Avicenna J Med Biochem. 2016 September; 4(2):e34104. doi: 10.17795/ajmb-34104.

Published online 2016 June 6. **Research Article**

Comparison of Phosphoinositide-Specific Phospholipase C Expression Panels of Human Osteoblasts Versus MG-63 and Saos Osteoblast-Like Cells

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Received 2015 October 29; **Revised** 2015 November 11; **Accepted** 2015 November 19.

Abstract

Background: A large number of phospholipase C (PLC) enzymes, both mRNA transcripts and proteins, have been detected in osteoblasts, corroborating the importance of calcium regulation in bone tissue. MG-63 and SaOS-2 human osteosarcoma cell lines are actually considered osteoblast-like cells, and are therefore widely used as experimental models for osteoblasts.
Objectives: Our aim was to verify whether MG-63 or SaOS-2 cells might also represent appropriate experimental osteoblast models for signal transduction studies, with special regard to the phosphoinositide (PI) pathway. We analyzed the expression and the subcellular distribution of enzymes related to calcium signal transduction (the PI-specific PLC family), which are known to possess high cell/tissue specificity.
Materials and Methods: The expression of PLC genes was analyzed by performing RT-PCR experiments. The presence of PLC enzymes and their subcellular distribution within the cells was analyzed with immunofluorescence experiments.
Results: Osteoblasts, MG-63 cells, and SaOS-2 cells have expression panels similar to those of PLC enzymes. However, slight differences were found in the expression of enzymes belonging to the PLCη subfamily.
Conclusions: MG-63 and SaOS-2 osteosarcoma cell lines might not represent appropriate experimental models for studies that aim to analyze signal transduction in osteoblasts.

Keywords: Signal Transduction, Phospholipase C, Osteosarcoma, Calcium, Osteoblasts

1. Background

Osteoblasts play the pivotal role of secreting the matrix components in osseous tissues. In addition to their involvement in bone formation and mineralization, osteoblasts can further differentiate into osteocytes and can provide crucial factors for osteoclast differentiation (1-3). Bone remodeling is strictly related to calcium metabolism. The complex network of signal transduction pathways that regulate calcium concentration has recently attracted great attention, including the phosphoinositide (PI) pathway and the related PI-specific phospholipase C (PLC) enzyme family.

PLC enzymes are involved in calcium-mediated regulation of osteoblast activity in a complex manner. Functional studies have demonstrated that increased calcium levels activate PLC, finally resulting in the sustained elevation of calcium concentration (4). Activated PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP2), a phosphorylated derivative of phosphatidylinositol that is mainly located in the inner half of the plasma membrane lipid bilayer (5-9), into inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces calcium release. DAG can be further cleaved to release arachidonic acid (10) or can activate serine/threonine calcium-dependent protein kinase C enzymes (PKC), also influenced by the IP3-induced calcium increase.

The mammalian PLC family comprises a related group of complex, modular, multi-domain enzymes that cover a broad spectrum of regulatory interactions, including direct binding to G protein subunits, small GTPases from Rho and Ras families, receptor and non-receptor tyrosine kinases, and lipid components of cellular membranes (11). PLC enzymes are thirteen isoforms classified on the basis of the amino acid sequence, domain structure, and mechanism of recruitment into six subfamilies: β (1-4), γ (1-2), δ (1, 3, 4), (1), (1), and η (1-2) (12). PLC was exclusively described

in testes and spermatic cell lines (12, 13).

Our previous studies suggested that selected PLC enzymes are involved in osteosarcoma progression (14-17), probably networking ezrin, a molecule that acts during metastatic spread (16, 17). PI-PLC enzymes are known to be strictly tissue-specific, and each cell type expresses selected isoforms (7, 9, 12). Knowledge of the expression panels of PI-PLC enzymes is a necessary and preliminary tool to address studies about their role in quiescent cells. Moreover, the expression panels of PI-PLC enzymes have been demonstrated to differ in quiescent cells with respect to the pathological or activated counterpart (18-30).

MG-63 (31, 32)and SaOS-2 (33-35) human osteosarcoma cell lines are considered to be osteoblast-like cells, and are widely used as experimental models for osteoblasts. However, no studies have been conducted in order to validate the use of MG-63 or SaOS-2 cell lines as osteoblast experimental models for signal transduction.

2. Objectives

The aim of this study was to investigate whether MG-63 or SaOS-2 cells might represent appropriate experimental osteoblast models for signal transduction studies, with special regard to the PI pathway. In the present paper, we compared the expression and the subcellular distribution of the PLC family enzymes in osteoblasts, MG-63 cells, and SaOS-2 cells.

3. Materials and Methods

3.1. Cell Cultures

We analyzed cultured human osteoblasts and two osteosarcoma cell lines, MG-63 and SaOS-2, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Osteoblasts were characterized using antibodies against osteonectin (ON) and osteocalcin (OC) (data not shown). The initial seeding number of cells was 250,000 for each experiment, and the cells were grown up to a level of 1×10^6 cells for the molecular biology experiments. The cells were cultured as previously described by Lo Vasco et al. in 2013. Briefly, the cells were grown under subconfluent or confluent conditions in medium at 37°C with 5% CO2. The cells were cultured in Dulbecco's minimum essential medium (Sigma) supplemented with 10% fetal bovine serum (GIBCO) with penicillin (100 μg/ml), streptomycin (100 U/ml), and sodium pyruvate. The cells were grown for 24 h, reaching a confluence of around 40% - 60%.

