Isolation and enzymic assay of choline and phosphocholine present in cell extracts with picomole sensitivity

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Increasing interest in receptor-regulated phospholipase C and phospholipase D hydrolysis of cellular phosphatidylcholine motivates the development of a sensitive and simple assay for the water-soluble hydrolytic products of these reactions, phosphocholine and choline respectively. Choline was partially purified from the methanol/water upper phase of a Bligh & Dyer extract by ion-pair extraction using sodium tetraphenylboron, and the mass of choline was determined by a radioenzymic assay using choline kinase and [32 P]ATP. After removal of choline from the upper phase, the mass of residual phosphocholine was determined by converting it into choline by using alkaline phosphatase, followed by radioactive phosphorylation. In addition to excellent sensitivity (5 pmol for choline and 10 pmol for phosphocholine), these assays demonstrated little mutual interference (phosphocholine $\rightarrow$ choline = 0%; choline $\rightarrow$ phosphocholine = 5%), were extremely reproducible (average S.E.M. of 3.5% for choline and 2.9% for phosphocholine), and were simple to perform with instrumentation typically available in most laboratories. In addition, the ability to apply the extraction technique to the upper phase of Bligh & Dyer extracts permitted simple analysis not only of choline and phosphocholine, but also of phosphatidylcholine and lipid products of phospholipase C and phospholipase D activity (1,2-diacylglycerol and phosphatidic acid respectively) from the same cell or tissue sample.

INTRODUCTION

Receptor stimulation in a variety of cells is associated with the hydrolysis of phosphatidylcholine by a variety of phospholipases [1–5]. Recently, increasing interest has developed with regard to the roles of receptor-mediated activation of phospholipase C (PLC) and phospholipase D (PLD). Activation of either of these pathways is thought to result in the formation of the second messenger 1,2-diacylglycerol, by direct hydrolytic formation via PLC, or by a two-step ‘indirect pathway’ involving hydrolysis of PLD-generated phosphatidic acid by phosphatidate phosphohydrolase [3–5]. Studies examining release of either labelled choline or phosphocholine from cells prelabelled with [3 H]choline have a number of inherent problems associated with the use of radioisotopes, particularly in cells which cannot be labelled sufficiently for analysis or to isotopic equilibrium (discussed in [6]). As a result, it would be of considerable value to be able to assay the mass of cellular choline and phosphocholine as a means to examine receptor-initiated phospholipase-mediated formation of these intermediates.

Quantitative analysis of choline and acetylcholine from cellular or tissue sources has been the focus of considerable investigation and has been reviewed [7]. Several early investigators utilized the spectrophotometric assessment of either choline reineckate or choline periodide to assess low- μ mol or low-nmol quantities of choline [8,9]. Enzymic methods have principally focused on the ability to detect the H_2O_2 generated by choline oxidase, and have resulted in assays possessing sensitivity in the low-nmol range [10–12]. More recently enhancements of the choline oxidase method have provided increased sensitivity (5 pmol), but require considerable h.p.l.c. expertise and costly instrumentation involving post-column immobilized enzymic detection of choline [11,12] or g.l.c./mass spectrometry [13]. Additionally, these sensitive h.p.l.c.-based techniques are time-consuming if large numbers of samples are involved.

In an assay first described in 1971 [14], choline kinase can be used to phosphorylate choline radioactively in the presence of [γ - 32 P]ATP. The 80 pmol sensitivity of the assay initially presented was excellent, but was not routinely employed, and the technique was rediscovered in 1983 in a brief report by Muma & Rowell [15].

In the present paper we report (a) significant enhancements and characterization of the choline kinase-based assay of choline, (b) an initial extraction of the water-soluble hydrolytic products choline and phosphocholine from the lipid products, (c) a simple separation of choline from phosphocholine by differential extraction, (d) the effective conversion of phosphocholine into choline by using alkaline phosphatase, and (e) the usefulness of this assay to quantify choline and phosphocholine, with the opportunity of concurrent determination of the related lipid products (diacylglycerol and phosphatidic acid) from limited cell sources.

