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Comparison of the phosphodiesterase inhibitory subunit interactions of frog and bovine rod outer segments

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The rod outer segments of the bovine and frog retina possess a cyclic GMP phosphodiesterase (PDE) that is composed of two larger subunits, α and β (P$\alpha\beta$), which contain the catalytic activity and a smaller γ (Pγ) subunit which inhibits the catalytic activity. We studied the binding of Pγ to P$\alpha\beta$ in both the bovine and frog rod outer segment membranes. Analysis of these data indicates that there are two classes of Pγ binding sites per P$\alpha\beta$ in both species. The activation of PDE by the guanosine 5′-[γ-thio]triphosphate form of the α subunit of transducin, Tα·GTPγS, was also studied. These data indicate that the two classes of Pγ binding sites contribute to the formation of two classes of binding sites for Tα·GTPγS. We demonstrate solubilization of a portion of the Pγ by Tα·GTPγS in both species. There is also present, in both species, a second class of Pγ which is not solubilized even when it is dissociated from its inhibitory site on P$\alpha\beta$ by Tα·GTPγS. The amount of full PDE activity which results from release of the solubilizable Pγ is about 50% in the frog PDE but only approx. 17% in the bovine PDE. We also show that activation of frog rod outer segement PDE by trypsin treatment releases the PDE from the membranes. This type of release by trypsin has already been demonstrated in bovine rod outer segments [Wensel & Stryer (1986) Proteins: Struct. Funct. Genet. **1**, 90–99].

INTRODUCTION

The dark-adapted rod photoreceptor is able to decrease its outer-segment membrane current by 3–5% in response to a single photon [1]. Cyclic GMP is known to regulate the light-sensitive conductance in the rod outer segment (ROS) [2]. The ROS cytoplasmic concentration of cyclic GMP is decreased as a result of photoisomerized rhodopsin acting through a GTP-binding protein which activates a cyclic GMP phosphodiesterase (PDE) (EC 3.1.4.17) [3–8]. Details of the molecular interactions of the light activation of PDE should contribute to the general understanding of the role of GTP-binding proteins in the regulation of signal transduction.

The ROS PDE is a peripheral membrane protein composed of three nonidentical subunits, including a dimer of two larger (α and β) subunits in which the catalytic activity resides (P$\alpha\beta$). There is also a smaller (γ) subunit (Pγ) which has been shown to function as the inhibitor of the catalytic activity of the enzyme [9] (M_r values as follows: for bovine PDE $\alpha = 88000$, $\beta = 84000$ and $\gamma = 11000$ [9,10]; for frog PDE $\alpha = 95000$ $\beta = 94000$ and $\gamma = 13000$ [3,11,12]. The inhibitory constraint imposed upon P$\alpha\beta$ by Pγ can be removed by the GTP form of the α subunit of the rod GTP-binding protein, transducin α (Tα) [13–16].

In this paper, we compare the interactions of Pγ with P$\alpha\beta$ between frog and bovine ROS PDEs. Previous studies have indicated interesting differences in the activation of PDE between the two species, specifically in terms of the GTP-dependent release of the Pγ subunit from the membrane associated P$\alpha\beta$ [13,15]. Both the frog and bovine PDE can be maximally activated by limited trypsin treatment of ROS membrane suspensions. This activation is the result of trypsin digestion of Pγ [9]. The physiological activator, Tα·GTP, appears to activate PDE by dissociating Pγ from its inhibitory site on P$\alpha\beta$ while forming a Tα·GTP·Pγ complex [15]. However, under experimental conditions where activation of PDE by Tα·GTP was studied, the Tα·GTP·Pγ complex appeared to be soluble in frog ROS [13] but to remain membrane-bound in bovine ROS [15]. In this paper we study aspects of the interaction of Pγ with P$\alpha\beta$ and ROS membranes in both species to understand these relationships better and the manner and extent to which they differ between the two species.

We have found two classes of binding sites for Pγ in both the frog and bovine PDEs. This is consistent with the recent finding that there are two Pγ associated with each P$\alpha\beta$ in the bovine PDE [17]. We also demonstrate release of a portion of the total inhibitory activity into the supernatant by Tα·GTPγS and hypothesize that this inhibitory activity is released predominantly from a single class of binding sites. This release occurs in both the bovine and amphibian systems. There is also present, in both species, a second class of Pγ which is dissociated from its inhibitory site on P$\alpha\beta$ but is not solubilized by Tα·GTPγS. A significant difference exists between the species in the amount of PDE activity which results from the release of the Pγ from the first site. This species difference may indicate a greater degree of allosteric regulation among the PDE subunits of the mammalian ROS.

