some of this effect may be secondary to the concomitant increase in the forskolin response. However, U73343 only produced a slight effect on the inhibition of forskolin-stimulated cAMP accumulation produced by CPA.

Effect of U73122 on Adenosine A_1 Receptor-stimulated [^{35}S]GTPγS Binding

An alternative means by which U73122 might inhibit the CPA-mediated suppression of forskolin-stimulated cAMP accumulation, and also the PI response to CPA, is by blocking the A_1 receptor-mediated activation of $G_{i/o}$ proteins. In human polymorphonuclear neutrophil membranes, it has been reported that U73122 can inhibit fMLP-stimulated GTPase activity at concentrations overlapping with its inhibitory effect on fMLP-stimulated PLC activity [27]. Given this precedence, the effects of U73122 on CPA-stimulated [^{35}S]GTPγS binding were also examined.

Under control conditions, CPA (1 μM) increased [^{35}S]GTPγS binding in CHO-A1 cell homogenates by 48 ± 3% over basal levels (Fig. 3, $P < 0.01$; EC_{50} = 3.5 ± 0.1 nM, N = 6). This response was completely abolished by pertussis toxin treatment (n = 3; data not shown), and is therefore mediated entirely by the A_1 receptors interacting with the $G_{i/o}$ subtype of G-proteins. U73343 marginally reduced both basal and CPA-stimulated [^{35}S]GTPγS binding at the highest concentrations (10–15 μM). U73122 also inhibited basal [^{35}S]GTPγS binding by up to 30% at the highest concentrations. However, it was much more effective in interfering with CPA-stimulated [^{35}S]GTPγS binding. The response to CPA was almost completely abolished by as low a concentration as 1 μM U73122 (Fig. 3). That this was not largely due to inhibition of receptor ligand binding was confirmed by measuring the specific binding of the A_1 receptor antagonist, [^{3}H]DPCPX (2 nM), in intact cell preparations. U73343 had no effect on total or nonspecific [^{3}H]DPCPX binding (N = 3; data not shown). U73122 caused a small but significant reduction ($P < 0.01$) in specific ligand binding, but only at the highest concentrations (10 μM: 20 ± 4%, 15 μM: 21 ± 2% decrease; N = 3).

Interestingly, we also found that U73122 produced a virtually identical inhibition of both basal and histamine (100 μM)-stimulated [^{35}S]GTPγS binding in parallel studies in CHO-H1 cells. At this concentration, histamine maximally stimulates [^{35}S]GTPγS binding through H_1 receptor activation (50 ± 3% increase over basal levels; EC_{50} = 8.7 ± 5.1 μM; n = 5), as well as PI hydrolysis.

Effect of U73122 on CHO-A1 Cell Viability

As U73122 is cytolytic to platelets at high concentrations (>50 μM, [18]), the cell viability of the CHO-A1 cells was

