PHOSPHOLIPASE C IS A TRANSMEMBRANE PROTEIN: IMMUNOCYTOCHEMICAL EVIDENCE

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Phospholipase C is a key enzyme for transmembrane signalling, since it generates phosphoinositide-derived intracellular messengers. The enzyme localization was studied by indirect immunofluorescent technique, using a rabbit monospecific antibody directed to the purified bacterial phospholipase C. This antibody, which is inhibitory *in vitro* and on phospholipase C of intact cells, cross-reacted with the enzyme from a variety of cell types (skeletal and smooth muscle cells, nerve cells, fibroblasts, leukocytes, erythrocytes) and species (frog, guinea-pig, rat, man). The reaction was specific, as demonstrated by using the F(ab')₂ fragment of the antibody molecule. Since the membrane-bound phospholipase C can be revealed by immunofluorescence on the surface of isolated, intact cells, it is concluded that the membrane-associated phospholipase C is a transmembrane protein.

There is a growing body of evidence that the breakdown of cell membrane polyphosphoinositides plays an important role in signal transduction. The key event is the receptor-induced activation of phospholipase C¹ (PLC), which hydrolyses membrane phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (4, 6, 23, 24). Inositol 1,4,5-trisphosphate causes a rapid rise in cytosolic free Ca²⁺ by releasing it from the endo(sarco)plasmic reticulum (4, 6) and by inhibiting the calcium-extrusion ATPase/pump of the plasma membrane (28). Moreover, inositol 1,4,5-trisphosphate is phosphorylated to inositol 1,3,4,5-tetrakisphosphate, which regulates Ca²⁺ entry into the cell from the external medium (16). Diacylglycerol activates protein kinase C, resulting in the phosphorylation of regulatory proteins (23, 24). Numerous reviews appeared in this field during the last three years (e.g. 1, 5, 10, 15, 18, 29, 33).

¹ Phospholipase C is a generic term for any enzyme that hydrolyses the diacylglycerol-phosphate bond in a glycerophospholipid. Although the synonym 'phosphodiesterase' is biochemically correct, it can obviously cause confusion with cyclic nucleotide phosphodiesterase. Therefore we preferred the term phospholipase C. Downes and Michell (10) proposed the name 'phosphoinositidase C', which in our opinion is suitable and attractive, but this term waits recognition.

PLC is a reasonably well characterized enzyme (30, 31, 34), and biochemical studies showed that PLC is located in the cytosol (2, 3, 18, 22) and the cell membrane (3, 9, 13, 32). The relationship between membrane-bound and cytosolic PLC(s) has been controversial, although it is believed that only the membrane-bound enzyme is involved in transmembrane signalling. Moreover, exogenous (bacterial) PLC was used in various experimental models to mimic the effect of agonist-induced phosphoinositide hydrolysis (7, 11, 26, 27).

The aim of this study was to answer two questions: (a) is PLC really located in the cell membrane and (b) is the membrane-bound PLC a transmembrane protein? Our results give a positive answer to both questions.

MATERIALS AND METHODS

Antibodies

Rabbit monospecific antibody directed to PLC from *Clostridium perfringens* (E.C. 3.1.4.3.; Calbiochem, Frankfurt/Main) was obtained as previously described (20, 21). The F(ab')₂ fragment of the antibody molecule was prepared by pepsin digestion as follows: after incubation of the antibody for 24 hr at 37°C with pepsin (3 × crystallized; Serva 3.4.4.1.; 2500 E/mg) at an antibody/enzyme ratio of 10/1 in sodium acetate buffer 0.1 M, pH 4.5, the reaction was stopped by raising pH to 8.0. The F(ab')₂ fragment was purified from the hydrolysate by gel-filtration on a Sephacryl S-200 column, elution buffer Tris 0.1 M, NaCl 0.5 M, pH 8.0, followed by affinity chromatography on a SpA-Sepharose column, elution buffer PBS, to remove the remnant Fc containing impurities.

