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Effect of the Aminosteroid U73122 on Prostaglandin E_2 Production in a Murine Clonal Osteoblast-like Cell Line, MC3T3-E1

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ABSTRACT. Prostaglandin E_2 production stimulated by various agents (arachidonic acid, prostaglandin $F_{2\alpha}$, ionomycin, the calcium ionophore A23187, and melittin) was investigated after pretreatment of murine osteoblast-like MC3T3-E1 cells with the putative phospholipase C blocker, U73122. The aminosteroid dose dependently inhibited prostaglandin E_2 production induced by all agonists, except arachidonic acid. The results suggest an inhibitory role of U73122 on phospholipase A_2 activity or activation. BIOCHEM PHARMACOL **60**;7: 899–904, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. U73122, phospholipase C, osteoblast

Prostaglandins are synthesized from arachidonic acid by cellular cyclooxygenases and have been shown to modulate several cellular functions in a variety of cell types [1, 2]. In osteoblasts, PGs† also act as local modulators [3], with PGE_2 in particular known to be a potent bone resorbing agent [4]. PGE_2 is the major eicosanoid produced by osteoblasts [4, 5] and clonal osteoblast-like cells derived from newborn mouse calvaria, MC3T3-E1 [6–9]. In the latter, PG synthesis can be attributed to both constitutive and inducible PG synthase enzymes (PGHS-1 and PGHS-2, respectively) [10]. It is well recognized that arachidonic acid release is the rate-limiting step of prostaglandin synthesis [11, 12]. Two major pathways may lead to AA liberation [2]: activation of phospholipase A_2, which directly liberates AA esterified to phospholipids, and the sequential hydrolysis of phosphoinositides by PLC and glycerol lipases. In MC3T3-E1 cells, AA release occurs predominantly via activation of PLA_2 [13, 14]. The aminosteroid U73122 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione) has recently been introduced as a specific blocker of PLC [15, 16]. Furthermore, the compound has been shown to inhibit effects of endothelin-1 and parathyroid hormone on signal transduction in UMR-106 rat ostesarcoma cells [17]. Although considered as specific, the action of U73122 may be more complex. Thus, it was cautioned that the compound may not be selective at concentrations required for maximal blockage of PLC, and that its selectivity is dependent on cell type [18]. There is accumulating evidence that U73122 has many non-specific actions besides its inhibitory effect on PLC, such as direct inhibitory action on PLD activity [19–21], an influence on tyrosine phosphorylation [22], and inhibition of thromboxane A_2 formation in platelets [23].

In the present study, we examined the effects of U73122 on PGE_2 synthesis by osteoblast-like MC3T3-E1 cells challenged with AA, $PGF_{2\alpha}$, ionomycin, the calcium ionophore A23187, and melittin, thereby evaluating the selectivity of the inhibitor.

MATERIALS AND METHODS

Reagents

U73122, PGE_2, $PGF_{2\alpha}$, ionomycin, indomethacin, quinacrine, thapsigargin, A23187, melittin, and HEPES buffer were from Sigma Chemical Co. α-Minimum essential medium and fetal bovine serum were obtained from Sera Lab. L-glutamine was from Serva. Trypsin–EDTA was purchased from Böhringer. Pentafluorobenzyl bromide, bis-(*N*,*O*-trimethylsilyl) trifluoroacetamide, silylation grade pyridine, acetonitrile, and *O*-methoxyamine hydrochloride were from Pierce Chemical Co. Culture dishes were from Falcon via Szabo. MC3T3-E1 cells were kindly donated by Dr. Klaushofer. Deuterated PGE_2 was obtained through MSD Isotopes via IC Chemikalien GmbH. [^{14}C]AA was from Amersham. All other chemicals and reagents were from Merck.

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† *Abbreviations:* PG, prostaglandin; PLA_2, phospholipase A_2; PGHS, prostaglandin endoperoxide synthase; AA, arachidonic acid; PLC, phospholipase C; and ET-1, endothelin-1.

