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Expression Pattern and Sub-Cellular Distribution of Phosphoinositide Specific Phospholipase C Enzymes After Treatment With U-73122 in Rat Astrocytoma Cells

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ABSTRACT

Phosphoinositide specific phospholipase C (PI-PLC) enzymes interfere with the metabolism of inositol phospholipids (PI), molecules involved in signal transduction, a complex process depending on various components. Many evidences support the hypothesis that, in the glia, isoforms of PI-PLC family display different expression and/or sub cellular distribution under non-physiological conditions such as the rat astrocytes activation during neurodegeneration, the tumoural progression of some neoplasms and the inflammatory cascade activation after lipopolysaccharide administration, even if their role remains not completely elucidated. Treatment of a cultured established glioma cell line (C6 rat astrocytoma cell line) induces a modification in the pattern of expression and of sub cellular distribution of PI-PLCs compared to untreated cells. Special attention require PI-PLC beta3 and PI-PLC gamma2 isoforms, whose expression and sub cellular localization significantly differ after U-73122 treatment. The meaning of these modifications is unclear, also because the use of this N-aminosteroid compound remains controversial, inasmuch it has further actions which might contribute to the global effect recorded on the treated cells. J. Cell. Biochem. 110: 1005–1012, 2010.

KEY WORDS: PHOSPHOLIPASE C; SIGNAL TRANSDUCTION; ASTROCYTOMA; C6 CELLS; U-73122; AMINOSTEROID

Phosphoinositide specific phospholipases C (PI-PLC) constitute a family of enzymes which interfering with the metabolism of inositol phospholipids, molecules involved in signaling and membrane trafficking [Suh et al., 2008]. A growing body of evidence highlighted the presence of nuclear signaling pathways based on lipid hydrolysis [Cocco et al., 2001; Irvine, 2002]. In the inositol lipid pathway, phosphatidylinositol 4,5-bisphosphate (PIP2) is hydrolyzed by means of PI-PLC in response to a wide panel of stimuli, including growth factors, hormones and neurotransmitters, that act on specific receptors localized at the plasma membrane. As a consequence 2 s messengers are generated: 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) [Hisatsune et al., 2005]. The former activates DAG-dependent protein kinase C (PKC) isoforms or is converted to phosphatidic acid, which has also signaling functions; the latter liberates calcium from intracellular stores. The PI-PLC family includes enzymes codified by separate genes and differ for protein structure and tissue distribution. Thirteen mammalian isoforms have been identified and classified into six sub families. Four isoforms belong to PI-PLC beta sub family, two to PI-PLC gamma [Suh et al., 2008], three to PI-PLC delta [Irino et al., 2004], one respectively to PI-PLC epsilon [Wing et al., 2003] and to PI-PLC zeta [Saunders et al., 2002], two to PI-PLC eta [Hwang et al., 2005; Zhou et al., 2005]. Sequence analysis studies demonstrate that each isoenzyme has more than one alternative splicing variant, probably having slightly different activity [Suh et al., 2008]. The distribution of PI-PLC isoenzymes seems strictly

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tissue specific in quiescent cells; this observation emphasizes the hypothesis that each isoenzyme bears a unique function in modulating physiological responses, but these potentialities are far to be completely highlighted. Many studies have been performed to identify the pathways in which PI-PLC isoforms are involved and the interacting molecules. In particular, PI-PLC isoforms have been detected in different tissues, being PI-PLC beta2 and PI-PLC gamma2 preferentially expressed in hematopoietic cell lines [Mao et al., 2000] and PI-PLC beta3 and PI-PLC beta4 in the nervous system [Tanaka and Kondo, 1994; Adamski et al., 1999]. PI-PLC delta1 is widely expressed in many tissues, but especially in cultured cells [Yamaga et al., 1999]. PI-PLC delta3 has been identified in many histotypes at low concentrations [Pawelczyk and Matecki, 1998]; PI-PLC delta4 is expressed in the brain and in regenerating tissues [Pawelczyk, 1999]. PI-PLC gamma1 is expressed in keratinocytes and foetal cartilage [Ananthanarayanan et al., 2002], found almost exclusively in the cytoplasm [McBride et al., 1991; Diakonova et al., 1997]; PI-PLC epsilon occurs in many tissues [Wing et al., 2003], while PI-PLC zeta has been identified only in spermatid cells [Saunders et al., 2002]. PI-PLC eta isoforms, the most recently identified and the less known until today, seem to be highly expressed in neuron-enriched brain regions, suggesting they may be involved in neuro-endocrine system regulation [Suh et al., 2008]. PI-PLCs are involved in myogenic [Faenza et al., 2004] and adipogenic differentiation [O'Carroll et al., 2009], in the progression of myeloid lineage malignancies [Lo Vasco et al., 2004] and other neoplasms [Cocco et al., 2009]. The inositol lipid cycle is involved in and contributes to glial activation during neurodegenerative processes [Vitale et al., 2004] and some isoforms seem to be involved also in the astrocyte response to inflammatory stimuli due to in vitro lypopolysaccharide administration [Lo Vasco et al., 2010]. U-73122 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]exyl]- 1H-pyrrole-2,5-dione), amphiphilic alkylating agent, is an aminosteroid homologue of the thiol reagent N-ethylmaleimide (NEM). U-73122 was developed as a specific inhibitor for PI-PLCs using neutrophils about 20 years ago [Bleasdale et al., 1990]. The inhibition of PI-PLC after U-73122 treatment is supposed to be possibly due to an action at G-protein coupling level [Smallridge et al., 1992]. U-73122 has been frequently used to define the role of PI-PLC mediated elevation of intracellular calcium concentration, indirectly used as a tool to investigate its involvement in signal transduction; some reports, by contrast, described that PI-PLC gamma2 was not inhibited by U-73122 [Hellberg et al., 1996]. In a previous study we obtained a panel of PI-PLC isoforms in neonatal rat astrocytes (supposed to be quiescent cells) and in an established rat glioma cell line (C6 rat astrocytoma cell line), confirming the absence of some isoforms in the quiescent cells and a different expression in the neoplastic counterpart [Lo Vasco et al., 2007]. In the present study, by using a combination of reverse transcriptase polymerase chain reaction (RT-PCR) and immunocytochemistry experiments performed in C6 rat astrocytoma cells, we analyzed the effect of U-73122 on the of PI-PLC isoforms expression and their sub-cellular localization, which also seems to modify their function in regulating cell life and apoptosis.