METHODS

Materials and equipment

Materials and their sources are as follows: unless otherwise indicated, all salts, small molecules and commercially prepared proteins and enzymes, Sigma Chemical Co. (St. Louis, MO, U.S.A.); organic solvents, Baker, Mallinkrodt and Burdick and Jackson, distributed by Sigma; male Sprague–Dawley rats, Southwest Breeding Labs (Midlothian, TX, U.S.A.); [3 H]choline, New England Nuclear (Boston, MA, U.S.A.); [γ - 32 P]ATP, ICN (Irvine, CA, U.S.A.); polypropylene plasticware from Sarstedt (St. Louis, MO, U.S.A.).

Isolation and purification of cells

Mast cells were obtained by lavage of the peritoneal and pleural cavities of rats and were purified to >98% purity by using discontinuous Percoll density gradients as described

Abbreviations used: PLC, phospholipase C; PLD, phospholipase D; TPB, sodium tetraphenylboron.

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in detail elsewhere [5]. Peripheral-blood polymorphonuclear leucocytes were isolated from heparinized blood of healthy volunteers [6].

Extraction of cellular choline

To the cellular pellet or a sample of authentic choline, 0.5 ml of methanol, 0.25 ml of chloroform and 0.2 ml of water were added in a modification of the Bligh & Dyer extraction [16]. After addition of 0.5 pmol of [^3H]choline (approx. 100 000 d.p.m.) for assessment of extraction efficiency, the extract was centrifuged at 200 *g* for 10 min to remove insoluble material, and phase separation of the supernatant was accomplished by adding 0.25 ml of chloroform and 0.25 ml of water. A 0.85 ml sample of the choline-containing upper methanol/water phase was removed for further analysis, the remaining upper phase removed by vacuum aspiration and the lower phase dried for future analysis of cellular lipids. After adding 0.65 ml of 12 mM-sodium phosphate (pH 7.0) to decrease the methanol concentration, choline was extracted from the upper methanol/water phase by using the ion-pairing reagent sodium tetraphenylboron (TPB) in heptan-4-one (1.0 ml of a 5 mg/ml solution of TPB). After vigorous mixing and phase separation by brief centrifugation, 0.9 ml of the heptanone phase was added to 0.3 ml of aq. 0.1 M-HCl to disrupt the ion pair and back-extract the free choline. After brief centrifugation in a Microfuge, the heptanone layer was removed by vacuum aspiration, and 0.25 ml of the aqueous phase dried by the use of a Speed Vac [evaporation in an oven at 65 °C (16 h) or under a gentle stream of N_2 (1 h at 40 °C) was found to be equally effective], and a 20 μl sample of the aqueous phase was removed and, after neutralization with 200 μl of 0.1 M- NaPO_4 (pH 7.0), counted for radioactivity to assess efficiency of choline extraction.

Extraction of cellular phosphocholine

Cells were treated exactly as described above, except that the following additional procedures were performed. First, after removal of the 0.9 ml sample of choline-containing TPB/heptanone upper phase from the methanol/water phase of the Bligh & Dyer extraction, the remaining upper phase was removed by vacuum aspiration. The phosphocholine-containing lower phase was re-extracted with TPB/heptanone, followed by 1 ml of heptanone further to remove remaining choline and TPB from the phosphocholine-containing lower phase. A 1.0 ml sample of the lower phase was then dried in a Speed Vac, in an oven at 65 °C, or under N_2 stream at 40 °C.

Conversion of phosphocholine into choline

After separation of phosphocholine from choline by differential extraction, phosphocholine was converted into choline by alkaline phosphatase (bovine intestinal mucosa; EC 3.1.3.1). To the phosphocholine-containing dried extract described in the previous section, the following materials were added: (a) 90 μl of a reaction buffer consisting of 20 mM-glycine buffer (pH 10.4), 1 mM- ZnCl_2 and 1 mM- MgCl_2 and (b) 10 μl of alkaline phosphatase (10 units; 1000 units/mg of protein dissolved in reaction buffer). After vortex-mixing, the hydrolytic reaction was allowed to proceed for 30 min at 37 °C. The reaction was terminated by placing tubes in a heating block at 105 °C or in a boiling-water bath for 10 min, and the choline produced by phosphocholine hydrolysis was extracted into the organic phase by adding 1.4 ml of 6 mM-sodium phosphate (pH 7.0) and 1 ml of TPB (5 mg/ml) in heptan-4-one as described above.