3.2. Molecular Biology

After the confluent monolayer was obtained, the cells were detached and suspended in TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA was isolated following the manufacturer's instructions. The purity of the RNA was assessed using a UV/visible spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA, USA). Next, 1 μg of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, RT buffer, dNTP mix, RT random primers, MultiScribe® reverse transcriptase, RNase inhibitor, and DEPC-treated distilled water were added to RNase-free tubes on ice, then the RNA sample was added. The thermal cycler was programmed as follows: 25°C for 10 minutes, then 37°C for 120 minutes, and the reaction was stopped at 85°C for 5 minutes. The final volume was 20 μL. For PCR reactions, the primer pairs (Bio Basic Inc., Amherst, NY, USA) are listed in Table 1.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control. The primers for GAPDH were: forward 5'-CGAGATCCCTCCAAAATCAA-3' and reverse 5'-GTCTTCTGGGTGGCAGTGAT-3'. The specificity of the primers was verified by searching in the NCBI database for possible homology to cDNAs of unrelated proteins. Each PCR tube contained the following reagents: 0.2 μM of both sense and antisense primers, 1 - 3 μL (approximately 1 μg) of template cDNA, 0.2 mM of dNTP mix, 2.5 U of REDTaq Genomic DNA polymerase (Sigma-Aldrich), and 1X reaction buffer. $MgCl_2$ was added at variable final concentrations (empirical determinations by setting the experiment). The final volume was 50 μL. The amplification was started with an initial denaturation step at 94°C for 2 minutes, followed by 35 cycles consisting of denaturation (30 seconds) at 94°C, annealing (30 seconds) at the appropriate temperature for each primer pair, and extension (1 minute) at 72°C. The PCR products were analyzed with 1.5% TAE ethidium bromide-stained agarose gel electrophoresis (Agarose Gel Unit, Bio-Rad Laboratories S.r.l., Segrate, Italy). A PC-assisted CCD camera with a UVB lamp (Vilber Lourmat, Marne-la-Valle, France) was used for gel documentation. Gel electrophoresis of the amplification products revealed single DNA bands with nucleotide lengths as expected for each primer pair. RNA samples were also amplified by PCR without RT. No band was observed, excluding DNA contamination during the procedure (data not shown). The reaction products were further quantified with the Agilent 2100 bioanalyzer using the DNA 1000 LabChip kit (Agilent Technologies, Germany).

Statistical analyses were applied in order to evaluate cell growth. For in vitro studies, differences were determined either with two-way repeated-measures analysis of

Table 1. PLC Gene Primers Used to Perform PCR Experiments

Genes Primers	Sequences
PLC β1 (PLCB1; OMIM *607120)	
Forward	5'-AGCTCTCAGAACAAGCCTCCAACA-3'
Reverse	5'-ATCATCGTCGTCGTCACTTTCCGT-3'
PLC β2 (PLCB2; OMIM *604114)	
Forward	5'-AAGGTGAAGGCCTATCTGAGCCAA-3'
Reverse	5'-CTTGGCAAACTTCCCAAAGCGAGT-3'
PLC β3 (PLCB3; OMIM *600230)	
Forward	5'-TATCTTCTTGGACCTGCTGACCGT-3'
Reverse	5'-TGTGCCCTCATCTGTAGTTGGCTT-3'
PLC β4 (PLCB4; OMIM *600810)	
Forward	5'-GCACAGCACACAAAGGAATGGTCA-3'
Reverse	5'-CGCATTTCCTTGCTTTCCCTGTCA-3'
PLC γ1 (PLCG1; OMIM *172420)	
Forward	5'-TCTACCTGGAGGACCCTGTGAA-3'
Reverse	5'-CCAGAAAGAGAG CGTGTAGTCG-3'
PLC γ2 (PLCG2; OMIM *600220)	
Forward	5'-AGTACATGCAGATGAATCACGC-3'
Reverse	5'-ACCTGAATCCTGATTTGACTGC-3'
PLC δ1 (PLCD1; OMIM *602142)	
Forward	5'-CTGAGCGTGTGGTTCCAGC-3'
Reverse	5'-CAGGCCCTCGGACTGGT-3'
PLC δ3 (PLCD3; OMIM *608795)	
Forward	5'-CCAGAACCACTCTCAGCATCCA-3'
Reverse	5'-GCCA TTGTTGAGCACGTAGTCAG-3'
PLC δ4 (PLCD4; OMIM *605939)	
Forward	5'-AGACACGTCCCAGTCTGGAACC- 3'
Reverse	5'-CTGCTTCCTCTTCCTCATATTC- 3'
PLC (PLCE; OMIM *608414)	
Forward	5'-GGGGCCACGGTCATCCAC-3'
Reverse	5'-GGGCCTTCATACCGTCCATCCTC-3'
PLC η1 (PLCH1; OMIM *612835)	
Forward	5'-CTTTGGTTCGGTTCCTTGTGTGG-3'
Reverse	5'-GGATGCTTCTGTCAGTCCTTCC-3'
PLC η2 (PLCH2; OMIM *612836)	
Forward	5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3'
Reverse	5'-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3'

variance (ANOVA) with Bonferroni's multiple-comparisons test or Student's t test, using Prism 5.0a software (GraphPad Software, San Diego, CA, USA). A p value of < 0.05 was considered significant.

3.3. Immunofluorescence Analysis

Immunofluorescence localization of all PLC isoforms was performed on coverslipped cultured cells. The cells were washed three times with PBS and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes at 4°C, followed by three washes with PBS. Cells were incubated with primary antibodies diluted in PBS for 1 hour at room temperature. The coverslips were then incubated with the specific secondary antibody Texas Red or fluorescein-conjugated for 1 hour at room temperature. The cells were washed twice with 1X PBS 5 minutes, then counterstained with 4',6-diamidino-2-phenylindole (DAPI) fluorescent staining. The slides were visualized and captured with an Olympus IX50 inverted fluorescence microscope (Olympus, Tokyo, Japan) and processed using Adobe Photoshop 7.0 software.

4. Results

No statistically significant differences were recorded in the cell growth rate in the analyzed cell lines that might invalidate the molecular biology results.

4.1. Molecular Biology

Osteoblasts: PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, PLCD4, and PLCE were expressed. PLCB2 was not expressed. PLCH1 and PLCH2 were inconstantly expressed (Figure 1 and Table 2).

Table 2. RT-PCR Results[a]

PLC	Osteoblasts	MG-63	SaOS-2
PLCB1	+	+	+
PLCB2	-	-	-
PLCB3	+	+	+
PLCB4	+	+	+
PLCG1	+	+	+
PLCG2	+	+	+
PLCD1	+	+	+
PLCD3	+	+	+
PLCD4	-	-	-
PLCE	+	+	+
PLCH1	+	-	+
PLCH2	+	-	-

[a](+) mRNA for PLC enzyme isoforms detected; (-) PLC gene not transcribed.