MATERIALS AND METHODS

CELL CULTURE

Rat astrocytoma cell line C6 (ATCC, Manassas, VA) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA). Cells were seeded into 75-cm^2 flasks at an initial density of $2–3 \times 10^6$ cells per flask and cultured to confluence (14 days) in a 5% CO_2 incubator at 37°C with media changes every 2–3 days. Upon confluence, cells were lifted from the flask surface using 0.25% trypsin/0.02% ethylen diamino tetra acetic acid (EDTA). Cells were then washed, plated on coverslips (15 mm) or in 6-well plates and grown 14 days. U-73122 (Sigma–Aldrich) was addicted to cultures at a concentration of 30 μmol in dimethyl sulfoxide (DMSO) and differently stopped 3, 6, and 24 h after the administration of the molecule depending on the use (suspension in TRIzol reagent for molecular biology analysis or chemical fixation for immunocytochemistry studies).

MOLECULAR BIOLOGY

Cells were suspended in TRIzol reagent 3, 6, and 24 h after U-73122 addiction to the cultures and sotred at −70°C until used. Total RNA was isolated from samples by using TRIzol reagent following the manufacturer's instructions. The obtained RNA was quantitated by the UV absorption ratio 260:280 nm by using a UV/visible spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA); 1 μg of total RNA was reverse transcribed by using SuperScriptIII reverse transcriptase according to manufacturer's instructions. Briefly, total RNA, oligo(dT), dNTP mix and DEPC-treated distilled water were heated for 5 min at 65°C and then buffer (250 mM Tris pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) containing DTT and recombinant RNase Inhibitor was added to the mixture. After incubation for 2 min at 42°C, SuperScript III reverse transcriptase (200 U) was added and incubated for 50 min. All reagents were obtained from Invitrogen. Reaction was stopped by heating for 15 min at 70°C. The final volume was 20 μl. For PCR reactions, the following primer pairs (M-Medical, Florence, Italy) were used: for PI-PLC beta1 reverse 5′-CTG AGC TCA CCC ATG ACA GA 3′, forward 5′-TGC ATA CGT GTC TGG GAC AT 3′; for PI-PLC beta2 reverse 5′-GAC ACA CTC GCT TTG GGA AG-3′, forward 5′-GGG CTC AGC TGC ATT TTA AG-3′; for PI-PLC beta3 reverse 5′-GGA GGA GGA GGA TCA TAG GC-3′, forward 5′-CCC AGA AAG AGT TGG AGC TG-3′; for PI-PLC beta4 reverse 5′-GGA TGC TCT ATC CGA TCC AA-3′, forward 5′-GCT CCT TTG CGT GTT TCT TC-3′; for PI-PLC delta1 reverse 5′-TCA TCC TGT CCC TGG AGA AC-3′, forward 5′-TCA GAC ACG TCA GTG GCT TC-3′; for PI-PLC delta3 reverse 5′-GGG TTG GAA CAG CAG ATT GT-3′, forward 5′-AGT GGG TAC ACT CGG GTC AG-3′; for PI-PLC delta4 reverse 5′-CCT CTG GGA ACA AGA GCA AG-3′, forward 5′-GCT TCA TGG GAT TGA AGG AA-3′; for PI-PLC gamma1 reverse 5′-TAC AGC AGG AAG GCC AGA CT-3′, forward 5′-CTC TCT GGG CCT TGT AGT CG-3′; for PI-PLC gamma2 reverse 5′-GGT TCT GAT GGG CTG TGT TT-3′, forward 5′-GGC AAG ATG GTC TTC AGC TC-3′for PI-PLC epsilon reverse 5′-CGG ATC AAA GGA GGA ATG AA-3′, forward 5′-GCC TCT GGT TGT CAG AAA GC-3′. The specificity of the primers was verified by searching in NCBI data base possible homology to cDNAs of unrelated proteins. Each PCR tube contained the following reagents: 0.2 μM of both sense

and antisense primers, 3–5 μl template cDNA, 0.2 mM dNTP mix, 2.5 U Platinum Taq DNA polymerase and 1× reaction buffer. $MgCl_2$ was added at variable (empirical determination by setting the experiment) final concentration. All reagents were obtained from Invitrogen. The final volume was 50 μl. The amplification was started with an initial denaturation step at 94°C for 2 min and was followed by 35 cycles consisting of denaturation (30 s) at 94°C, annealing (30 s) at the appropriate temperature for each primer pairs and extension (1 min) at 72°C. The PCR products were analyzed by 1.5% TBE agarose gel electrophoresis (Submarine Agarose Gel Unit, Hoefer, San Francisco, CA). Gels were previously stained with ethidium bromide (50 ng/100 ml). A PC-assisted CCD camera (GelDoc 2000 System/Quantity One Software; Bio-Rad) was used for gel documentation and quantification. Optical densities were normalized to the mRNA content of glyceraldehyde 3 phosphate dehydrogenase (GAPDH), a typical reference constitutive transcript. Gel electrophoresis of the amplification products revealed single DNA bands with nucleotide lengths as expected for all primer pairs. To exclude possible DNA contamination during the RT-PCR, RNA samples were amplified by PCR without reverse transcription. No band was observed, suggesting that there was no DNA contamination in the RNA preparation procedure.

