

*Short Communication***A SIMPLE TECHNIQUE FOR ESTIMATION OF PHOSPHOLIPASE D AND
PHOSPHOLIPASE C ENZYMES IN SMOOTH MUSCLE AND ITS CELLS**

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ABSTRACT: This research article describes two novel and simple techniques for the estimation of phospholipase D and phospholipase C enzymes in aortic smooth muscle and cells cultured from the bovine aorta. The techniques encompass the use of ion exchange chromatography and liquid scintillation spectrophotometry.

Key words: Smooth muscle, phospholipase D, phospholipase C, DMEM, LiCl

INTRODUCTION

The importance of phospholipase D (PLD) and phospholipase C (PLC) mediated signal transduction mechanisms is an important area of life sciences. Phospholipids like the phosphatidylcholine can be hydrolysed by PLD and PLC leading to the release of arachidonic acid via the conversion of phosphatidic acid and diacylglycerol (1). Another key phospholipids messenger, PtdOH is a general name for all glycerolipids with a phosphate at the *sn*-3 position of the glycerol backbone. Depending on the nature of the fatty acids that are linked by an ester bond to the first and second hydroxyls of the glycerol, the molecules are going to be quite different and might exhibit different biological properties (2). It was obvious that the major part of the PtdOH formed was low in polyunsaturated fatty acids, as it originated for the major part from PtdInsP₂ (through PLC activity) rather than PtdCho or PtdEtn (through PLD activity). However, this analysis did not allow us to determine whether PLC preferred some precise PtdInsP₂ molecular species. This article describes a simple way of estimating both the PLD and PLC in bovine aorta and its cells.

METHODS**Culture of bovine aortic smooth muscle cells:**

Primary cultures of bovine smooth cells can be obtained from calf thoracic aorta as described previously with some modifications (3, 4). Three separate isolations of primary cultures of bovine smooth muscle cells need to be performed. Cells are then cultured with Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose with 10% heat inactivated calf serum (Gibco BRL, Grand Island, NY). And third to eighth passage cells are used for experiments. After the cells reached confluence in DMEM plus 10% calf serum (5). Cells are maintained in these conditions for four days with replacement of medium every day in order to maintain consistency and confluence.

Estimation of PtdOH and PLD activation

BA cells are labeled for 44 hours with 3 μ Ci/ml of Oleic acid and washed twice with DMEM media. The cells were then incubated for 1 hour at 37°C in DMEM media containing 200 mM EtOH (11.7 μ l/min).

The reaction is terminated with 1.5 ml cold Methanol/2M HCl (9/1) and cells are then scraped and removed into polypropylene tubes. To assess the PLD activation, transphosphatidylation in the presence of ethanol, resulting in the formation of the PLD specific product phosphatidylethanol (PtdEtoH) is measured. PtdEtoH is isolated by thin layer chromatography on heat activated silica gel plates that is later exposed to iodine to demark the spots on the plate. The mobile phase consisted of chloroform/Acetone/Methanol/Acetic acid (10:4:3:5:2) is used for the thin layer chromatography. Radioactive PtdEtoH spots are identified along with unlabelled standards and separated from the plate and quantified by liquid scintillation spectrophotometry. These are based on modification of earlier established techniques (6,7).

Phospholipase C assay

The bovine aortic strips are labeled with myo-[³H] inositol (12 μ Ci/ml) for 4 hours and rinsed for 25 minutes in non radioactive solution containing 10Mm LiCl. ET-1 is then applied in the presence of LiCl. The reaction is stopped by freeze clamping the strips with tongs pre-chilled in liquid N₂. Inositol phosphates are extracted with 5 % trichloroacetic acid (TCA, Sigma) with the assistance of 15-30 minutes sonication. The samples are then washed four times with 3 ml diethyl ether to extract. TCA is neutralized with 100mM imidazole and 0.1N NaOH, and stored then at -20^oC. On the next day, inositol phosphates are separated by ion exchange chromatography (Bio-Rad) according to Berridge et al (8). The values are calculated as counts per minute [³H] inositol per millimeter of the vessel length.

Conclusion

Lipid headgroups have been shown to modulate the activity of proteins such as transporters, receptors, cytoskeletal elements, and other effector molecules (9). The two key phospholipase enzymes, PLD and PLC play a pivotal role in phospholipid breakdown mediated signal transduction. This article describes a very easy way of estimating the PLD and PLC enzymes in bovine aorta and cells. These techniques can be of great use in developing countries as these are relatively cost effective techniques used by conventional methods.

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