Quantification of choline

Assaying choline obtained either by direct ion-pair extraction of cellular aqueous extracts or from cellular phosphocholine was

accomplished by radioactive phosphorylation with [$\gamma\text{-}^{32}\text{P}$]ATP. Final reactions conditions were: 5–20 μCi /assay, 1.25 mM-ATP, 50 mM-sodium phosphate (pH 7.0), 15 mM- MgCl_2 , 1 mg of dithiothreitol/ml and 2.5 m-units of choline kinase (from yeast; EC 2.7.1.32) in a final volume of 50 μl . Although the pH optimum of choline kinase is 8.5, a pH of 7.0 was utilized to decrease the possibility of base hydrolysis of the phosphocholine product. The reaction was allowed to proceed for 30 min at room temperature. The reaction was stopped on ice, and the labelled ATP that had not reacted was removed by precipitation of the insoluble barium salt formed by adding 20 μl of 0.3 M-barium acetate (freshly made or from frozen batches), 80 μl of phosphocholine (2.5 mg/ml) and 100 μl of distilled water to the reaction mixture. [^{32}P]Phosphocholine was separated from remaining labelled ATP by passage of a 200 μl sample over 300 μl of Dowex AG-1 X8 (Bio-Rad; formate form, 200–400 mesh) in a 1 ml pipettor tip or polypropylene mini-column, by using an LSC vial rack (Isolabs, Akron, OH, U.S.A.), eluting with 900 μl of 75 mM-ammonium acetate (pH 10). The phosphocholine-containing eluate was collected into a 5 ml scintillation vial containing 225 μl of 2 M-HCl (to adjust the mixture to near-neutral pH), and radioactivity was measured after adding 3.3 ml of ACS II (Amersham, Arlington Heights, IL, U.S.A.) or without neutralization by adding 5 ml of Tru-Count (IN/US, Fairfield, NJ, U.S.A.).

Analysis of data

Each experiment was performed in triplicate and repeated 2–3 times as indicated in appropriate legends. Unless otherwise indicated, data represent the mean $\pm$ S.E.M. of data obtained from a representative experiment. Absence of error bars indicates a S.E.M. smaller than the size of the symbol.

RESULTS

Overview of approach

Scheme 1 illustrates the approach utilized for the quantitative analysis of choline and phosphocholine in cellular extracts.

Scheme 1. Overview of approach used to extract and quantify cellular choline and phosphocholine

Abbreviation: P-choline, phosphocholine.

Particularly notable aspects include (a) the effective extraction of choline into heptanone with the ion-pair-forming reagent TPB, (b) conversion of phosphocholine into choline with alkaline phosphatase and (c) efficient detection of choline by its conversion into [^{32}P]phosphocholine by choline kinase in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP.

