NEUROPROTOCOLS: A Companion to Methods in Neurosciences 3, 125–133 (1993)

Use of U-73122 as an Inhibitor of Phospholipase C-Dependent Processes

John E. Bleasdale* and Stephen K. Fisher†

*Metabolic Diseases Research, Upjohn Laboratories, Kalamazoo, Michigan 49001; and †Neuroscience Laboratory and Department of Pharmacology, University of Michigan, Ann Arbor, Michigan 48104-1687

1-[6-[[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]-hexyl]1*H*-pyrrole-2,5-dione (U-73122) is an aminosteroid that was identified initially as a potent inhibitor of platelet activation by receptor-specific agonists. U-73122 inhibits receptor-coupled generation of inositol 1,4,5-trisphosphate (but not cyclic AMP) and intracellular mobilization of Ca^{2+} in a variety of cell types. U-73122 inhibits phosphoinositide-specific phospholipase C (PI-PLC) activity in cell-free systems, but exhibits little or no direct inhibition of phospholipases A_2 and D. Structure–activity analysis revealed that the maleimide group of U-73122 is essential, but not sufficient, for inhibitory activity. The succinimide analog of U-73122 (U-73343) has negligible inhibitory activity and is a useful control compound. On the basis of information derived from the use of U-73122 in a variety of cell types, procedures for storing, dissolving, and presenting U-73122 to cells are recommended. While knowledge of the mechanism of action of U-73122 would extend the utility of this compound, U-73122 has already been employed successfully to examine PI-PLC involvement in a variety of cellular processes. The application of U-73122 in an investigation of muscarinic receptor sequestration in SK-N-SH neuroblastoma cells is illustrated. © 1993 Academic Press, Inc.

The discovery of a second-messenger function of inositol 1,4,5-trisphosphate ($InsP_3$) a decade ago (1) provided the impetus for much subsequent research into a variety of cell signaling processes in which phosphoinositide-specific phospholipases C (PI-PLC) are critically involved. These enzymes are a family of phosphodiesterases that catalyze the hydrolysis of phosphatidylinositol (PI), or one of its derivatives such as phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), to yield diacylglycerol (DG) and a variety of inositol phosphates as products. Several of the products of PI-PLC action are bioactive and participate in signal transduction mechanisms. Comprehensive reviews of the structure, function, and regulation of PI-PLC are available (2–4). Because the products of PI-PLC are important not only in normal cell physiology but also in pathologic processes, the potential of PI-PLC-dependent processes as drug targets has recently received increased attention (5). Indeed, the possible therapeutic uses of PI-PLC inhibitors are as diverse as the various cellular functions of PI-PLC that have been identified. For example, PI-PLC inhibitors have potential utility in preventing neutrophil recruitment and activation in inflammation, as anticancer agents, and as novel anti-obesity therapeutics (5, 6). Unfortunately, the goal of identifying compounds that selectively inhibit various PI-PLC isozymes by defined molecular mechanisms has yet to be achieved. Nevertheless, a variety of compounds that inhibit PI-PLC-dependent processes (with varying degrees of specificity and via apparently diverse mechanisms) have been described (5). One of these compounds, 1-[6-[[17β-3-methoxy-estra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122, Fig. 1) has been used to probe PI-PLC involvement in a variety of cellular processes (Table 1).

In this article, we first summarize our experience with U-73122 as an inhibitor of receptor-specific activation of human polymorphonuclear neutrophils (PMN) and platelets, emphasizing those aspects that are applicable to the successful use of this compound with other cells. Second, we illustrate the use of U-73122 in an investigation of PI-PLC involvement in muscarinic receptor sequestration in SK-N-SH neuroblastoma cells.

DISCOVERY AND CHARACTERIZATION OF U-73122

U-73122 was synthesized as an amphiphilic alkylating agent that might be expected to partition into biomembranes and interfere with enzymes that act on membrane lipids (5, 7). It was expected that this compound, like *p*-bromophenacyl bromide, would inhibit phospholipase A_2. It was observed, however, that the action of U-73122 on cells was more consistent with a selective inhibition of PI-PLC-dependent processes (7, 8). U-73122 was found

1058-6741/93 $5.00
Copyright © 1993 by Academic Press, Inc.
All rights of reproduction in any form reserved.

to inhibit the activation of platelets and PMN by receptor-specific agonists at an early step in signal transduction. U-73122 did not affect the binding of ligands to the platelet thromboxane A_2 receptor (7). This finding, together with the observation that this compound inhibited the action of a diverse group of receptor-specific agonists, suggested that a postreceptor mechanism common to a variety of receptors was the target of U-73122 (7). Agonist-induced increases in $[Ca^{2+}]_i$ in platelets and PMN were found to be inhibited by U-73122 at submicromolar concentrations (7, 8). Receptor-coupled production of IP_3 and DG in platelets and PMN was also inhibited by U-73122 (7, 8). Because it inhibited agonist-induced DG production in PMN, U-73122 prevented the subcellular translocation and activation of protein kinase C (8). U-73122, however, had no direct action on protein kinase C *in vitro* under assay conditions where inhibition by staurosporine was clearly demonstrable (8). Activity of PI-PLC either in subcellular fractions of platelets and PMN or partially purified from human amnion was inhibited by U-73122 (7–9), whereas the activities of phospholipases A_2 purified from either snake venom or porcine pancreas were not (7). Neither phospholipase D-dependent production of DG in PMN elicited by a phorbol ester (7) nor phospholipase D activity in erythroleukemia cells (10) was inhibited by U-73122. Agonist-stimulated high-affinity GTPase activity in plasma membranes isolated from human PMN was inhibited by U-73122 at concentrations $> 1\ \mu M$ (8). Receptor-coupled changes in cyclic AMP in a variety of cell types, however, were unaffected by U-73122 (7, 8, 11). PI-PLC-dependent mobilization of arachidonic acid from platelet PtdIns was inhibited by U-73122 (7). Although these data do not preclude the possibility that U-73122 may interfere with other cellular processes, they support the proposition that U-73122 is an acute, potent, and selective inhibitor of PI-PLC-dependent processes.

