1-Palmitoyl-2-(*p*-benzoyl)benzoyl phosphatidylcholine, a photoactive phospholipid for the labelling of membrane components

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The preparation and use of a new photoactivatable phosphatidylcholine derivative [1-palmitoyl-2-(*p*-benzoyl)benzoyl phosphatidylcholine] is described. The reagent is shown to be effective in labelling different membrane proteins under irradiation conditions that preserve their enzymic activity. The properties of this new phospholipid analogue suggest that it can be useful in studying several aspects of membrane structure and function.

INTRODUCTION

Hydrophobic photolabelling with photoactive phospholipid analogues is a powerful method for the investigation of several aspect of membrane biology (Brunner, 1981; Bayley, 1983; Bisson & Montecucco, 1985, 1986). It is based on the introduction of a suitable light-sensitive group at a defined position of one fatty acid chain. On illumination, a highly unstable intermediate is generated that is able to react and form covalent derivatives with nearly all molecules present in its immediate environment. If the reagent is radioactive, its association with the various membrane components can be easily traced, thus providing information on their localization and organization in the membrane.

Frequently that part of the hydrophobic domain of integral proteins exposed to lipids is formed by very unreactive aliphatic residues. The nitrene- and carbene-generating photolabels introduced so far do show selectivity (Hoppe *et al.*, 1983, 1984). Sequencing data are still too scanty to construct a scale of reactivity and only allow one to draw the conclusion that both nitrenes and carbenes react much more quickly with sulphur-containing amino acids than with the other residues and that the aliphatic side chains are the least modified (Bisson & Montecucco, 1985, 1986). A lack of labelling does not provide any information on a system, since it can be interpreted in several alternative ways and hence reagents able to overcome this limitation of the method are clearly desirable.

Here we report on the preparation of a phosphatidylcholine analogue based on the photoreactive benzophenone group (see Scheme 1 below). Illumination excites the benzophenone to a triplet state able to attack unactivated C—H bonds, and this may then result in the formation of a cross-linked derivative (Hammond *et al.*, 1961; Walling & Gibian, 1965). The phospholipid derivative has been made radioactive at the choline moiety to produce a photoactive phospholipid with the highest specific radioactivity so far reported. As a consequence of the very low amount of probe needed, the possibility of perturbative effects is greatly reduced.

EXPERIMENTAL

p-Benzoylbenzoic acid was from Janssen Chimica and carbonyldi-imidazole from Sigma (St Louis, MO, U.S.A.). [³H]Methyl iodide (specific radioactivity 85 Ci/mmol) was obtained from Amersham International. 4-Pyrrolidinopyridine was prepared as reported by Patel & Sparrow (1979). 1-Palmitoyl lysophosphatidylcholine was prepared as described by Wells & Hanahan (1969). Snake-venom phospholipase A₂ and cabbage phospholipase D were the products of Boehringer (Mannheim, Germany). Sarcoplasmic-reticulum Ca²⁺-activated ATPase and *Electrophorus electricus* (Na⁺ + K⁺)-activated ATPase were prepared and assayed as described previously (Bisson & Montecucco, 1981; Montecucco *et al.*, 1981). Protein concentration was determined by the Lowry method, with bovine serum albumin as standard, and phospholipid phosphorus as described by Bartlett (1959). Phospholipids were detected on t.l.c. plates (silica-gel 60 F; Merck) by the Dittmer (Dittmer & Lester, 1964) spray reagent, by u.v. absorbance and for phosphatidylethanolamine also by ninhydrin spraying. Solvent systems for t.l.c. were as follows: chloroform/methanol/water (65:35:4, by vol.) (solvent A); chloroform/methanol/water/acetic acid (65:35:7:3, by vol.) (solvent B); chloroform/methanol/7M-NH₃ (65:35:4, by vol.) (solvent C). All operations involving photoactive materials were performed in a red-lighted room.

Preparation of PC-BB

A 77 mg portion of carbonyldi-imidazole was added to 106 mg of *p*-benzoylbenzoic acid in 5 ml of dry, freshly distilled, chloroform under dry N₂. After 30 min of stirring (when no more bubbles are formed), 50 mg of 1-palmitoyl lysophosphatidylcholine were added, followed by 60 mg of 4-pyrrolidinopyridine. The vial was stoppered under N₂ and the reaction allowed to proceed overnight to 42 °C. The reaction went to completion, as estimated by t.l.c., showing no residual phosphatidylcholine. A 10 ml portion of chloroform/methanol (1:1, v/v) was added and the mixture extracted with 5 ml of

Abbreviations used: PC-BB, 1-palmitoyl-2-(*p*-benzoyl)benzoyl phosphatidylcholine [1-palmitoyl-2-(*p*-benzoyl)benzoyl-*sn*-glycero-3-phosphocholine]; PE-BB, 1-palmitoyl-2-(*p*-benzoyl)benzoyl-*sn*-glycero-3-phosphoethanolamine.

high yields were also obtained for the preparation of arylazido phospholipids. The exceptionally high yield of this reaction may be of great help in the preparation of related phosphatidylcholine analogues containing spin labels, fluorescent labels and n.m.r., as well as radioactive isotopes. The reaction proceeds under mild conditions and hence can be exploited for the preparation of phosphatidylcholines containing highly unsaturated fatty acid chains.