Extraction of choline and phosphocholine

Choline and phosphocholine are freely soluble in water and require no extraction, but, since experiments involving the activity of phospholipases might often involve analysis of cellular lipids, methods were developed to permit both lipid extraction and efficient recovery of choline and phosphocholine. As expected, neither choline nor phosphocholine is found to any significant degree in the chloroform-containing lower phase of the Bligh & Dyer [16] extraction (results not shown). A number of investigators have used TPBs to form complexes with naturally occurring trimethylammonium-containing compounds such as choline, carnitine and acetylcholine [17–19]. We exploited this phenomenon and avoided the need to dry the upper phase of a Bligh & Dyer extract by modestly decreasing the methanol concentration of the upper methanol/water phase of the Bligh & Dyer extract from approx. 50% (v/v) to approx. 28% by adding additional water to the upper phase before extraction with TPB-containing heptanone. In the absence of added water, recovery of choline extracted into the TPB-containing heptanone upper phase was $67 \pm 5\%$, compared with $80 \pm 3\%$ when the methanol concentration was decreased (recoveries corrected upwardly based on the fraction lost by intentionally incomplete sampling, 30%). Although the adequate recovery of choline was accomplished with a single extraction by TPB-containing heptanone, a second extraction was employed in situations where vigorous depletion of choline was sought before assessing the mass of phosphocholine and resulted in $>95\%$ removal. Free choline was isolated from the heptanone-soluble TPB–choline complex by back-extracting free choline into water into 0.3 ml of aq. 0.1 M-HCl. Heptan-4-one was found to provide modestly, but significantly, increased recoveries than heptan-2-one or heptan-3-one (results not shown). Although a Speed Vac was initially employed to dry the final acidic aqueous choline-containing extract, drying was equally effective when the samples were placed in a drying oven at 65°C for approx. 16 h or within 1 h when a gentle stream of N_2 at 40°C was used. Phosphocholine in the upper methanol/water phase of the Bligh & Dyer cellular extract was obtained and separated from choline by using a modification of the method described for choline (Scheme 1). In addition to the first extraction of choline with TPB/heptanone, nearly all remaining choline was removed from the water-enriched Bligh & Dyer upper phase by a second extraction with TPB-containing heptanone. After removal of the heptanone by vacuum aspiration, the phosphocholine-containing methanol/water extract was dried. Although previous investigators have reported the use of acid phosphatase to convert phosphocholine into choline [20], we used the well-characterized and readily available alkaline phosphatase to convert phosphocholine efficiently into choline. This phosphocholine-derived choline was extracted with TPB-containing heptanone as described above. Recovery of choline originally present as phosphocholine was $30 \pm 4\%$ of the total, which corresponds to 65% of the maximum theoretical recovery (owing to intentionally incomplete sampling at several steps).

Depending on the needs of the experiment, 0.5 pmol of either [^3H]choline or [^3H]phosphocholine was added during the extraction procedures as an internal standard to permit appropriate correction for modest variability of recovery. Although recovery of unlabelled choline mass was identical with recovery of

Fig. 1. Effect of added NaCl on the efficiency of ion-pair extraction of choline

Portions (500 pmol) of [^3H]choline was placed in a series of tubes and extracted with 1 ml of TPB (5 mg/ml in heptan-4-one) in the presence of 1.5 ml of water/methanol (18:7, v/v) containing 10 mM-sodium phosphate (pH 7.0) and various concentrations of NaCl. A sample of the TPB/heptanone phase was removed and the choline back-extracted in aqueous HCl as described in the Methods section. Data are normalized to 100% recovery with no added NaCl, and represent means $\pm$ S.E.M. of triplicate values obtained in one of two experiments (actual recovery was 83% after correction for intentionally incomplete sampling).

[^3H]choline, an unexplained finding was that the labelled phosphocholine standard was consistently recovered to a greater extent than was unlabelled phosphocholine mass. Since no isotope effect was detected with regard to extraction of either choline or phosphocholine, we suspect that this effect may be due to preferential cleavage and recovery of [^3H]phosphocholine rather than unlabelled phosphocholine by alkaline phosphatase.

Increasing ionic strength decreased the effectiveness of choline extraction into TPB/heptanone, presumably as a result of disrupting the formation of the ion pair. Fig. 1 demonstrates the impact of additional NaCl in the aqueous phase on the recovery of [^3H]choline in TPB/heptanone. Thus, in situations in which NaCl either is used during the Bligh & Dyer extraction to enhance extraction of lipids associated with proteins or is present as a result of the extraction of cell-free supernatants containing physiological concentrations of saline, several compensatory procedures should be considered: (a) correcting for incomplete recovery when the salt concentration is modest, (b) decreasing the salt concentration by dilution with additional water during the TPB/heptanone extraction of choline, or (c) increasing the concentration of TPB.

