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Light-induced membrane protein phosphorylation in the bovine rod outer segment

A magic angle spinning ^{31}P-NMR study

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Magic angle spinning ^{31}P-NMR (MAS ^{31}P-NMR) spectra of bovine rod outer segments, unphosphorylated and phosphorylated, were obtained. In the phosphorylated samples the spectra showed new resonances not assignable to phospholipids. These signals were present only when stimulation of receptor phosphorylation occurred. These resonances were not due to exogenous, soluble phosphorus-containing compounds. Limited proteolysis to remove the carboxyl-terminal region of the photoreceptor that contains the phosphorylation sites removed these resonances. The chemical shifts were in the usual range for serine phosphate and threonine phosphate. The pK_a obtained from a pH titration of the ^{31}P chemical shift was typical of serine phosphate. Therefore, these ^{31}P-NMR resonances were assigned to the phosphorylation sites on membrane proteins in the rod outer segment disk membranes. Static ^{31}P-NMR measurements revealed that at least some of these sites gave rise to relatively narrow resonances, indicative of considerable motional freedom of the carboxyl-terminal segment of the photoreceptor when phosphorylated. These data indicate that it is possible to study phosphorylation sites on membrane proteins using MAS ^{31}P-NMR, and that using in vivo ^{31}P 'spin labelling' one can study directly and selectively regions of receptors crucial to receptor function.

1. Introduction

Receptor phosphorylation, in the case of G-protein mediated responses, has been suggested to be involved in the desensitization to agonists of the receptor response system [1]. According to this model, a stimulated receptor becomes substrate for an endogenous protein kinase. Phosphorylation of the receptor inhibits the ability of the receptor to activate the G-protein that mediates the signal transduction between the agonist and the cellular response to that agonist. The mechanism of this inhibition of coupling between the G-protein and receptor is unknown. However, because of the extensive homology among some receptor systems utilizing G-proteins (such as the photoreceptor, β-adrenergic receptor, and muscarinic receptor), a common mechanism may be predicted.

In the case of the photoreceptor, phosphorylation of the receptor, rhodopsin, occurs in response to light activation. The extent of phosphorylation appears from ^{32}P labelling studies to be proportional to the light stimulus, or the percent bleach [2–4]. In turn, the extent of phosphorylation appears to be directly correlated with inhibition of the coupling between rhodopsin and the G-protein, or transducin [4].

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To be able to study the state of the receptor and to have a non-perturbing probing method sensitive to regions of the receptor potentially involved in interactions between the receptor and the G-protein would open a useful new avenue of investigation to expand present knowledge of receptor biochemistry. Magic angle spinning ^{31}P-NMR (MAS ^{31}P-NMR) of bovine rod outer segments containing phosphorylated rhodopsin would appear to offer such an opportunity. Previous studies have shown the power of the MAS ^{31}P-NMR approach in other systems [5–8], but none have been reported using this approach to study phosphorylated proteins. The object in this report was to investigate the ability of MAS ^{31}P-NMR to detect phosphorylation sites on membrane proteins in the rod outer segment. Several lines of evidence were obtained that particular ^{31}P-NMR resonances observed in these experiments arose from light-stimulated phosphorylation of serine and threonine sites on membrane proteins, including rhodopsin.

2. Materials and methods

2.1. Materials

Frozen, dark-adapted retinas were obtained from J. Lawson, Inc. (Lincoln, NE).

2.2. Preparation of rod outer segments

Bovine rod outer segments were prepared according to the procedure described in ref. 9. All preparations were carried out under dim red light. All buffers were purged with N_2. The thawed retinas were suspended in 70 mM phosphate buffer (pH 7), 1 mM $MgCl_2$, 50 μM diethylenetriaminepentraacetic acid (DTPA), 5 mM dithiothreitol, 45% (w/w) sucrose and gently shaken. This material was placed in centrifuge tubes, overlayered with buffer minus the sucrose and centrifuged at 25 000 rpm in a Beckman SW28 rotor, for 30 min at 4°C. The supernatant was harvested, resuspended in 15% sucrose, and centrifuged at 5000 rpm for 5 min in a Sorvall SS34 rotor at 4°C. The pellet was resuspended and centrifuged on a discontinuous sucrose density gradient of four layers (1.10, 1.11, 1.13 and 1.15 mg/ml sucrose) in the Beckman SW28 rotor at 27000 rpm for 30 min. The rod outer segments were harvested and washed by centrifugation at 5000 rpm in the Sorvall SS34 rotor for 5 min at 4°C.