IMMUNOCYTOCHEMISTRY

C6 rat astrocytoma cells grown on coverslips were fixed in 3:1 MetOH/Acetic acid 3, 6, and 24 h after the addiction of U-73122 to the cultures. After fixation, cells were washed several times in phosphate buffer (PBS) and first saturation was performed with 1% bovine serum albumin (BSA) in PBS; then incubation with primary antibodies was performed, following the manufacturer's indications with regard to the dilutions, lasting overnight at +4°C. The used primary antibodies (purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were: mouse monoclonal anti PI-PLC beta1, rabbit polyclonal anti PI-PLC beta3, rabbit polyclonal anti-PI-PLC beta4, mouse monoclonal anti-PI-PLC gamma1, rabbit polyclonal anti-PI-PLC gamma2, goat polyclonal anti-PI-PLC delta3, goat polyclonal anti-PI-PLC delta4, goat polyclonal anti-PI-PLC epsilon. As negative controls, also rabbit polyclonal anti-PI-PLC beta2 and goat polyclonal anti-PI-PLC delta1 were used. After several washes with PBS, a second incubation was performed with the secondary fluorochrome conjugated antibody 60 min at room temperature in humified dark chamber. The secondary antibodies were purchased from: Sigma (for FITC conjugated anti-mouse and anti-rabbit); Santa Cruz Biotechnologies (for Texas Red conjugated anti-goat); negative controls were performed by omitting the primary antibody (for the experiments testing PI-PLC beta3, beta4, delta3, and delta4) or by using normal goat IgG (for the experiments testing PI-PLC beta1, gamma1, and epsilon) (data not shown). After several washes performed with PBS in dark chamber, the coverslips were counterstained with diamino phenyl indole (DAPI), mounted on slides and observed with a fluorescence microscope (Nikon Eclipse 800) equipped with NIS-Element-Advanced Research image analysis and acquisition program (Nikon Corporation, Japan).

RESULTS

RT-PCR

Three hours after U-73122 treatment no PI-PLC isoforms were expressed. Six hours after U-73122 treatment PI-PLC beta1, PI-PLC beta2, PI-PLC beta3, PI-PLC beta4, PI-PLC gamma1, and PI-PLC delta1 were not expressed, while the remaining isoforms (PI-PLC gamma2 PI-PLC delta3, PI-PLC delta4, and PI-PLC epsilon) were expressed. Twenty-four hours after U-73122 treatment all the PI-PLC isoforms were expressed excepting for PI-PLC beta2, PI-PLC gamma2, and PI-PLC delta1 (Table I). PI-PLC beta2 and PI-PLC delta1 resulted not expressed at any time of analysis.

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PI-PLC beta1, PI-PLC beta4, and PI-PLC gamma1 were not detected in the nucleus and detected in the cytoplasm after 3 h; they were not detected in any cellular compartment after 6 h; they were not detected in the nucleus and detected in the cytoplasm 24 h after U-73122 treatment. PI-PLC beta3 was absent both in the nucleus and the cytoplasm after 3 and 6 h; it was absent in the nucleus, but was detected in the cytoplasm 24 h after U-73122 treatment. PI-PLC gamma2 was absent in the nucleus, but detected in the cytoplasm after 3 and 6 h, while it was absent in both compartments 24 h after U-73122 treatment. PI-PLC delta3 was detected both in the nucleus and the cytoplasm after 3 and 6 h; it was absent in the nucleus and detected in the cytoplasm 24 h after U-73122 treatment. PI-PLC delta4 was detected both in the nucleus and the cytoplasm after 3 h; it was detected in the nucleus, but absent in the cytoplasm after 6 h; it was detected both in the nucleus and the cytoplasm 24 h after U-73122 treatment. PI-PLC epsilon was detected in the nucleus, but absent in the cytoplasm after 3 h; it was detected both in the nucleus and the cytoplasm 6 and 24 h after U-73122 treatment (Fig. 1) (Table II). PI-PLC beta2 and PI-PLC delta1 were not detected in any cellular compartment at any time of observation (Table II).

DISCUSSION

Signal transduction is a complex process depending on various components including lipid signaling molecules, in particular

TABLE I. RT-PCR Results RT-PCR Expression of PI-PLC Isoforms in Untreated Rat Astrocytoma C6 Cells (Left Column) and in U-73122 Treated Rat Astrocytoma C6 Cells at Different Times After Treatment (Right Columns)

		U 73122 treated C6 cells		
UT C6 cells	PI-PLC	3 h	6 h	24 h
+	Beta1	−	−	+
−	Beta2	−	−	−
+	Beta3	−	−	+
+	Beta4	−	−	+
+	Gamma1	−	−	+
+	Gamma2	−	+	−
−	Delta1	−	−	−
+	Delta3	−	+	+
+	Delta4	−	+	+
+	Epsilon	−	+	+