Quantification of extracted choline by its conversion into [^{32}P]phosphocholine

As initially described by Reid *et al.* [14] and rediscovered by Muma & Rowell [15], choline can be very efficiently radioactively phosphorylated by using choline kinase. As illustrated in Fig. 2, the formation of labelled phosphocholine is linearly related to the quantity of choline added over a 1000-fold range (10 pmol–10 nmol), and the assay is useful over a 10000-fold range. The sensitivity of the assay (5 pmol) is limited not by the ability to achieve sufficient specific radioactivity, but rather by the quantity of ^{32}P -labelled material eluted from the ion-exchange column. Because increasing the amount of ion-exchange resin and changing a variety of chromatographic parameters were unsuccessful at decreasing the elution of radioactive compounds (results not shown), it is suspected that the [^{32}P]ATP contained minor quantities of labelled compounds failing to bind to the resin. The elution of labelled compounds represented only

Fig. 2. Extraction and quantification of authentic choline

Various amounts of unlabelled choline were subjected to Bligh & Dyer extraction, and choline in the water/methanol phase was extracted with TPB/heptanone as described in detail in the Methods section. Partially purified choline was quantified by radioactive phosphorylation by using choline kinase and [γ - 32 P]ATP. Data represent means $\pm$ S.E.M. of triplicate observations obtained in one of four experiments. Choline detected represents the recovery of [32 P]phosphocholine obtained after correction for intentionally incomplete sampling (70%).

Fig. 3. Extraction and quantification of authentic phosphocholine

Various amounts of unlabelled phosphocholine were subjected to Bligh & Dyer extraction and, after removal of choline from the water/methanol phase by double-ion-pair extraction, phosphocholine was converted into choline by using alkaline phosphatase. Phosphocholine-derived choline was quantified radioenzymically as described in detail in the Methods section. Data represent means $\pm$ S.E.M. of triplicate values obtained in one of two experiments. Choline detected represents the recovery of [32 P]phosphocholine obtained after correction for intentionally incomplete sampling (46.7%).

0.0026 % of the [32 P]ATP initially added. Of note is that the use of 100–200 mesh (instead of 200–400 mesh) was considerably less successful and required approx. 3-fold more resin to achieve adequate binding of labelled ATP. Increased sensitivity could likely be achieved by further scaling-down of the assay from 50 μ l to 10–20 μ l, or by purification of the labelled ATP.

Quantification of extracted phosphocholine

Fig. 3 demonstrates the linear relationship of choline kinase-catalysed formation of labelled phosphocholine from authentic phosphocholine subjected to the extraction procedures, enzymic hydrolysis and radioactive phosphorylation described above. Although the sensitivity of the assay for phosphocholine (10 pmol) was not as good as that for choline, the typical presence of approx. 10-fold more phosphocholine than choline in cells makes this limitation of trivial significance.

Utility of assaying mixtures of choline and phosphocholine

Table 1 illustrates the results obtained when defined mixtures of choline and phosphocholine were prepared and subjected

Table 1. Cross-interference of choline and phosphocholine

Values for choline mass detected have been corrected for decreased overall recovery of choline owing to incomplete sampling (70%) and efficiency of extraction (80%). Values for phosphocholine mass detected have been corrected for decreased overall recovery of phosphocholine-derived choline secondary to intentional incomplete sampling (46.7%) and alkaline phosphatase efficiency (81%). Net choline mass has been corrected by deducting the labelling obtained in the absence of added choline or phosphocholine (9.3 pmol in this experiment). Net phosphocholine has been corrected by deducting the labelling obtained in the absence of added choline or phosphocholine (38.3 pmol in this experiment).

