Biochem. J. (1990) 265, 655–658 (Printed in Great Britain)

The effect of the γ-subunit of the cyclic GMP phosphodiesterase of bovine and frog (*Rana catesbiana*) retinal rod outer segments on the kinetic parameters of the enzyme

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Rod-outer-segment cyclic GMP phosphodiesterase (PDE) (subunit composition $\alpha\beta\gamma_2$) contains catalytic activity in $\alpha\beta$. The γ-subunits are inhibitors. Removal of the γ-subunits increases $V_{max.}$ without affecting the K_m. The inhibitory effect of a single γ-subunit ($\alpha\beta\gamma$) on the $V_{max.}$ of $\alpha\beta$ is much greater in bovine than in frog (*Rana catesbiana*) PDE. Bovine PDE in the $\alpha\beta\gamma_2$ state has a $V_{max.}$ that is $2.6\pm0.4\%$ of the $V_{max.}$ of $\alpha\beta$. The removal of one γ-subunit to give $\alpha\beta\gamma$ results in a $V_{max.}$ $5.2\pm1\%$ of that for maximal activity. Frog $\alpha\beta\gamma_2$ has a $V_{max.}$ $10.8\pm2\%$, and $\alpha\beta\gamma$ has a $V_{max.}$ $50\pm18\%$, of the $V_{max.}$ of $\alpha\beta$. These data suggest that a single γ-subunit can inhibit the catalytic activity of active sites on both α- and β-subunits in bovine, but not in frog, rod-outer-segment PDE.

INTRODUCTION

The outer segment of the retinal rod is known to contain a cyclic GMP-specific phosphodiesterase (PDE) (EC 3.1.4.17) (Miki *et al.*, 1975; Baehr *et al.*, 1979). Photoisomerized rhodopsin interacts with the rod GTP-binding protein, transducin (T), and stimulates binding of GTP to its α-subunit (Tα) (Fung & Stryer, 1980). The rod PDE is activated as a result of interacting with Tα in the GTP form (Tα·GTP) (Godchaux & Zimmerman, 1979; Fung *et al.*, 1981; Kohnken *et al.*, 1981). The decrease in rod-outer-segment (ROS) cyclic GMP concentration, as a result of the light-initiated activation of PDE, diminishes the conductance across the ROS plasma membrane by the closing of cyclic GMP-activated cation channels (Fesenko *et al.*, 1985).

The ROS PDE is a peripheral-membrane protein composed of two larger (α and β) subunits in which the catalytic activity resides (Pαβ) (Hurley & Stryer, 1982). There is also a smaller (γ) subunit (Pγ) which has been shown to function as the inhibitor of the catalytic activity of Pαβ (Hurley & Stryer, 1982). M_r values reported for various subunits are as follows: bovine PDE α-subunit 88000; β-subunit 84000; γ-subunit 11000 (Baehr *et al.*, 1979; Hurley & Stryer, 1982); frog PDE α-subunit 95000; α-subunit 94000; γ-subunit 13000 (Miki *et al.*, 1975; Hamm & Bownds, 1986; Yamazaki *et al.*, 1987). The inhibitory constraint imposed on Pαβ by Pγ is removed by the interaction of Pγ with Tα·GTP (Yamazaki *et al.*, 1983; Wensel & Stryer, 1986; Deterre *et al.*, 1986).

It has recently been shown that there are two Pγ per Pαβ in bovine ROS PDE (Deterre *et al.*, 1988). We have demonstrated that there are also two Pγ per Pαβ in frog ROS PDE. Additionally we found that the PDE of both frog and bovine ROS has a class of Pγ which is released into the supernatant as a soluble complex with Tα·GTP (releasable Pγ, rPγ) and another class of Pγ which remains membrane-associated (mPγ), even when dissociated from its inhibitory site on Pαβ by Tα·GTP (Whalen & Bitensky, 1989). In this paper we quantify that fraction of Pγ which is mPγ. We also compare the kinetic parameters of the ROS PDE in three different states: before removal of rPγ ($P\alpha\beta\gamma_2$), after its removal (postulated $P\alpha\beta\gamma$) and when both Pγ have been removed (Pαβ).