Cells and tissues

Rat cerebellar and striated muscle (*adductor femoris magnus*) fragments were dissected from ether-anaesthetized animals, cryofixed and sectioned at 10 µm thickness. Single smooth muscle cells from guinea-pig *taenia coli* were obtained by enzymatic digestion with collagenase type XI (Sigma; lot 46F-6838) according to the method of Momose and Gomi (19) modified by Obara (25). Intact striated muscle fibers were isolated from guinea-pig *adductor femoris magnus* according to the method described by Zuurveld *et al.* (34). Single muscle fibers were also mechanically isolated by microdissection from the ventral head of *semitendinosus* muscle of *Rana temporaria*, using the method of Edman and Kiessling (12), modified as previously described (17). The muscle fibers were mounted in a Perpex chamber containing normal Ringer solution. Isometric twitches were obtained by electrical stimulation using 0.2 ms pulses, and trains of pulses (1 s) with a frequency of 16–20 Hz were used to get tetanic contractions. Tension was recorded using a mechano-electrical transducer and signals were displayed on an ENDIM x-y recorder. Conventional glass microelectrodes (about 12 MOhm) were used for intracellular recording of membrane potentials. Human peripheral blood leukocytes from healthy volunteers were obtained and cultured as previously described (21). Monolayers of human quiescent embryo fibroblasts were obtained according to established methods (14).

Indirect immunofluorescent technique

Cryosections or isolated cells were incubated 20 min at room temperature with anti-PLC antibody (5 mg/ml) or F(ab')₂ fragment (0.71 mg/ml) and then washed twice (with PBS containing 1% bovine serum albumin). After an additional incubation of

20 min at room temperature with goat anti-rabbit IgG fluorescein isothiocyanate (FITC) labelled antibody (IgG fraction of serum; Cantacuzino Institute, Bucharest) and two washings, isolated cells and tissue sections were examined with a Leitz Wetzlar Orthoplan fluorescence microscope with epi-illumination, and photographed on Agfa Pan 400 professional film. The goat anti-rabbit IgG FITC labelled antibody was diluted to half the highest concentration that did not yield any significant nonspecific fluorescence (in the absence of incubation with primary antibody). To test the integrity of the plasma membrane of isolated cells, controls were done using human antinuclear antibodies (histone and dsDNA specific; IgG fraction of serum) as primary antibody.

RESULTS

All the investigated cells and tissues, i.e. human fibroblasts, peripheral blood leukocytes and erythrocytes, guinea-pig smooth (Fig. 1) and striated muscle cells, rat striated muscle cells and cerebellar Purkinje cells, frog striated muscle cells (Fig. 2), showed a positive immunofluorescence when incubated with anti-PLC antibody (whole molecule or F(ab')₂ fragment).

The specificity of the fluorescent labelling was shown by the following controls:

(1) Normal rabbit IgG failed to bind to the specimens, giving no detectable fluorescence.

(2) The antigen-specific F(ab')₂ fragment of the monospecific anti-PLC antibody produced an identical labelling to the one obtained when using the whole antibody molecule.

(3) In the case of smooth muscle cells and lymphocytes, an additional proof was the inhibition of membrane PLC activity in the living cells after treatment with the anti-PLC antibody (20, 21; see also Discussion).

Cell viability and implicitly, cell membrane integrity, were tested. For instance, more than 95% of isolated smooth muscle cells (300 cells counted) were capable of trypan blue exclusion, and more than 99% showed no detectable staining with antibodies directed to truly intracellular antigens (histone and dsDNA specific antinuclear antibodies). Isolated frog skeletal muscle fibers had an intact sarcolemma because: (a) a normal resting potential (-80.0 ± 2.3 mV; $n=5$) was recorded, (b) a characteristic action potential could be generated (maximum rate of rise 166 ± 13 mV/ms; $n=5$), and (c) typical isometric twitches and tetanic contractions were recorded after electrical stimulation (Fig. 3) or K⁺ depolarization.

The images of the cryofixed and sectioned tissues showed a strongly fluorescent cell periphery, clearly distinguishable in the case of striated muscle cross-sections (results not shown). An intracellular fluorescence of lower intensity was also visible, but no specific intracellular structures, e.g. the nuclei, were recognized.