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Cell Culture

MC3T3-E1 cells (passage number 10–20) were cultured routinely in α-minimum essential medium containing 5% fetal bovine serum, gentamycin sulfate (83.4 mg/L), 50 μg/mL of ascorbate, and L-glutamine (0.584 g/L) in a humidified atmosphere of 5% CO_2in 80-cm^2 flasks (initial plating density 2×10^4 cells/cm^2) and transferred to 4-cm^2 12-well culture dishes before experiments. Experiments were carried out at confluency (day 7 of culture).

PGE_2 Analysis

Medium (1 mL) was removed and the cell monolayer incubated in 1 mL of HEPES-buffered (20 mM) Hank's balanced salt solution, containing 3 mM calcium. Incubations with test compounds or vehicle were carried out for 30 min. Preincubations with U73122 were carried out for 10 min. The incubation buffer was removed and PGE_2 measured (GC-NICI-MS) [24]. Briefly, PGE_2 was converted to its pentafluorobenzyl ester-trimethylsilyl ether-*O*-methyloxime derivative. Quantitation was carried out by use of tetradeuterated PGE_2. A Fisons Trio 1000 GC–MS system was used. GC was performed on a 15-m DB-5MS fused silica capillary column (Fisons Instruments). The temperature of the splitless Grob injector was kept at 290° and the initial column temperature was 160° for 1 min, followed by an increase of 40°/min to 310°. Negative ion chemical ionization was carried out in the single ion recording mode with methane as a moderating gas.

Cytosolic Calcium Determination

MC3T3-E1 cells were cultured and grown to confluency as described above. Medium was changed to serum-free α-minimum essential medium containing 2 μM Fura-2/AM. After 45 min, cells were washed twice with the incubation buffer (10 mM HEPES, Ca^{2+}-free, containing 120 mM NaCl, 5 mM KCl, 10 mM glucose, 0.1% BSA, pH 7.4) at 37° and equilibrated in serum-free medium for 15 min before trypsinization. After harvesting, cells were resuspended in incubation buffer and adjusted to a final concentration of 3×10^6 cells/mL. The cell suspension was transferred to a stirred thermoregulated (37°) cuvette. Fluorescence was monitored with excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm on a Shimadzu RF 5001 PC dual-wavelength spectrofluorophotometer. R_{max} (maximum fluorescence ratio) was measured after addition of 50 μg/mL of digitonin and R_{min} (minimum fluorescence ratio) was determined in the presence of EGTA (4 mM). The 340/380 nm ratio was used to determine $[Ca^{2+}]_i$.

[^{14}C]Arachidonate Release

MC3T3-E1 cells were cultured as described and prelabeled with [^{14}C]AA (0.1 μCi/well) for 24 hr. After removal of the culture medium, the cell layer was washed three times with PBS. Incubations with agents or vehicle were carried out for 30 min in 1 mL of HEPES-buffered (20 mM) Hank's balanced salt solution, containing 3 mM $CaCl_2$. Preincubations with U73122 were carried out for 10 min. The supernatant was collected and counted for radioactivity in a Beckman LSC.

Statistical Methods

Statistical analysis was performed with Student's *t*-test for unpaired samples. All data shown are representative of at least 3 independent repeat experiments.

RESULTS

Effect of U73122 on $PGF_{2\alpha}$- Induced PGE_2 Production in MC3T3-E1 Cells

As shown in Fig. 1A, PGE_2 production was dose dependently blocked by U73122. At 25 μM, only basal PG production was observed, the stimulatory effect of $PGF_{2\alpha}$ being totally abolished.

Effect of U73122 on ET-1- Induced PGE_2 Production in MC3T3-E1 Cells

Stimulation of PGE_2 production by ET-1 was also dose dependently blocked by U73122 with an IC_{50} of 5.2 μM, as shown in Fig. 1C. U73122 had no stimulatory action on PG production up to 25 μM.

Effect of U73122 on ET-1- Induced $[Ca^{2+}]_i$ in MC3T3-E1 Cells

Elevation of intracellular calcium induced by ET-1 was also dose dependently blocked by U73122 with a calculated IC_{50} of 3.6 μM, as shown in Fig. 1B.