Compounds added		Compounds detected	
Choline (pmol)	Phosphocholine (pmol)	Net choline (pmol)	Net phosphocholine (pmol)
0	0	0 $\pm$ 0.7	0 $\pm$ 2.7
0	50	-1.4 $\pm$ 0.5	54.1 $\pm$ 3.1
0	500	-0.2 $\pm$ 0.7	514 $\pm$ 3.7
50	0	49.4 $\pm$ 0.9	3.5 $\pm$ 2.9
50	50	50.9 $\pm$ 1.7	54.4 $\pm$ 1.9
50	500	50.6 $\pm$ 2.5	523 $\pm$ 13.0
500	0	493 $\pm$ 5.0	21.8 $\pm$ 2.7
500	50	534 $\pm$ 2.9	54.0 $\pm$ 3.5
500	500	529 $\pm$ 9.8	505 $\pm$ 11.6

to the extraction and analytical procedures described above. No interference by phosphocholine on choline detection was observed, but approx. 5% of the choline crossed over into the phosphocholine assay, largely owing to incomplete extraction of choline by TPB/heptanone. However, owing to the greater pool size of phosphocholine (compared with choline), the choline not removed by double extraction of cell samples with TPB/heptanone (and therefore included in the phosphocholine-containing lower phase) represents a modest contamination of little consequence. More stringent analyses could employ either a third TPB/heptanone extraction or acetylation of remaining choline with acetic anhydride before alkaline-phosphatase-mediated hydrolysis of phosphocholine.

Interference by other amino alcohols

Several experiments were performed in order to determine whether cellular ethanolamine and/or serine would interfere with the detection of choline. Addition of up to 5 nmol of either serine or ethanolamine caused neither an increase nor a decrease in the elution of 32 P-labelled material from the ion-exchange column. In previous experiments, Reid *et al.* [14] demonstrated that phosphoethanolamine failed to be eluted from Dowex AGX1 anion-exchange columns at pH 11, but was at pH 8.5, presumably owing to the ability to remove its positive charge at higher pH. Similarly, phosphoserine would be expected to be retained on the column. Thus, although choline kinase might phosphorylate ethanolamine and/or serine, their phosphorylated derivatives were not detected in the anion-exchange-column eluate and thus do not provide a functional interference with the measurement of cellular choline and phosphocholine. The differential elution of phosphoethanolamine from that of phosphocholine might allow assay of cellular ethanolamine by secondary elution of the anion-exchange column with a buffer of lower pH.

Quantification of choline and phosphocholine from cellular sources

This assay was used to characterize the levels of choline and

Fig. 4. Quantification of choline from mast cells

Different numbers of mast cells were extracted, and the choline was obtained and quantified in the cell pellet as described in detail in the Methods section. Data points represent means $\pm$ S.E.M. of triplicate values obtained in one of two experiments joined by the line obtained by linear regression analysis. Choline detected represents the recovery of [32 P]phosphocholine obtained after correction for recovery of [3 H]choline used as an internal standard, but was not corrected for control formation of labelled phosphocholine observed in the absence of added cells.

phosphocholine from mast cells and neutrophils. Fig. 4 illustrates the linear relationship of the formation of labelled phosphocholine with the number of mast cells extracted and subjected to assay. The slope of this curve indicates that choline is present in unstimulated mast cells at 17.8 pmol/ 10^6 cells. Levels of choline and phosphocholine in unstimulated mast cells in a series of experiments were found to be 13.1 ± 1.2 pmol of choline/ 10^6 cells (11 experiments) and 182 ± 19 pmol of phosphocholine/ 10^6 cells (2 experiments). Similar experiments in neutrophils result in values of 14.6 ± 1.2 pmol of choline/ 10^6 cells and 178 ± 9 pmol of phosphocholine/ 10^6 cells for choline (2 experiments) and phosphocholine (3 experiments) respectively. As $> 95\%$ of the labelled choline incubated with sonicated neutrophils for 30 min at 37°C is recovered unchanged (results not shown), metabolism of choline does not affect the levels measured. Aside from the content of cellular choline, no interference by the cellular extract was observed on exogenously added choline at the cell numbers utilized in these experiments (10^6 – 10^7).

DISCUSSION

Taken together, these data illustrate the sensitivity, wide assay range and relative ease of quantifying both choline and phosphocholine from a single cellular extract. Principal improvements over existing methods include: (a) increased sensitivity, speed and ease in the assay of choline, (b) simpler and more effective conversion of phosphocholine into choline, (c) the ability to assay both choline and phosphocholine from a single cellular extract by separating choline and phosphocholine by simple differential extraction in the presence of an ion-pairing reagent, and (d) the possibility of concurrent determination of lipid products such as diacylglycerol and phosphatidic acid from the same sample.