DISCUSSION

First to be mentioned is the cross-reactivity between the antibody raised against the purified bacterial PLC and PLC from very different species and cell types. Clark *et al.* (8) also found cross-reactivity between an antibody directed to PLC from *Bacillus cereus* and a phosphatidylcholine-preferring PLC from human monocytes. The en-

FIG. 1. Intact, isolated smooth muscle cell from guinea-pig *taenia coli* free-floating in physiological saline solution. Stained by indirect immunofluorescent method with monospecific antibody against phospholipase C. Anti-phospholipase C antibodies decorate the cell membrane.

FIGS. 2a, b. Single striated muscle fiber isolated by microdissection from frog *semitendinosus* muscle (fiber length: 11 mm). a. Light microscope image. b. The same field stained by indirect immunofluorescent method with monospecific antibody against phospholipase C. Note the strong fluorescence on the sarcolemma.

FIG. 3. Functional control of the isolated striated muscle fiber shown in Figs. 2a, b, prior to immunofluorescent processing. Isometric recording of the twitch (t) and the tetanic contraction (T) induced by electrical stimulation.

zyme probably carries a (some) common epitope(s) during phylogenesis and differentiation. This is supported also by the seemingly contradictory results obtained by Rhee *et al.* (30) and Ryu *et al.* (31), who, after purifying three PLC isozymes of mammalian origin, did not find cross-reactivity, using isozyme-specific monoclonal antibodies. However, the clonal analysis of the isozymes showed two regions in the primary structure having a high degree of homology (>60%) between different PLCs, putatively related to the catalytic site (30), but probably not to the epitopes recognized by the monoclonals used. These regions of homology could explain the results obtained with our polyclonal antibody. If so, one should expect cross-reactivity to occur on a large scale—which makes this anti-PLC antibody a useful tool in the investigation of PLC role or localization in various cells, as we have previously suggested (20, 21). Additional proof was provided by cooperative research with Drs. M. Hirata and F. Murad (Stanford Univ., California, USA), who showed that our antibody inhibited the agonist-induced PLC catalysed inositolphosphate (total or inositol 1,4,5-trisphosphate fraction) generation in cultured smooth muscle (bovine aorta) and kidney (LLC-PK₁) cells (manuscript in preparation), which also directly demonstrates the binding specificity of the antibody to membrane PLC. Moreover, Dr. Phyllis Gardner and collaborators (also from Stanford Univ.) showed that our antibody inhibited the anti-T3 antibody-induced Ca^{2+} rise (which is inositol 1,4,5-trisphosphate dependent) in Jurkat T lymphocytes (personal communication). That our antibody specifically inhibits PLC-depending phenomena was also found by Drs. N. Flavahan and P. Vanhoutte (Mayo Clinic, Rochester, Minnesota, USA) (submitted).

The cross-sections showed that, as expected, there is an important membrane-associated fraction of cellular PLC. Presumably, the weak intracellular fluorescence can be ascribed to the cytosolic PLC pool.

Immunofluorescence results on isolated cells (e.g. Figs. 1, 2) cannot be ascribed to the penetration of anti-PLC antibodies inside the cells, because IgG molecules (MW ~ 150,000 Da) normally do not penetrate the plasmalemma, and control tests (see Results) showed that indeed the plasma membrane of isolated cells was intact.

Therefore, the antibody molecules decorated the surface of the cells by reacting with a (some) epitope(s) of PLC which is (are) exposed on the outer side of the

plasmalemma. Keeping in mind that when activated, PLC hydrolyses membrane polyphosphoinositides to release two intracellular second messengers, these results are strong evidence for PLC being a transmembrane protein with an ectodomain able to bind the antibody. This is also supported by the recent data of Bennett and Crooke (3), showing that ~80% of the membrane-associated fraction of PLC cannot be extracted without using a detergent for complete solubilization, i.e. the interaction with the membrane is not ionic.