2.3. Light-stimulated phosphorylation

The rod outer segments were resuspended in 10 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$. These were centrifuged at 5000 rpm for 5 min at 4°C in the Sorvall SS34 rotor. The concentration was adjusted to 0.5 mg/ml protein and 3 mM ATP. The material was gently homogenized in a hand homogenizer, then mildly sonicated in a brown vial in a bath sonicator for 30 s. The material was then incubated at 30°C for 30 min before stimulation with light.

2.4. Papain proteolysis

Papain proteolysis was carried out according to the procedures described in detail by Albert and Litman [10].

2.5. MAS ^{31}P-NMR

MAS ^{31}P-NMR spectra were obtained on a Nicolet NT-150 NMR spectrometer using home-built MAS probes. Spinning at 3200 rps (revolutions per second) yielded spectra with no detectable spinning side bands. ^{1}H decoupling ($\gamma B_1/2\pi = 30$ kHz) during data acquisition resulted in minor but significant line narrowing of the ^{31}P signals.

3. Results and discussion

Nonspinning FT ^{31}P-NMR spectra of biological membranes consist primarily of an anisotropic powder pattern. This axially symmetric powder pattern is about 40–45 ppm wide ($\sigma_\perp - \sigma_\parallel$) [11,12]. Such a powder pattern is characteristic of phospholipids in a bilayer. The powder pattern results from axial diffusion of the phospholipid phosphate-containing head group, with the director for

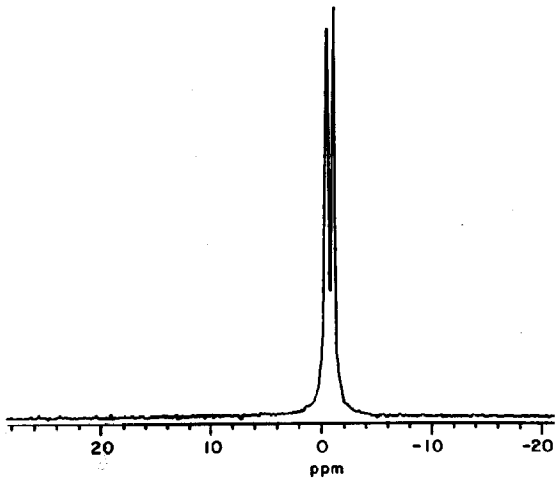

Fig. 1. 60 MHz MAS ^{31}P-NMR spectrum of an unsonicated aqueous dispersion of multilamellar liposomes containing a mixture of phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The downfield resonance arises from PE, that upfield being from PC.

axial diffusion oriented perpendicular to the bilayer surface [13]. MAS ^{31}P-NMR spectroscopy is capable of collapsing the ^{31}P powder pattern of the membrane phospholipid bilayer to the isotropic ^{31}P-NMR resonances characteristic of the individual phospholipid head group classes.

Fig. 1 shows the MAS ^{31}P-NMR spectrum of an unsonicated aqueous dispersion of multilamellar liposomes containing a mixture of phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Two isotropic resonances are observed, which correspond to the isotropic chemical shifts of PE (the downfield resonance) and PC (the upfield resonance). Although the isotropic resonances for these two phospholipids are separate by less than 1 ppm, they are resolved by the MAS technique. In the absence of the MAS, this same sample gives rise to an axially symmetric powder pattern in the ^{31}P-NMR spectrum with $(\sigma_{\perp} - \sigma_{\parallel}) = -45$ ppm.

