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Binding of inositol phosphates to arrestin

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Arrestin binds to phosphorylated rhodopsin in its light-activated form (metarhodopsin II), blocking thereby its interaction with the G-protein, transducin. In this study, we show that highly phosphorylated forms of inositol compete against the arrestin–rhodopsin interaction. Competition curves and direct binding assays with free arrestin consistently yield affinities in the micromolar range; for example, inositol 1,3,4,5-tetrakisphosphate (InP_4) and inositol hexakisphosphate (InP_6 bind to arrestin with dissociation constants of 12 μM and 5 μM, respectively. Only a small control amount of inositol phosphates is bound, when arrestin interacts with phosphorylated rhodopsin. This argues for a release of bound inositol phosphates by interaction with rhodopsin. Transducin, rhodopsin kinase, or cyclic GMP phosphodiesterase are not affected by inositol phosphates. These observations open a new way to purify arrestin and to inhibit its interaction with rhodopsin. Their physiological significance deserves further investigation.

Signal transduction; G-protein; Rhodopsin; Arrestin; Inositol phosphate

1. INTRODUCTION

The rhodopsin/transducin/phosphodiesterase cascade of the rod is a well-studied example of a receptor/G-protein/effector system. Absorption of light leads to the formation of metarhodopsin II (MII) that activates transducin by catalysing GDP/GTP exchange [1,2]. Transducin activates a cyclic GMP phosphodiesterase and lowers cytoplasmic cyclic GMP. Since cyclic GMP keeps Na^+/Ca^{2+} channels open for the influx of these ions, the channels close, the cell hyperpolarizes, and the Ca^{2+} level drops (reviewed in ref. 3).

Quenching of the light signal occurs at least at three points. When metarhodopsin II is phosphorylated by a specific kinase (reviewed in ref. 4), it binds a regulatory protein, arrestin, which blocks further activation of the G protein [5]. A protein with analogous function, β-arrestin, was recently described for the β-adrenergic system [6]. The G protein is de-activated by hydrolysis of bound GTP to GDP. In a third quenching pathway, the lowering of calcium stimulates guanylate cyclase via a calcium-binding protein and restores the cyclic GMP level (reviewed in ref. [7]).

Recently, we have found that heparin is a potent inhibitor of the arrestin–rhodopsin interaction and that heparin and phosphorylated rhodopsin induce the same distinctive pattern in the limited proteolysis of arrestin [8]. We tested other potential ligands of arrestin, including sulfonated or phosphorylated analogues of carbohydrates, nucleotides, and sulfonated or phosphorylated inositol derivatives. A digestion pattern similar to heparin was obtained only for phosphorylated analogues of inositol.

This finding, the role of inositol phosphates in invertebrate phototransduction [9] and evidence of the complete pathway in rods [10] motivated the present study. We present evidence that arrestin is a soluble receptor for inositol phosphates. Binding of the phosphates and of phosphorylated metarhodopsin II compete against one another.

2. MATERIALS AND METHODS

2.1. *Reagents*

Inositol phosphates were in D-myo conformation. [inositol-1-^{3}H(N)]1,3,4,5-Tetrakisphosphate (17 Ci/mmol) and [inositol-2-