Effect of U73122 on PGE_2 Production in MC3T3-E1 Cells after PLA_2 Activation

The effect of U73122 on stimulation of PGE_2 synthesis by the calcium ionophore A23187 is demonstrated in Fig. 2A. The compound completely blocked PGE_2 production above 14 μM, with an IC_{50} of approximately 5 μM. In Fig. 2B, the effect after melittin activation is shown. Again, the half-maximal effective dose for U73122 was at 4–5 μM, but inhibition of PGE_2 production was not complete (83% inhibition). Partial inhibition was also observed after ionomycin stimulation (73% reduction in maximal values), as indicated in Fig. 2C. The half-maximal effective dose was 4–5 μM.

Effect of U73122 on AA-Induced PGE_2 Production in MC3T3-E1 Cells

AA-stimulated PGE_2 synthesis was markedly potentiated by U73122 in a dose-dependent manner (Fig. 3). In

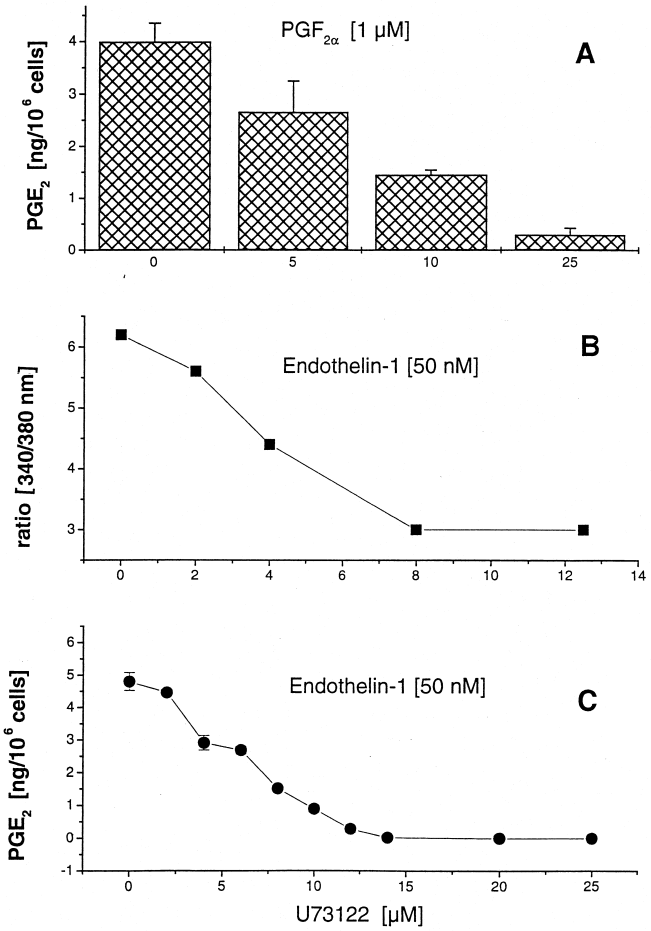

FIG. 1. Effect of U73122 on $PGF_{2\alpha}$ - (A) and ET-1 - (C) induced PGE_2 production and ET-1 -induced elevation of intracellular calcium (B) in MC3T3-E1 cells. Cells were cultured as described in Methods. Experiments were conducted at confluency (day 7 of culture) in HEPES-buffered salt solution as described in Methods. Preincubation with the indicated doses of U73122 was carried out for 10 min, followed by stimulation with $PGF_{2\alpha}$ (1 μM) or ET-1 (50 nM) for 30 min. PGE_2 in the incubation medium and intracellular calcium elevation were measured as described in Methods. Values are given as means ± SEM of 6 determinations.

