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Short Communication

Lyophilization can generate artifacts in chromatographic profiles of inositol phosphates

Elizabeth A. Woodcock*, Karen E. Anderson and Sarah L. Land

Cellular Biochemistry Laboratory, Baker Medical Research Institute, Commercial Road, Prahran 3181 (Australia)

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ABSTRACT

Lyophilized extracts of [^{3}H]inositol-labelled rat heart or renal tubule preparations were found to contain unidentified ^{3}H-labelled compounds in addition to the inositol phosphates. The appearance of these labelled substances was caused by the presence in the extracts of compounds which bound [^{3}H]inositol when lyophilized together with it. These studies demonstrate a previously undescribed source of [^{3}H]inositol-labelled compounds which can complicate chromatographic profiles of inositol phosphates. These problems can be overcome either by not lyophilizing the samples or by lyophilizing in the presence of 0.3 *M* urea, which prevents the association with [^{3}H]inositol and does not interfere with the chromatography.

INTRODUCTION

The phosphatidylinositol (PtdIns) turnover pathway is a complex signalling system initiated by stimulation of phospholipase C specific for phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P_2] and resulting in the generation of two well established second messengers, *sn*-1,2-diacylglycerol (DAG) and inositol(1,4,5)trisphosphate [Ins(1,4,5)P_3]. DAG activates protein kinase C and Ins(1,4,5)P_3 initiates release of Ca^{2+} from specific intracellular stores [1]. In addition, the pathway is associated with the activation of a number of other phospholipases [2], lipid kinases [3] and protein kinases [4]. The overall pattern of the response varies between different cell types [5] and depends, to some extent, on the nature of the stimulus [6], providing a complex, flexible control system which can initiate and sustain a wide variety of cellular responses. The inositol phosphate response itself is complex and variable. In addition to metabolism by dephosphorylation, released Ins(1,4,5)P_3 is metabolized by phosphorylation to Ins(1,3,4,5)P_4 [7] which may itself have a signalling function [8] and is the precursor of the isomers of InsP_5 as well as other isomers of InsP_4 [7]. Some of these compounds likely also have a signalling function [9].

Understanding inositol phosphate metabolism requires reliable methods for the extraction and identification of the numerous isomers present in most tissues. This is most often achieved by labelling tissues with [^{3}H]inositol, extracting with trichloroacetic acid (TCA) or perchloric acid (PCA) and separating the labelled inositol phos-

* Corresponding author.

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phates using anion-exchange HPLC [10]. Accurate quantitation depends on the absence of background labelled material and on the correct identification of all inositol phosphate isomers.

Most detailed studies of the PtdIns turnover pathway have been conducted using immortalized cell lines [11,12], and the available extraction procedures have been developed accordingly. However, it cannot be assumed that long-term cultured cells will always provide a reliable model of activity *in vivo* and it is essential that some studies are performed with freshly isolated tissue, if the physiological importance of the pathway is to be evaluated. Therefore, it is essential that extraction methods appropriate for such studies be developed. Extraction procedures employing acidic chloroform–methanol mixtures have been shown to generate labelled compounds by methanolysis of the inositol phospholipids [13]. In addition, we have previously demonstrated that extraction of heart tissue with perchloric acid introduces artifacts generated from the inositol phosphates themselves [14].

However, extraneous peaks in inositol phosphate profiles can be generated other than from inositol phospholipids or inositol phosphates. In the current study, we show that some tissues contain material which binds [^{3}H]inositol irreversibly when lyophilized together with it. This causes the appearance of large, heterogeneous peaks in inositol phosphate profiles. Lyophilization is a convenient procedure for removal of remaining acid and also serves to reduce extraction volume so that entire extracts can be loaded onto HPLC columns.

We show that inclusion of urea (0.3 *M*), prior to lyophilization, prevents the formation of these complexes and does not itself interfere with the chromatography. Thus, acceptable chromatographic profiles can be obtained even from tissues containing this inositol-binding material.

EXPERIMENTAL

Materials

[^{3}H]Inositol was obtained from the Radiochemical Center (Amersham, UK). Cell culture medium was from the Commonwealth Serum Labs. (Parkville, Australia). Collagenase was Worthington type III.

All other chemicals were AR grade and reagents were dissolved in highly purified Milli Q water.