MATERIALS AND METHODS

Enzyme and protein assays

Phosphodiesterase activity was measured at 30°C with cyclic [8-^{3}H]GMP. The 5′-GMP product was converted

Abbreviations used: ROS, rod outer segments; PDE, phosphodiesterase; GTPγS, guanosine 5′-[γ-thio]triphosphate; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide-gel electrophoresis.

to guanosine by the addition of snake venom 5'-nucleotidase and separated from unreacted substrate by the addition of anion exchange resin (which binds only unreacted substrate). An aliquot of the supernatant was counted in a liquid-scintillation counter [18]. The concentration of cyclic GMP in the assay was 1 mM. Protein was determined by the method of Bradford [19] or with the BCA protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.) using bovine serum albumin (Sigma) as the standard.

Preparation of bovine ROS PDE

The 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 [20]. In our preparations, we added 100 mM-KCl to the diluting buffer used in the procedure. ROS were bleached with room light, and washed to remove most soluble and some peripheral membrane proteins [21,22]. ROS were suspended in 100 mM-Tris/HCl, pH 7.5, containing 5 mM-$MgSO_4$, 5 mM-DTT and 0.1 mM-PMSF (Buffer A) by passage through a 21-gauge needle (8×) and then centrifuged at 48000 *g* to remove soluble proteins (repeated 3×). ROS membranes were then resuspended (as above) in 10 mM-Tris/HCl, pH 7.5, containing 5 mM-$MgSO_4$, 5 mM-DTT and 0.1 mM-PMSF (Buffer B) to remove some loosely associated membrane proteins (repeated 2×). The proteins of interest remain membrane-bound in the presence of 5 mM-Mg^{2+}, or in the case of Tα, in the absence of GTP. Finally, the ROS membranes were washed (4×) with buffer A containing 0.1 mM-GTPγS in order to elute a large fraction of Tα from the membranes, which were then used as the source of PDE. Since we used ROS membranes rather than purified PDE it was not possible to measure the amount of PDE directly. Thus, we determined the amount of PDE based on its known ratio to a quantifiable ROS protein from the same membranes, or from the turnover number of trypsin-activated PDE. The PDE concentration on the membranes was determined from the amount of purified Tα obtained from a given ROS preparation (T:PDE $\simeq$ 10:1) [10,23]. PDE concentration was also determined from PDE activity of the trypsin-treated membranes using a turnover number of 1000 s^{-1} [23]. The two independent determinations of PDE concentration gave results which agreed within 20% and the two values were routinely averaged. The membranes were divided into portions and stored at −80 °C.

Preparation of frog ROS PDE

Frog ROS were prepared from dark adapted (12 h) *Rana catesbiana* by flotation on 45% (w/v) sucrose [25] and the ROS membranes were washed and stored in the same way as the bovine ROS membranes.

Preparation of bovine PDE inhibitory subunit (Pγ)

Bovine ROS membranes were initially washed with buffer A (3×), buffer B (2×) and buffer A containing 0.1 mM-GTPγS (4×). The membranes were then washed (4×) with 10 mM-Tris/HCl, pH 7.5, containing 0.5 mM-$MgSO_4$, 5 mM-DTT and 0.1 mM-PMSF (buffer C). Exposure of the ROS membranes to low-Mg^{2+} buffer elutes the PDE [3,9,10,22]. The combined low-Mg^{2+} supernatants were then lyophilized, which resulted in destruction of PDE activity, leaving the inhibitory activity of Pγ intact. The lyophilized PDE preparation was resuspended in approx. 500 μl of water, centrifuged to remove insoluble material and applied to an h.p.l.c. molecular sieve column (TSK-125 with a fractionation range 60000–500) obtained from BioRad, Richmond, CA, U.S.A. The column was eluted with 20 mM-phosphate, pH 6.8, containing 50 mM-Na_2SO_4, 10 mM-$MgSO_4$ and 0.005% Brij 35 (buffer D). Column fractions were assayed for ability to inhibit trypsin-treated PDE. Fractions with inhibitory activity were eluted from the column at an estimated M_r of 12000. The column was calibrated with bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), chymotrypsinogen A (M_r 25000) and ribonuclease A (M_r 13700). The Pγ prepared by this TSK-125 column procedure sometimes showed additional bands on SDS/PAGE (8–20% acrylamide) [24]. These contaminants were removed by a subsequent run through the same column. After the second column run the Pγ gave a single band at M_r approx. 11000 on SDS/PAGE. Inhibitory fractions were pooled and stored at −20 °C. γ-subunit concentrations were determined by the BCA protein assay.