MG-63: PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, and PLCE were expressed. PLCB2 was inconstantly expressed. PLCD4, PLCH1, and PLCH2 were not expressed (Figure 1, Table 2).

SaOS-2: PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, PLCE, and PLCH1 were expressed. PLCB2, PLCD4, and PLCH2 were not expressed (Figure 1, Table 2).

4.2. Immunofluorescence

In the osteoblasts, the following proteins were detected in the cytoplasm: PLC β1, PLC β4, PLC γ1, PLC δ1, and PLC η1. PLC β3 was detected at the perinuclear level, while PLC η2 was detected at the membrane level (Figure 2).

In the MG-63 cells, the following proteins were detected in the cytoplasm: PLC β1, PLC β3, PLC β4, PLC γ1, PLC δ1, PLC δ3, PLC , PLC η1, and PLC η2 (Figure 3).

In the SaOS cells, the following proteins were detected in the cytoplasm: PLC β1, PLC δ1 and (weakly) PLC γ1. The fluorescence signal for PLC β1 was strong. PLC β4 was detected in the cytoplasm as a perinuclear halo. PLC β2 was also detected as a weak perinuclear halo. PLC η1was detected in the cytoplasm in vesicles with slight membrane reinforcements. PLC η2 was detected in the perinuclear region, distributed in vesicles (Figure 4).

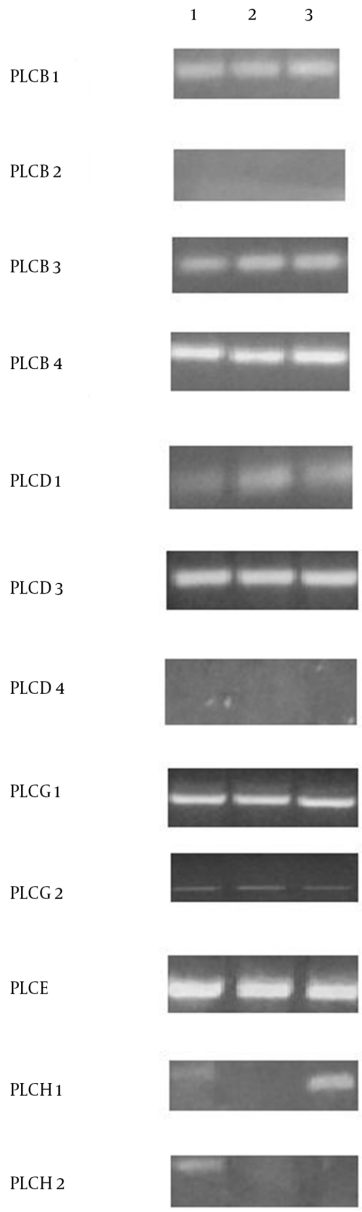
5. Discussion

Bone remodeling is a cyclic and continuous physiological process, which ensures conservation and renewal of the bone matrix. Research efforts were addressed to analyze the metabolism of bone cells and the dynamic nature of the mineralized tissue. Osteosynthesis of the bone matrix is achieved by osteoblasts, strictly related to and coordinated with osteoclast activity, which resorbs the extracellular bone matrix.

Osteoblasts are specialized bone-forming cells with high metabolic activity. In fact, osteoblasts play several important roles in bone remodeling, such as activation of osteoclasts expressing specific factors, production of bone matrix proteins, and bone mineralization (36). Osteoblasts derive from mesenchymal cells and can further differentiate in osteocytes (37). Under specific conditions and depending on many factors, osteoblasts can also differentiate into chondroid bone-forming cells (38). Great attention has been given to the wide number of signal transduction pathways acting in osteoblasts, which coordinate and regulate osteoblasts' complex contribution to bone remodeling. Various signal transduction pathways occurring in osteoblasts have been actively studied (39-43), with special regard to the systems related to complex calcium metabolism (44).

The use of MG-63 or SaOS cell lines as experimental models for human osteoblasts has been widely reported.