SYNTHESIS AND PHYSICAL PROPERTIES OF U-73122 AND U-73343

U-73122 is synthesized from 3-methoxyestrone, 1,6-hexanediamine, and maleic anhydride as detailed elsewhere (7). Substitution of maleic anhydride by succinimide in the synthetic procedure yields 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione (U-73343), a close but inactive analog of U-73122 (Fig. 1) (7). Both compounds are now commercially available (Biomol Research Laboratories, Inc., Plymouth Meeting, PA). U-73122 ($C_{29}H_{41}N_2O_3$, FW465.3) is a pale yellow solid with a melting point of 123 to 124°C and is stable when stored at either room temperature or −20°C over desiccant. Vials of U-73122 stored at −70°C should be allowed to reach room temperature before being opened to avoid reaction of the maleimide group with water that may condense from the air. U-73343 ($C_{29}H_{43}N_2O_3$, FW467.3) is a white solid with a melting point of 138.5 to 140°C and is stable at room temperature. Both U-73122 and U-73343 are freely soluble in chloroform and methylene chloride and are soluble in dimethyl sulfoxide (DMSO) (to at least 5 mM) and absolute ethanol (to at least 2 mM). Concentrated solutions of U-73122 in DMSO turn pink when left at room temperature overnight. The pink derivative, which is likely a product of a reaction between the maleimide group and a trace contaminant in the DMSO, has not been identified but is biologically inactive. U-73122 to be added to cells should be dissolved

TABLE 1

Some Reported Applications of U-73122

Cell type	Cellular process	Ref.
Human platelets	Receptor-specific activation	7
Human PMN	Receptor-specific activation	8, 18
Human amnion cells	Agonist-induced PGE_2 production	9
SK-N-SH neuroblastoma cells	Sequestration of muscarinic receptors	11
	G-protein regulation of PI-PLC	14
Rat anterior pituitary cells	GnRH-induced release of LH	13, 19
Rat pancreatic acinar cells	Calcium oscillations propagated by receptor-specific agonists and sphingosine	20–22
Human erythroleukemia cells	PGE-induced Ca^{2+} mobilization and activation of phospholipase D	10
Human umbilical vein endothelial cells	Flow-induced NO production	23
NIH 3T3 cells	Cell growth	6
GH_3 pituitary cells	TRH-induced Ca^{2+} mobilization and prolactin secretion	24
N1E-115 neuroblastoma cells	Neurotensin receptor down-regulation	25
Rat hepatocytes	Insulin receptor down-regulation	26
Chicken granulosa cells	Rapid, nongenomic estrogen effect on Ca^{2+} mobilization	27
Human myometrial cells	Agonist-induced Ca^{2+} mobilization	28
Bovine artery endothelial cells	Bradykinin-induced increase in transcellular permeability	29

in DMSO immediately before use, and unused portions of U-73122 solutions should be discarded.

ADMINISTRATION OF U-73122 TO CELLS

U-73122 is sparingly soluble in aqueous media and so is generally added as a solution (in DMSO or ethanol) to an aqueous medium that usually contains either serum or serum albumin. When exposure of the cells to organic solvents must be avoided, it is possible to deliver U-73122 to suspensions of cells from a dry film as follows. A solution of U-73122 (10 mM) is prepared in methylene chloride, and a measured volume is delivered to the vessel that will receive cells. The methylene chloride is evaporated under N_2 and the cell suspension is added to the vessel with mixing. This procedure has been used successfully to deliver U-73122 from glass aggregometer cuvettes to PMN and to platelets. Delivery from glass to platelets in platelet-rich plasma is apparently rapid because, even with amounts of U-73122 calculated to yield submicromolar final concentrations, maximal effects on platelet activation are observed within 2 min.

The choice of concentrations of U-73122 to be used with a particular cell type is influenced by several considerations.

Cell Type

Confluent attached cells may require a concentration of U-73122 greater than that required for cells in suspension. This may be an indication of restricted entry of U-73122 into attached cells (perhaps involving partitioning of U-73122 into the apical plasma membrane followed by lateral diffusion to other parts of the plasma membrane and intracellular membranes). Experience with a variety of cell types that contain PI-PLC isozymes in various relative abundances would indicate that U-73122 is not selective for a particular PI-PLC isozyme.

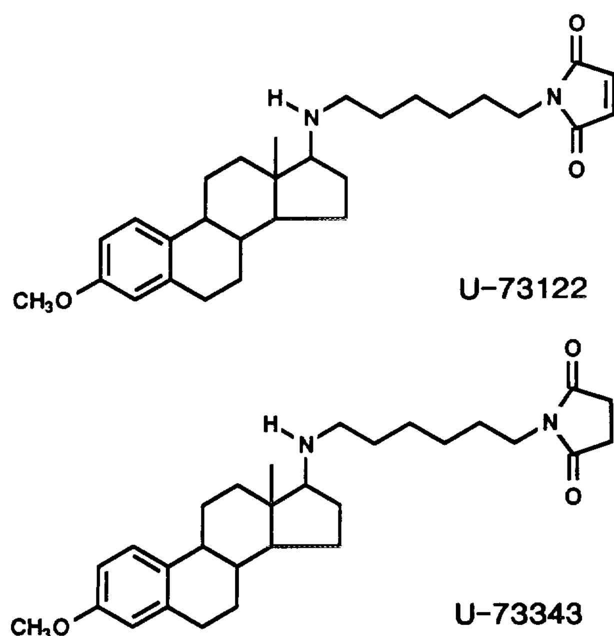

FIG. 1. Structures of U-73122 and U-73343.

Cell Density

U-73122, as an amphiphilic cation, partitions into cell membranes. When cells are used at high density, the extent of membrane partitioning may be sufficient to deplete U-73122 from the bulk medium. Effects of U-73122 on human PMN (8) and SK-N-SH neuroblastoma cells (11) that are dependent upon cell density have been described and are considered later in this article. Special considerations in delivering amphiphilic compounds to cells are discussed in detail elsewhere in this issue [see Buxser (35)].

Medium Composition

The maleimide group of U-73122 is reactive with water and with several chemical groups that are potentially found in components of the medium. U-73122 will react with thiol compounds, and treatment of U-73122 with β-mercaptoethanol destroys its ability to inhibit receptor-specific activation of PMN (8). Activity of U-73122 does not appear to be compromised in media with a high protein content because the activation of human platelets in platelet-rich plasma was inhibited by U-73122 at submicromolar concentrations (7). This may also indicate that removal of U-73122 from cells, e.g., by adsorption to albumin, may be difficult.