Preparation of frog inhibitory subunit (Pγ)

A portion of the inhibitory activity elutes with the GTP binding protein in the amphibian [13]. The frog ROS membranes were washed with buffer A (3×) and buffer B (2×). Buffer A containing 0.1 mM-GTP (4×) was then used to elute Tα and accompanying inhibitory activity from the membranes. The GTP supernatants were lyophilized, applied to an h.p.l.c. TSK-125 column and eluted with buffer D. The inhibitory activity elutes from this column with an apparent M_r of 13 000. We pooled the inhibitory fractions from a single column run. The Pγ was either homogeneous or showed a contaminating band on SDS/PAGE (8–20% acrylamide) [24]. The contaminant was removed upon a subsequent run through the same column, giving a single band at M_r approx. 13000 on SDS/PAGE. Frog Pγ concentrations were determined as they were for the bovine Pγ.

Preparation of bovine and frog Tα·GTPγS

The ROS membranes were initially washed with buffer A (3×) and buffer B (2×). Tα with GTPγS bound to it is rather selectively eluted from the ROS by washing with buffer A (containing 0.1 mM-GTPγS) [22]. Supernatants from the GTPγS washes were concentrated to a volume of 300 μl using an Amicon CF-25 filtration cone. The concentrated supernatants were applied to h.p.l.c. TSK-250 (BioRad) molecular sieve column (fractionation range 300000–1000). The column was eluted with 20 mM-phosphate, pH 6.8, containing 50 mM-Na_2SO_4, 10 mM-$MgSO_4$ and 1 mM-DTT [22]. Column fractions were monitored for Tα 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 eluted at an apparent M_r of 43000. Tα-containing fractions were pooled and stored in 50% glycerol in the presence of 10 mM-$MgSO_4$ (which keeps the GTPγS bound to Tα [26]) at −20 °C. The pooled Tα showed a single band on SDS/PAGE (8–20% acrylamide) [24] stained with Coomassie Brilliant Blue. The protein concentrations of these preparations were determined by the method of Bradford [19].

Trypsin-activated PDE

Bovine or frog membrane PDE (prepared as described above) were exposed to tosylphenylalanine chloromethane ('TPCK')-treated trypsin (20 μg/ml with activity of 222 unit/mg) at 30 °C. Times of exposure were selected to give maximal activation of PDE activity, and were 3 min for bovine PDE and 1.5 min for frog PDE preparations. The reaction was stopped by the addition of a 5-fold excess of soybean trypsin inhibitor (1 mg inhibits 1.7 mg of trypsin).

Each experiment was repeated one or more times. The Figures show the results of a representative experiment.

RESULTS

Inhibition of frog ROS PDE activated by trypsin

Since limited trypsin treatment of ROS PDE results in proteolysis of Pγ and complete activation of PDE in bovine ROS [9], this preparation was used in studying inhibition of frog P$\alpha\beta$ by Pγ. The fully trypsin-activated frog PDE was assayed in the presence of increasing concentrations of Pγ. The inhibition data are shown in Fig. 1. The Hill coefficient (Fig. 1*c*) is 0.70 ± 0.06. Hill coefficients of less than one indicate negative co-operativity or two non-interacting sites of different affinities. Fig. 1(*b*) is a linear transformation [14] of the inhibition data. Here, the inverse of the slope gives an apparent dissociation constant for Pγ. The binding equilibrium equation from which the linear transformation is derived:

$$P\alpha\beta + P\gamma \underset{K_{D1}}{\rightleftharpoons} P\alpha\beta\gamma$$

initially assumes a single Pγ subunit binding to P$\alpha\beta$. This transformation of the data, however, yields two slopes (two apparent dissociation constants), indicating that two Pγ subunits bind per PDE:

$$P\alpha\beta + P\gamma \underset{K_{D1}}{\rightleftharpoons} P\alpha\beta\gamma + P\gamma \underset{K_{D2}}{\rightleftharpoons} P\alpha\beta\gamma_2$$

Although the linear transformation is useful in emphasizing the biphasic nature of inhibition of P$\alpha\beta$ by Pγ, it is not possible to calculate an accurate K_{D1} and K_{D2} from it. Accurate K_{D1} and K_{D2} determinations would require a knowledge of [P$\alpha\beta\gamma$/P$\alpha\beta$] for K_{D1} and [P$\alpha\beta\gamma_2$/P$\alpha\beta\gamma$] for K_{D2} at each concentration of free Pγ.

