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The presence of a calcium-sensitive p26-containing complex in bovine retina rod cells

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A protein with apparent M_r 26 kDa (p26 [(1991) Biokimya 56, 225–228] or recoverin [(1991) Science 251, 915–918]) was suggested to activate GC in a calcium dependent manner in bovine retina rod cells [(1991) Science 251, 915–918; (1991) EMBO J, 10, 793–798]. However, according to our present data homogeneous p26 preparations do not activate the enzyme. At the same time we have revealed a complex of p26 with an unidentified protein, presumably RK, in bovine ROS. Calcium favours formation of the complex whereas EGTA addition (which corresponds to a low free Ca^{2+} concentration) leads to its dissociation.

Photoreception; Recoverin; Guanylate cyclase; Rhodopsin kinase; Bovine retina rod cell

1. INTRODUCTION

Signal transduction in retina rod cells involves light activation of the cascade Rh–transducin–PDE responsible for light-dependent hydrolysis of the photoreceptor second messenger, cGMP. To bring the cell back to the dark state the activated cascade components should be switched off and the cGMP dark level in ROS should be restored ([4] for review).

Rhodopsin switch-off mechanisms include: (1) phosphorylation of Rh* (but not Rh) by RK (it should be stressed that the mechanisms of RK control are poorly known); (2) a 48-kDa protein which binds to phosphorylated Rh* and fully suppresses its catalytic activity. Transducin (and PDE) inactivation is achieved due to its intrinsic GTPase that transforms active (GTP-containing) transducin to its inactive (GDP-containing) form; the state of PDE depends directly on the state of transducin ([4] for review).

In contrast to the cascade swich-off mechanisms, control of the cGMP dark level recovery in the ROS cytoplasm is poorly understood. The recovery key enzyme, GC, is tightly bound with ROS structures [5–8] and controlled by a soluble protein factor in a calcium-dependent manner [9]. A Ca^{2+}-binding protein with apparent M_r 26 kDa (p26 [1] or recoverin [2]) was suggested to be a candidate for the role of the GC regulator in bovine ROS [2,3].

We communicate in this work that: (1) GC activity in bovine ROS membranes, depleted of p26, cannot be restored by addition of homogeneous p26; (2) Ca^{2+}-sensible complex of p26 and other protein(s) is present in bovine ROS extract. We suggest that a possible function of p26 in bovine rods can be control of RK and thus regulation of the cascade Rh–transducin–PDE.

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Abbreviations: ROS, rod outer segments; cGMP, 3',5'-cyclic guanosine monophosphate; Rh and Rh*, 'dark' and photobleached rhodopsin, respectively; GC, guanylate cyclase; PDE, cGMP-phosphodiesterase; RK, rhodopsin kinase.

2. EXPERIMENTAL

ROS prepared from fresh bovine retinas [10] under dim red light were frozen in liquid nitrogen and stored at −70°C. The Rh concentration was determined by the difference in the optical densities of the ROS suspension at 500 nm before and after illumination taking $\varepsilon = 42{,}000$ [11].

The procedure of p26 purification (our modification of the original method [1,2]) included fractionation by ammonia sulphate, affinity chromatography on the immobilized monospecific antibody to p26 and FPLC-chromatography on Mono-Q HR 5/5 (Pharmacia-LKB). p26 was identified using immunoblotting as described in [1,2]. Protein concentrations were determined by the Coomassie blue binding method [12]. SDS/PAGE was performed according to [13] using 12.5% gel and 1% SDS.

As GC preparation was used ROS suspension (30–50 μg Rh per sample) in 10 mM MOPS, pH 7.1, containing 5 mM dithiotreitol, and a pellet obtained by centrifugation (150,000 × g, 10 min, 4°C) of 1 mg Rh/ml ROS suspension in the same buffer. GC activity was determined at 25°C in almost complete darkness (under very week red light) in the reaction mixture [9] at a 'high' (about 400 nM) or 'low' (< 10 nM) concentration of free Ca^{2+} which was achieved by adding 0.9 mM $CaCl_2$ + 1 mM EGTA or 1 mM EGTA, respectively. To inhibit PDE activity 0.1 mM zaprinast and 1 mM isobutylmethylxanthine (Serva) were added to the reaction mixture. The reaction was started by addition of GTP and after 10 min was stopped by adding 0.5 N HCl with subsequent boiling for 5 min. The amount of cGMP in the samples was determined using columns with Al_2O_3, Brockman II (Serva) [14]. Thin-layer chromatography on PEI-cellulose (Sigma) [9]

was used to estimate cGMP hydrolysis in similar samples containing [γ-^{3}H]cGMP (Amersham); PDE activity during the reaction did not exceed 10% of that of GC.