Cellular Response of Interest

There is evidence that some cellular responses are more sensitive to U-73122 than others. For instance, FMLP-induced changes in $[Ca^{2+}]_i$ were inhibited by U-73122 at concentrations of 100 nM or less, with an IC_{50} of approximately 500 nM (Fig. 2) (8). In contrast, U-73122 inhibited FMLP-induced accumulation of IP_3 and DG with an IC_{50} of approximately 2 μM (Fig. 2) (8). Possible reasons for this differential sensitivity to U-73122 include the following:

(i) The existence of multiple sites of action of U-73122.

(ii) Cooperativity in the IP_3-dependent mobilization of Ca^{2+} (12) results in large changes in $[Ca^{2+}]_i$ over a relatively narrow range of IP_3 concentrations. Conversely, a small decrease in IP_3 concentration imposed by U-73122 may be sufficient to block completely the IP_3-dependent increase in $[Ca^{2+}]_i$.

(iii) When $[Ca^{2+}]_i$ measurements were made, a maximally effective FMLP concentration of 10 nM was used. For measurement of IP_3 production, a greater concentration of FMLP (100 nM) was required for the maximal effect.

(iv) $[Ca^{2+}]_i$ measurements were made at a cell density (2.5×10^6 cells/ml) much lower than those used for IP_3 measurements (10^7 to 5×10^7 cells/ml) and, as discussed above, partitioning of U-73122 into cell membranes may deplete the concentration of U-73122 in the bulk medium at high cell densities.

Cytotoxicity

U-73122 at a concentration of 100 μM caused the release of approximately 17% of lactate dehydrogenase activity from human PMN (7). This concentration is more than 10 times that at which PI-PLC-dependent processes in PMN are completely inhibited, and so cytotoxicity does not appear to compromise the use of U-73122 with these cells. Cytotoxicity is likely to be dependent upon the cell type and should be determined experimentally, especially when concentrations of U-73122 greater than 20 μM are used or when exposure to the compound is prolonged.

In general, we recommend that U-73122 be administered as a freshly prepared 1000× solution in DMSO (or ethanol) and used at a final concentration in the range 0.1 to 10 μM, and that unnecessarily prolonged exposure to the compound be avoided. Exposure of human PMN to U-73122 (300 nM) for only 60 s was sufficient to produce maximal inhibition (>80%) of FMLP-induced degranulation (Fig. 3) (8). Even when added simultaneously with FMLP, U-73122 inhibited degranulation approximately 50%, but was without effect when added 30 s *after* FMLP (Fig. 3) (8).

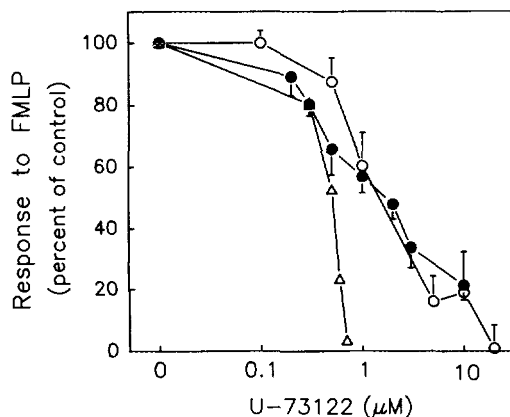

FIG. 2. Inhibition by U-73122 of FMLP-induced increases in $InsP_3$, DG, and $[Ca^{2+}]_i$ in human PMN. PMN were preincubated for 3 min with U-73122 at various concentrations before the addition of FMLP and cytochalasin B. $[Ca^{2+}]_i$ was measured in PMN loaded with the calcium-sensitive dye Fura-2, as described (8). $Ins(1,4,5)P_3$ was quantitated in TCA extracts of PMN by use of a competitive radiobinding assay, and DG was measured as $[^{32}P]PtdOH$ in a reaction with $AT^{32}P$ catalyzed by DG kinase (7, 8). The maximal change in $[Ca^{2+}]_i$ within 30 s of FMLP addition, FMLP-induced $InsP_3$ production at 20 s, and FMLP-induced DG accumulation at 5 min are each expressed as 100% of the control response. Inhibition by U-73122 is expressed as a percentage of the control response. U-73343, at concentrations up to 10 μM, did not alter FMLP-induced changes in $[Ca^{2+}]_i$, $InsP_3$, or DG by more than 10%. Data are mean values ± SEM from three experiments ($InsP_3$, ●), four experiments (DG, ○), and a typical experiment ($[Ca^{2+}]_i$, △) (7, 8).

MECHANISM OF ACTION OF U-73122

While U-73122 has already proven to be a useful research tool, full exploitation of this compound awaits the elucidation of its mechanism of action. Some of the structural features of U-73122 that are required for inhibitory activity have been identified by comparing a series of aminosteroid analogs of U-73122. This structure–activity analysis supported the following conclusions.

(i) Inhibition of PI-PLC-dependent processes is not a general property of all diaminosteroids.

(ii) The maleimide group of U-73122 is essential, but not sufficient, for inhibitory activity. U-73343, a close analog that differs only in the substitution of a succinimide group for the maleimide moiety (Fig. 1), is inactive (or much less active) in most systems and has been a useful control compound whenever U-73122 is used (7, 8). Reaction of U-73122 with β-mercaptoethanol destroys inhibitory activity (7). On the other hand, a variety of nonsteroidal water-soluble or lipophilic N-substituted maleimides do not mimic U-73122 in inhibiting PI-PLC-dependent processes (7).

(iii) Removal of the 3-methoxy group from U-73122 greatly reduces inhibitory activity (i.e., increased IC_{50} for inhibition of platelet activation) as does saturation of the A ring.