Inositol phosphate response in renal papillary tubules

Papillary tubules were prepared in a manner similar to that described elsewhere [15]. Isolated tubules were resuspended in medium 199 containing 10% foetal calf serum and 100 U/ml each of penicillin and streptomysin and incubated 3.5 h at 37°C to allow attachment of the cells to 3-cm dishes. After this time, medium was replaced with fresh medium 199 containing 2% bovine serum albumin (BSA), antibiotics and [^{3}H]inositol (10 μCi/ml) for 24 h to label the inositol phospholipids.

Inositol phosphate generation was terminated by adding 1 ml of ice-cold 5% TCA containing 5 m*M* EDTA followed by sonication. Wells were washed with a further 1 ml of TCA, and the combined extracts were centrifuged at 200 *g*. Excess TCA was removed using freon–tri-N-octylamine [16]. Extracts were passed through 1-ml columns of Dowex-50 cation-exchange resin and eluted with 1 ml of water [17].

Inositol phosphate studies in intact rat heart

Adult male Sprague–Dawley rats (300–350 g) were injected with heparin (1000 U/kg). After 30 min the animals were killed by decapitation and the hearts rapidly removed into ice cold saline. After thorough chilling, the hearts were cannulated via the aorta according to the method of Langendorff, perfused with HEPES-buffered Krebs' medium and allowed to warm up gradually to 37°C. Perfusion was continued until all blood was removed. After this time, [^{3}H]inositol (5 μCi/ml) was added and the perfusion medium was recirculated. After 2 h labelling, radioactive medium was replaced with medium containing propranolol (10^{-6} *M*), mercaptoethanol ($5 \cdot 10^{-3}$ *M*) and LiCl (10^{-2} *M*) and perfusion continued for 10 min. Norepinephrine (10^{-4} *M*) was

then added. Release of inositol phosphates was terminated by dropping the hearts into liquid nitrogen.

Frozen hearts were homogenized in 5% TCA containing 5 m*M* EDTA and 5 m*M* phytic acid. Phytic acid was added to protect the inositol phosphates from metabolizing enzymes and from non-specific attachment to proteins [10]. Two 10-s passes of a Polytron homogenizer (3/4 maximum speed) were used separated by 15-s chilling on ice. The homogenate was then sonicated and centrifuged at 2000 *g*. The TCA pellet was re-extracted with TCA, EDTA, phytic acid and the pooled supernatants were treated as described above.

Analysis of inositol phosphate isomers

Analysis of the isomers of the inositol phosphates was carried out using anion-exchange HPLC employing a 10 cm × 1 cm I.D. Whatman 10-μm Partisil SAX column in a Waters Radial compression unit. Inositol phosphates were eluted with a complex gradient of ammonium phosphate pH 3.8, essentially as described elsewhere [14]. The gradient program involved the use of gradients from 0 to 0.08 *M* over 22 min, from 0.2 to 0.28 *M* over 30 min and from 0.5 to 0.56 *M* over 25 min for the separation of the isomers of $InsP_1$, $InsP_2$ and $InsP_3$, respectively. Higher inositol phosphates and other tightly retained compounds were separated using isocratic elution with 2 *M* ammonium phosphate. ^{3}H-Labelled compounds were detected and quantitated using an on-line β-counter, Model CR from Radiomatic Instruments. This provided retention times and integrated peak values for each of the isomers of the inositol phosphates. The minimum peak area was set to 100 cpm. The identities of the various isomers were established using appropriate standards. Nucleotide standards ATP, ADP and AMP (20 μg each) were added to each sample prior to injection to monitor any change in chromatographic performance.

RESULTS

Profiles of [^{3}H]inositol-labelled compounds in extracts of renal papilla

Papillary tubule cells were attached to tissue culture plates and labelled for 24 h with [^{3}H]inositol. After removal of the [^{3}H]inositol, cells were stimulated with endothelin-1 (ET-1) plus LiCl or LiCl alone for 30 min and inositol phosphates extracted using TCA followed by lyophilization as described in the Experimental section. As is shown in Fig. 1, TCA extracts contained compounds identifiable as inositol phosphates. However, in addition, profiles contained large amounts of other compounds with retention times between inositol and $InsP_1$. Another group of compounds with retention times between those of $InsP_1$ and $InsP_2$ was also observed.