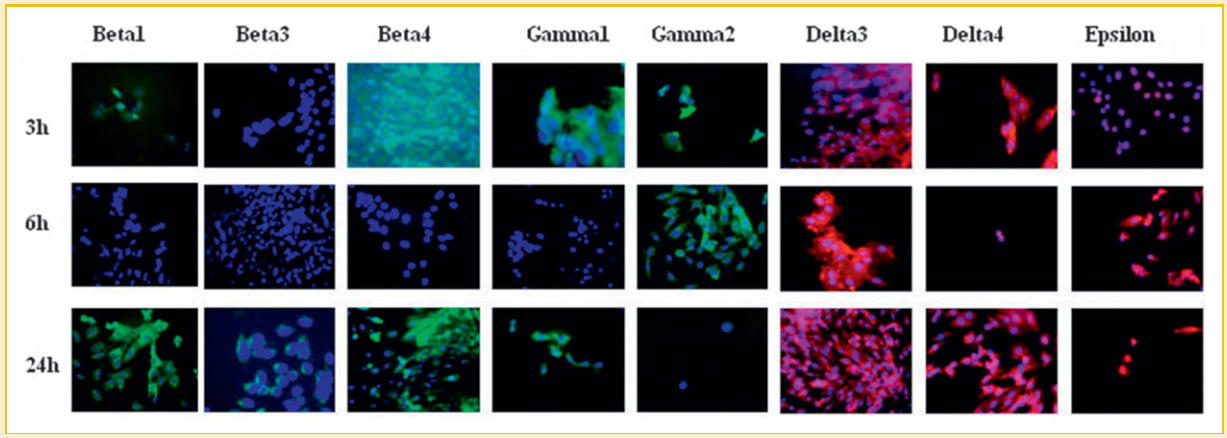

Fig. 1. Microscopy immunofluorescence imaging after immunocytochemistry analysis in U-73122 treated rat astrocytoma C6 cells (40× magnification). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

phosphoinositides and their related enzymes, such as PI-PLCs. The occurrence of different PI-PLC isoforms expression and sub-cellular compartmentalization has been identified in normal and pathological tissues, as nervous tissue (containing mainly beta isoforms) [Martelli et al., 1996; Vitale et al., 2004], liver (PI-PLC beta1, PI-PLC gamma1 and PI-PLC delta1) [Martelli et al., 2004], smooth, skeletal and heart muscle cells (PI-PLC beta1) [Labelle et al., 2002; Faenza et al., 2004]. Various PI-PLC isoforms have also been found in primary human colon carcinoma (PI-PLC gamma1, PI-PLC beta,1 and PI-PLC delta1) [Nomoto et al., 1995] and in hepatoma cell lines (Morris hepatoma H7795 cells—HI and H3924A cells—HII); different expression of some of them has been identified in slow or fast growing hepatoma cells [Santi et al., 2003]. The inositol lipid cycle has also been hypothesized to be involved in numerous neural and glial functions such as glial activation during the neurodegenerative processes [Vitale et al., 2004]. PI-PLCs expression varies after lypopolysaccharide inflammatory stimulation of the astrocytes [Lo Vasco et al., 2010], a glial cell type which under physiological conditions provides nutrient support to neurons, also playing a pivotal role in the response to brain injury in terms of glia activation. In untreated neonatal (supposed to be quiescent) rat astrocytes, our previous study [Lo Vasco et al., 2007] reported that various PI-PLC isoforms are expressed excepting for PI-PLC beta1, PI-PLC beta2, and PI-PLC gamma2. The absence of PI-PLC beta2 and PI-PLC gamma2 accorded to literature data suggesting their strictly restricted expression to hematopoietic lineage. By contrast, in C6 rat astrocytoma cells, PI-PLC beta1, and PI-PLC gamma2 were expressed, while PI-PLC beta2 was not. Also PI-PLC delta1 was not expressed in C6 rat astrocytoma cells, while it was expressed and microscopically detected in neonatal rat astrocytes [Lo Vasco et al., 2007]. U-73122 is considered and experimentally used as a specific inhibitor of PI-PLC enzymes, acting probably by interfering with G-protein coupling [Smallridge et al., 1992]. The electrophilic maleimide side chain of U-73122 is essential, since either its substitution with succinimide or its reaction with dithiotreitol results in loss of the inhibitory activity upon PI-PLCs [Bleasdale et al., 1990; Smith et al., 1990]. U-73122 is known to have further actions, some of these shared to U-73343, an analogue having the electrophilic maleimide group substituted with a succinimide moiety, less active or totally inactive upon PI-PLC [Bleasdale et al., 1990]. Both U-73122 and U-73343 have been reported to block the calcium influx and pore formation induced by maitotoxin in bovine aortic endothelial cells, suggesting a PI-PLC independent inhibitory mechanism for these compounds [Estacion and

TABLE II. Immunocytochemistry Results in Untreated Rat Astrocytoma C6 Cells (Left Columns) and in U-73122 Treated Rat Astrocytoma C6 Cells at Different Times After Treatment (Right Columns)

			Treated C6 cells					
UT C6 cells			3 h		6 h		24 h	
Nucleus	Cytoplasm	PI-PLC	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
−	+	Beta1	−	+	−	−	−	+
−	−	Beta2	−	−	−	−	−	−
+	+	Beta3	−	−	−	−	−	+
+	+	Beta4	−	+	−	−	−	+
−	+	Gamma1	−	+	−	−	−	+
+	+	Gamma2	−	+	−	+	−	−
−	−	Delta1	−	−	−	−	−	−
+	+	Delta3	+	+	+	+	−	+
+	+	Delta4	+	+	+	−	+	+
+	+	Epsilon	+	−	+	+	+	+