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Abbreviations: InP_3, D-myo-inositol 1,4,5-triphosphate; InP_4, D-myo-inositol 1,3,4,5-tetrakisphosphate; InP_5, D-myo-inositol 1,3,4,5,6-pentakisphosphate; InP_6, inositol hexakisphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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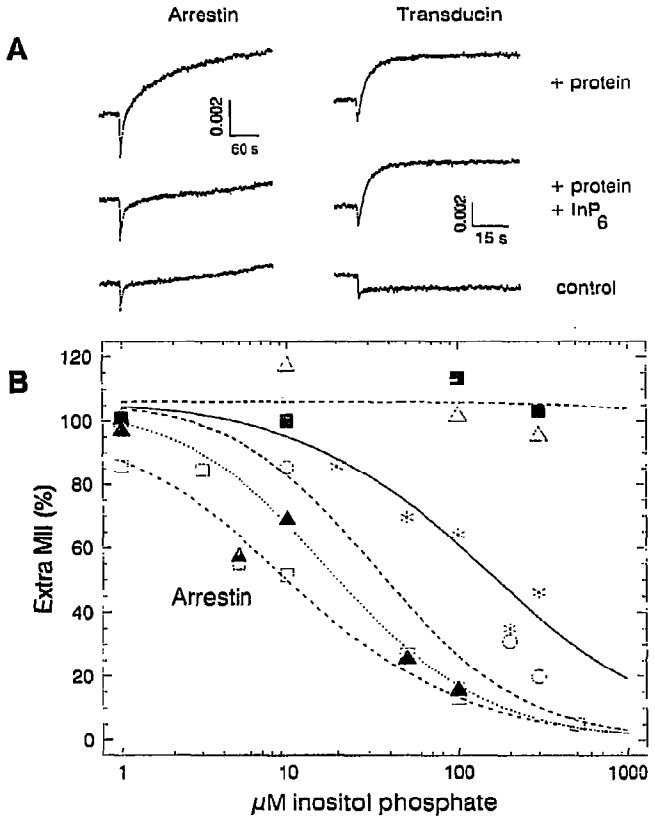

Fig.1. Inhibition of the interaction between metarhodopsin II (MII) and arrestin by InP_6 and other phosphorylated derivatives of inositol. A. Effect of InP_6 on the stability of metarhodopsin II (MII) in complex with arrestin or transducin. The records are the increase in absorption at 380 nm (A_{max} of MII photoproduct). Undisturbed interaction of MII with arrestin or transducin leads to enhanced MII formation (extra MII). For arrestin the enhancement is inhibited by IP_6 (left, middle), and only the control amount of MII is formed. B. Level of extra MII (MII with arrestin and inositol analogues, minus the control MII) as a function of ligand concentration. The symbols are: inositol 1-phosphate (InP_1), closed square; inositol 1,4-bisphosphate (InP_2), open triangle; InP_3, asterisk; InP_4, open circle; InP_5, closed triangle; and InP_6, open square. The lines through the data are computer fits with hyperbolic functions (exponents between 0.8 and 1.1).

^{3}H(N)]hexakisphosphate(12 Ci/mmol) were obtained from NEN (DuPont). Inositol 1-phosphate, inositol 1,4-bisphosphate, InP_3, InP_4, inositol 1,2,5,6-tetrakisphosphate, inositol 1,4,5,6-tetrakisphosphate, and InP_5 were purchased from Boehringer Mannheim. InP_6 and other chemicals were obtained from Sigma Chemical Co..

2.2. *Proteins*

Phosphorylated rhodopsin was prepared from urea-washed ROS as described previously [11]. Arrestin was prepared from bovine retinas using a modification of the previously described method [12].

40 bovine retinas were glass–glass homogenized with ice-cold 10 mM HEPES buffer, pH 7.5, containing 1 mM benzamidine and 15 μg/ml leupeptin. Soluble proteins were separated by centrifugation for 25 min at 43 700 × g. The extract was applied to a 1.6 × 14 cm column of DEAE-cellulose (Whatmann DE52) which had been equilibrated with 10 mM HEPES, pH 7.5. The column was washed with HEPES buffer containing 15 mM NaCl, at the flow rate 30 ml/h until absorbance at 280 nm dropped below 0.1 and a major hemoglobin band was eluted from the column. Arrestin was eluted by a NaCl gradient (240 ml total, from 0 to 150 mM NaCl containing 0.5 mM benzamidine). Arrestin was eluted at about 75 mM NaCl as determined by SDS-PAGE. Fractions containing arrestin were applied to a 1 × 7 cm column of Heparin-Sepharose which had been equilibrated with 10 mM HEPES and 100 mM NaCl, pH 7.5. The column was washed with HEPES buffer containing 150 mM NaCl, at a flow rate of 12 ml/h until the absorbance at 280 nm dropped below 0.01. Arrestin was eluted by a InP_6 gradient (70 ml total from 0 to 8 mM InP_6 in the washing buffer). Arrestin was eluted at 2.5 μM of InP_6 and with a purity higher than 90%.