Inhibition of bovine ROS PDE activated by trypsin

We also studied the effects of increasing concentrations of Pγ on the bovine PDE which had been fully activated by trypsin (Fig. 2*a*). Hill plots of these data (Fig. 2*c*) give a Hill coefficient of 1.85 ± 0.07. As with the Hill coefficients generated in the preceding experiments, these data also indicate that there are at least two classes of binding sites for Pγ. The linear transformation (Fig. 2*b*) gives two slopes indicating two different Pγ binding sites on the bovine PDE. These data are consistent with the finding of Deterre *et al.* that two Pγ are associated with each P$\alpha\beta$ [17].

Tα·GTPγS activation of the ROS PDE

The demonstration of two classes of inhibitory subunit binding sites from the above studies with Pγ would predict biphasic activation of PDE by Tα·GTPγS, since Tα interacts directly with Pγ in the activation of PDE [14,15]. We measured the percentage of maximum PDE activity (trypsin-activated) produced by increasing concentrations of purified Tα·GTPγS added to bovine ROS PDE (Fig. 3*a*). A linear transformation for these activation data (Fig. 3*b*) is analogous to the transformation made for the inhibition data (see legend to Fig. 3). The transformation gave two slopes, indicating that activation by Tα·GTPγS occurred at two classes of binding sites each with a different apparent affinity. The Hill coefficient (Fig. 3*c*) for activation by Tα·GTPγS was 1.57 ± 0.11, indicating at least two classes of sites for Tα interaction with the inhibited bovine PDE. The two classes of Tα·GTPγS binding sites would likely correspond to Pγ bound at two different classes of binding sites on P$\alpha\beta$. A biphasic activation by Tα·GTPγS is also seen with the frog PDE (results not shown).

Is biphasic inhibition due to two species of ROS PDE?

An alternative explanation for the biphasic inhibition of ROS PDE is that the two classes of Pγ binding sites are the result of two species of PDE, P$\alpha\beta$ and P$\alpha'\beta'$, each

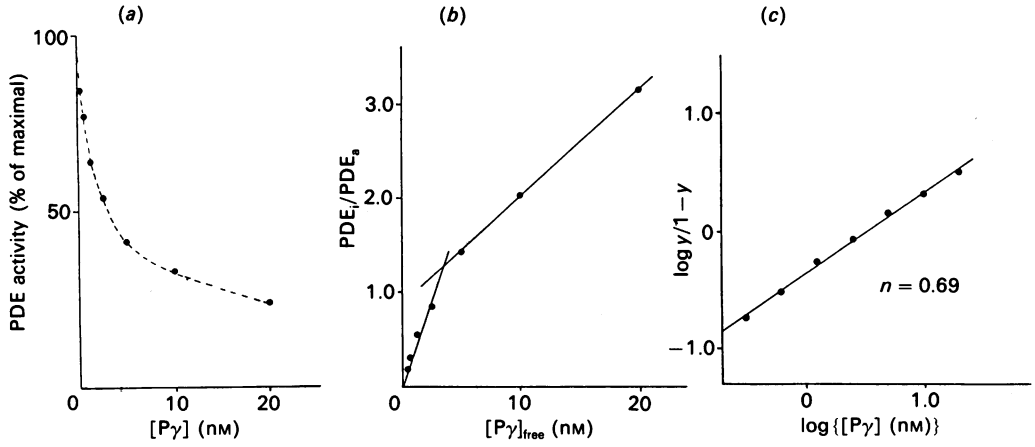

Fig. 1. Inhibition of frog ROS PDE activated by trypsin

(*a*) PDE activity was plotted as a function of Pγ concentration; [PDE] = 5×10^{-10} M. (*b*) Ratio of inhibited PDE (PDE_i) to activated PDE (PDE_a) was plotted as a function of free Pγ concentration; *r* values for calculated lines = 0.983 and 1.00. (*c*) Hill plot of inhibition data, $r = 0.997$.