(iv) Changes in the length of the side chain at C17 of U-73122 alter inhibitory activity somewhat, but all com-

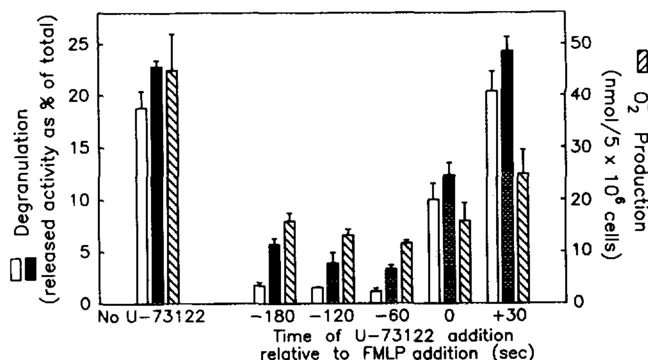

FIG. 3. Kinetics of inhibition by U-73122 of FMLP-induced degranulation and superoxide production in PMN. PMN were preincubated with U-73122 (300 nM) for up to 3 min before the addition of FMLP (10 nM for degranulation or 100 nM for O_2^- production) and cytochalasin B (5 μg/ml). Degranulation was measured as the release of myeloperoxidase (□) and vitamin B_{12}-binding protein (■) 2 min after FMLP addition (8). Superoxide anion (▨) generated after 5 min of exposure of the PMN to FMLP was measured as the reduction of ferricytochrome c inhibitable by superoxide dismutase. Data are adapted from (8) and are mean values ± SEM from three experiments.

pounds tested in the range of 2 to 8 carbons had inhibitory activity.

Because U-73122 was active against a variety of receptor-specific agonists, it seemed unlikely that U-73122 inhibited ligand binding to cell surface receptors. U-73122 did not inhibit the cell surface binding of either a thromboxane antagonist (7) or a muscarinic antagonist (11). Indeed, when receptors are bypassed and inositol phosphate production is stimulated with AlF_4^-, U-73122 is still inhibitory (11, 13, 14). This suggests that the site of action of U-73122 is at the level of either the PI-PLC or the interaction of PI-PLC with a regulator molecule. Inhibition by U-73122 of *in vitro* PI-PLC activity in a cytosolic fraction of human platelets (7), a plasma membrane-enriched fraction from human PMN (8), and of a partially purified PI-PLC from human amnion (9) has been described. U-73343, under identical conditions, is either inactive or much less active than U-73122. Unfortunately, the use of any of the above systems to elucidate the mechanism of action of U-73122 is complicated by the interaction of this amphiphilic cation with the substrate (either PtdIns or $PtdIns(4,5)P_2$) used in the *in vitro* assays. Calcium also binds to the substrate and the calcium requirement for *in vitro* PI-PLC activity is dependent upon the concentration of substrate (15). This suggests that the binding of calcium to the small vesicles or micelles of substrate reduces the negative charge on these substrate particles and facilitates their interaction with PI-PLC. As an amphiphilic cation, U-73122 may be even better than calcium in altering the net charge on substrate particles. Indeed, at molar ratios of Ca^{2+}:PtdIns in the range of 4 to 12, hydrolysis of PtdIns by a partially purified soluble PI-PLC from human amnion is submaximal and is *increased* by the addition of U-73122. At Ca^{2+}:PtdIns molar ratios < 2, U-73122 inhibits, and at ratios > 12 U-73122 is without affect. In either isolated PMN membranes (8) or permeabilized SK-N-SH cells (11), PI-PLC activity induced by either GTPγS or receptor-specific agonists is relatively susceptible to inhibition by U-73122, whereas PI-PLC activity driven by excess calcium is poorly inhibited. These observations support (but do not prove) the proposition that U-73122 may act at the level of PI-PLC interaction with regulatory G-proteins. Inhibition by U-73122 of FMLP-stimulated high-affinity GTPase activity in PMN membranes (8) would also support such a proposition. Interestingly, another maleimide (*N*-ethylmaleimide) has been reported to selectively uncouple α-adrenoreceptor-mediated inhibition of adenylate cyclase and stimulation of GTPase in human platelets (16). The possibility that the mechanism of action of U-73122 may involve G-proteins is considered further below.

U-73122 does not appear to be specific for a particular PI-PLC isozyme because it suppresses PDGF-induced PI-PLC activity in NIH 3T3 cells (predominantly a γ isozyme regulated by tyrosine phosphorylation) (6) as well as it does FMLP-induced PI-PLC activity in PMN (8) and thrombin-induced PI-PLC activity in platelets (7) [predominantly β-like and ϵ-like isozymes regulated by G-proteins (17)].

The absolute requirement for the electrophilic maleimide moiety of U-73122 for inhibitory activity suggests that U-73122 may react with a nucleophile in either PI-PLC or a regulator molecule. However, attempts to demonstrate covalent modification of a PI-PLC by U-73122 have been limited and unsuccessful (unpublished observations). Nevertheless, this does not preclude the possibility of a covalent modification of a protein by U-73122. Such a modification could be reversible, and even water bound at the catalytic site is a candidate nucleophile. In view of the finding that reaction of U-73122 with thiol compounds such as β-mercaptoethanol destroys inhibitory activity, it was curious to observe that a variety of dithio compounds (e.g., dithiothreitol, dithioerythritol) but not mono-thiol compounds (e.g., β-mercaptoethanol, thiourea, thioglycolic acid, L-cysteine, glutathione) greatly potentiated the inhibition by U-73122 of PI-PLC activity in a cytosolic fraction from human platelets (unpublished observations).

Because the mechanism of action of U-73122 has not been defined, caution should be exercised in the interpretation of data, especially when a cellular response not previously thought to be PI-PLC-dependent is altered by U-73122. Ignorance of the mechanism of action of U-73122 is not an impediment when this compound has been used to inhibit receptor-coupled IP_3 production without affecting the cellular response of interest. Thus, U-73122 was very useful in demonstrating dissociation of receptor-coupled IP_3 production from Ca^{2+}-dependent LH secretion from anterior pituitary cells (13) and in defining the relationship between receptor-coupled IP_3 production and muscarinic receptor sequestration in SK-N-SH cells (11).