Schilling, 2002]. In a study performed on an immortalized C57BL/6 mouse microglial cell line (MG6-1), a low concentration of U-73122 (1–10 μM) inhibited the ATP related activation of P2X7, ATP gated ion channel receptors playing important roles in brain microglial function. Upon the same cell line U-73343 did not inhibit the transient increase of calcium induced by ATP [Takenouchi et al., 2005]. This evidence suggested that U-73122, by using a PI-PLC dependent pathway, specifically inhibits the calcium increase following ATP administration. In the same study, both U-73122 and U-73343 inhibited the sustained calcium increase induced by both ATP (1.5 mM) and BzATP (300 μM), suggesting that this second effect may be attributed to a PI-PLC independent process [Takenouchi et al., 2005]. In many studies U-73343 has been used as selective negative control of U-73122 anti PI-PLC activity. U-73343 exerts effects similar to U-73122 in the affection of many cellular functions, but some results remain controversial and studies suggest that the action of U-73122 and U-73343 may not be as specific as originally thought [Muto et al., 1997; Bosch et al., 1998]. U-73122 has recently been identified as a potent and selective inhibitor of human telomerase, especially in hematopoietic cancer cells [Chen et al., 2006]. The inhibition of human telomerase was attributed primarily to the pyrrole-2,5-dione group, which seems to be a determinant because highly thiol reactive [Chen et al., 2006]. The structural analogue of U-73122, U-73343, did not inhibit telomerase [Chen et al., 2006], demonstrating that the activity of the two compounds does not differ only for the role played as PI-PLCs inhibitors and partially modifying the meaning of their experimental comparative use. Moreover reports describe that U-73122 inhibits or someway affects several cellular functions not involving or that could not be clearly connected to PI-PLC signaling pathway [Jin et al., 1994; Mogami et al., 1997; Walker et al., 1998]. Evidences have also highlighted that molecular targets for U-73122 other than PI-PLCs may exist [Alter et al., 1994; Grierson and Meldolesi, 1995; Wang, 1996; Walker et al., 1998; Hughes et al., 2000]. U-73122 inhibits the dephosphorylation and translocation of cofilin, an actin binding protein playing an important role in the migration, phagocytosis and superoxyde production of activated phagocytes [Matsui et al., 2001] and also inhibits cholecystokinin-(26-33)-peptide amide (CCK-8) induced phospholipase D (PLD) activation in Chinese hamster ovary cells expressing the cholecystokinin-A receptor [Bosch et al., 1998]. U-73122 acts a direct effect at the histamine H1 receptor, also in the concentration range at which it inhibits PI-PLC activity [Hughes et al., 2000] and exhibits strong estrogenic activity possibly by binding to estrogen receptors alfa and beta [Cenni and Picard, 1999].

In a previous study we obtained a panel of PI-PLC isoforms in neonatal rat astrocytes and C6 rat astrocytoma cells, confirming the absence of some isoforms in the quiescent cells and further differences in their expression in the neoplastic counterpart [Lo Vasco et al., 2007]. In the present study, by using RT-PCR, we tested the effect of U-73122 on the expression of PI-PLC isoforms in tumoural cultured cells (C6 rat astrocytoma cell line). After treatment with U-73122 we observed modifications in the expression panel of PI-PLC isoforms with respect to untreated control C6 cells in the immediate period after the administration of the molecule (3–6 h). In fact, C6 rat astrocytoma cells expressed no PI-PLC isoforms' mRNA 3 h after U-73122 treatment. Treated C6 cells expressed the mRNA of only some isoforms (PI-PLC gamma2, PI-PLC delta3, PI-PLC delta4 and PI-PLC epsilon) 6 h after treatment, while the remaining isoforms still resulted unexpressed. A further control, performed 24 h after U-73122 treatment, showed that the PI-PLCs expression pattern returned to that observed in untreated C6 cells, excepting for PI-PLC gamma2, which was unexpressed. This observation opens the way to the hypothesis that a transitory decrease/blockade in the transcription of the PI-PLC genes may be possible.

Also the sub-cellular localization of PI-PLCs is different depending on the tissue and the isoforms, being some of them, such as PI-PLC beta1, mainly nuclear [Cocco et al., 2001], suggesting a different function or timing of action during the cell cycle. To delineate the sub-cellular localization of the isoforms we had found expressed in C6 rat astrocytoma cells after U-73122 treatment, we performed immunocytochemical analysis.

PI-PLC beta2 and PI-PLC delta1 were not detected at any time of the microscopy observations after U-73122 treatment, accordingly to RT-PCR results, confirming their absence in untreated C6 rat astrocytoma cell line [Lo Vasco et al., 2007 and untreated controls of the present experiment].

PI-PLC beta1, which was absent in the nucleus, but present in the cytoplasm of untreated C6 rat astrocytoma cells, 3 h after U-73122 administration had the same distribution that it had before treatment; after 6 h was absent both in the nucleus and in the cytoplasm. After 24 h it was absent in the nucleus and present in the cytoplasm, resembling the distribution that it had before the U-73122 treatment.

PI-PLC beta3, which was present both in the nucleus and in the cytoplasm of untreated C6 rat astrocytoma cells, was absent in both cell compartments after 3 and 6 h, while it was absent in the nucleus and present in the cytoplasm 24 h after U-73122 treatment.

PI-PLC beta4, which was present both in the nucleus and in the cytoplasm of untreated C6 rat astrocytoma cells, was absent in the nucleus and present in the cytoplasm 3 h after U-73122 treatment, being absent in both compartments after 6 h and again absent in the nucleus and present in the cytoplasm after 24 h.

PI-PLC gamma1, which was absent in the nucleus and detected in the cytoplasm of untreated C6 rat astrocytoma cells, had the same distribution that it had before treatment 3 h after U-73122 treatment, was absent in both compartments after 6 h and absent in the nucleus, but present in the cytoplasm 24 h after U-73122 administration, resembling the distribution that it had before U-73122 treatment.