Before gel-chromatography the ROS suspension (about 8 mg Rh/ml) in buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulphonylfluoride, 1 mM dithiotreitol) with or without 100 μM $CaCl_2$ was incubated for 30 min in the dark at 4°C. Then the suspension was centrifuged (27,000 × g, 15 min, 4°C) and the supernatant was used for FPLC chromatography on a Superose 12 HR 10/30 column (Pharmacia-LKB) in buffer A with 100 μM $CaCl_2$ or 1 mM EGTA or without additions.

3. RESULTS AND DISCUSSION

The data on the Ca^{2+}-dependent effect of p26 upon GC were first obtained using purified p26 and ROS membranes washed only once with a low salt buffer [2,3]. As the membranes thus washed retained a large amount of extractable proteins we removed the major portion of them by washing many times the membranes with a low salt buffer and attempted to reconstitute Ca^{2+}-sensitive GC activity using homogeneous p26 and the membranes thus obtained. Since in this case the reconstitution was not obtained (data not shown) we reproduced the conditions of the experiments described earlier [2,3].

One can see (Fig. 1) that GC in the ROS suspension depends on free Ca^{2+} concentration in the reaction mixture. Centrifugation of the ROS suspension produces a pellet (P) with a low GC level and a supernatant (S) with no GC activity. Combination P + S gives almost full restoration of Ca^{2+}-sensitive GC activity of the pellet. Contrary to this result the addition of homogeneous p26, instead of supernatant S, to pellet P does not reconstitute their Ca^{2+}-sensitive GC activity.

The experiment described gives us grounds to believe that GC is not the functional target for p26. The same conclusion has recently been made [15] by the other authors of the work [2]. Therefore, the seemingly solved problem on the p26 function has been raised again.

One of the approaches to answer this question can be a search for a complex between p26 and its target protein(s). To reveal this hypothetical complex we used FPLC-chromatography of an isotonic bovine ROS extract on a Superose 12HR 10/30 column (Fig. 2). One can see that homogeneous p26 was eluted from the column as a single peak with apparent M_r about 28 kDa (A,B). However, two fractions of p26 with apparent M_r about 28 and 94 kDa were present in the eluate after gel-chromatography of the isotonic ROS extract under the same conditions (C). The high-molecular fraction of p26 was revealed in this experiment both in the presence of 100 μM $CaCl_2$ during ROS extraction and gel-chromatography (see Fig. 2C) or without addition of Ca^{2+} (data not shown). However, quite a different picture was obtained when the elution buffer contained 1 mM EGTA: in this case the only low-molecular fraction of p26 was found (Fig. 2D) just as during gel-chromatography of homogeneous p26 (see Fig. 2B).

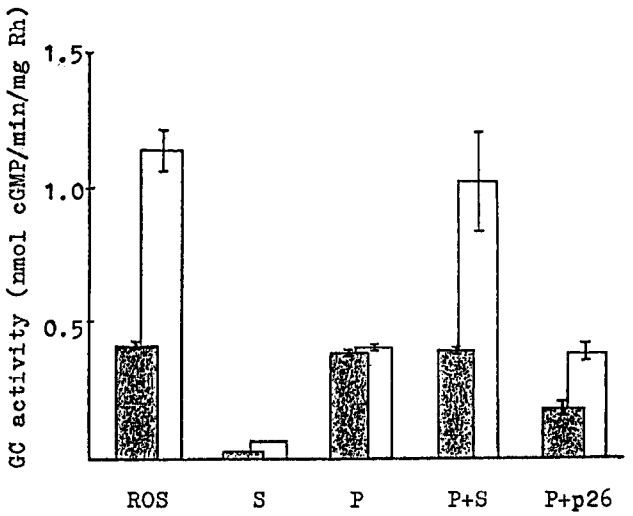

Fig. 1. GC activity in the ROS suspension and the reconstituted systems 'P + S' and P + p26' in the presence of high (full bars) or low (empty bars) free Ca^{2+} concentrations. Pellet 'P' and supernatant 'S' were obtained by centrifuging the ROS suspension (see section 2). 2–4 μg of p26 were added to the sample. Average magnitudes of the two values obtained in one of the 6 separate experiments are presented.

SDS-electrophoresis of the 94 kDa fraction (Fig. 2C, track b) revealed a major band of the 67 kDa protein (p67) apparent M_r of which coincided with that of RK [16]. In addition, several minor bands, apparently the 48 kDa protein (arrestin) and α- and β-subunits of transducin, were found in the fraction. This set of proteins differs from that obtained by the cross-linking method in [16].