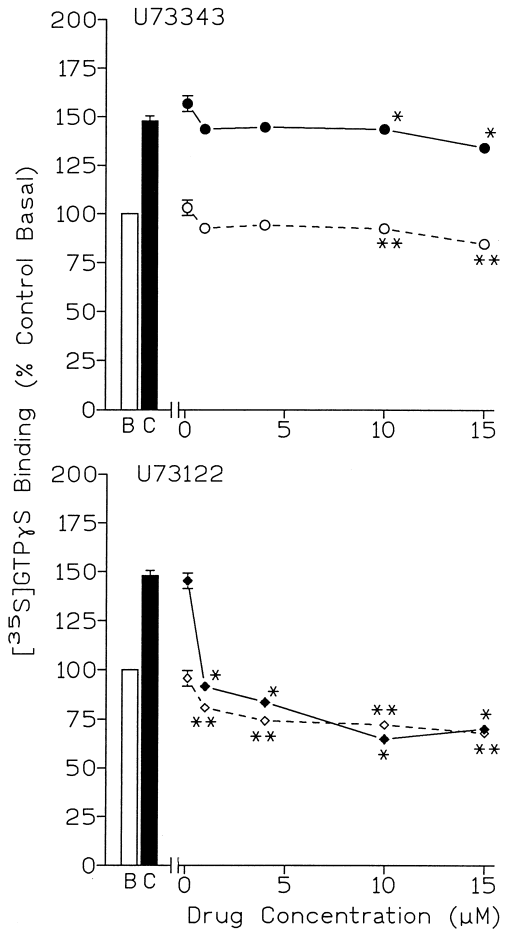

FIG. 3. Effect of U73122, and its inactive analogue U73343, on CPA-stimulated [^{35}S]GTPγS binding to CHO-A1 cell homogenates. The bars represent responses measured under control conditions (B, basal; C, 1 μM CPA). Basal and CPA-stimulated responses, measured in the presence of U73343 (circles) or U73122 (diamonds), are shown by open and closed symbols, respectively. Data are the means ± SEM for observations from three independent experiments. Values significantly lower than **control basal or *control CPA-stimulated responses ($P < 0.05$).

checked following treatment with this amphiphilic cation, by trypan blue exclusion. Confluent CHO-A1 cells under control conditions showed the characteristic wedge-like morphology of CHO-K1 cells, with cell nuclei and cytoplasmic inclusions just discernible under dark contrast. This morphology remained unchanged in the presence of U73343 and up to 1 μM U73122 (Fig. 4). At 4 μM U73122, a change in the morphology of a proportion of the cells was evident. A number of these cells became phase bright, taking up a rounded appearance. Under the conditions described so far, all of the cells maintained their ability to exclude trypan blue. At 10–15 μM, all the CHO-A1 cells displayed the rounded-cell morphology. At 15 μM, and to a much lesser extent at 10 μM, a number of the cells had detached from the culture dish surface and a significant proportion were no longer viable as judged by trypan blue permeability (Fig. 4).

DISCUSSION

The initial aim of this study was to selectively block the G_i-mediated interaction of adenosine A_1 receptors with PLC so that other consequences of G_i activation could be examined in isolation. This would be of use in establishing the dependence of downstream processes on the individual transduction events immediately following receptor activation. Antibody or antisense knockout of effector activity represent two approaches which could be employed to ablate specific pathways. Antisense oligonucleotides to PLC-β have been used in a small number of studies [28, 29], but pharmacological agents, due to their technical ease, are of much more universal appeal. U73122 has been used most extensively as a specific PLC inhibitor and could be active at both PLC-β and PLC-γ isoforms [30].

The mechanism of action of U73122 in inhibiting PLC activity has not been clearly defined. An inhibition of PIP_2 and phosphatidylinositol hydrolysis by a soluble PLC fraction prepared from platelets suggested that U73122 might act by direct interaction with the PLC protein [18]. However, in a study on PI metabolism [31], evidence has suggested that U73122 might also inhibit the synthesis of phosphatidylinositol 4-phosphate and PIP_2 by blocking the phosphatidylinositol and phosphatidylinositol 4-phosphate kinases. Therefore, decreased substrate availability could also contribute to U73122's mode of action [31]. Thompson *et al.* [32] have additionally suggested that G-proteins could be the site of U73122's inhibitory activity. This conclusion was primarily based on the observation that activation of PLC by Ca^{2+} was less affected by U73122 than activation by G-proteins using either receptor agonist or GTPγS as a stimulus. Apparently irreversible inhibition of PLC-dependent processes [32, 33], as well as the observation that *N*-ethylmaleimide can inactivate $G_q\alpha$ [34], indicated that the maleimide side chain of U73122 (substituted for a succinimide group in U73443) could be covalently modifying a thiol (or other nucleophilic) group on G-proteins essential for the regulation of PLC activity [32]. Our findings from the [^{35}S]GTPγS binding studies in CHO-H1 cells might be interpreted as further evidence supporting the inactivation of G_q proteins by U73122.