PI-PLC gamma2, which was present both in the nucleus and in the cytoplasm of untreated C6 rat astrocytoma cells, was absent in the nucleus and present in the cytoplasm after 3 and 6 h, while it was absent 24 h after U-73122 treatment, accordingly to RT-PCR results. In the present experiment, PI-PLC gamma2 was the only isoform that resulted unexpressed at the long lasting period of observation after the treatment with U-73122 (24 h).

PI-PLC delta3, which was present both in the nucleus and in the cytoplasm of untreated C6 rat astrocytoma cells, showed the same distribution that it had before U-73122 treatment after 3 and 6 h,

while it was absent in the nucleus and present in the cytoplasm 24 h after U-73122 administration.

PI-PLC delta 4, which was present both in the nucleus and in the cytoplasm of untreated C6 rat astrocytoma cells, shows the same distribution that it had before U-73122 treatment after 3 h, was present in the nucleus and absent in the cytoplasm after 6 h, and again present both in the nucleus and in the cytoplasm 24 h after U-73122 addiction to the cultures, resembling the distribution that it had before treatment.

PI-PLC epsilon, which was present both in the nucleus and in the cytoplasm of untreated C6 rat astrocytoma cells, was present in the nucleus and absent in the cytoplasm after 3 h; it was present both in the nucleus and in the cytoplasm 6 and 24 h after U-73122 treatment, resembling the distribution that it had before the U-73122 administration.

The absence of the mRNA of all the analyzed isoforms in the period briefly following (3 h after) the treatment with U-73122 suggests the possibility that this molecule may someway interact with the transcription of the genes codifying for the enzymes. The detection of the isoforms within the cells at immunofluorescence microscopy observation (all excepting for PI-PLC beta3) 3 h after the addiction of U-73122 to the cultures might be due to the presence of storage proteins synthesized before the treatment. In fact, the sub-cellular distribution of some of these isoforms (PI-PLC beta1, PI-PLC delta3 and PI-PLC delta4) is the same detected in untreated C6 cells. Our results do not allow to confirm that the expression and/or the sub-cellular distribution of PI-PLCs is significantly modified by the administration of U-73122 in C6 rat astrocytoma cell line, excepting for PI-PLC beta3 and PI-PLC gamma2, whose behavior requires further comments. PI-PLC beta3 is expressed both in quiescent astrocytes and in C6 rat astrocytoma cells (in our previous study and in the controls of the present experiment), suggesting that its expression does not probably contribute to the progression of the neoplastic phenotype in astrocytes, even if its sub-cellular distribution resulted different. In fact PI-PLC beta3 is localized in the nucleus of neonatal quiescent rat astrocytes, but both in the nucleus and in the cytoplasm of C6 rat astrocytoma cells [Lo Vasco et al., 2007]. In the present experiment, as all the other isoforms, the mRNA for PI-PLC beta3 was not found after 3 h from the U-73122 treatment, but, differently from all the other isoforms, also the protein was not found within the treated cells, probably indicating the complete depletion of the cell storage of this isoform. PI-PLC beta3 is the only one isoform undetectable at immunofluorescence observation 3 h after U-73122 treatment; it was undetected also after 6 h and was detected 24 h after U-73122 treatment localized in the cytoplasm of treated cells. Upon the other side, PI-PLC gamma2 is the only one isoform that resulted unexpressed after the long lasting period of observation after the treatment with U-73122 (24 h). Moreover, interestingly, PI-PLC gamma2 isoform had resulted unexpressed in neonatal rat astrocytes [Lo Vasco et al., 2007], accordingly to literature data that suggest its tissue specificity for hematopoietic lineages, but it was found to be expressed in C6 rat astrocytoma cells, both in our previous study [Lo Vasco et al., 2007] and in the untreated control cells of the present experiment. We found that 3 h after U-73122 treatment, no mRNA of PI-PLC gamma2 was expressed; regarding its sub-cellular distribution, in U-73122 treated C6 cells, PI-PLC gamma2 was detected in the cytoplasm, being absent in the nucleus, showing a different pattern of distribution with respect to untreated C6 cells, in which this isoform was localized both in the nucleus and the cytoplasm. The mRNA of PI-PLC gamma2 was expressed and the protein was detected, localized in the cytoplasm, 6 h after the treatment, but surprisingly no mRNA was found and no sub-cellular detection was possible 24 h after U-73122 addiction to the cultures. In our previous study, the appearance of PI-PLC gamma2 and PI-PLC beta1 (absent in neonatal rat astrocytes) and the disappearance of PI-PLC delta1 (present in neonatal rat astrocytes) in C6 rat astrocytoma neoplastic counterpart [Lo Vasco et al., 2007] suggested the hypothesis that a relationship might exist with the neoplastic progression of the cell phenotype, even if it was not possible to speculate any further consideration about the causality, the consequentiality or the role played. In the present experiment, the behaviors of PI-PLC beta3, absent in the treated C6 cells 3 h after U-73122 administration, and PI-PLC gamma2, whose mRNA is unexpressed and the protein is absent in the cell 24 h after the treatment, open the way to further investigations. These results remain controversial both because the expression of the mRNA of PI-PLC gamma2 6 h after U-73122 treatment supports the hypothesis that the transcription had restarted, while it seems to be stopped after 24 h and also because data literature [Hellberg et al., 1996] described that PI-PLC gamma2 might not be inhibited by U-73122, suggesting that mechanisms other than the action of this N-aminosteroid may be implicated. These evidences require further investigations n order to obtain a more accurate definition of the timing both of PI-PLC gamma2 expression and of the presence of this protein within the cell compartments. The observation that no mRNA of any isoform of PI-PLC was found expressed 3 and, for some isoforms, also 6 h after the administration of U-73122 also requires further investigations to evaluate the possibility that U-73122 acts or interferes at gene transcription level.