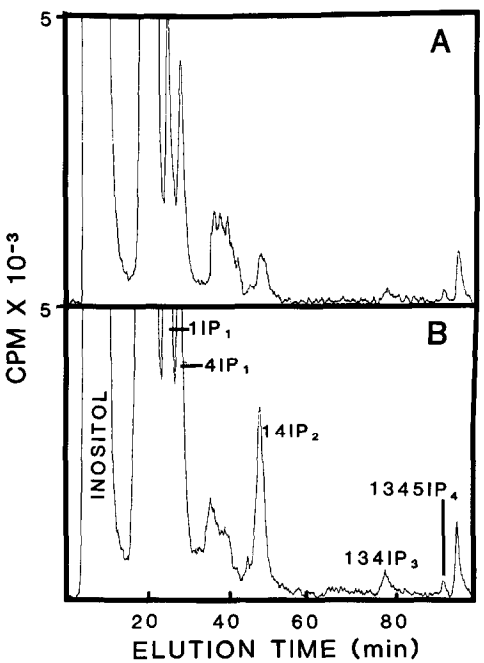

Fig. 1. [^{3}H]Inositol-labelled compounds in lyophilized extracts of renal papillary tubules and the effect of stimulation with endothelin-1. [^{3}H]Inositol-labelled tubules were incubated in the (A) absence or (B) presence of endothelin (100 n*M*) for 30 min and then extracted and lyophilized. Extracts were chromatographed as described in the Experimental section. Peaks identifiable as inositol phosphates are indicated. The experiment was performed three times with similar results.

Complex chromatographic profiles were observed in the presence or absence of ET-1. ET-1 increased the levels of compounds identified as inositol phosphates but did not alter the profile of these other [^{3}H]inositol-labelled compounds (Fig. 1). Extraction procedures did not influence the generation of these unidentified compounds. Similar profiles were observed when excess TCA was removed using diethyl ether instead of the freon–octylamine mixture or when extraction was performed using PCA instead of TCA.

Effects of lyophilization and of urea

[^{3}H]Inositol-labelled papillary tubules were washed to remove excess label and extracted with TCA as described in the Experimental section. The extract was divided into three aliquots. One of these was lyophilized as usual. Urea (0.3 *M*) was added to a second aliquot prior to lyophilization. The third aliquot was lyophilized and urea was added subsequently. HPLC profiles of the three aliquots are shown in Fig. 2. Extracts lyophilized without urea contained large amounts of material chromatographing between 15 and 25 min. Addition of urea (0.3 *M*) to the TCA extract eliminated most of this extraneous material. To be effective, urea had to be present prior to lyophilization. Addition of urea after lyophilization did not affect the appearance of this ^{3}H-labelled material.

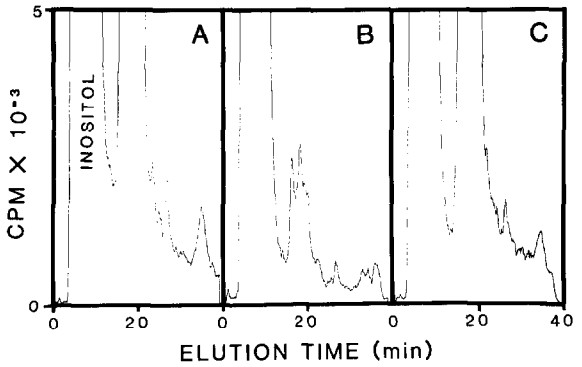

Fig. 2. Effects of lyophilization and urea on [^{3}H]inositol-labelled compounds in renal papillary extracts. Extracts of [^{3}H]inositol-labelled tubules were: (A) lyophilized, (B) lyophilized in the presence of urea (0.3 *M*) and (C) lyophilized and 0.3 *M* urea was added subsequently. Extracts were chromatographed as described in the Experimental section. The experiment was performed three times with similar results.

The most likely interpretation of these findings is that renal papillary tubules contain compounds which bind [^{3}H]inositol irreversibly when lyophilized together with it and that this binding is prevented in the presence of urea. This interpretation was tested further by preparing unlabelled TCA extracts of renal papillary tubules and lyophilizing these in the presence of [^{3}H]inositol (2.5 μCi/ml). Such lyophilized extracts contained substances which chromatographed between 15 and 25 min, $49.8 \pm 16.8 \cdot 10^3$ cpm/well (mean ± S.D., $n = 3$). The inclusion of urea (0.3 *M*) prior to lyophilization decreased the amount of this material to $1.8 \pm 0.18 \cdot 10^3$ cpm/well. Addition of [^{3}H]inositol to culture medium which had not been in contact with cells did not generate such labelled material following lyophilization.