MATERIALS AND METHODS

Enzyme and protein assays

PDE activity was measured at 30 °C with cyclic [8-^{3}H]GMP. The 5′-GMP product was converted into guanosine by the addition of snake-venom 5′-nucleotidase and separated from substrate that had not reacted by the addition of anion-exchange resin (which binds only the unchanged substrate). A portion of the supernatant was counted for radioactivity in a liquid-scintillation counter (Thompson & Appleman, 1971). PDE activity was determined at six different concentrations of cyclic GMP from 39 μM to 1.25 mM. Protein was determined by the method of Bradford (1976) or by the BCA protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.), with bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) as the standard.

Quantification of Pγ on ROS

Solid-phase radioimmunoassays were carried out on ROS preparations using an antibody to a 15-amino-acid sequence from the *C*-terminus (amino acids 73–87) of bovine Pγ (Cunnick *et al.*, 1988). ROS were applied to glutaraldehyde-coated polystyrene tubes and were then

Abbreviations used: GTP[S], guanosine 5′-[γ-thio]triphosphate; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide-gel electrophoresis; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane ('TPCK'); ROS PDE, rod-outer-segment cyclic GMP phosphodiesterase.

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incubated with Pγ antibody. Antibody bound to ROS was quantified by treatment with ^{125}I-Protein A (Suter, 1982; Cunnick *et al.*, 1988).

Preparation of bovine ROS PDE

ROS were prepared from frozen bovine retina (Hormel, Austin, MN, U.S.A.) under dim red light, by a minor modification of the sucrose-flotation method of Papermaster (1982). We added 100 mM-KCl to the diluting buffer used in the procedure. ROS were bleached with room light, and washed, as follows, to remove most soluble and some peripheral-membrane proteins (Kuhn, 1981; Yamazaki *et al.*, 1988). ROS were suspended in 100 mM-Tris/HCl (pH 7.5)/5 mM-$MgSO_4$/5 mM-DTT/0.1 mM-PMSF (buffer A) by passage eight times through a 21-gauge needle and centrifuged at 48000 ***g*** to remove soluble proteins (repeated three times). ROS membranes were next resuspended (as described above) in 10 mM-Tris/HCl (pH 7.5)/5 mM-$MgSO_4$/5 mM-DTT/0.1 mM-PMSF (buffer B) (repeated twice). This preparation of ROS PDE has no Pγ removed. ROS membranes were finally washed (four times) with buffer A containing 0.1 mM-guanosine 5′-[γ-thio]triphosphate (GTP[S]) to remove Tα·GTP[S]·rPγ and Tα·GTP[S] (Whalen & Bitensky, 1989). Since we used membranes rather than purified PDE, we estimated the amount of PDE from the activity of trypsin-treated membranes using a catalytic-centre activity of 1000/s (Sitaramayya *et al.*, 1986). Membranes were divided into portions and stored at −80 °C.

Preparation of frog ROS PDE

ROS were prepared from dark-adapted (12h) frogs (*Rana catesbiana*) by flotation on 45% (w/v) sucrose (Halliday *et al.*, 1984), followed by the same treatment as described for bovine ROS membranes.

Trypsin-activated PDE

Bovine or frog ROS PDE was exposed to Tos-Phe-CH_2Cl-treated trypsin (20 μg/ml with an activity of 222 units/mg) at 30 °C. Times of exposure were selected to give maximal activation of PDE (by digestion of Pγ). These were 3 min for bovine PDE and 1.5 min for frog PDE. The reaction was stopped by the addition of a 5-fold excess of soybean trypsin inhibitor (1 mg inhibits 1.7 mg of trypsin).

Preparation of bovine and frog Tα·GTP[S]

The ROS membranes were initially washed with buffer A (three times) and buffer B (twice). Tα·GTP[S] was then eluted from membranes by washing with buffer A containing 0.1 mM-GTP[S] (Yamazaki *et al.*, 1988). Supernatants from the GTP[S] washes were concentrated to a volume of 0.30 ml with an Amicon CF-25 filtration cone. Concentrated supernatants were applied to a TSK-250 molecular-sieve h.p.l.c. column (fractionation range 300000–1000 M_r; Bio-Rad, Richmond, CA, U.S.A.), and eluted with 20 mM-sodium phosphate (pH 6.8)/50 mM-Na_2SO_4/10 mM-$MgSO_4$/1 mM-DTT (Yamazaki *et al.*, 1988). Column fractions were monitored for Tα·GTP[S] activity by their ability to stimulate PDE activity. The column was calibrated with aldolase (M_r 158000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000) and chymotrypsinogen A (M_r 25000). Tα·GTP[S] was eluted at an apparent M_r of 43000. Tα·GTP[S]-containing fractions were pooled and stored in 50% (v/v) glycerol at −20 °C. The Tα gave a single band on SDS/PAGE (Laemmli, 1970) when the gel was stained with Coomassie Brilliant Blue.