APPLICATION OF U-73122 IN AN INVESTIGATION OF MUSCARINIC RECEPTOR SEQUESTRATION

Agonist occupancy of G-protein-linked receptors is frequently reported to induce the sequestration of cell surface receptors as part of an adaptive response whereby the cell reduces its ability to respond to chronic receptor activation. For muscarinic cholinergic receptors (mAChRs) in human SK-N-SH neuroblastoma cells, two lines of evidence suggest that the activation of PI-PLC and receptor sequestration are interrelated events. First, the concentrations required for half-maximal activation of the two processes are similar and, second, differences in muscarinic agonist efficacy for activation of PI-PLC are reflected in the extent of mAChR sequestration (30). In this section,

we describe experiments in which U-73122 has been utilized to evaluate further the relationship between mAChR activation of PI-PLC and sequestration of cell surface receptors in SK-N-SH cells.

Inhibition of mAChR-Stimulated Inositol Phosphate Production in Intact Cells by U-73122

SK-N-SH cells are grown (in the absence of antibiotics) for 10–14 days in the presence of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) in an atmosphere of 10% CO_2/air. Cells are fed three times weekly by replacement of 50% of existing medium with fresh medium. On Days 11–14, the cells are radiolabeled in DMEM/FCS medium supplemented with 10 μCi/ml of [^{3}H]inositol. At the end of the radiolabeling period, the radioactive medium is aspirated and 10 ml of Puck's D_1 solution is added to detach the cells (30). This isotonic solution lacks the Ca^{2+} and Mg^{2+} ions which are necessary for cell attachment. Unlike many cell types, SK-N-SH cells are not tightly substratum-adherent and thus do not require the use of trypsin for their removal. Following a 10-min incubation in Puck's D_1 solution at 37°C, cells detach from the flask after it is sharply tapped and are then collected and centrifuged at 300*g* for 1 min. The cell pellet is then washed once with D_1 solution prior to resuspension in a physiological buffer (Buffer A: 142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 30 mM Na^+ Hepes buffer, pH 7.4, and 5.6 mM D-glucose) at a protein concentration of 4 mg/ml. Li^+ (20 mM final concentration) is added at this stage and prevents the breakdown of released inositol phosphates. Because the ability of U-73122 to effectively inhibit oxotremorine-M (Oxo-M, a muscarinic agonist)-stimulated inositol phosphate production is markedly dependent upon protein concentration (11), assays are routinely conducted at a final protein concentration of 0.2–0.3 mg/ml in a final volume of 1 ml. In most experiments, reactions are allowed to proceed for 30 min at 37°C and are terminated by the addition of 1 ml of 20% trichloroacetic acid. Labeled inositol phosphates in neutralized extracts are quantitated by batch-wise anion-exchange chromatography using Dowex formate resins (11). Inclusion of U-73122 at a concentration of 10 μM results in a 70% inhibition of muscarinic agonist-stimulated [^{3}H]inositol phosphate release at all time points examined (Fig. 4). The extent of inhibition is not dependent upon preincubation with the aminosteroid, and the concentration of U-73122 required to elicit a 50% inhibition is 3–4 μM. The ability of U-73122 to inhibit mAChR-stimulated phosphoinositide hydrolysis is the same regardless of whether full agonists (e.g., Oxo-M or carbachol) or partial agonists (e.g., arecoline or bethanechol) are employed (11). The succinimide derivative of U-73122, i.e., U-73343, is much less inhibitory (13% inhibition at 10 μM).

Inhibition of mAChR-Stimulated Inositol Phosphate Production in Digitonin-Permeabilized Cells by U-73122

To probe the mechanism of action of U-73122, the ability of U-73122 to inhibit GTPγS, Oxo-M, or Ca^{2+}-activated PI-PLC activity can be investigated using digitonin-permeabilized cells. SK-N-SH cells (one or two flasks) that have been prelabeled with [^{3}H]inositol are detached and washed once with Puck's D_1 solution. They are then resuspended in a buffer that approximates the intracellular milieu (KGEH: 139 mM K^+ glutamate, 2 mM ATP, 4 mM $MgCl_2$, 10 mM LiCl, 10 mM EGTA, and 30 mM Na^+ Hepes buffer, pH 7.4) containing 20 μM digitonin. Cells are permeabilized for 5 min at 37°C at a protein concentration of 3–4 mg/ml, centrifuged at 600*g* for 5 min, and washed in an equal volume of KGEH buffer (minus digitonin). Cells are then resuspended in KGEH with sufficient Ca^{2+} (3 mM) added to maintain a free [Ca^{2+}] of 60 nM (Ca^{2+}:EGTA molar ratio of 0.3). Permeabilized cells are then routinely incubated for 30 min in the presence or absence of U-73122 and the stimulatory agent (Oxo-M, GTPγS, or Ca^{2+}). As shown in Table 2, the amino-

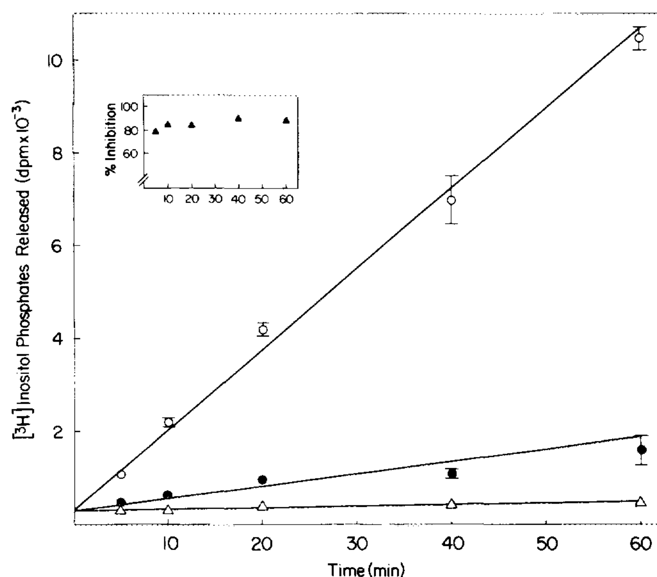

FIG. 4. Time course of U-73122 inhibition of Oxo-M-stimulated phosphoinositide hydrolysis. Intact cells (0.2–0.3 mg protein/ml), prelabeled with [^{3}H]inositol, were incubated either with Buffer A (△) or with 1 mM Oxo-M in the absence (○) or presence (●) of 10 μM U-73122 (added simultaneously) at 37°C for the times indicated. Reactions were terminated by the addition of trichloroacetic acid and a total inositol phosphate fraction was isolated. Results are expressed as inositol phosphate release as a function of time. Values are the means ± SEM for triplicate replicates from one of four experiments that gave similar results. No further inhibition was observed when cells were preincubated with U-73122 for 5 min at 37°C prior to the addition of Oxo-M. Inclusion of U-73122 had no discernible effect on basal release of inositol phosphates. Inset: Calculated percentage inhibition of Oxo-M-stimulated phosphoinositide hydrolysis observed in the presence of U-73122 as a function of time. [Data taken from Ref. (11) with permission.]