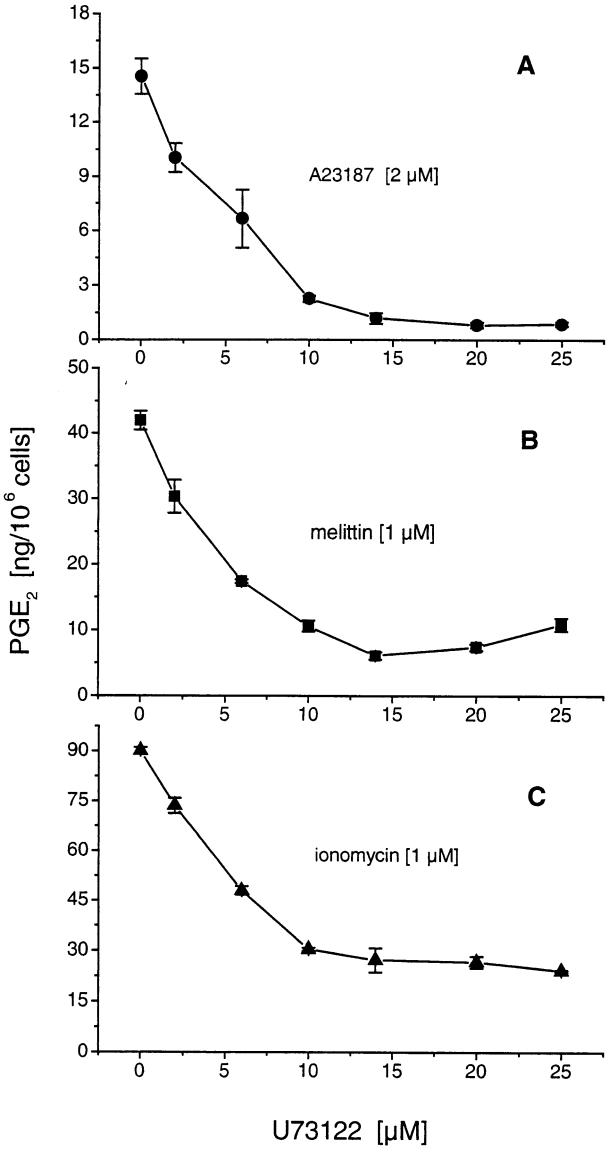

FIG. 2. Effect of U73122 on PGE_2 production induced by (A) A23187 (2 μM), (B) melittin (1 μM), and (C) ionomycin (1 μM) in MC3T3-E1 cells. Cells were cultured as described in Methods. Experiments were conducted at confluency (day 7 of culture) in HEPES-buffered salt solution as described in Methods. Preincubation with the indicated doses of U73122 was carried out for 10 min, followed by stimulation with the agonists for 30 min. PGE_2 in the incubation medium was measured as described. Values are given as means ± SEM of 6 determinations.

absence of inhibitor, AA stimulation produced PGE_2 levels of 16 ng/10^6 cells. At 25 μM U73122, however, PGE_2 values reached 50 ng/10^6 cells (312.5% of untreated cells). Quinacrine (30 μM) did not alter the basal and U73122-induced PGE_2 synthesis. Thapsigargin (100 nM) showed a potentiation of PGE_2 synthesis comparable to U73122. There was no synergism between the latter two agents.

Effect of U73122 on [^{14}C]AA Release in MC3T3-E1 Cells

The results are shown in Fig. 4. There was no significant change in released radioactivity after U73122 treatment in unstimulated cells. In cells challenged with the agonists, radioactivity in the incubation medium was reduced to basal levels by U73122 addition. Pretreatment with indomethacin (0.5 μM) did not show any effect.