Preliminary results using our anti-PLC antibody as primary antibody in indirect immunoelectron microscopy with colloidal gold (10 nm particles) showed gold particles attached to the outer surface of isolated human blood cells (in preparation).

Finally, we can conclude that: (1) our data provide direct evidence that PLC is localized in the cell membrane as a transmembrane protein, and (2) the cross-reactivity between our antibody and the PLC from various cell types and species and its binding to the enzyme ectodomain of intact, living cells, recommend their use to unequivocally establish the contribution of membrane-bound PLC in the triggering of various cell responses to extracellular stimuli.

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REFERENCES

1. Abdel-Latif, A. A.: Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacol. Rev.* 38; 227-272, 1986.
2. Banno, Y., Nakashima, S. and Nozawa, Y.: Partial purification of phosphoinositide phospholipase C from human platelet cytosol: characterization of its three forms. *Biochem. Biophys. Res. Commun.* 136; 713-721, 1986.
3. Bennett, C. F. and Crooke, T.: Purification and characterization of a phosphoinositide-specific phospholipase C from guinea-pig uterus. *J. Biol. Chem.* 262; 13789-13797, 1987.
4. Berridge, M. J.: Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* 212; 849-858, 1984.
5. Berridge, M. J.: Inositol lipids and cell proliferation. *Biochim. Biophys. Acta* 907; 33-45, 1987.
6. Berridge, M. J. and Irvine, R. F.: Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 312; 315-321, 1984.
7. Cernescu, C., Constantinescu St. N., Baltă, F., Popescu, L. M. and Cajal, N.: Interferon-induced antiviral state is inhibited by neomycin and mimicked by diacylglycerol. *Biochem. Biophys. Res. Commun.* 151; 402-407, 1988.
8. Clark, M. A., Schorr, R. G. L. and Bomalaski, J. S.: Antibodies prepared to *Bacillus cereus* phospholipase C cross-react with a phosphatidylcholine preferring phospholipase C in mammalian cells. *Biochem. Biophys. Res. Commun.* 140; 114-120, 1986.
9. Cockcroft, S.: Polyphosphoinositide phosphodiesterase: regulation by a novel guanine nucleotide binding protein, G_p. *Trends Biochem. Sci.* 12; 75-78, 1987.
10. Downes, C. P. and Michel, R. H.: Inositol phospholipid breakdown as a receptor-controlled generator of second messengers. In *Molecular Mechanisms of Transmembrane Signalling*, ed. by P. Cohen and M. D. Houslay, Elsevier Science Publishers, Amsterdam, 1985, p. 3.