In order to further eliminate contaminating proteins (less than 5%) and InP_6, arrestin was briefly dialyzed against 10 mM HEPES buffer, pH 7.5, containing 100 mM NaCl. Next, arrestin was applied to a 1 × 3.5 cm column of Heparin-Sepharose, equilibrated with 10 mM HEPES and 100 mM NaCl, pH 7.5. The column was washed with HEPES buffer containing 200 mM NaCl (8 ml), at a flow rate of 12 ml/h. Arrestin was eluted by 400 mM NaCl in the HEPES buffer. About 4 mg of homogeneous arrestin was obtained. All procedures were performed at 5°C.

Transducin was isolated from rod outer segments according to Kühn [13].

2.3. *Assays*

Extra-MII—interaction with transducin [2] or arrestin [8,14] stabilizes the 380 nm photoproduct metarhodopsin II (MII) at the expense of its tautomeric forms MI and MIII, which absorb in the 460–480 nm spectral range. The flash photolysis apparatus measures the difference between the absorbance changes at 380 nm (A_{max} of MII) and 417 nm (isosbestic point of the MI/MII transition) [14].

Equilibrium dialysis was performed using 10 mM HEPES buffer, pH 7.5, containing 100 mM NaCl, essentially as described elsewhere [15]. The dialysis cells contain Spectrapor 1 membranes (molecular weight cutoff 10 000). 130 μl of arrestin solutions (0.1–1 mg/ml) were placed in one compartment and 130 μl of 2–705 μM [inositol-1-^{3}H(N)]1,3,4,5-tetrakisphosphate or 4–44 μM [inositol-2-^{3}H(N)]hexa-

TABLE 1

Binding of inositol derivatives to arrestin

Inositol derivative	IC_{50} (μM)	K_D (μM)
1. InP_1	102 000	3 400
2. InP_2	3 360	1 120
3. InP_3	170	57
4. InP_4 (1,3,4,5 isomer)	38	14
5. InP_4 (1,2,5,6 isomer)	134	45
6. InP_4 (1,4,5,6 isomer)	73	25
7. InP_5	20	7
8. InP_6	10	3

IC_{50} were determined employing the 'extra Meta II' assay(Fig. 1; section 2.

K_D values were estimated assuming the following scheme of reactions:

$$MI \rightleftharpoons MII + A \rightleftharpoons MII\,A$$
$$+ InP_n$$
$$\vdots$$
$$AInP_n$$

where A is arrestin, MI and MII is metarhodopsin I and II, respectively. The equation for IC_{50} derived from the scheme is $IC_{50} = \{[K_{D1}/(K_{D1} + 1]\times[A_{total} - R_{total})/K_{D2}] - 1\}\times K_{D3}$, where R is rhodopsin; $K_{D1} = [MII]/[MI]$; $K_{D2} = [MII - A]/[MII]\times[A]$; $K_{D3} = [A - InP_n]/[A]\times[InP_n]_{total}$. Abbreviations: D-myo-inositol 1-phosphate (InP_1); D-myo-inositol 1,4-bisphosphate (InP_2).