REFERENCES

Adamski FM, Timms KM, Shieh BH. 1999. A unique isoform of phospholipase Cbeta4 highly expressed in the cerebellum and eye. Biochim Biophys Acta 1444:55–60.

Alter CA, Amagasu M, Shah K, Jolly YC, Major C, Wolf BA. 1994. U-73122 does not specifically inhibit phospholipase C in rat pancreatic islets and insulin-secreting beta-cell lines. Life Sci 54:107–112.

Ananthanarayanan B, Das S, Rhee SG, Murray D, Cho W. 2002. Membrane targeting of C2 domains of phospholipase C-delta isoforms. J Biol Chem 277:3568–3575.

Bleasdale JE, Thakur NR, Gremban RS, Bundy GL, Fitzpatrick FA, Smith RJ, Bunting S. 1990. Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. J Pharmacol Exp Ther 255:756–768.

Bosch RR, Patel AM, Van Emst-de Vries SE, Smeets RL, De Pont JJ, Willems PH. 1998. U73122 and U73343 inhibit receptor-mediated phospholipase D activation downstream of phospholipase C in CHO cells. Eur J Pharmacol 346:345–351.

Cenni B, Picard S. 1999. Two compounds commonly used for phospholipase C inhibition activate the nuclear estrogen receptors. Biochem Biophys Res Commun 261:340–344.

Chen YJ, Sheng WY, Huang PR, Wang TC. 2006. Potent inhibition of human telomerase by U-73122. J Biomed Sci 13(5):667–674.

Cocco L, Martelli AM, Barnabei O, Manzoli FA. 2001. Nuclear inositol lipid signaling. Adv Enzyme Regul 41:361–384.

Cocco L, Faenza I, Follo MY, Billi AM, Ramazzotti G, Papa V, Martelli AM, Manzoli L. 2009. Nuclear inositides: PI-PLC signaling in cell growth, differentiation and pathology. Adv Enzyme Regul 49(1):2–10.

Diakonova M, Chilov D, Arnaoutov A, Alexeyev V, Nikolsky N, Medvedeva N. 1997. Intracellular distribution of phospholipase C gamma 1 in cell lines with different levels of transformation. Eur J Cell Biol 73:360–367.

Estacion M, Schilling WP. 2002. Blockade of maitotoxin-induced oncotic cell death reveals zeiosis. BMC Physiol 2:2.

Faenza I, Bavelloni A, Fiume R, Santi P, Martelli AM, Billi AM, Lo Vasco VR, Manzoli L, Cocco L. 2004. Expression of phospholipase C beta family isoenzymes in C2C12 myoblasts during terminal differentiation. J Cell Physiol 200(2):291–296.

Grierson JP, Meldolesi J. 1995. Calcium homeostasis in mouse fibroblast cells affected by U-73122 a putative phospholipase C b blocker, via multiple mechanisms. Br J Pharmacol 115:11–14.

Hellberg C, Molony L, Zheng L, Andersson T. 1996. Ca2+ signaling mechanisms of the b2 integrin on neutrophils: Involvement of phospholipase Cg2 and Ins(1,4,5)P3. Biochem J 317(Pt 2):403–409.

Hisatsune C, Nakamura K, Kuroda Y, Nakamura T, Mikoshiba K. 2005. Amplification of Ca2+ signaling by diacylglycerol-mediated inositol 1,4,5-trisphosphate production. J Biol Chem 280(12):11723–11730.

Hughes SCF, Gibson WJ, Young JM. 2000. The interaction of U-73122 with the histamine H1 receptor: Implications for the use of U-73122 in defining H1 receptor-coupled signalling pathways. Naunyn-Schmiedebergs Arch Pharmacol 362:555–558.

Hwang JI, Oh YS, Shin KJ, Kim H, Ryu SH, Suh PG. 2005. Molecular cloning and characterization of a novel phospholipase C, PLC-eta. Biochem J 389:181–186.

Irino Y, Cho H, Nakamura Y, Nakahara M, Furutani M, Suh PG, Takenawa T, Fukami K. 2004. Phospholipase C delta-type consists of three isozymes: Bovine PLCdelta2 is a homologue of human/mouse PLCdelta4. Biochem Biophys Res Commun 320:537–543.

Irvine RF. 2002. Nuclear lipid signalling. Sci STKE 150:RE13.

Jin W, Lo TM, Loh HH, Thayer SA. 1994. U-73122 inhibits phospholipase C-dependent calcium mobilization in neuronal cells. Brain Res 642: 237–243.

LaBelle EF, Wilson K, Polyak E. 2002. Subcellular localization of phospholipase C isoforms in vascular smooth muscle. Biochim Biophys Acta 1583: 273–278.

Lo Vasco VR, Calabrese G, Manzoli L, Palka G, Spadano A, Morizio E, Guanciali-Franchi P, Fantasia D, Cocco L. 2004. Inositide-specific phospholipase c beta1 gene deletion in the progression of myelodysplastic syndrome to acute myeloid leukemia. Leukemia 18(6):1122–1126.

Lo Vasco VR, Fabrizi C, Artico M, Cocco L, Billi AM, Fumagalli L, Manzoli FA. 2007. Expression of phosphoinositide-specific phospholipase C isoenzymes in cultured astrocytes. J Cell Biochem 100:952–959.

Lo Vasco VR, Fabrizi C, Fumagalli L, Cocco L. 2010. Expression of phosphoinositide specific phospholipase C isoenzymes in cultured astrocytes activated after stimulation with Lipopolysaccharide. J Cell Biochem 109: 1006–1012.