Figure 1. RT- PCR Results

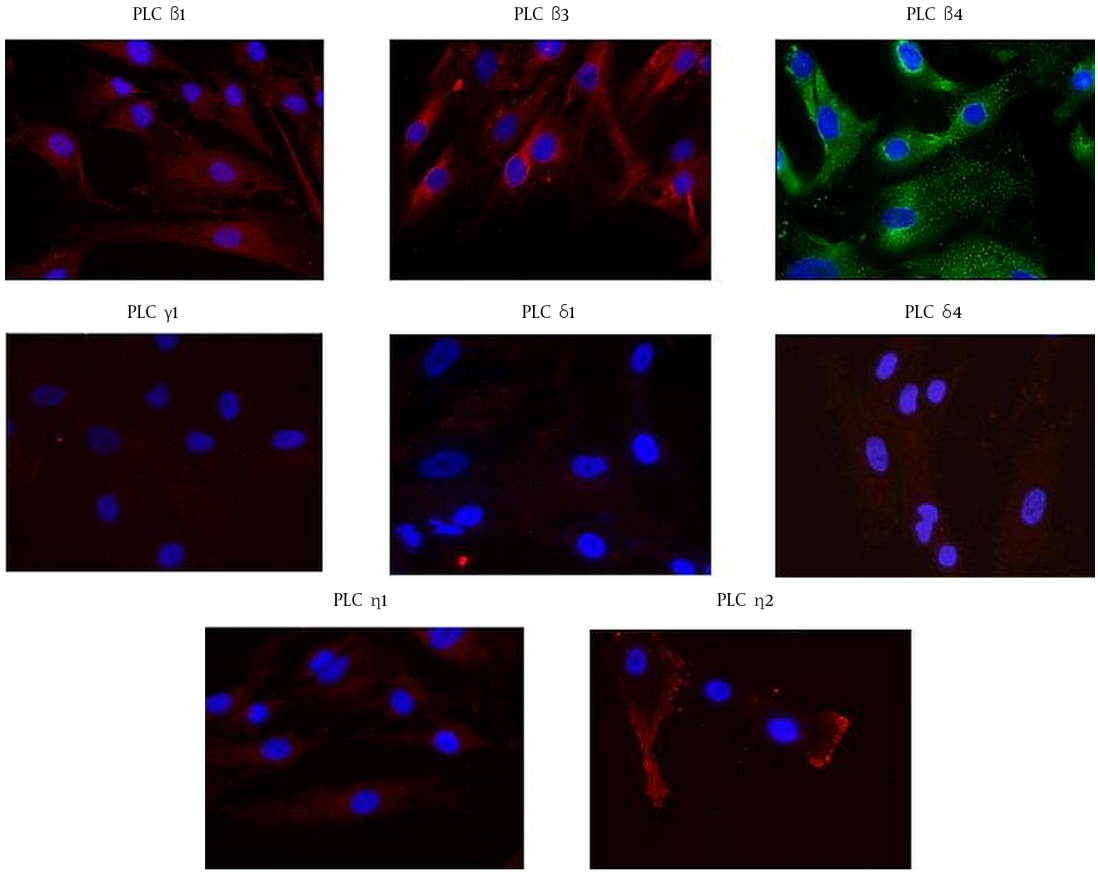


Line 1, osteoblasts; line 2, MG-63 cells; line 3, SaOS-2 cells.

The MG-63 osteosarcoma cell line is commonly used as an experimental model for human osteoblasts (31-33). SaOS is an established epithelial-like osteosarcoma cell line that is also used as an experimental model of osteoblasts (34-36). The aim of the present experiments was to verify whether MG-63 or SaOS-2 cells might also represent appropriate experimental osteoblast models for signal transduction studies, with special regard to the calcium-related PI pathway.

In the present experiments, we analyzed the expression panel and subcellular distribution of PLC enzymes. PLC enzymes are described as highly tissue-specific in normal tissues (18, 19, 21). However, the PLC expression panels in tissues vary under abnormal conditions compared to normal counterparts (20, 21, 23, 24, 26-28). Moreover, the subcellular distribution of PLC enzymes seems to play

Figure 2. Immunofluorescence Microscopy-Acquired Images of PLC Enzymes Within Osteoblasts



Blue: diamino-phenylindole (DAPI) counterstain for nuclei. Green or red: anti-human PLC conjugated antibody.

a role in influencing their activity (45-52).

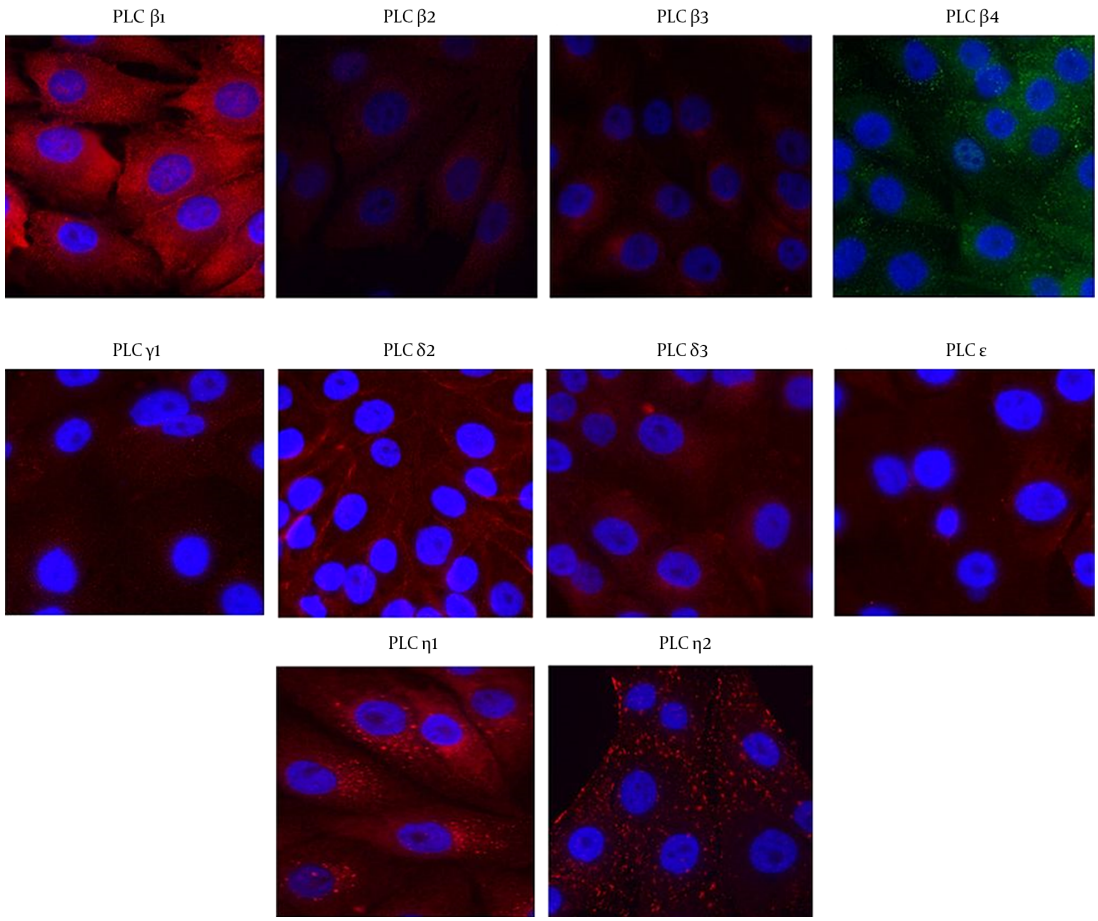
The present results confirm our previous findings regarding the expression of PLC enzymes in MG-63 and SaOS-2 cell lines (14). The present findings also confirm the presence of a wide number of PLC enzymes, both mRNA transcripts and proteins, in osteoblasts, corroborating the importance of calcium regulation in bone tissue. Our present results indicate that osteoblasts, MG-63 cells, and SaOS-2 cells share expression panels similar to those of PLC enzymes. However, slight differences were found in the expression of selected isoenzymes, which deserve some comment.

According to our previous report (14), both MG-63 and SaOS-2 cells express a number of PLC enzymes. MG-63 expressed PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, PLCE, and, inconstantly, PLCB2. SaOS-2 expressed PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, PLCE, and PLCH1. In osteoblasts, PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, and PLCE were expressed, while PLCB2 and PLCD4 were not, and PLCH1 and PLCH2 were only inconstantly expressed.