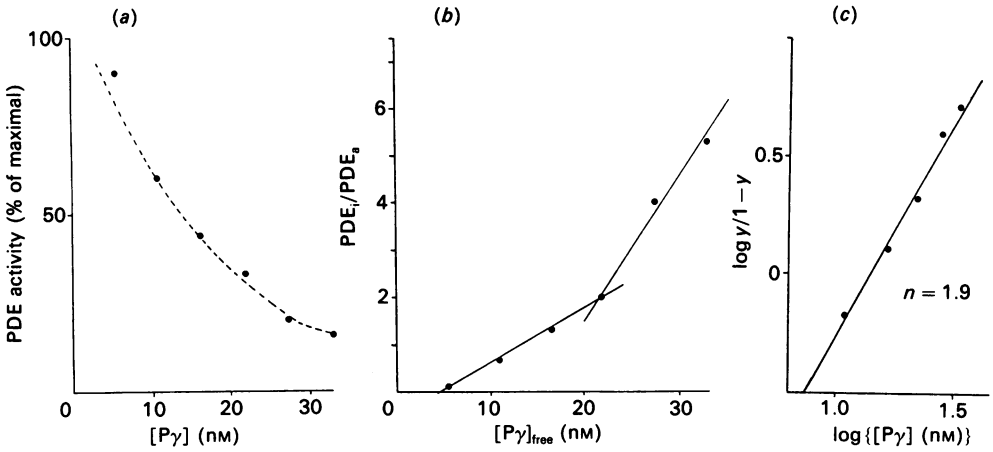

Fig. 2. Inhibition of bovine ROS PDE activated by trypsin

(*a*) PDE activity was plotted as a function of γ subunit concentration; [PDE] = 3×10^{-10} M. (*b*) Ratio of inhibited PDE (PDE_i) to active PDE (PDE_a) as a function of the concentration of free Pγ; *r* values for the calculated lines = 0.997 and 0.994. (*c*) Hill plot of inhibition data, $r = 0.994$.

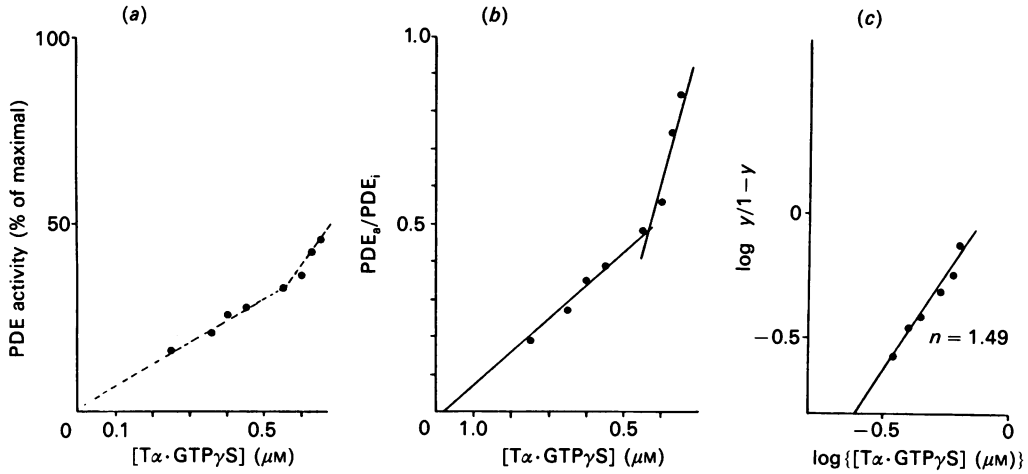

Fig. 3. Activation of bovine PDE by bovine Tα·GTPγS

(*a*) PDE activity was plotted as a function of Tα·GTPγS concentration; [PDE] = 2.5×10^{-10} M. (*b*) Ratio of activated PDE (PDE_a) to inhibited PDE (PDE_i) was plotted as a function of Tα·GTPγS (PDE_a = fraction maximal trypsin-activated activity for 2.5×10^{-10} M-PDE); *r* values for the calculated lines = 0.996 and 0.947. (*c*) Hill plot of activation data, $r = 0.972$.

of which has a different affinity for binding a single Pγ.

To address this question we looked at the M_r profile of trypsin-treated frog ROS PDE. Activation of ROS PDE by trypsin results in dissociation of essentially all PDE activity from the ROS membranes (see below). We applied frog PDE which had been released from the ROS membranes by trypsin to a TSK-250 molecular sieve and assayed the column fraction for PDE activity. The PDE activity eluted as a single peak at M_r approx. 160000 (Fig. 4). Bovine ROS PDE released from the membranes by low-Mg^{2+} buffer (see the Materials and methods section), also gave a single peak of activity (M_r approx. 160000) when chromatographed on the TSK-250 molecular sieve.