Two previous authors have described the use of choline kinase-mediated radioactive phosphorylation as a method for the determination of choline [14,15]. In the original description [14], sensitivity of 80 pmol was observed, and relatively large columns were utilized to separate the labelled phosphocholine product from ATP. Further, because choline kinase was not commercially available, a longer reaction time was required for quantitative phosphorylation of choline. The more recent report [15] provides only a sketchy description of the methods, but claims sensitivity

down to approx. 10 pmol, although information about the values obtained in the absence of added choline is not provided.

The differential extraction of choline and phosphocholine with TPB in ketonic solvents is a variation of that employed by Tucek *et al.* [19], using TPB to separate acetylcholine from acetylcarnitine. This differential extraction is particularly important in the separation of choline from phosphocholine in order to facilitate the quantification of both compounds from the same sample.

Although a variety of other sensitive assays exist for quantifying choline [7,10–13], the radioenzymic assay described in the present paper has some distinct advantages for certain applications. The most commonly used assay having sensitivity in the low-pmol range is based on the electrochemical detection of H_2O_2 produced by choline oxidase. The practical lower limit of this assay with tissue samples is 5 pmol of choline, similar to the sensitivity described in the current assay. The choline oxidase-based method has distinct practical drawbacks: (a) it requires costly equipment, including an h.p.l.c. instrument with an autoinjector (or very considerable technician time) and an electrochemical detector, and (b) in order to avoid the expense and time required for multiple assays, the authors covalently immobilized the enzyme on a vinyl sulphone-hydroxyethyl methacrylate support in a post-column reactor, a procedure not necessarily within the reach of all analytical laboratories. Thus the choline oxidase-based assay of choline requires considerable chromatographic expertise. From the perspective of analysis time, frequent analysis of a small number of samples favours the use of the h.p.l.c. technique, whereas the assessment of 50–60 samples would favour the radioenzymic assay described in the present paper. A similarly sensitive and analytically intensive assay has been developed recently involving g.l.c./mass spectrometry [13]. The present method for assaying phosphocholine provides the same sensitivity and cellular values in neutrophils as our recently described method utilizing a much more tedious assay involving enzymic conversion of phosphocholine into [32 P]CDP-choline [6].

The availability of this sensitive and relatively simple assay will become increasingly important in light of the emerging need to distinguish the activities of various receptor-dependent phospholipid-specific PLCs and PLDs in cells subjected to physiological stimulation. Coupled with existing sensitive methods for the analysis of the mass of lipid products of these reactions, diacylglycerol [21,22] and phosphatidic acid [23], the ability to quantify choline and phosphocholine provides the opportunity to use mass-based analyses to assess the importance of phosphatidylcholine-specific PLC and PLD during cell activation (reviewed in [3,24]). In addition to a preliminary report in neutrophils [25], the methods described in the present paper have been utilized in mast cells to demonstrate that IgE-receptor-dependent stimulation causes a 10-fold increase in choline mass, whereas that of phosphocholine declines with stimulation (T. T. Dinh & D. A. Kennerly, unpublished work), results that support the activation of PLD, but not PLC, towards phosphatidylcholine during cell activation.

Note added in proof (received 28 June 1990)

Because some preparations of glycine contain impurities that act as substrates for choline kinase, highly purified glycine or alternative buffers should be used during alkaline-phosphatase-mediated hydrolysis of phosphocholine to avoid encountering high backgrounds in the quantification of phosphocholine.

This work was supported by grants from the National Institutes of Health (AI-22277 for D.A.K. and RR-05424 for J.J.M.). D.A.K. was also supported by the Burroughs Wellcome/American Academy of

Allergy Developing Investigator Award in Immunopharmacology of Allergic Diseases. J.J.M. was also supported by an RJR Nabisco Scholar's Award.

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Received 23 March 1990/26 April 1990; accepted 1 May 1990