The most relevant difference among the analyzed cells is represented by the behavior of enzymes belonging to the PLC η subfamily, which comprises two isoforms and is the most recently identified among the PLC families (53-60). Structurally, PLCη enzymes contain long C-terminal extensions with unknown activity (53-56).

PLC η1 acts as a signal amplifier in G protein-coupled receptor (GPCR)-mediated calcium signaling. Knocking down PLC η1, but not PLC-η2, significantly reduces ionomycin-induced PLC activity. Intracellular calcium stores can efficiently activate PLC η1, which suggests that intracellular calcium mobilization from the endoplasmic reticulum plays a pivotal role in PLC η1 activation (59). Similarly, PLC η2 contributes to temporal and spatial calcium dynamics within the cell (58, 61) probably transducing signals arising from mitochondrial calcium. Increased calcium concentration can modulate PLC η2 activity, which

Figure 3. Immunofluorescence Microscopy-Acquired Images of PLC Enzymes Within MG-63 Cells



Blue: diamino-phenylindole (DAPI) counterstain for nuclei. Green or red: anti-human PLC conjugated antibody.

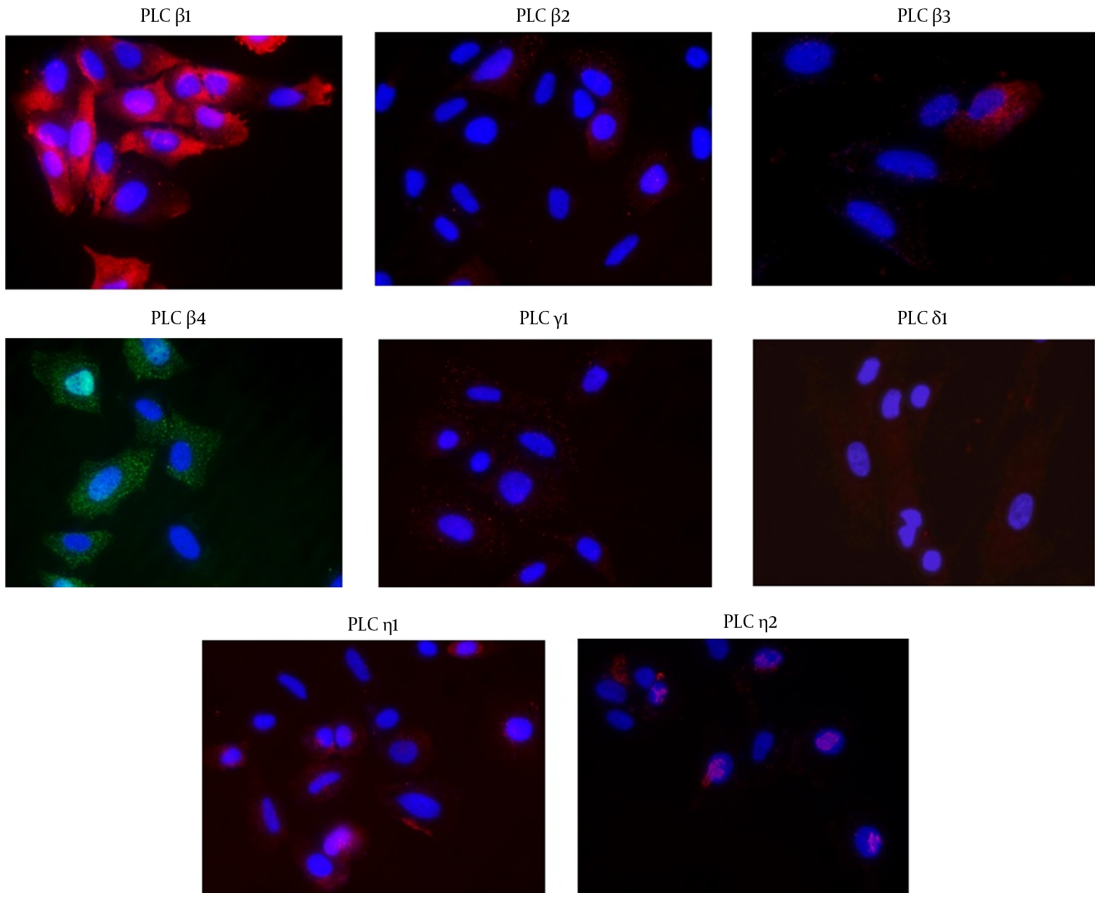
probably indicates that PLC η2 may help regulate calcium-signaling, coordinating both intracellular and extracellular stimuli. The sensitivity of PLC η2 to calcium might favor the amplification of intracellular calcium transients and/or crosstalk between storage compartments. In the present experiments, PLCH1 and PLCH2 were differently, although inconstantly, expressed in osteoblasts compared to MG-63 and SaOS-2 cells. PLCH1 and PLCH2 were transcribed in osteoblasts, while they were not transcribed in MG-63. In SaOS-2 cells, the PLCH1 transcript was detected. Our findings might confirm the highest sensitivity of PLC η subfamily enzymes to calcium concentration. The presence of both PLC η enzymes in osteoblasts might be due to the critical role of calcium regulation in osteoblasts, due to their role in bone remodeling. Our findings will require further studies that address and analyze the specific role of PLC η enzymes in osteoblasts, and their relationship and coordination with enzymes belonging to other PLC subfamilies.

The present results indicate that osteoblasts, MG-63 cells, and SaOS-2 cells share very similar expression panels with PLC enzymes. However, significant differences were identified in the expression of the most newly identified and lesser-known among the PLC subfamilies, the PLC η subfamily. Therefore, the MG-63 and SaOS-2 osteosarcoma cell lines might not represent appropriate experimental models for studies that analyze signal transduction in osteoblasts.

Acknowledgments

The authors thank Serena Talarico Onlus for her encouragement.

Figure 4. Immunofluorescence Microscopy-Acquired Images of PLC Enzymes Within Saos-2 Cells



Blue: diamino-phenylindole (DAPI) counterstain for nuclei. Green or red: anti-human PLC conjugated antibody.