steroid was more effective as an inhibitor of Oxo-M, GTPγS, or Oxo-M plus GTPγS-stimulated inositol phosphate production than when PI-PLC was directly activated by the addition of 2 mM free Ca^{2+}. These results indicate that U-73122 may preferentially inhibit the guanine nucleotide regulation of PI-PLC activity. However, the distinction is not absolute, nor is it yet determined whether a G-protein itself or a region of PI-PLC regulated by G-protein interaction is involved.

U-73122 Blocks the mAChR-Mediated Rise in Cytosolic Ca^{2+}

SK-N-SH cells from one confluent flask (containing approximately 10 mg of protein) are first loaded with 1 μM Fura 2/AM for 15 min at 37°C in 10 ml of Buffer A. The cells are then centrifuged at 300*g* for 1 min and the excess dye is removed by washing with a further 40 ml of Buffer A at room temperature. One-milliliter aliquots of dye-loaded cells (0.2–0.3 mg protein/ml) are then incubated in a dual-wavelength Shimadzu RF-500 spectrofluorometer, equipped with a thermostatically controlled cuvette holder maintained at 37°C. The cells (continuously stirred) are allowed to equilibrate for 2–3 min prior to the addition of the agonist. When included, U-73122 (dissolved in DMSO) is present at a concentration of 10 μM and an equivalent volume of DMSO (10 μl) is added to the control incubations (both added 1 min prior to addition of Oxo-M). The fluorescence at excitation wavelengths of 340 and 380 nm (λ emission = 505 nm) is monitored every 1.5 s and changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) are followed for the next 5–10 min. U-73122 (10 μM) blocks (>90%) the rise in $[Ca^{2+}]_i$ induced by Oxo-M addition, whereas the addition of U-73343 at the same concentration has little effect. Neither U-73122 nor U-73343 has any effect on basal $[Ca^{2+}]_i$ under these conditions (Fig. 5).

TABLE 2

Inhibition of Stimulated Hydrolysis of Phosphoinositides in Digitonin-Permeabilized SK-N-SH Cells by U-73122

Agent	Percentage inhibition by U-73122
Oxo-M	86 ± 3
GTPγS	66 ± 5
Oxo-M + GTPγS	69 ± 3
2 mM Ca^{2+}	24 ± 8

Note. Digitonin-permeabilized SK-N-SH cells (0.2–0.3 mg protein/ml) were incubated in KGEH buffer with 1 mM Oxo-M, 50 μM GTPγS, or 2 mM Ca^{2+} (without EGTA) in the absence or presence of 10 μM U-73122. Reactions were terminated after 30 min and a total inositol phosphate fraction was isolated. Results are expressed as the percentage inhibition of inositol phosphate release observed in the presence of U-73122 relative to that in control (DMSO alone) incubations. Values shown are the means ± SEM for seven separate experiments. Release of inositol phosphates in control, Oxo-M, GTPγS, Oxo-M + GTPγS, or 2 mM Ca^{2+} incubations was approximately 250, 2000, 2000, 4500, and 5000 dpm, respectively. In the same series of experiments, inclusion of 10 μM U-73343 resulted in a 25 ± 8% (n = 4) inhibition of Oxo-M-stimulated inositol phosphate release. [Data taken from Ref. (11) with permission.]

U-73122 Inhibits the Agonist-Induced Sequestration of mAChRs

For determination of the ability of U-73122 to disrupt mAChR sequestration, intact SK-N-SH cells are incubated in 50-ml conical centrifuge tubes at a protein concentration of 0.2–0.3 mg/ml in a total volume of 20 ml. This large volume is necessary to ensure that a reasonably sized cell pellet (approx 4–6 mg) can be obtained following the centrifugation and washing procedures. Cells in Buffer A, supplemented with either DMSO or U-73122, are first preincubated at 37°C for 5–10 min. Oxo-M (1 mM final concentration) is then added to initiate the reactions, which are allowed to proceed for 30 min, a time point at which mAChR sequestration is maximal (28). At the end of the incubation period, the cells are centrifuged at 300*g* for 1 min and the pellets are then washed with 20 ml of ice-cold Buffer A. The low temperature of the washing

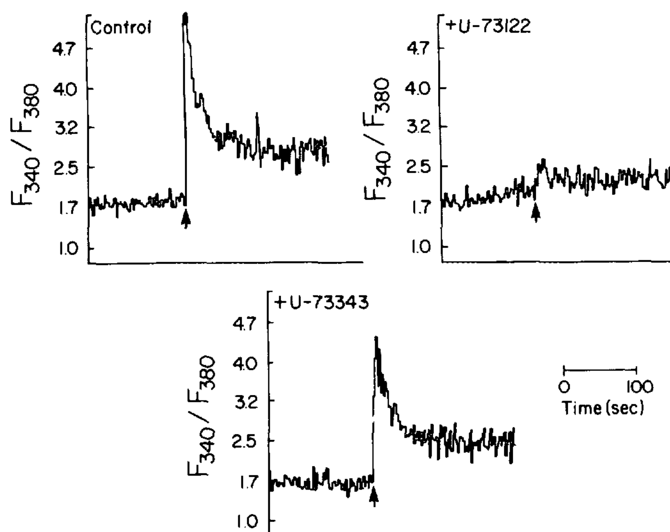

FIG. 5. Effects of U-73122 and U-73343 on the Oxo-M-stimulated rise in $[Ca^{2+}]_i$. Intact cells, loaded with 1 μM Fura 2/AM for 15 min at 37°C, were incubated with 1 mM Oxo-M in the presence of DMSO (control), 10 μM U-73122, or 10 μM U-73343 at a protein concentration of 0.2–0.3 mg/ml. In each trace, the arrow indicates the point of addition of the agonist. The ratios of fluorescence obtained at 340 and 380 nm are shown. Basal $[Ca^{2+}]_i$ values (62 ± 7 nM, n = 9) were unaltered by the presence of either aminosteroid. In the experiment shown, the peak value for $[Ca^{2+}]_i$ upon exposure to Oxo-M was 638 nM, whereas in the presence of U-73122 this was reduced to 80 nM. In contrast, addition of 10 μM U-73343 had little effect on Oxo-M stimulation (peak $[Ca^{2+}]_i$ = 509 nM). In three separate experiments, the addition of Oxo-M increased $[Ca^{2+}]_i$ to 648 ± 23 nM. In the presence of U-73122 and U-73343, the corresponding values were 106 ± 15 and 406 ± 52 nM, respectively. [Data taken from Ref. (11) with permission.]