The photoactivatable group has been placed here as close as possible to the phospholipid head-group region because superficial labelling has provided most valuable information (Bisson & Montecucco, 1985, 1986). However, phospholipid derivatives with the photoreactive group at different distances from the glycerol moiety can be prepared with the same procedure starting from the corresponding fatty acid derivatives, prepared as described by Breslow *et al.* (1978).

The presence of the *p*-benzoylbenzoic group in position 2 of the glycerol backbone of phosphatidylcholine may explain the relatively low conversion of PC-BB into the corresponding phosphatidylethanolamine. However, owing to the low cost of the reagents, this does not constitute a drawback in the synthetic sequence.

Also the third step has been modified to prepare a highly radioactive reagent. The exhaustive methylation of ethanolamine to choline was performed with [^3H]methyl iodide of specific radioactivity 85 Ci/mmol, leading to a phosphatidylcholine derivative of specific radioactivity ($85 \times 3 =$) 255 Ci/mmol. The attainment of such a high specific activity allows one to introduce very minute amounts of the probe in the biological system under study in such a way as to minimize possible perturbative effects and self-quenching reactions.

As shown in Scheme 1(b), long-wave u.v. radiation promotes the benzophenone group to its triplet state, which does not react with water and does not rearrange intramolecularly (Galardy *et al.*, 1973), thus lowering the possibility of the non-productive decays of the photo-generated intermediate that occur with several nitrenes and carbenes (Bisson & Montecucco, 1985, 1986). Rather it can abstract a hydrogen atom from a neighbouring molecule generating two radicals that either recombine to form a cross-linked derivative or react with themselves; this latter reaction is the less likely one the smaller is the molar fraction of the probe in the membrane.

Fig. 1 shows the absorbance spectrum of PC-BB before and after irradiation with long-wave u.v. radiation. This treatment appears to be able to activate the reagent and yet not to alter the enzymic properties of the ATPases used here.

The reactivity properties of the benzophenone triplet probe are very different from those of carbenes and nitrenes. These groups are strong electrophiles and, for this reason, nitrene- and carbene-based reagents may fail to react with protein residues lacking nucleophilic groups (Montecucco *et al.*, 1983a,b). The reagent described here is expected not to suffer from this limitation; rather its usefulness in membrane-protein studies may be limited by a predominant labelling of the hydrocarbon chains of lipid with respect to protein.

Fig. 2 shows the distribution of the radioactivity along a gel of the ($\text{Na}^+ + \text{K}^+$)-activated ATPase (a) and of the Ca^{2+} -activated ATPase (b) reconstituted with [^3H]PC-BB and illuminated. Radioactivity is found only at the level of the protein and at the gel front where phospholipids

Fig. 1. Absorption spectrum in ethanol of PC-BB before (—) and after (---) irradiation for 2 h in a 1 cm-path-length cuvette with a UVL-58 lamp

The inset shows on an expanded scale the effect of irradiation for 0 h (—), 2 h (---) and 4 h (····).

migrate. All proteins are labelled in agreement with their known integral nature, showing that [^3H]PC-BB is effective in cross-linking to intrinsic proteins. The yield of labelling is less than 0.1% for both proteins. Low yields of protein cross-linking are typically found in hydrophobic photolabelling, owing to competing reactions such as self-quenching and covalent binding to lipids. This latter reaction has already been studied in great detail for the benzophenone group and shown to occur with high yields with steroids, detergents, long-chain alcohols and phospholipids (Breslow *et al.*, 1973, 1978; Czarniecki & Breslow, 1979; Breslow, 1980). Cross-linking to lipids appears to be the main reason for the low yield of protein labelling with PC-BB, since much higher values were previously found with the arylazido reagents, which show a large preference for protein residues with respect to hydrocarbon chains (Bisson & Montecucco, 1981). For this reason, [^3H]PC-BB may be best used in lipid studies such as lateral-phase segregation in phospholipid mixtures. However, the low yield of protein labelling does not hamper the study of lipid-protein interaction and of protein folding in the membrane because, as shown by the patterns of Fig. 2, the radioactivity associated with the protein can be clearly identified at the protein level. Benzophenone-based photoaffinity labels have already been proved to be useful in studying hormone- and ATP-binding sites (Galardy *et al.*, 1974; Williams & Coleman, 1982;

Fig. 2. Pattern of labelling with $[{}^3\text{H}]\text{PC-BB}$ of $(\text{Na}^+ + \text{K}^+)\text{-activated ATPase (a)}$ and $\text{Ca}^{2+}\text{-activated ATPase (b)}$ after 60 min of irradiation as described in the Experimental section

Irradiations of longer than 60 min caused a progressive loss of ATPase active. The gel photographs show Coomassie Blue staining profiles.

Abbott *et al.*, 1984; Bar-zvi & Shavit, 1984; Mahmood & Yount, 1984).

An important control in protein labelling with benzophenone derivatives is the determination of the amount of reagent bound after incubation in the dark, since we found that the $\text{Ca}^{2+}\text{-activated ATPase}$ binds, via a chemical reaction, about a quarter of the probe bound after illumination. This may be due to the formation of a Schiff base between a lysine residue and the aromatic ketone group.

In conclusion, a method is described for the preparation of a highly radioactive photoactivatable derivative of phosphatidylcholine. This method may be very valuable for the preparation of other phospholipid analogues as well. This new reagent has been shown here to label effectively two integral membrane proteins. For

its specificity of reaction it may be most useful in the study of the behaviour of lipid mixtures and in membrane-protein labelling as a reagent complementary to the already established nitrene- and carbene-generating phospholipid probes.

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