Footnote

Authors' Contribution: Experimental design, data elaboration, writing the article, Vincenza Rita Lo Vasco; experiments, Martina Leopizzi; helping in revision of material and new experiment, Anna Scotto d'Abusco; critical discussion, Carlo Della Rocca.

References

1. Mackie EJ. Osteoblasts: novel roles in orchestration of skeletal architecture. *Int J Biochem Cell Biol.* 2003;**35**(9):1301–5. [PubMed: 12798343].
2. Raggatt LJ, Partridge NC. Cellular and molecular mechanisms of bone remodeling. *J Biol Chem.* 2010;**285**(33):25103–8. doi: 10.1074/jbc.R109.041087. [PubMed: 20501658].
3. Matsuo K, Irie N. Osteoclast-osteoblast communication. *Arch Biochem Biophys.* 2008;**473**(2):201–9. doi: 10.1016/j.abb.2008.03.027. [PubMed: 18406338].
4. Hu F, Pan L, Zhang K, Xing F, Wang X, Lee I, et al. Elevation of extracellular Ca2+ induces store-operated calcium entry via calcium-sensing receptors: a pathway contributes to the proliferation of osteoblasts. *PLoS One.* 2014;**9**(9):107217. doi: 10.1371/journal.pone.0107217. [PubMed: 25254954].
5. Berridge MJ, Dupont G. Spatial and temporal signalling by calcium. *Curr Opin Cell Biol.* 1994;**6**(2):267–74. [PubMed: 7517689].
6. Divecha N, Irvine RF. Phospholipid signaling. *Cell.* 1995;**80**(2):269–78. [PubMed: 7834746].
7. Hisatsune C, Nakamura K, Kuroda Y, Nakamura T, Mikoshiba K. Amplification of Ca2+ signaling by diacylglycerol-mediated inositol 1,4,5-trisphosphate production. *J Biol Chem.* 2005;**280**(12):11723–30. doi: 10.1074/jbc.M409535200. [PubMed: 15637078].
8. Bunney TD, Katan M. PLC regulation: emerging pictures for molecular mechanisms. *Trends Biochem Sci.* 2011;**36**(2):88–96. doi: 10.1016/j.tibs.2010.08.003. [PubMed: 20870410].
9. Fukami K, Inanobe S, Kanemaru K, Nakamura Y. Phospholipase C is a key enzyme regulating intracellular calcium and modulating the phosphoinositide balance. *Prog Lipid Res.* 2010;**49**(4):429–37. doi: 10.1016/j.plipres.2010.06.001. [PubMed: 20553968].
10. Tang CH, Yang RS, Fu WM. Prostaglandin E2 stimulates fibronectin expression through EP1 receptor, phospholipase C, protein kinase Calpha, and c-Src pathway in primary cultured rat osteoblasts. *J Biol Chem.* 2005;**280**(24):22907–16. doi: 10.1074/jbc.M500130200. [PubMed: 15833739].