DISCUSSION

The results of the present study clearly demonstrate pronounced effects of the putative PLC inhibitor U73122 on PGE_2 synthesis in osteoblast-like MC3T3-E1 cells. Inhibition of $PGF_{2\alpha}$- and ET-1-induced PG formation can certainly be explained by PLC inhibition, since $PGF_{2\alpha}$ exerts

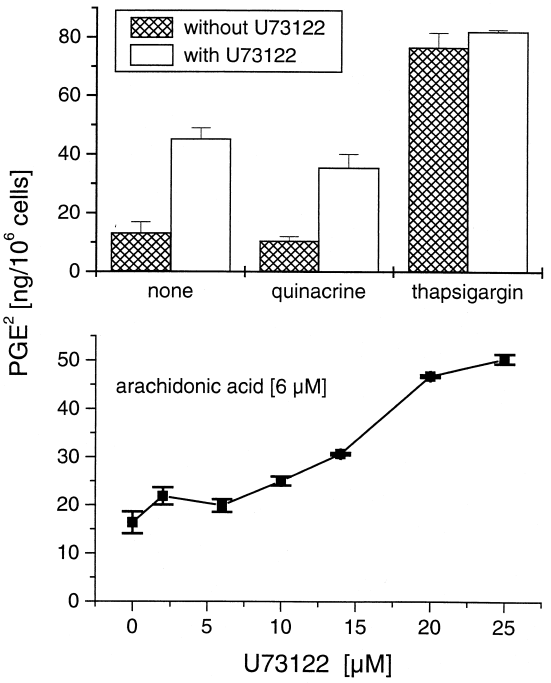

FIG. 3. Effect of U73122 on AA - induced PGE_2 production in MC3T3-E1 cells. Cells were cultured as described in Methods. Experiments were conducted at confluency (day 7 of culture) in HEPES-buffered salt solution as described in Methods. (Upper panel) Preincubation with U73122 (10 μM) was carried out for 10 min, followed by addition of quinacrine (30 μM) or thapsigargin (100 nM). After an additional 10 min, stimulation with AA (6 μM) was carried out for 30 min. (Lower panel) Preincubation with the indicated doses of U73122 was carried out for 10 min, followed by stimulation with AA (6 μM) for 30 min. PGE_2 in the incubation medium was measured as described. Values are given as means ± SEM of 6 determinations.

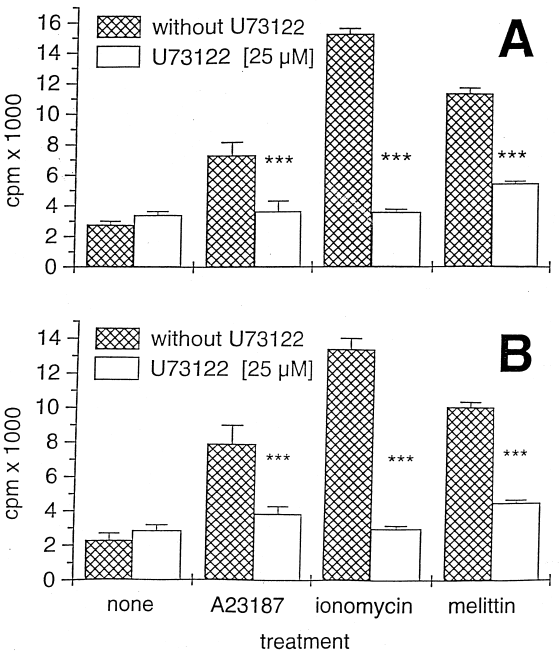

FIG. 4. Effect of U73122 on [^{14}C]AA release in MC3T3-E1 cells in the absence (A) and presence (B) of indomethacin (0.5 μM). Cells were cultured and prelabeled with [^{14}C]AA (0.1 μCi/Well) as described in Methods. Preincubation with the indicated doses of U73122 was carried out for 10 min, followed by stimulation with melittin (2 μM), A23187 (2 μM), ionomycin (2 μM) or vehicle for 30 min. Indomethacin pretreatment was for 10 min. Radioactivity released into the medium was measured by liquid scintillation counting. Values are given as means ± SEM of 6 determinations. ***Significantly different from samples not treated with U73122 ($P < 0.001$).