Fig. 2 shows the MAS ^{31}P-NMR spectra from a rod outer segment preparation. Within the mixed phospholipid population of the rod outer segment disk membranes, one can identify the resonances due to PE and PC by comparison to fig. 1. These two phospholipids are the major phospholipid components of the rod outer segment disk membranes [14]. However, phosphatidylinositol and phosphatidylserine are also present in the disk membranes, and their ^{31}P-NMR isotropic chemical shifts fall between those of PC and PE [13]. The presence of the two latter phospholipids obscures some of the resolution between the ^{31}P-NMR resonances of the two major phospholipids of the membrane, PE and PC, compared to fig. 1.

Exposure of rod outer segments to light under the appropriate conditions stimulates the phosphorylation of disk membrane proteins [2,3,15]. Rhodopsin constitutes 90% of the disk membrane proteins and its phosphorylation under these conditions has been well documented using ^{32}P labelling [4]. Rod outer segments were illuminated in the presence of ATP as described in section 2 and then washed to remove soluble phosphate-containing compounds. This preparation was used for the spectrum that is shown in fig. 2. This spectrum contains a resonance in addition to the two prominent phospholipid resonances, deshielded compared with the chemical shifts of the phospholipids. This downfield resonance is absent in dark-adapted control membranes that were not illuminated before measurement (and thus not phosphorylated).

So far, the data have indicated the appearance of new ^{31}P-NMR resonances after the application of the light-induced phosphorylation protocol to

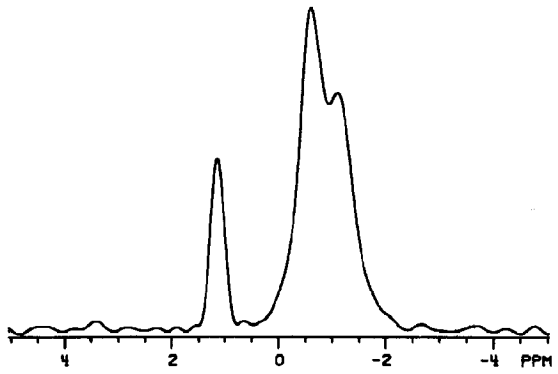

Fig. 2. 60 MHz MAS ^{31}P-NMR spectra of phosphorylated bovine rod outer segments. 4500 transients were collected at a repetition rate of 4 s and 18° C. 10 kHz spectra were obtained and a 16K transform calculated with no exponential filtering. An observation pulse of 6 μs was employed. Gated (on during acquisition only) high-power (30 kHz) ^{1}H decoupling was used.

the rod outer segments. Therefore, it is reasonable to investigate whether the new ^{31}P-NMR resonances correspond to light-induced protein phosphorylation. The following characteristics of the light-induced phosphate resonances are consistent with the phosphorylation of membrane protein in the rod outer segments.

First, it is important to note that these new ^{31}P-NMR resonances appear only after the phosphorylation protocol. Unbleached rod outer segments and rod outer segment disk preparations do not show these resonances (data not shown).

Second, the ^{31}P chemical shift demonstrates that these resonances do not correspond to common soluble phosphorus compounds, such as ATP and ADP. These latter compounds were removed by a centrifugation procedure to wash the membranes free of soluble phosphorus-containing compounds prior to accumulation of the NMR spectrum.

Third, the variation of the ^{31}P chemical shift of these resonances as a function of pH reveals a pK_a that is different from that of inorganic phosphate. In this experiment, the rod outer segments were stimulated by light to undergo phosphorylation in the presence of ATP. The membranes were then washed, and the pH adjusted to the desired value, prior to MAS ^{31}P-NMR spectroscopy. The chemical shift of the light-induced ^{31}P resonance was then measured as a function of pH. Fig. 3 shows the results. The pK_a was determined to be 5.9.