Table 1. Michaelis constants and maximum velocities of bovine ROS PDE

Postulated subunit composition of PDE (treatment)	$V_{max.}$* (μmol/min per mg)	K_m* (μM)	Antibody binding† (%)
$P\alpha\beta\gamma_2$ (no Pγ removed)	7.7±1.3	196±45‡	100
$P\alpha\beta\gamma$ (GTP[S]-washed)	15.6±3.9	202±62‡	55±4
$P\alpha\beta$ (assayed with Tα·GTP[S]‖)	277±28	202±75§	ND¶
$P\alpha\beta$ (trypsin)	300±10	110±21‡	0

* From Eadie–Hofstee plots; r values were routinely > 0.97.
† Values corrected for antibody binding in blanks and normalized to antibody binding of ROS with no Pγ removed (in order to compare antibody binding data from three different experiments).
‡ Eight or more determinations.
§ Three determinations.
‖ Pγ removal by assaying PDE activity in the presence of 2–4 μM purified Tα·GTP[S] using ROS membranes from which endogenous Tα and rPγ had already been removed by GTP[S] washing.
¶ ND, not determined.

RESULTS

Pγ content of ROS membranes

Antibody binding was compared between bovine ROS membranes from which rPγ had been removed (GTP[S]-washed ROS) and those where both Pγ were still present (Table 1). ROS from which rPγ had been removed bound 55±4% as much Pγ antibody as those from which it had not been removed.

The presence of 2–4 μM purified Tα·GTP[S], during the assay of GTP[S]-washed-ROS PDE, fully activates the enzyme from both bovine and frog ROS (Whalen & Bitensky, 1989). However, if this high concentration of Tα·GTP[S] is removed before assay, the PDE activity remains inhibited.

Consistent with this observation, GTP[S]-washed bovine ROS bind the same amount of Pγ antibody, whether or not they have been previously exposed to 2–4 μM purified Tα·GTP[S]. These facts indicate that concentrations of Tα·GTP[S] sufficient to remove mPγ from inhibition of Pαβ do not remove it from the ROS membranes.

The antibody to bovine Pγ was also used to study the Pγ content of frog ROS. Removal of rPγ from frog ROS (by washing with GTP[S]) resulted in 44±2% as much Pγ-antibody binding (Table 2).

Kinetic parameters of bovine ROS PDE at various levels of inhibition

We determined the K_m and $V_{max.}$ of bovine ROS PDE with no exposure to GTP[S] ($P\alpha\beta\gamma_2$), after removal of rPγ (predominantly $P\alpha\beta\gamma$), after maximal activation by trypsin digestion ($P\alpha\beta$) and maximal activation by Tα·GTP[S] ($P\alpha\beta$) (i.e. 2–4 μM purified Tα·GTP[S] present during the assay of GTP[S]-washed ROS) (Table 1).

Table 2. Michaelis constants and maximum velocities of frog ROS PDE

Postulated subunit composition of PDE (treatment)	$V_{max.}$* (μmol/min per mg)	K_m* (μM)	Antibody† binding (%)
$P\alpha\beta\gamma_2$ (no Pγ removed)	32.5±6.1	79.6±14.6‡	100
$P\alpha\beta\gamma$ (GTP[S]-washed)	150±54	74.8±10.1‡	44±2
$P\alpha\beta$ (assayed with Tα·GTP[S]‖)	277±34	116±67§	ND¶
$P\alpha\beta$ (trypsin)	300±20	116±40‡	0

* As in Table 1 footnote.
† As in Table 1 footnote.
‡ Five or more determinations.
§ As in Table 1 footnote.
‖ As in Table 1 footnote.
¶ ND, not determined.