buffer is necessary to prevent the recovery of sequestered mAChRs back to the cell surface (30). The washing procedure is repeated once more and supernatant is then aspirated. Cell pellets are then resuspended in Buffer A (2 mg protein/ml) and 200-μl samples are taken for radioligand binding assays. The latter are performed in triplicate in a final volume of 2 ml of Buffer A, which contains 6 nM N-[^{3}H]methylscopolamine (NMS), a radiolabeled probe for cell surface mAChRs, in the presence or absence of 50 μM atropine. [^{3}H]NMS binding is routinely assayed at 2–4°C and equilibrium is achieved within 24 h. Reactions are terminated by vacuum filtration through Whatman GF/B glass fiber filters. Radioactivity bound to the cells is trapped on the filter, whereas unbound radioligand (>98% of radioactivity added) passes through and is discarded. Bound radiolabel monitored in the presence of atropine indicates nonspecific binding and is subtracted from the total binding to obtain specific binding. In control cells, the addition of 1 mM Oxo-M results in a 40–50% sequestration of cell surface mAChRs. As indicated in Fig. 6, addition of U-73122 results in a dose-dependent inhibition of mAChR sequestration (IC_{50}, 4.1 μM; maximal inhibition, 65–70%). Inclusion of U-

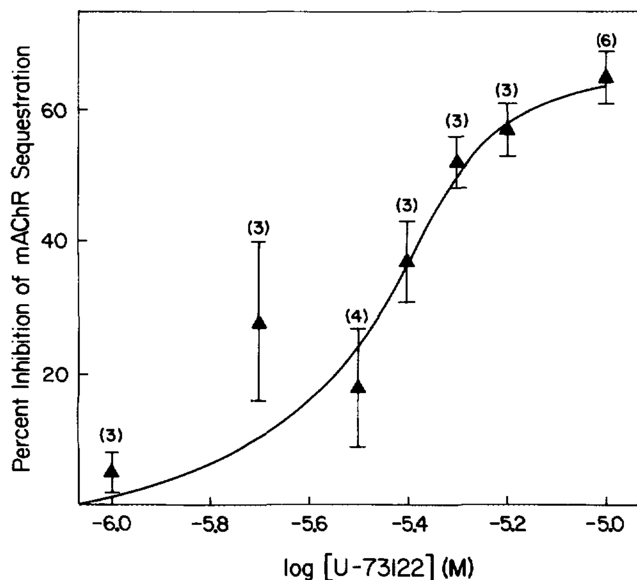

FIG. 6. Dose dependence of U-73122 inhibition of Oxo-M-stimulated mAChR sequestration. Intact cells (0.2–0.3 mg/ml) were first pretreated with U-73122 at the concentrations indicated for 10 min at 37°C and then incubated in the absence or presence of 1 mM Oxo-M for an additional 30 min at 37°C. Cells were washed twice with 20 ml of ice-cold Buffer A and incubated for 18 h at 0°C with 6 nM [^{3}H]NMS. Maximal sequestration of cell surface receptors after a 30-min incubation with 1 mM Oxo-M was 49 ± 2% (n = 20). Results are expressed as the percentage inhibition of maximal sequestration as a function of U-73122 concentration. Values shown are the means ± SEM for the number of separate experiments shown in parentheses. GraphPad analysis indicated an IC_{50} of 4.1 μM and an extrapolated value for maximal inhibition of 65%. [Data taken from Ref. (11) with permission.]

73343 resulted in only a 20% reduction of mAChR sequestration.

The ability of U-73122, at similar concentrations, to inhibit both mAChR sequestration and activation of PI-PLC provides further evidence for a link between these two cellular events. It should be emphasized that mAChR-stimulated inositol phosphate production and receptor sequestration can be dissociated based on their differential sensitivities to chelation of extracellular Ca^{2+} (11). Thus, removal of extracellular Ca^{2+} substantially inhibits mAChR-stimulated inositol phosphate production (~60%) without adversely affecting mAChR sequestration (11). In contrast, U-73122 inhibits both events to similar extents and within the same concentration range. Taken together, results indicate (a) that inositol phosphate production is not required for mAChR sequestration and (b) that U-73122 acts at a site common to the two cellular processes. Based upon the results obtained from permeabilized cells (Table 2), possible sites of action for U-73122 could include either the G-protein that regulates PI-PLC activity or a region of the PI-PLC molecule required for G-protein interaction. From this, one could speculate that G-protein activation is required for the sequestration of mAChRs, as postulated for the internalization of β- and α_2-adrenergic receptors [(31, 32); but see also Ref. (33)]. Further indication of the involvement of a G-protein in mAChR sequestration was obtained from experiments in which inclusion of GDPγS, a nonhydrolyzable analog of GDP, was observed to inhibit the Oxo-M-induced loss of [^{3}H]NMS sites from permeabilized SK-N-SH cells (11). In contrast, GTPγS was without effect. Taken collectively, these results and those obtained with U-73122 point to the possible involvement of a G-protein in mAChR internalization in these cells.