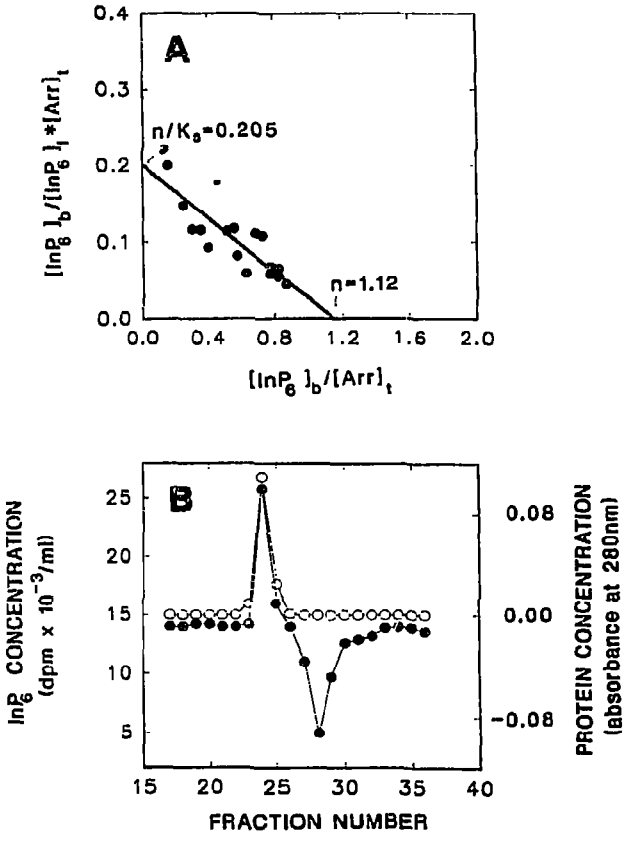

Fig.2. Binding of InP_6 to arrestin. A. Scatchard plot of equilibrium dialysis [12,15].[inositol-2-^{3}H(N)]hexakisphosphate and arrestin were placed in two different compartments of a dialysis cell and dialysed to equilibrium as described in section 2. B. Gel filtration according to ref. [16]. [inositol-2-^{3}H(N)]hexakisphosphate and arrestin were loaded on a Superose 6 column as described in section 2. Protein profile from 280 nm absorption (open circle), inositol from radioactive counting (closed circle).

kisphosphate in the other. Dialysis was conducted for 50 h at 5°C with continuous shaking. Controls showed that the system reached equilibrium. Inositol derivative concentrations were determined from specific radioactivity.

Gel filtration—Gel filtration was carried out according to Ackers [16] on a Superose 6 column (Pharmacia) equilibrated with 10 mM HEPES, pH 7.5, 100 mM NaCl, in the presence of 0.2 μM [inositol-1-^{3}H(N)]1,3,4,5-tetrakisphosphate or 0.2 μM [inositol-2-^{3}H(N)]hexakisphospate. 0.24 mg of arrestin was loaded per run. The protein profile was determined by the absorption at 280 nm; inositol concentration was determined by radioactive counting.

3. RESULTS

3.1. *Inositol phosphates compete against arrestin–rhodopsin interaction*

Fig. 1 shows original records (A) and competition curves (B) for the 'extra MII' assay. Under control conditions, a flash of light forms a small amount of metarhodopsin II (MII). Additional MII arises from the interaction of arrestin or transducin with MII. The amount of extra MII (MII level with arrestin minus control) is a stoichiometric measure of the complexes formed [14]. As Fig. 1A shows, only formation of the complex with arrestin, but not with transducin, is blocked by IP_6. All inositol phosphates gradually suppress extra-MII with increasing concentration (Fig. 1B), with a potency that increases roughly with the degree of phosphorylation. Among the three tested isomers of inositol tetrakisphosphate the isomer (1,3,4,5) is most effective (data not shown), tri-, di- and mono-phosphorylated derivatives of inositol are much less effective (Table I). The data for all inositol phosphates fit well to linear hyperbolic competition curves (lines through the data).

The MII stabilization data are consistent with the interpretation that one molecule of inositol phosphate

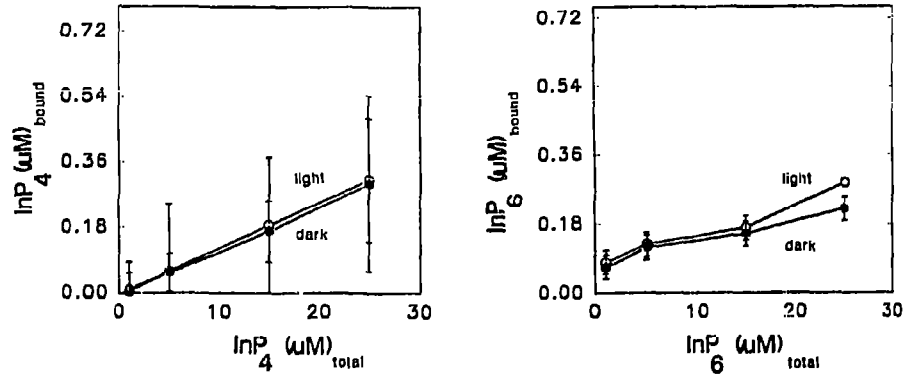

Fig.3. Binding of InP_4 and InP_6 to phosphorylated membranes and arrestin. The experiments analyse the trace amounts of inositol phosphates that bind to phosphorylated membranes when arrestin is bound to excess phosphorylated photoactivated rhodopsin. The sample of pre-phosphorylated rhodopsin, arrestin and different amounts of the inositol phosphates was illuminated (light) or incubated in the dark (dark) and centrifuged as described in section 2. It is seen that the illuminated sample (containing the rhodopsin arrestin complex) does not bind more inositol than the dark control.

interacts with arrestin and competes with phosphorylated metarhodopsin II for the same binding site.