Mao GF, Kunapuli SP, Koneti Rao A. 2000. Evidence for two alternatively spliced forms of phospholipase C-beta2 in haematopoietic cells. Br J Haematol 110:402–408.

Martelli AM, Lach S, Grill V, Gilmour RS, Cocco L, Narducci P, Bareggi R. 1996. Expression and immunohistochemical localization of eight phospholipase C isoforms in adult male mouse cerebellar cortex. Acta Histochem 98:131–141.

Martelli AM, Manzoli L, Cocco L. 2004. Nuclear inositides: Facts and perspectives. Pharmacol Ther 101:47–64.

Matsui S, Adachi R, Kusui K, Yamaguchi T, Kasahara T, Hayakawa T, Suzuki K. 2001. U-73122 inhibits the dephopshorylation and translocation of cofilin in activated macrophage-like U937 cells. Cell Signal 13:17–22.

McBride K, Rhee SG, Jaken S. 1991. Immunocytochemical localization of phospholipase C-gamma in rat embryo fibroblasts. Proc Natl Acad Sci USA 88(16):7111–7115.

Mogami H, Lloyd Mills C, Gallacher DV. 1997. Phospholipase C inhibitor, U73122, releases intracellular Ca2+, potentiates Ins(1,4,5)P3-mediated Ca2+ release and directly activates ion channels in mouse pancreatic acinar cells. Biochem J 324:645–651.

Muto Y, Nagao T, Urushidani T. 1997. The putative phospholipase C inhibitor U73122 and its negative control, U-73343, elicit unexpected effects on the rabbit parietal cell. J Pharmacol Exp Ther 282:1379–1388.

Nomoto K, Tomita N, Miyake M, Xhu DB, LoGerfo PR, Weinstein IB. 1995. Expression of phospholipases gamma 1, beta 1, and delta 1 in primary human colon carcinomas and colon carcinoma cell lines. Mol Carcinog 12:146–152.

O'Carroll SJ, Mitchell MD, Faenza I, Cocco L, Gilmour RS. 2009. Nuclear PLCbeta1 is required for 3T3-L1 adipocyte differentiation and regulates expression of the cyclin D3-cdk4 complex. Cell Signal 21(6):926–935.

Pawelczyk T. 1999. Isozymes delta of phosphoinositide specific phospholipase C. Acta Biochim Pol 46:91–98.

Pawelczyk T, Matecki A. 1998. Localization of phospholipase C delta3 in the cell and regulation of its activity by phospholipids and calcium. Eur J Biochem 257:169–177.

Santi P, Solimando L, Zini N, Santi S, Riccio M, Guidotti L. 2003. Inositol-specific phospholipase C in low and fast proliferating hepatoma cell lines. Int J Oncol 22:1147–1153.

Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. 2002. PLC zeta: A sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development 129:3533–3544.

Smallridge RC, Kiang JG, Gist ID, Fein HG, Galloway RJ. 1992. U-73122, an aminosteroid phospholipase C antagonist, non-competitively inhibits thyrotropin-releasing hormone effects in GH3 rat pituitary cells. Endocrinology 131:1883–1888.

Smith RJ, Sam LM, Justen JM, Bundy GL, Bala GA, Bleasdale JE. 1990. Receptor-coupled signal transduction in human polymorphonuclear neutrophils: Effects of a novel inhibitor of phospholipase C-dependent precesses on cell responsiveness. J Pharmacol Exp Ther 253:688–697.

Suh PG, Park J, Manzoli L, Cocco L, Peak JC, Katan M, Fukami K, Kataoka T, Yuk S, Ryu SH. 2008. Multiple roles of phosphoinositide-specific phospholipase C isozymes. BMB Rep 41:415–434.

Takenouchi T, Ogihara K, Sato M, Kitani H. 2005. Inhibitory effects of U73122 and U73343 on Ca2+ influx and pore formation induced by the activation of P2X7 nucleotide receptors in mouse microglial cell line. Biochim Biophys Acta 1726:177–186.

Tanaka O, Kondo H. 1994. Localization of mRNAs for three novel members (beta 3, beta 4 and gamma 2) of phospholipase C family in mature rat brain. Neurosci Lett 182:17–20.

Vitale M, Rezzani R, Gobbi G, Ponti C, Matteucci A, Cacchioli A, Ruggeri A, Jr., Cocco L. 2004. Phospholipase-C beta1 is predominantely expressed in the granular layer of rat cerebellar cortex. Int J Mol Med 14(2):161–164.

Walker EM, Bispham JR, Hill SJ. 1998. Nonselective effects of the putative phospholipase C inhibitor, U73122, on adenosine A1 receptor-mediated signal transduction events in Chinese hamster ovary cells. Biochem Pharmacol 56:1455–1462.

Wang JP. 1996. U-73122, an aminosteroid phospholipase C inhibitor, may also block Ca2+ influx through phospholipase C-independent mechanism in neutrophils activation. Naunyn-Schmiedebergs Arch Pharmacol 353:599–605.

Wing MR, Bourdon DM, Harden TK. 2003. PLC-epsilon: A shared effector protein in Ras-, Rho-, and G alpha beta gamma-mediated signaling. Mol Interv 3:273–280.

Yamaga M, Fujii M, Kamata H, Hirata H, Yagisawa H. 1999. Phospholipase C-delta1 contains a functional nuclear export signal sequence. J Biol Chem 274:28537–28541.

Zhou Y, Wing MR, Sondek J, Harden TK. 2005. Molecular cloning and characterization of PLC-eta2. Biochem J 391:667–676.