In this study we have shown that low concentrations of U73122 cannot clearly discriminate between the G_i-mediated coupling of the A_1 receptors to adenylyl cyclase and the G_i-mediated $G_{\beta\gamma}$/PLC-β pathway. Strong inhibition of A_1 receptor-mediated PI hydrolysis was only observed at concentrations where effects on A_1 receptor-mediated inhibition of forskolin-stimulated cAMP accumulation are apparent. The effect of U73122 on receptor ligand binding has not been evaluated in most studies. However, in the few studies where it has, ligand binding to G-protein-coupled receptors which promote PI hydrolysis has not been reduced by this compound [18, 32]. In this study, U73122 only reduced ligand binding to A_1 receptors at 10–15 μM, and then by a small extent. This suggests that the receptor blockade is not greatly involved in the inhibitory effects of

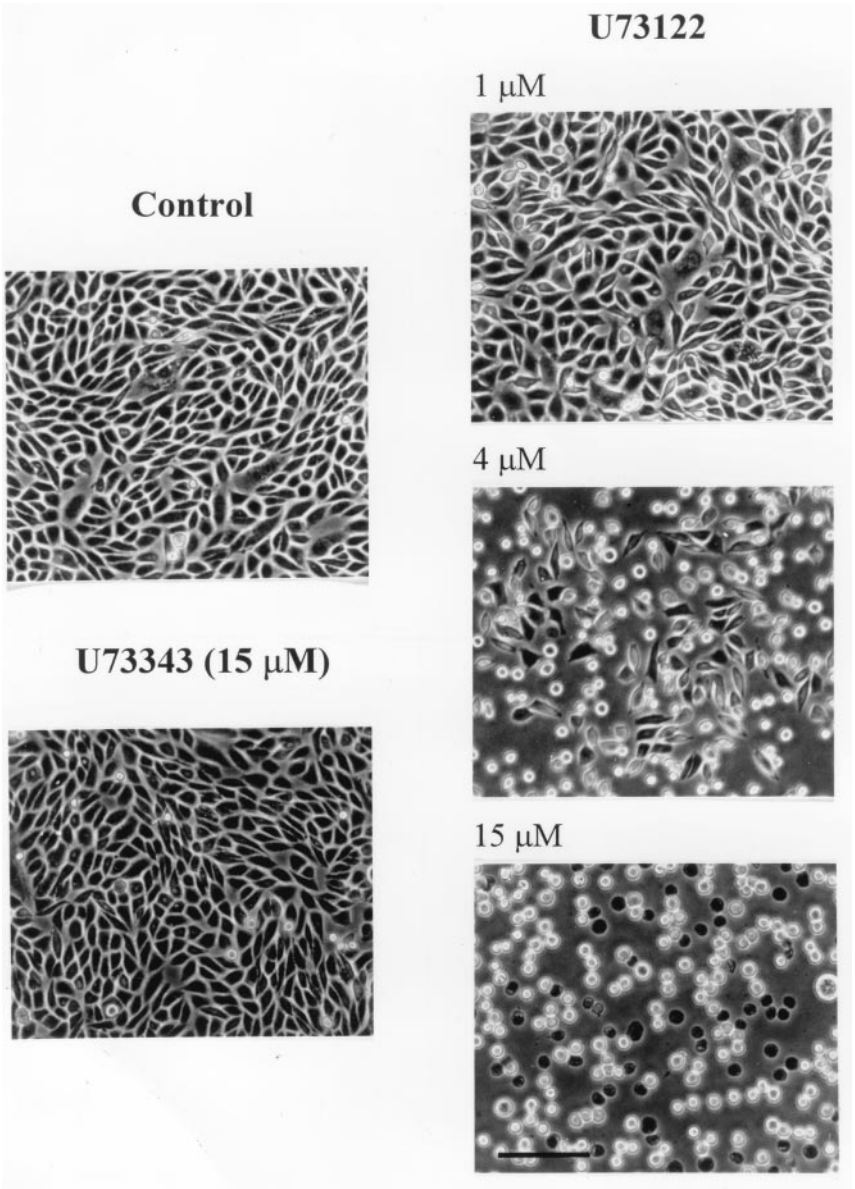

FIG. 4. The effect of U73122, and its inactive analogue U73343, on CHO-A1 cell morphology and integrity. Phase contrast photographs of CHO-A1 cells preincubated (30 min) with dimethylsulfoxide, as the vehicle control, U73343, or U73122, and then exposed to the cell impermeant dye trypan blue. The dark rounded cells at 15 μM U73122 are the only cells shown presenting trypan blue uptake. The bar represents 100 μm.