These data indicate that we are studying Pγ binding to a single M_r species of PDE. Since the trypsin-activated PDE is no longer associated with the membranes it is also unlikely that there is heterogeneity in Pαβ due to different orientations in the membrane or by association with other membrane components. Thus two species of ROS PDE (Pαβ and Pα′β′) each with a single Pγ binding site, with Pαβ having a different affinity for Pγ than Pα′β′, seems unlikely in our preparations. In light of the demonstration of two Pγ per Pαβ in bovine ROS PDE by Deterre *et al.* [17], we think the simplest interpretation of the biphasic inhibition and activation data in both the frog and bovine ROS is the existence of two Pγ binding sites per Pαβ.

The magnitude of Tα·GTPγS activation of bovine and frog PDE is equivalent to trypsin activation

When either frog or bovine PDE is assayed in the presence of concentrations of Tα·GTPγS greater than 4 μM the level of activity is that of the trypsin-activated enzyme (Table 1). Equivalence of trypsin and Tα·GTPγS

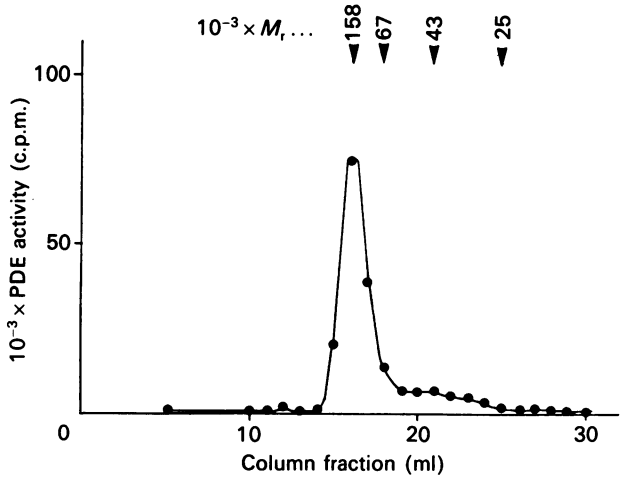

Fig. 4. Elution profile of frog PDE activity (released from ROS by trypsin) run on a TSK-250 column

Supernatants from trypsin-treated frog ROS were concentrated in an Amicon CF-25 filtration cone and applied to a TSK-250 molecular sieve. The column was eluted with 20 mM-phosphate, pH 6.8, containing 50 mM-Na_2SO_4, 10 mM-$MgSO_4$ and 1 mM-DTT. An aliquot of the column fractions was assayed for PDE activity. The PDE activity eluted at M_r approx. 160000.

activation has already been demonstrated in the bovine enzyme [5,23]. The data in Table 1 show that this is also true in the frog PDE. However, when Tα·GTPγS is removed by centrifugation, the PDE activity of frog ROS membranes returns to approx. 50% of maximal activity (Table 1) while the PDE activity of bovine ROS membranes returns to approx. 17% of maximal activity ([14], Table 1). This indicates that a portion of the Pγ in both species remains bound to the membrane and is able to rebind its inhibitory site on P$\alpha\beta$ once the Tα·GTPγS has been removed by centrifugation. Tα·GTPγS levels between 0.1 and 1 μM are sufficient to solubilize the first class of Pγ when it is dissociated from its inhibitory binding site on P$\alpha\beta$. A concentration of Tα·GTPγS near 4 μM is needed to fully dissociate the remaining Pγ from its inhibitory site on P$\alpha\beta$ but will not dissociate this Pγ from the ROS membranes in either species. If the Pγ which is released from the membrane constitutes half of the inhibitor sites (the most parsimonious model in view of the subunit composition and data presented below) then activation by Tα·GTPγS in the 0.1–1 μM concentration range is probably removing approximately half the total inhibitor in both species but gives between 10 and 20% of full activity in bovine PDE and approx. 50% in frog PDE.

Inhibitor elutes from the bovine ROS membranes with Tα·GTPγS

The experiments described in the previous section indicate that there is a population of Pγ, in both frog and bovine, which is not solubilized under experimental conditions which dissociate this Pγ from its inhibitory site on the P$\alpha\beta$ of the ROS membranes.