The $V_{max.}$ of $P\alpha\beta\gamma_2$ is 2.6±0.4% of the $V_{max.}$ of $P\alpha\beta$ (trypsin treatment). Removal of rPγ results in an increase only to 5.2±1% of maximum, with no change in K_m. $P\alpha\beta$, produced by using 3 μM-Tα·GTP[S], has 92±9% of the $V_{max.}$ resulting from trypsin treatment, with no change in K_m. The K_m of $P\alpha\beta$ formed by trypsin treatment, however, is approximately half that of $P\alpha\beta$ produced by Tα·GTP[S].

Kinetic parameters of frog ROS PDE at various levels of inhibition (Table 2)

Frog $P\alpha\beta\gamma_2$ has 10.8±2.0% of the $V_{max.}$ of $P\alpha\beta$ (trypsin treatment). The removal of rPγ results in 50±18% of maximal activity, with no change in the K_m. The $V_{max.}$ of $P\alpha\beta$ produced by Tα·GTP[S] is 92±11% of $V_{max.}$ produced by trypsin treatment, with no statistically significant change in K_m (according to Student's t test). Unlike the bovine enzyme, $P\alpha\beta$ produced by trypsin removal of Pγ has the same K_m as that produced by Tα·GTP[S].

DISCUSSION

There are two Pγ subunits per $P\alpha\beta$ in both bovine and frog ROS (Deterre *et al.*, 1988; Whalen & Bitensky, 1989). The Pγ-antibody-binding data presented here indicate that approximately half of the total Pγ, in both bovine and frog ROS PDE, is released from the ROS membranes by Tα·GTP[S]. The other half of the Pγ in the ROS remains on the membranes (mPγ), although its inhibitory effect can be removed by high concentrations of Tα·GTP[S]. Since approximately half of the total Pγ is rPγ, it is reasonable to assume that removal of rPγ results in the formation of $P\alpha\beta\gamma$.

The K_m of the frog enzyme is essentially the same in all forms tested, and the K_m of the bovine enzyme is unchanged by activation with Tα·GTP[S] *in vitro*. However, digestion of both Pγ from the bovine enzyme by trypsin decreases the K_m by approximately half. Thus trypsin alters bovine $P\alpha\beta$ in a way that normal activation does not. Trypsin has previously been shown to have effects which are not simply the result of Pγ digestion [for example, trypsin treatment releases $P\alpha\beta$ from ROS membranes by a mechanism apparently not related to the loss of Pγ (Wensel & Stryer, 1986)].

These data indicate that, in both bovine and frog ROS, Pγ inhibits PDE activity by decreasing $V_{max.}$ rather than by increasing the K_m for cyclic GMP. These results also indicate a significant difference between the two species in the allosteric inhibition of $P\alpha\beta$ by mPγ. The data for both the frog and bovine PDE fit a model where there are two active sites per $P\alpha\beta$ (most simply, one on each subunit). In frog PDE, one Pγ inhibits only one active site. However, in bovine PDE it appears that one Pγ inhibits both active sites. This model is consistent with the fact that when half of the Pγ is removed from frog PDE, half of the full enzyme activity is expressed. However, in bovine PDE removal of half of the Pγ results in the expression of only 5% of maximal activity. Thus the data suggest that, *in vivo*, bovine PDE requires removal of both Pγ in order to achieve significant activation.

The results indicate that mPγ remains with the ROS membranes, even at concentrations of Tα·GTP[S] that remove its inhibition of $P\alpha\beta$, and is able to re-inhibit $P\alpha\beta$ upon removal of Tα·GTP[S]. *In vivo*, concentrations of Tα·GTP sufficient to remove all inhibition of $P\alpha\beta$ can be readily achieved [Tα·GTP may reach as high as 500 μM (Chabre, 1985)]. If mPγ remains membrane-associated, even with high concentrations of Tα·GTP *in vivo*, it may be part of a mechanism for rapid PDE turn-off. As Tα·GTP is inactivated by hydrolysis of GTP, mPγ could readily re-inhibit $P\alpha\beta$ (resulting in as much as 95% turn-off of PDE) without having to re-attach to the membrane.

We thank Dr. Gaynor Wild for critical reading of the manuscript. The work was supported by grant EYO6816 from the National Institutes of Health.

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Received 25 April 1989/30 August 1989; accepted 11 September 1989