11. Rhee SG, Kim H, Suh PG, Choi WC. Multiple forms of phosphoinositide-specific phospholipase C and different modes of activation. *Biochem Soc Trans.* 1991;**19**(2):337–41. [PubMed: 1653736].
12. Suh PG, Park JI, Manzoli L, Cocco L, Peak JC, Katan M, et al. Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Rep.* 2008;**41**(6):415–34. [PubMed: 18593525].
13. Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, et al. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development.* 2002;**129**(15):3533–44. [PubMed: 12117804].
14. Lo Vasco VR, Leopizzi M, Chiappetta C, Puggioni C, Di Cristofano C, Della Rocca C. Expression of Phosphoinositide-specific phospholipase C enzymes in human osteosarcoma cell lines. *J Cell Commun Signal.* 2013;**7**(2):141–50. doi: 10.1007/s12079-013-0194-6. [PubMed: 23430511].
15. Lo Vasco VR, Leopizzi M, Stoppoloni D, Della Rocca C. Silencing of phosphoinositide-specific phospholipase C epsilon remodulates the expression of the phosphoinositide signal transduction pathway in human osteosarcoma cell lines. *Anticancer Res.* 2014;**34**(8):4069–75. [PubMed: 25075031].
16. Lo Vasco VR, Leopizzi M, Puggioni C, Della Rocca C. Ezrin silencing remodulates the expression of Phosphoinositide-specific Phospholipase C enzymes in human osteosarcoma cell lines. *J Cell Commun Signal.* 2014;**8**(3):219–29. doi: 10.1007/s12079-014-0235-9. [PubMed: 25073508].
17. Lo Vasco VR, Leopizzi M, Della Rocca C. Ezrin-related Phosphoinositide pathway modifies RhoA and Rac1 in human osteosarcoma cell lines. *J Cell Commun Signal.* 2015;**9**(1):55–62. doi: 10.1007/s12079-015-0265-y. [PubMed: 25618778].
18. Lo Vasco VR, Pacini L, Di Raimo T, D'Arcangelo D, Businaro R. Expression of phosphoinositide-specific phospholipase C isoforms in human umbilical vein endothelial cells. *J Clin Pathol.* 2011;**64**(10):911–5. doi: 10.1136/jclinpath-2011-200096. [PubMed: 21742750].
19. Lo Vasco VR, Leopizzi M, Chiappetta C, Puggioni C, Di Cristofano C, Della Rocca C. Expression of phosphoinositide-specific phospholipase C enzymes in human skin fibroblasts. *Connect Tissue Res.* 2013;**54**(1):1–4. doi: 10.3109/03008207.2012.712584. [PubMed: 22800439].
20. Lo Vasco VR, Polonia P. Molecular cytogenetic interphase analysis of Phosphoinositide-specific Phospholipase C beta1 gene in paraffin-embedded brain samples of major depression patients. *J Affect Disord.* 2012;**136**(1-2):177–80. doi: 10.1016/j.jad.2011.07.023. [PubMed: 21880371].
21. Lo Vasco VR, Fabrizi C, Artico M, Cocco L, Billi AM, Fumagalli L, et al. Expression of phosphoinositide-specific phospholipase C isoenzymes in cultured astrocytes. *J Cell Biochem.* 2007;**100**(4):952–9. doi: 10.1002/jcb.21048. [PubMed: 17063484].
22. Lo Vasco VR, Fabrizi C, Panetta B, Fumagalli L, Cocco L. Expression pattern and sub-cellular distribution of phosphoinositide specific phospholipase C enzymes after treatment with U-73122 in rat astrocytoma cells. *J Cell Biochem.* 2010;**110**(4):1005–12. doi: 10.1002/jcb.22614. [PubMed: 20564200].
23. Lo Vasco VR, Fabrizi C, Fumagalli L, Cocco L. Expression of phosphoinositide-specific phospholipase C isoenzymes in cultured astrocytes activated after stimulation with lipopolysaccharide. *J Cell Biochem.* 2010;**109**(5):1006–12. doi: 10.1002/jcb.22480. [PubMed: 20082315].
24. Lo Vasco VR, Leopizzi M, Chiappetta C, Businaro R, Polonia P, Della Rocca C, et al. Expression of phosphoinositide-specific phospholipase C enzymes in normal endometrium and in endometriosis. *Fertil Steril.* 2012;**98**(2):410–4. doi: 10.1016/j.fertnstert.2012.04.020. [PubMed: 22608312].
25. Lo Vasco VR, Leopizzi M, Della Rocca C, Fais P, Montisci M, Cecchetto G. Impairment and reorganization of the phosphoinositide-specific phospholipase C enzymes in suicide brains. *J Affect Disord.* 2015;**174**:324–8. doi: 10.1016/j.jad.2014.12.006. [PubMed: 25532079].
26. Lo Vasco VR, Leopizzi M, Puggioni C, Della Rocca C, Businaro R. Neuropeptide Y reduces the expression of PLCB2, PLCD1 and selected PLC genes in cultured human endothelial cells. *Mol Cell Biochem.* 2014;**394**(1-2):43–52. doi: 10.1007/s11010-014-2079-2. [PubMed: 24903829].
27. Lo Vasco VR, Leopizzi M, Puggioni C, Della Rocca C, Businaro R. Fibroblast growth factor acts upon the transcription of phospholipase C genes in human umbilical vein endothelial cells. *Mol Cell Biochem.* 2014;**388**(1-2):51–9. doi: 10.1007/s11010-013-1898-x. [PubMed: 24242047].
28. Lo Vasco VR, Leopizzi M, Chiappetta C, Puggioni C, Della Rocca C, Polonia P, et al. Lypopolysaccharide downregulates the expression of selected phospholipase C genes in cultured endothelial cells. *Inflammation.* 2013;**36**(4):862–8. doi: 10.1007/s10753-013-9613-3. [PubMed: 23420070].
29. Lo Vasco VR, Longo L, Polonia P. Phosphoinositide-specific Phospholipase C beta1 gene deletion in bipolar disorder affected patient. *J Cell Commun Signal.* 2013;**7**(1):25–9. doi: 10.1007/s12079-012-0182-2. [PubMed: 23161413].
30. Lo Vasco VR, Cardinale G, Polonia P. Deletion of PLCB1 gene in schizophrenia-affected patients. *J Cell Mol Med.* 2012;**16**(4):844–51. doi: 10.1111/j.1582-4934.2011.01363.x. [PubMed: 22507702].
31. Bonewald LF, Schwartz Z, Swain LD, Boyan BD. Stimulation of matrix vesicle enzyme activity in osteoblast-like cells by 1,25(OH)2D3 and transforming growth factor beta (TGF beta). *Bone Miner.* 1992;**17**(2):139–44. [PubMed: 1611299].
32. Maillard C, Berruyer M, Serre CM, Dechavanne M, Delmas PD. Protein-S, a vitamin K-dependent protein, is a bone matrix component synthesized and secreted by osteoblasts. *Endocrinology.* 1992;**130**(3):1599–604. doi: 10.1210/endo.130.3.1531628. [PubMed: 1531628].
33. Schwartz Z, Dennis R, Bonewald L, Swain L, Gomez R, Boyan BD. Differential regulation of prostaglandin E2 synthesis and phospholipase A2 activity by 1,25-(OH)2D3 in three osteoblast-like cell lines (MC-3T3-E1, ROS 17/2.8, and MG-63). *Bone.* 1992;**13**(1):51–8. [PubMed: 1581109].
34. Fukayama S, Tashjian AJ. Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone (PTH) and PTH-related protein. *Endocrinology.* 1989;**124**(1):397–401. doi: 10.1210/endo-124-1-397. [PubMed: 2909373].
35. Fukayama S, Bosma TJ, Goad DL, Voelkel EF, Tashjian AJ. Human parathyroid hormone (PTH)-related protein and human PTH: comparative biological activities on human bone cells and bone resorption. *Endocrinology.* 1988;**123**(6):2841–8. doi: 10.1210/endo-123-6-2841. [PubMed: 2848688].
36. Rodan SB, Imai Y, Thiede MA, Wesolowski G, Thompson D, Bar-Shavit Z, et al. Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. *Cancer Res.* 1987;**47**(18):4961–6. [PubMed: 3040234].
37. Karsenty G. Transcriptional control of skeletogenesis. *Annu Rev Genomics Hum Genet.* 2008;**9**:183–96. doi: 10.1146/annurev.genom.9.081307.164437. [PubMed: 18767962].
38. Franz-Odendaal TA, Hall BK, Witten PE. Buried alive: how osteoblasts become osteocytes. *Dev Dyn.* 2006;**235**(1):176–90. doi: 10.1002/dvdy.20603. [PubMed: 16258960].
39. Li M, Amizuka N, Oda K, Tokunaga K, Ito T, Takeuchi K, et al. Histochemical evidence of the initial chondrogenesis and osteogenesis in the periosteum of a rib fractured model: implications of osteocyte involvement in periosteal chondrogenesis. *Microsc Res Tech.* 2004;**64**(4):330–42. doi: 10.1002/jemt.20088. [PubMed: 15481050].
40. Partridge NC, Bloch SR, Pearman AT. Signal transduction pathways mediating parathyroid hormone regulation of osteoblastic gene expression. *J Cell Biochem.* 1994;**55**(3):321–7. doi: 10.1002/jcb.240550308. [PubMed: 7962163].
41. ten Dijke P, Fu J, Schaap P, Roelen BA. Signal transduction of bone morphogenetic proteins in osteoblast differentiation. *J Bone Joint Surg Am.* 2003;**85-A Suppl 3**:34–8. [PubMed: 12925607].
42. Yavropoulou MP, Yovos JG. The role of the Wnt signaling pathway in osteoblast commitment and differentiation. *Hormones (Athens).*