A population of Pγ which becomes soluble when dissociated from its inhibitory site on P$\alpha\beta$ by Tα·GTP has already been demonstrated in frog [13]. However, solubilization of Pγ by Tα·GTP has not been observed

Table 1. Activation of bovine and frog ROS PDE by Tα·GTPγS trypsin and GTPγS

Activator	Activation (% of full)* Bovine	Frog	Postulated number of Pγ bound per P$\alpha\beta$
Trypsin*	100†	100‡	0
> 4 μM-Tα·GTPγS (present during assay)	101±10	103±8	0§
> 4 μM-Tα·GTPγS (removed prior to assay)	17±5	52±4	1

* Treatment is described in the Materials and methods section.
† 100% = 761±14 μmol/min per mg.
‡ 100% = 493±6 μmol/min per mg.
§ 1 Pγ still associated with membrane.

in bovine ROS. In our studies, we have found (Fig. 5) that inhibitory activity is solubilized with Tα·GTPγS in bovine ROS. The concentrated GTPγS supernatant from bovine ROS was chromatographed as described above for Tα·GTPγS purification. The column fractions were assayed for Tα·GTPγS activity (solid line, Fig. 5). Since Tα is labile and Pγ is stable at 90 °C for 3 min, an aliquot of each column fraction was then heated under these conditions and assayed for inhibitory activity. The major inhibitory peak (broken line, Fig. 5) overlaps with the Tα·GTPγS activation peak. There is a minor inhibitory peak (apparent M_r 160000) which corresponds to Pγ associated with P$\alpha\beta$ (PDE is a minor contaminant in the material applied to the column). The major inhibitory activity elutes at an apparent M_r of 38000. This is somewhat lower than the indicated M_r of the Tα·GTPγS (43000) activation peak, but a much higher M_r than the uncomplexed Pγ would give, thus indicating that the Pγ is travelling in a complex of M_r near that of the Tα. Since Pγ is known to form a membrane-associated complex with Tα [15] it seems likely this soluble inhibitory activity may also be in a complex with Tα·GTPγS. The fact that the fractions that inhibit and those that activate overlap but are not superimposed is consistent with the fact that the complex between Tα·GTPγS and Pγ would be expected to travel at a different apparent M_r than Tα. It is also consistent with the observation that Tα which is complexed with Pγ does not activate inhibited PDE [15].

We have also prepared bovine Pγ from the GTP washes of the ROS by the procedure described for preparing frog Pγ (application of lyophilized iso-osmotic GTP washes to a TSK-125 column). The inhibitory activity eluted from the molecular sieve column with an apparent M_r of 12000. Preparation of bovine Pγ by this procedure would not be possible unless a portion of bovine Pγ is solubilized with the Tα·GTP just as it is in the frog system. Furthermore, the amount of bovine inhibitory activity solubilized by Tα·GTP is approximately equal to that which remains on the membrane.

PDE is released from frog ROS membranes by limited trypsin treatment

It has been demonstrated that PDE is released from ROS membranes by limited trypsin treatment in bovine

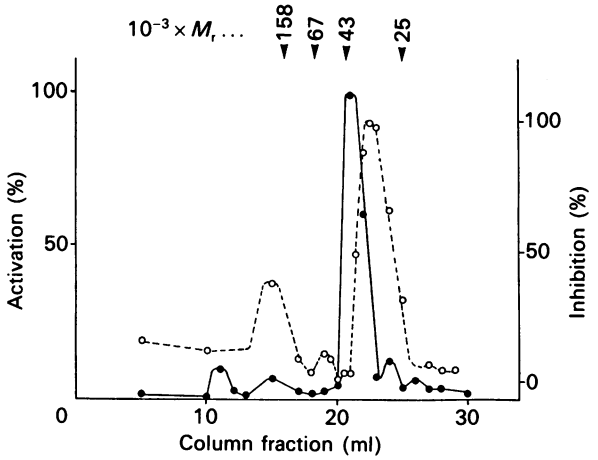

Fig. 5. Tα·GTPγS elution from TSK-250 column

Concentrated GTPγS supernatants were applied to a TSK-250 molecular sieve and the column was eluted with 20 mM-phosphate, pH 6.8, containing 50 mM-Na_2SO_4, 10 mM-$MgSO_4$ and 1 mM-DTT. The presence of Tα·GTPγS (solid line) was indicated by testing an aliquot of each column fraction for stimulation of inhibited PDE activity; inhibitory activity (broken line) was indicated by testing a heated (90 °C, 3 min) aliquot of the same column fractions for the ability to inhibit trypsin-activated PDE in a separate PDE assay.

ROS [14]. The release of bovine PDE from the ROS membranes was apparently not due to digestion of Pγ by trypsin, because readdition of intact Pγ did not put Pαβ back on the membranes [14]. This showed that Pγ is probably not responsible for anchoring PDE to the membrane in bovine ROS. Thus it is likely that there is a trypsin-sensitive portion of Pαβ [3] which is responsible for attachment of PDE to the ROS membranes.