its effects via G-protein-coupled receptors and subsequent PLC activation in these cells [25]. The resulting formation of inositol trisphosphate leads to release of calcium from intracellular stores and hence to PLA_2 activation. Inhibition of calcium release upon ET-1 stimulation correlates well with the inhibition of prostanoid synthesis, thus suggesting blockage of PLC. This can be extrapolated to the inhibition of bradykinin-induced PGE_2 formation by U73122 in these cells (results not shown). On the other hand, inhibition at the PLC site or prior to this at the level of the heterotrimeric G-proteins would definitely exclude any effects associated with direct activation of PLA_2. Melittin, a known activator of PLA_2, should therefore be unaffected by U73122, as well as PLA_2 stimulation via intracellular calcium elevation by calcium ionophores (A23187 and ionomycin). Single activation of PLA_2 and not the sequential hydrolysis of phosphoinositides by PLC and glycerol lipases has been demonstrated to be responsible for AA liberation in MC3T3-E1 cells [13, 14], a concept basically also favored by others [26, 27]. Interestingly, ionomycin-stimulated PGE_2 production is much more pronounced as compared to A23187. The same effect is observed with both ionophores for [^{14}C]AA release, the values obtained with ionomycin being about twice the levels measured with A23187. This correlates well with the cation-complexing properties of the compounds, since ionomycin binds calcium ions in a 1:1 molar ratio, whereas the calcium–A23187 complex has a 1:2 stoichiometry [28]. There is no alteration of [^{14}C]AA release in the presence of indomethacin, thus excluding any influence of prostanoid-stimulated PLC activity, which has been described to occur in bone cells [29]. Decreased PGE_2 synthesis is definitely not due to PGHS-1 inhibition, as our experiments with AA stimulation revealed. In contrast, AA metabolism is strongly potentiated by U73122, whereas the agent itself had no effect on basal PG levels (results not shown). Explanations for this effect could be that U73122 blocks lipoxygenase activity, thus shunting AA substrate into prostanoid production. In our experiments, however, we were not able to detect any lipoxygenase metabolism in this cell line that could account for the potentiating effect. Effects associated with PGHS-2 induction cannot be ruled out on the basis of our data, but are rather unlikely to account for the strong potentiation by U73122 due to the short incubation period of 10 + 30 min. We have recently shown that PGE_2 production associated with PGHS-2 induction with endothelin reaches its maximum after 3 hrs [10]. PLA_2 activity is required for the redistribution of AA

into phospholipids. This rapidly occurring uptake could be blocked by PLA_2 inhibition via U73122 and thus provide the substrate AA for PGHS enzymes. Failing to produce similar results using the PLA_2 inhibitor quinacrine, however, rules out this possibility of activation.

U73122 has been shown to transiently increase the cytosolic free calcium concentration in rabbit pancreatic acinar cells by inhibition of the internal Ca^{2+} pump [30]. These calcium transients could be too low for PLA_2 activation *per se*, but could possibly be involved in triggering the above-mentioned potentiating activity. This is underlined by the fact that thapsigargin, an inhibitor of the calcium store calcium-ATPase, and U73122 produced a nearly identical potentiation of AA-induced PGE_2 production, and that no synergism was observed.

There is convincing evidence in the literature that U73122 cannot be considered a selective inhibitor of PLC [19–23]. In human platelets, the compound increased tyrosine phosphorylation of platelet protein, but inhibited all further tyrosine phosphorylation on addition of thrombin or other agonists [22]. Since phosphorylation of protein tyrosine kinases, in particular mitogen-activated protein kinases, is crucial to PLA_2 activation, selective inhibition of these kinases could be responsible for the observed effects. This does not seem to be restricted to cells of the osteoblastic phenotype, since in the rat perfused heart a similar behavior with regard to prostacyclin production was observed after stimulation with bradykinin and AA [31].

In conclusion, significant inhibitory effects of the aminosteroid U73122 on PGE_2 synthesis in osteoblast-like MC3T3-E1 cells have been demonstrated. These cannot be attributed to the sole inhibition of PLC. Inhibitory effects on PGHS-1 can be ruled out as well. It is highly probable from our data and those in the literature that U73122 exerts its effects via PLA_2, either directly or via blockage of PLA_2 activation by mitogen-activated protein kinases.

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