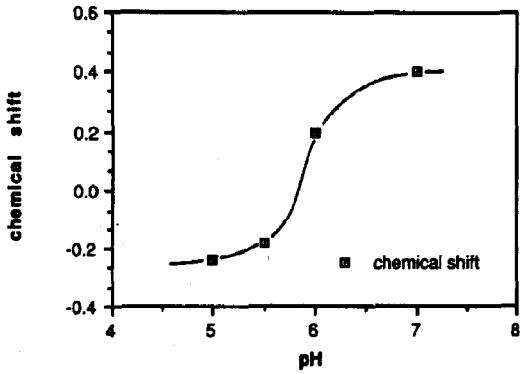

Fig. 3. Variation with pH of the chemical shift of the main resonance arising from the MAS ^{31}P-NMR spectrum of phosphorylated rod outer segments.

The pK_a of serine phosphate is 6.19 [16], similar to the pK_a determined here.

Fourth, the ^{31}P chemical shift is in the range expected for serine (threonine) phosphate [17] which constitute(s) the phosphorylation sites on rhodopsin.

Fifth, the resonances induced by light-stimulated phosphorylation can be removed by limited proteolysis of the membranes. Papain treatment of rod outer segment disk membranes produces a limited cleavage of the photoreceptor [10]. One cleavage site removes the carboxyl terminal of the protein containing a high concentration of threonines and serines that are the putative phosphorylation sites on rhodopsin for the endogenous rhodopsin kinase. Rod outer segments were phosphorylated as for the experiments described above. The ^{31}P-NMR resonances arising from the phosphorylation protocol were observed. Then the membranes were treated with papain and washed by centrifugation as described in section 2. The ^{31}P-NMR spectra showed no evidence of the resonances induced by light-stimulated phosphorylation. This result is consistent with removal of the phosphorylation sites on rhodopsin by papain proteolysis.

If these resonances are due to serine phosphates on rhodopsin, arising from light-induced phosphorylation, then changes in these resonances as a function of light stimulation of the photoreceptor would be important to examine. With respect to the phosphorylation-induced ^{31}P-NMR resonances, changes were observed in the resonance intensity with variations in the level of photoreceptor stimulation.

The intensity of the phosphorylation-induced ^{31}P-NMR resonances could be expressed in terms of the number of phosphate groups per rhodopsin molecule to which that resonance intensity corresponded. This was done by comparing the intensity of the phosphorylation-induced resonances with those arising from the phospholipid in the membranes. The respective resonance intensities were determined by integration and/or by computer-based spectral deconvolution using lineshape fitting routines. Using a value of 65 phospholipids/rhodopsin [18], the intensity of the phosphorylation-induced resonances could be

converted to effective phosphates per rhodopsin. The effective number of phosphates per rhodopsin was found to vary from a low of 2 at low stimulation of the photoreceptor, to near 10 at high stimulation of the photoreceptor. This level of phosphorylation was in the range reported previously for rhodopsin using radiolabelled phosphate [2–4].

The MAS experiments effectively separate the resonances due to the phospholipids and those induced by the phosphorylation protocol. However, the details of the motion characterizing the sites giving rise to those resonances are lost under the influence of MAS. We therefore performed preliminary static ^{31}P-NMR experiments on phosphorylated rod outer segments. The experiments revealed the presence of a relatively narrow (about 300 Hz) resonance in the static experiment, corresponding to the phosphorylation sites already identified in the MAS experiments. This resonance appeared superimposed on the broad asymmetric powder pattern which arose from the phospholipid bilayer in the disk membranes in the rod outer segment. Apparently, when the photoreceptor was phosphorylated, at least a portion of the carboxyl terminal of the protein experienced considerable motional freedom.

The present data suggest that the ^{31}P-NMR resonances induced by the phosphorylation protocol correspond well to the light-induced phosphorylation of the photoreceptor. This opens possibilities for extraction of further information about the phosphorylation of the photoreceptor and the properties of that portion of the rhodopsin molecule that contains the phosphorylation sites. The new experimental approach, described here, to studying desensitization of the photoreceptor by phosphorylation shows promise for elucidation of mechanistic details of the interaction between rhodopsin and transducin.

Acknowledgments

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