2007;**6**(4):279–94. [PubMed: 18055419].
43. Lausson S, Cressent M. Signal transduction pathways mediating the effect of adrenomedullin on osteoblast survival. *J Cell Biochem.* 2011;**112**(12):3807–15. doi: 10.1002/jcb.23311. [PubMed: 21826707].
44. Tatrai A, Lee SK, Stern PH. U-73122, a phospholipase C antagonist, inhibits effects of endothelin-1 and parathyroid hormone on signal transduction in UMR-106 osteoblastic cells. *Biochim Biophys Acta.* 1994;**1224**(3):575–82. [PubMed: 7803518].
45. Wang S, Lukinius A, Zhou Y, Stalberg P, Gobl A, Oberg K, et al. Subcellular distribution of phospholipase C isoforms in rodent pancreas and gastric mucosa. *Endocrinology.* 2000;**141**(7):2589–93. doi: 10.1210/endo.141.7.7533. [PubMed: 10875262].
46. Liu N, Fukami K, Yu H, Takenawa T. A new phospholipase C delta 4 is induced at S-phase of the cell cycle and appears in the nucleus. *J Biol Chem.* 1996;**271**(1):355–60. [PubMed: 8550586].
47. Fukami K, Takenaka K, Nagano K, Takenawa T. Growth factor-induced promoter activation of murine phospholipase C delta4 gene. *Eur J Biochem.* 2000;**267**(1):28–36. [PubMed: 10601847].
48. Hwang SC, Park KH, Ha MJ, Noh IS, Park TB, Lee YH. Distribution of phospholipase C isozymes in normal human lung tissue and their immunohistochemical localization. *J Korean Med Sci.* 1996;**11**(4):305–13. doi: 10.3346/jkms.1996.11.4.305. [PubMed: 8878798].
49. Bertagnolo V, Mazzoni M, Ricci D, Carini C, Neri LM, Previati M, et al. Identification of PI-PLC beta 1, gamma 1, and delta 1 in rat liver: subcellular distribution and relationship to inositol lipid nuclear signalling. *Cell Signal.* 1995;**7**(7):669–78. [PubMed: 8519596].
50. Nishida A, Kaiya H, Tohmatsu T, Banno Y, Wakabayashi S, Nozawa Y. Regional distribution of phosphoinositide-specific phospholipase C activity in rat brain. *Neurosci Lett.* 1988;**90**(3):354–7. [PubMed: 2843800].
51. Graber R, Losa GA. Subcellular localization of inositide enzymes in established T-cell lines and activated lymphocytes. *Anal Cell Pathol.* 1993;**5**(1):1–16. [PubMed: 8093839].
52. Behl B, Sommermeyer H, Goppelt-Strube M, Resch K. Phospholipase C in rabbit thymocytes: subcellular distribution and influences of calcium and GTP gamma S on the substrate dependence of cytosolic and plasma membrane-associated phospholipase C. *Biochim Biophys Acta.* 1988;**971**(2):179–88. [PubMed: 2901860].
53. Hwang JI, Oh YS, Shin KJ, Kim H, Ryu SH, Suh PG. Molecular cloning and characterization of a novel phospholipase C, PLC-eta. *Biochem J.* 2005;**389**(Pt 1):181–6. doi: 10.1042/BJ20041677. [PubMed: 15702972].
54. Nakahara M, Shimozawa M, Nakamura Y, Irino Y, Morita M, Kudo Y, et al. A novel phospholipase C, PLC(eta)2, is a neuron-specific isozyme. *J Biol Chem.* 2005;**280**(32):29128–34. doi: 10.1074/jbc.M503817200. [PubMed: 15899900].
55. Zhou Y, Wing MR, Sondek J, Harden TK. Molecular cloning and characterization of PLC-eta2. *Biochem J.* 2005;**391**(Pt 3):667–76. doi: 10.1042/BJ20050839. [PubMed: 16107206].
56. Stewart AJ, Mukherjee J, Roberts SJ, Lester D, Farquharson C. Identification of a novel class of mammalian phosphoinositol-specific phospholipase C enzymes. *Int J Mol Med.* 2005;**15**(1):117–21. [PubMed: 15583837].
57. Kanemaru K, Nakahara M, Nakamura Y, Hashiguchi Y, Kouchi Z, Yamaguchi H, et al. Phospholipase C-eta2 is highly expressed in the habenula and retina. *Gene Expr Patterns.* 2010;**10**(2-3):119–26. doi: 10.1016/j.gep.2009.12.004. [PubMed: 20060933].
58. Popovics P, Beswick W, Guild SB, Cramb G, Morgan K, Millar R. P. . Phospholipase C-η2 is activated by elevated intracellular Ca2+ levels. Cell Signal. *Cell Signal.* 2011;**23**:1777.
59. Kim JK, Choi JW, Lim S, Kwon O, Seo JK, Ryu SH, et al. Phospholipase C-eta1 is activated by intracellular Ca(2+) mobilization and enhances GPCRs/PLC/Ca(2+) signaling. *Cell Signal.* 2011;**23**(6):1022–9. doi: 10.1016/j.cellsig.2011.01.017. [PubMed: 21262355].
60. Zhou Y, Sondek J, Harden TK. Activation of human phospholipase C-eta2 by Gbetagamma. *Biochemistry.* 2008;**47**(15):4410–7. doi: 10.1021/bi800044n. [PubMed: 18361507].
61. Popovics P, Lu J, Nadia Kamil L, Morgan K, Millar RP, Schmid R, et al. A canonical EF-loop directs Ca(2+) -sensitivity in phospholipase C-eta2. *J Cell Biochem.* 2014;**115**(3):557–65. doi: 10.1002/jcb.24690. [PubMed: 24123053].