U73122 on adenosine A_1 receptor signalling, particularly at low aminosteroid concentrations. However, a reduction in $G_{i/o}$ protein activation might play a more significant role.

U73122 strongly inhibited the [^{35}S]GTPγS binding response to A_1 receptor agonist mediated by $G_{i/o}$ in CHO-A1 cells. This suggests that U73122 might interrupt receptor/$G_{i/o}$ interactions or more directly prevent GDP/GTP exchange on these G-proteins. U73122 also inhibits fMLP-stimulated GTPase activity [27]. Hence, the ability of this agent to inactivate $G_{i/o}$ as well as G_q proteins might not be unprecedented. A potential counter-argument to U73122 interfering with A_1 receptor-mediated signal transduction responses by such a mechanism is that U73122 was most potent in inhibiting CPA-stimulated [^{35}S]GTPγS binding. However, without even calling on the issue of signal amplification between G-protein and effector activity, this discrepancy could still be explained on methodological grounds. [^{35}S]GTPγS binding is measured in homogenate preparations, and the other assays in intact cells. U73122 might simply have better access to G-proteins on the internal surface of the plasma membrane in the [^{35}S]GTPγS binding assay. Nevertheless, if U73122 does inactivate $G_{i/o}$ proteins, such an effect may be cell- or $G_{i/o}$ isoform-specific since U73122 did not affect the G_i-mediated μ-opioid receptor inhibition of forskolin-stimulated cAMP in SK-N-SH cells [32].

At higher concentrations, U73122 produces marked effects on cell morphology and integrity. It is not inconceivable that the inhibitory effects of this compound and its ability to potentiate forskolin-stimulated cAMP accumulation in our CHO-A1 cells might be associated partly or generally to events associated with these morphological changes. These observations may also point to effects of U73122 on small molecular weight G-proteins such as Rho-A, since toxins (*Clostridium difficile* toxin A and B) which glucosylate Rho-A produce similar morphological

changes in these cells.* A degree of apparent lost signal transduction activity can certainly be attributed to changes in CHO-A1 cell morphology and integrity at 15 μM of U73122, since the detachment of cells from the assay culture plates was evident at this concentration. The concentration at which U73122 induces cytolysis could be cell-dependent, as in platelets concentrations in excess of 50 μM were required before such effects were observed [18].

In addition to our findings, evidence from a number of studies has now accumulated indicating that, rather than being a selective antagonist of PLC activity, U73122 can produce other undesired effects. In pancreatic acinar cells and NG105-15 cells, U73122 produces slow increases or oscillations in intracellular Ca^{2+} levels which have been attributed to release from intracellular stores or changes in plasma membrane permeability to calcium ions [35, 36, 33]. U73122 has also been shown to activate (unidentified) cation channels, giving rise to inward currents in pancreatic acinar cells and thus generating implications for plasma membrane potentials and intracellular electrolyte content [37]. Tyrosine phosphorylation of proteins is also a reported action of U73122 in platelets, suggesting an activation of tyrosine kinases or inhibition of tyrosine phosphatases [30]. Other reported inhibitory effects of U73122 include inhibition of voltage-sensitive Ca^{2+} channels [38, 33], store-regulated Ca^{2+} entry into cells [39], noninactivating K^+ currents [36], and agonist-evoked protein tyrosine phosphorylation [30], though these effects are mimicked less effectively by U73343 [38–40, 36]. It can be concluded that the effects of U73122 may be multiple, and therefore its use as a tool in establishing the dependence of stimulated cellular events on PLC activity may be limited without the use of careful controls.

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