Like transducin, rhodopsin kinase is not inhibited by up to 1 mM InP_6 (nor by InP_3, see ref. Palczewski et al. 1988b); cyclic GMP phosphodiesterase is not inhibited either by 250 μM InP_6 (data not shown).

3.2. *Inositol phosphates bind to free arrestin*

The interaction between inositol phosphates and arrestin can be directly monitored by equilibrium dialysis and gel filtration. Fig. 2A shows a Scatchard plot of equilibrium dialysis data for IP_6. One obtains a dissociation constant of K_D = 5.5 μM and a number of binding sites n = 1.12 For IP_4, the values are 12.5 μM and n = 1.02 (dat not shown). These values fit to the K_D's from the competition curves in Fig. 1 (see Table I).

Elution profiles of gel filtration are shown in Fig. 2B. The positive and negative peaks are expected for binding of InP_4 and InP_5 to arrestin. A comparison of the column profiles with the standard proteins bovine serum albumin (67 000 Da) and ovalbumin (43 000) ensured that arrestin dit not aggregate in the presence of the inositols (data not shown). When Heparin-Sepharose is used as an affinity matrix for arrestin, arrestin is the only protein eluted with InP_6 (see section 2). This argues for an at least partial overlap of the binding sites at arrestin for heparin and the inositol phosphates.

3.3. *Inositol phosphates do not bind to the rhodopsin-arrestin complex*

When an excess of pre-phosphorylated metarhodopsin II is formed, more than 90% of the arrestin in the sample is bound at μM concentration. As shown in Fig. 3, the amount of InP_4 and InP_6 recovered with the membranes is as small as in the dark control. Together with the data of the preceding section, this shows that bound inositol phosphates are released when arrestin binds to rhodopsin.

On the other hand, the data show a small, light-independent capacity of disc membranes to bind inositol derivatives.

4. DISCUSSION

Direct binding assays and inhibition of complex formation between arrestin and phosphorylated active metarhodopsin II (MII) have shown that arrestin binds inositol phosphates with micromolar affinity. The binding is specific, and is not even seen with rhodopsin kinase which shares with arrestin a high affinity for heparin. The binding site of inositol phosphates appears to overlap the binding domain of phosphorylated rhodopsin. Identification of inositol phosphates as specific ligands of arrestin, which decouple it from phosphorylated rhodopsin, provides a simple tool for studying arrestin function in the rod cell and simplifies its isolation. β-Arrestin [6] can be expected to be inhibited as well.

Further conclusions will depend on determination of the amount of higher inositol phosphates in the rod cell. InP_4, InP_5 and InP_6 are present in μM concentration in various mammalian cells, including neuronal cell lines [17,18]. A receptor for IP_6 was recently identified in rat brain [19]. Not only IP_3 but also higher phosphates are involved in the control of calcium [20].

Arrestin is abundant (100–300 μM) in rods [21] and the binding of the inositol phosphates (K_D in the 10 μM range, Table I) is tight enough to complex the accessible cellular inositol with arrestin. Since the complex of arrestin with phosphorylated metarhodopsin II is much tighter (K_D in the 50 nM range [14]), a change in the InsP concentration should not influence the rhodopsin-arrestin complex. However, bleaching of a large fraction of the rhodopsin (3 mM in the rod outer segment) could considerably affect the inositol pool. This is the condition of bleaching adaptation (see for example ref. [22]), a regulation of rod sensitivity that appears to bypass the G-protein [23,24]. Whether a regulatory pathway exits, which originates from the light-dependent release of arrestin-bound inositol phosphates, remains to be investigated.

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