CONCLUSION

In summary, the evidence derived from a variety of cell types, both neural and nonneural, strongly supports the proposition that U-73122 is a relatively specific inhibitor of PI-PLC-dependent events. Because the precise mechanism of inhibition remains unknown, results obtained with this compound should always be interpreted cautiously and the possibility of other nonspecific effects needs to be considered. In the latter regard, the availability of the succinimide derivative, U-73343, is a distinct advantage. Regardless of the detailed mechanism of action, U-73122 has already been of great use in determining the role, if any, played by PI-PLC in certain cell signaling events. To date, the most noteworthy examples of this are in the generation of Ca^{2+} oscillations and transients (13, 20–22) or receptor internalization (11, 25, 26). Now that both PI-PLC and G_q have been purified and successfully reconstituted *in vitro* (34), an appropriate means

for the delineation of the mechanism of inhibition of U-73122 may now be available.

ACKNOWLEDGMENTS

The authors thank Jo Ann Kelsch for secretarial assistance. S.K.F. was supported by NIMH 42652 and NIH 23831.

REFERENCES

1. Streb, H., Irvine, R. F., Berridge, M. J., and Schulz, I. (1983) *Nature* **306,** 67-69.
2. Rhee, S. G., and Choi, R. D. (1992) *Adv. Second Messenger Phosphoprotein Res.* **26,** 35-61.
3. Meldrum, E., Parker, P. J., and Carozzi, A. (1991) *Biochim. Biophys. Acta* **1092,** 49-71.
4. Sternweis, P. C., and Smrcka, A. V. (1992) *Trends Biochem. Sci.* **17,** 502-506.
5. Bleasdale, J. E. (1992) *Drugs of the Future* **17,** 475-487.
6. Powis, G., Lowry, S., Forrai, L., Secrist, P., and Abraham, R. (1991) *J. Cell Pharmacol.* **2,** 39-44.
7. Bleasdale, J. E., Thakur, N. R., Gremban, R. S., Bundy, G. L., Fitzpatrick, F. A., Smith, R. J., and Bunting, S. (1990) *J. Pharmacol. Exp. Therap.* **255,** 756-768.
8. Smith, R. J., Sam, L. M., Justen, J. M., Bundy, G. L., Bala, G. A., and Bleasdale, J. E. (1990) *J. Pharmacol. Exp. Therap.* **253,** 688-697.
9. Bala, G. A., Thakur, N. R., and Bleasdale, J. E. (1990) *Biol. Reprod.* **43,** 704-711.
10. Wu, H., James-Kracke, M. R., and Halenda, S. P. (1992) *Biochemistry* **31,** 3370-3377.
11. Thompson, A. K., Mostafapour, S. P., Denlinger, L. C., Bleasdale, J. E., and Fisher, S. K. (1991) *J. Biol. Chem.* **266,** 23856-23862.
12. Spät, A., Bradford, P. G., McKinney, J. S., Rubin, R. P., and Putney, J. W. (1984) *Nature* **319,** 514-516.
13. Hawes, B. E., Waters, S. B., Janovick, J. A., Bleasdale, J. E., and Conn, P. M. (1992) *Endocrinology* **130,** 3475-3483.
14. Fisher, S. K., McEwen, E., Kunkle, C., Thompson, A. K., and Slowiejko, D. M. (1993) *J. Neurochem.* **60,** 1800-1805.
15. Sagawa, N., Bleasdale, J. E., and Di Renzo, G. C. (1983) *Biochim. Biophys. Acta* **752,** 153-161.
16. Jakobs, K., Lasch, P., Minuth, M., Aktories, K., and Schultz, G. (1982) *J. Biol. Chem.* **257,** 2829-2833.
17. Cockroft, S., and Thomas, G. M. H. (1992) *Biochem. J.* **288,** 1-14.
18. Smith, R. J., Sam, L. M., Leach, K. L., and Justen, J. M. (1992) *J. Leukocyte Biol.* **52,** 17-26.
19. Stojilkovic, S. S., Kukuljan, M., Tomic, M., Rojas, E., and Catt, K. J. (1993) *J. Biol. Chem.* **268,** 7713-7720.
20. Yule, D. I., and Williams, J. A. (1992) *J. Biol. Chem.* **267,** 13830-13835.
21. Yule, D. I., Essington, T. E., and Williams, J. A. (1993) *Am. J. Physiol.,* G786-G791.
22. Yule, D. I., Wu, D., Essington, T. E., Shayman, J. A., and Williams, J. A. (1993) *J. Biol. Chem.* **268,** 12,353-12,358.
23. Frangos, J. A., and Kuchan, M. J. (1992) *FASEB. J.,* A1820.
24. Smallridge, R. C., Kiang, J. G., Gist, I. D., Fein, H. G., and Galloway, R. J. (1992) *Endocrinology* **131,** 1883-1888.
25. Yamada, M., Yamada, M., and Richelson, E. (1992) *Eur. J. Pharmacol.* **226,** 187-188.
26. Galan, J., Tramkina, M., Noel, R., Sprague, E., and Ward, W. (1991) *FASEB J.* **5,** A757.
27. Morley, P., Whitfield, J. F., Vanderhyden, B. C., Tsang, B. K., and Schwartz, J. L. (1992) *Endocrinology* **131,** 1305-1312.
28. Molnar, M., and Hertelendy, F. (1992) *J. Maternal-Fetal Med.* **1,** 1-6.
29. Aschner, J. L., Kendall, P. A., Fletcher, P. W., Lum, H., and Malik, A. B. (1992) *Am. Rev. Respir. Dis.* **145,** A836.
30. Thompson, A. K., and Fisher, S. K. (1990) *J. Pharmacol. Exp. Ther.* **252,** 744-752.
31. McKernan, R. M., Howard, J. J., Motulsky, H. J., and Insel, P. A. (1987) *Mol. Pharmacol.* **32,** 258-265.
32. Cheung, A. H., Sigal, I. S., Dixon, R. A. F., and Strader, C. D. (1989) *Mol. Pharmacol.* **34,** 132-138.
33. Campbell, P. T., Hnatowich, M., O'Dowd, B. F., Caron, M. G., Lefkowitz, R. J., and Hausdorff, W. P. (1991) *Mol. Pharmacol.* **39,** 192-198.
34. Berstein, G., Blank, J. L., Smrcka, A. V., Higashijima, T., Sternweis, P. C., Exton, J. H., and Ross, E. M. (1992) *J. Biol. Chem.* **267,** 8081-8088.
35. Buxser, S. (1993) *NeuroProtocols* **3,** 165-174.