11. Drummond, A. H.: Bidirectional control of cytosolic free calcium by thyrotropin-releasing hormone in pituitary cells. *Nature* 315; 752-755, 1985.
12. Edman, K. A. P. and Kiessling, A.: The time course of the active state in relation to sarcomere length and movement studied in single skeletal muscle fibres of the frog. *Acta Physiol. Scand.* 81; 182-196, 1971.
13. Fülle, H.-J., Höer, D., Lache, W., Rosenthal, W., Schultz, G. and Oberdisse, E.: *In vitro* synthesis of ³²P-labelled phosphatidylinositol 4,5-bisphosphate and its hydrolysis by smooth muscle membrane-bound phospholipase C. *Biochem. Biophys. Res. Commun.* 145; 673-679, 1987.
14. Hayflick, L. and Moorhead, P. S.: The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 25; 585-621, 1961.
15. Hirasawa, K. and Nishizuka, Y.: Phosphatidylinositol turnover in receptor mechanism and signal transduction. *Ann. Rev. Pharmacol. Toxicol.* 25; 147-170, 1985.
16. Irvine, R. F. and Moor, R. M.: Microinjection of inositol(1,3,4,5)-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca²⁺ entry. *Biochem. J.* 240; 917-920, 1986.
17. Isac, M. and Isac, R. M.: Effects of 2,4-dichlorophenoxyacetic acid on the excitation-contraction coupling in frog single muscle fibres. *Rev. Roum. Biol.* 30; 141-148, 1985.
18. Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, Th. S., Bross, T. E., Ishii, H., Bansal, V. S. and Wilson, D. F.: The metabolism of phosphoinositide-derived messenger molecules. *Science* 24; 1519-1526, 1986.
19. Momose, K. and Gomi, Y.: Studies on isolated smooth muscle cells. VI. Dispersion procedures for acetylcholine-sensitive smooth muscle cells of guinea-pig. *Jpn. J. Smooth Muscle Res.* 16; 29-36, 1980.
20. Moraru, I. I., Popescu, L. M., Vidulescu, C. and Tzigaret, C.: Antibodies against phospholipase C inhibit smooth muscle contraction induced by acetylcholine and histamine. *Eur. J. Pharmacol.* 138; 427-431, 1987.
21. Moraru, I. I., Manciulea, M., Călugăru, A., Ghyka, Gr. and Popescu, L. M.: Anti-phospholipase C antibodies inhibit the lectin-induced proliferation of human lymphocytes. *Biosci. Rep.* 7; 731-736, 1987.
22. Nakanishi, H., Nomura, H., Kikkawa, U., Kishimoto, A. and Nishizuka, Y.: Rat brain and liver soluble phospholipase C: resolution of two forms with different requirements for calcium. *Biochem. Biophys. Res. Commun.* 132; 582-590, 1985.
23. Nishizuka, Y.: The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* 308; 693-697, 1984.
24. Nishizuka, Y.: Studies and perspectives of proteinkinase C. *Science* 233; 305-312, 1986.
25. Obara, K.: Isolation and contractile properties of single smooth muscle cells from guinea-pig taenia caeci. *Jpn. J. Physiol.* 34; 41-54, 1984.
26. Pace, C. S. and Goldsmith, K. T.: Phospholipase C and mellitin enhancement of glucose-induced electrical activity. *Endocrinology* 118; 102-107, 1986.
27. Popescu, L. M., Popescu, M. and Moraru, I. I.: Phospholipase C contracts visceral smooth muscle. *Eur. J. Pharmacol.* 131; 149-152, 1986.
28. Popescu, L. M., Hinescu, M. E., Musat, S., Ionescu, M. and Pistritzu, F.: Inositol trisphosphate and the contraction of vascular smooth muscle cells. *Eur. J. Pharmacol.* 123; 167-169, 1986.
29. Putney, J. W.: A model for receptor regulated calcium entry. *Cell Calcium* 7; 1-12, 1986.
30. Rhee, S. G., Suh, P. G., Ryu, S. H., Moon, K. H., Suh, H. W., Choi, W. C. and Lee, K. Y.: Complete primary structure and tissue specific distribution of three immunologically distinct phosphoinositide-specific phospholipase C isozymes. *Abstracts, 14th Intern. Congr. Biochem.*, FR: 425, 149, 1988.
31. Ryu, S. H., Suh, P. G., Cho, K. S., Lee, K. Y. and Rhee, S. G.: Bovine brain cytosol contains three immunologically distinct forms of inositolphospholipid-specific phospholipase C. *Proc. Natl. Acad. Sci. USA* 84; 6649-6653, 1987.

32. Sasaguri, T., Hirata, M. and Kuriyama, H.: Dependence of Ca^{2+} of the activities of phosphatidylinositol 4,5-bisphosphate phosphodiesterase and inositol 1,4,5-trisphosphate phosphatase in smooth muscles of the porcine coronary artery. *Biochem. J.* 231; 497–503, 1985.
33. Sekar, M. Ch. and Hokin, L. E.: The role of phosphoinositides in signal transduction. *J. Membrane Biol.* 89; 193–210, 1986.
34. Waite, M.: Approaches to the study of mammalian cellular phospholipases. *J. Lipid. Res.* 26; 1379–1388, 1985.
35. Zuurveld, J. G. E. M., Veerkamp, J. H. and Wirtz, P.: Isolated myofibers from rat skeletal muscle in suspension: cellular morphology and calcium homeostasis. *Muscle & Nerve* 8; 750–759, 1985.