Therefore, a complex of p26 with unidentified protein(s) is present in bovine ROS. Calcium favours formation of the complex whereas EGTA addition (which corresponds to a low free Ca^{2+} concentration) leads to its dissociation.

The following indirect evidence allows us to suggest that p67, presumably RK, might be a partner of p26 in the complex: (1) apparent M_r of the complex (94 kDa) coincides with the M_r sum of p26 and p67; (2) according to our unpublished data, p26 is present as a main admixture during RK purification up to the last stage of the purification procedure [17]; (3) S-modulin, a p26-like Ca^{2+}-binding protein from frog ROS, is capable of modulating the level of Rh phosphorylation in frog ROS [18].

At present, we are carrying out a detailed research on the p26–p67 interaction and verifying the suggestion that p67 is identical to RK.

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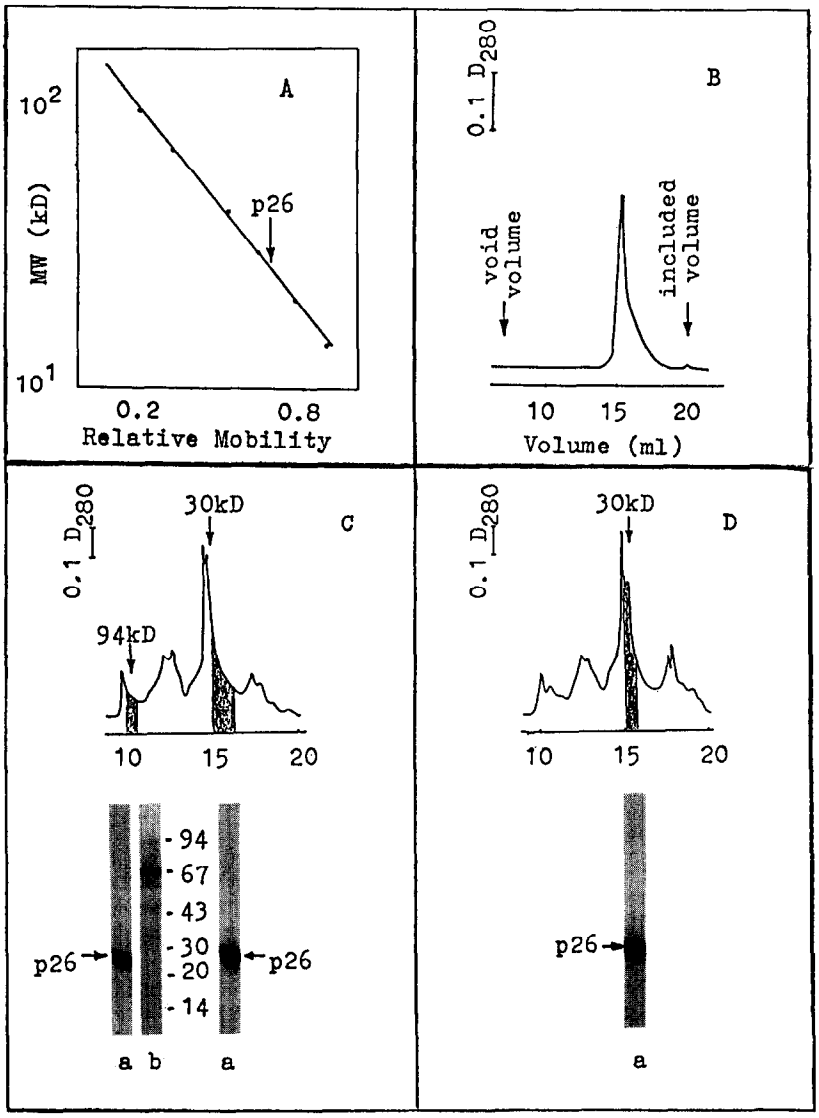

Fig. 2. FPLC chromatography of the ROS extract on a Superose 12HR 10/30 column. (A) The molecular weight standards used (Da) were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), α-lactalbumin (14,400) with blue dextran used to indicate the void volume and with sodium azide to mark the included volume. (B) The elution profile of homogeneous p26. (C) The elution profile of the ROS extract in the presence of 100 μM Ca^{2+}. (D) The same as (C) but in the presence of 1 mM EGTA. The hatched fractions were analyzed by immunoblotting (tracks a) and SDS/PAGE (track b).

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