Profiles of [^{3}H]inositol-labelled material in heart

[^{3}H]Inositol-labelled hearts were stimulated for 20 min with norepinephrine in the presence of LiCl and then subjected to TCA extraction. Extracts were divided into three aliquots. One sample was chromatographed without lyophilization, one was lyophilized without urea and one was lyophilized in the presence of urea (0.3 *M*). Results are shown in Fig. 3. Lyophilized heart extracts contained heterogeneous material chromatographing between 15 and 25 min and between 30 and 40 min. This material was not present either in extracts which had not been lyophilized or in extracts which had been lyophilized in the presence of urea. Peaks identifiable as inositol phosphates were not influenced either by lyophilization or by addition of urea. Urea itself did not alter chromatographic profiles at concentrations up to 3.0 *M*.

DISCUSSION

Results presented here demonstrate the presence in heart and renal papilla of compounds which bind [^{3}H]inositol irreversibly when lyophilized together with it. The labelled compounds

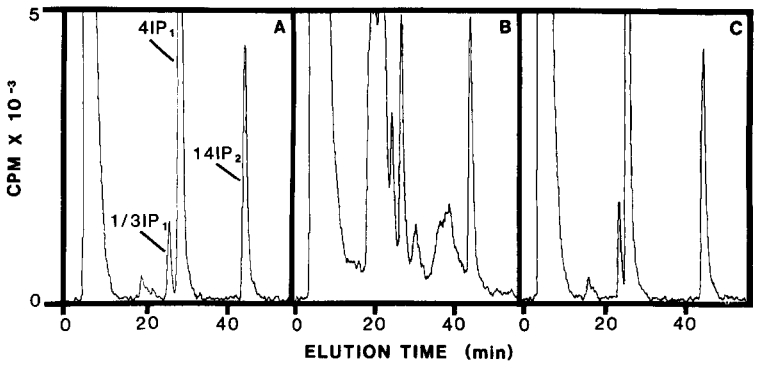

Fig. 3. [^{3}H]Inositol-labelled compounds in extracts of isolated rat heart. Extracts of [^{3}H]inositol-labelled hearts were divided into three aliquots and (A) chromatographed without lyophilization, (B) lyophilized and then chromatographed and (C) lyophilized in the presence of urea (0.3 *M*) and then chromatographed. Chromatography was performed as described in the Experimental section. The experiment was performed three times with similar results.

in extracts of heart and kidney appeared to be similar because they had similar chromatographic properties and both were formed during the lyophilization process. Furthermore, their formation was inhibited by urea. Apparently similar compounds are found in extracts of renal proximal tubules. However, we have never observed such material in preparations of adrenal glomerulosa cells, vascular smooth muscle cells or isolated atria. Thus, the inositol-binding compounds appear to be restricted to a limited number of cell types.

Lyophilization is a convenient procedure for the preparation of samples for anion-exchange separation of inositol phosphates. First, lyophilization removes any remaining acid which can cause phosphate group migration and dephosphorylation during storage [10]. The alternative procedure, neutralization, increases the ionic strength and this can be detrimental to the resolution of the isomers of $InsP_1$ [10]. Second, lyophilization reduces sample volume so that entire extracts can be analyzed. This is especially important in tissues such as heart which require large volumes for effective extraction. While lyophilization of extracts is a suitable procedure for extracts of most tissues, it is obviously not suitable for studies of PtdIns turnover either in intact heart or in renal tubule preparations unless urea is added.

The generation of artifacts in chromatographic profiles of inositol phosphates by extractions employing acidic chloroform–methanol mixtures has been described previously [13]. In that case the artifacts were generated from the inositol phospholipids. Studies in our own laboratory have shown that PCA extraction can cause the generation of artifacts from the inositol phosphates themselves, if the PCA is not rapidly removed [14]. The artifacts described here are not generated either from the inositol phosphates or the phospholipids. Instead, certain tissues contain compounds which have been shown to bind to [^{3}H]inositol during the process of lyophilization generating large and heterogeneous peaks in the inositol phosphate profiles. Such compounds are clearly unrelated to the PtdIns turnover pathway, are not influenced by receptor stimulation and can make examination of inositol phosphate responses impossible. Such problems can be overcome either by not lyophilizing the samples or by adding urea prior to lyophilization.

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