We looked at the effect of limited trypsin treatment on the membrane association of PDE in the ROS membranes of the frog. Frog ROS membranes were treated with trypsin for 1.5 min, which corresponded to maximal activation of the PDE. The trypsin-treated membranes were centrifuged and both the membranes and supernatant were assayed for PDE activity. The supernatant contained $96 \pm 5\%$ ($n = 8$) of the total PDE activity and the membranes had $4 \pm 5\%$ ($n = 8$) of the total PDE activity. This procedure was carried out on membranes from two different frog ROS preparations. The same determinations were also made in two different bovine ROS membrane preparations and they showed $99 \pm 1\%$ ($n = 4$) of PDE activity in supernatant. Our data show that attachment of PDE to the membranes of frog ROS is also trypsin-sensitive. In analogy to the bovine PDE, it is likely that this attachment is not through Pγ but through some trypsin-sensitive portion of Pαβ.

DISCUSSION

The comparisons of frog and bovine ROS PDE presented in these studies indicate that the PDEs of the two species are similar in several respects. They are both inhibited by their Pγ subunits in a biphasic manner. Both frog and bovine ROS PDEs release a portion of their Pγ as a soluble complex with Tα·GTP. The ROS of both species also retain a portion of their Pγ on the membranes even after exposure to high concentrations of Tα·GTPγS which are sufficient to release all Pγ from inhibitory sites on Pαβ. A class of Pγ which was not released from the membranes by association with Tα·GTPγS has already been demonstrated in bovine PDE [14,15]. However, the two species differ in the percentage of full PDE activity that results when the first Pγ dissociates from the membrane as a soluble complex with Tα·GTP. The frog enzyme exhibits approx. 50% of its full activity after removal of the Tα·GTP-releasable Pγ, whereas the bovine ROS PDE is only about 17% activated after release of that portion of Pγ. This may indicate that there is a greater degree of allosteric interaction among the PDE subunits in the bovine ROS. Frog and bovine PDEs are also activated by purified Tα·GTPγS in a biphasic manner that is consistent with the existence of two classes of Pγ binding sites. The existence of two Pγ sites per Pαβ has already been demonstrated in bovine and is a likely explanation of the data we present for the frog.

Thus, in both species, the membrane-associated Pγ would appear to remain in close physical proximity to the PDE so that when Tα·GTPγS concentrations are lowered, Pγ can rapidly reassociate with its inhibitory site on Pαβ. The affinity of Pγ for Pαβ is at least 1000 times higher than the affinity of Tα·GTP for Pγ [14]. This may be important for a rapid turn-off of light-activated PDE. Thus, bovine and frog PDEs may be more similar with respect to Pγ interactions than previous studies have indicated. However, a greater degree of allosteric regulation of the inhibition of Pαβ by Pγ may exist in the mammalian ROS.

Deterre *et al.* [17] proposed a possible role for the two Pγ per Pαβ in the turn-off of fully activated bovine PDE. They postulated that $P\alpha\beta\gamma_2$ would mix with Pαβ to form Pαβγ and lead to a decrease in total PDE activity, if Pαβγ has less than half the activity of Pαβ. Our data support their proposal that Pαβγ has less than half the activity of Pαβ (Pαβγ has 10–20% of Pαβ) in the bovine. However, our data would indicate that in frog PDE such a mixing, if it occurs, would not be useful since Pαβγ may have about half the activity of Pαβ.

A possible physiological role for the two classes of Pγ binding sites could be in the graded response of the PDE to bleaches of varying intensity. A small number of photons (low level of rhodopsin bleaching) may generate Tα·GTP concentrations in the interdiskal space sufficient only to release Pγ from the membrane-dissociable class of binding sites. Larger numbers of photons (extensive rhodopsin bleaching) resulting in significantly higher Tα·GTP concentrations would then begin to remove Pγ from the other inhibitory site (it has been estimated that Tα concentrations could reach 500 μM in the interdiskal space, due both to the small volume of the space and the relatively large number of Tα·GTP [27]). The removal of the second Pγ site in bovine PDE may result in a greater than 4-fold increase in PDE activity. However, if Tα·GTP concentrations become very high over a very small portion of the disk membrane then fully activated PDE could result even with low bleaches. This situation might present the opportunity for the turnoff of Pαβ by diffusional mixing with $P\alpha\beta\gamma_2$ as proposed by Detterre *et al.* [17]. The two classes of sites could also be relevant for the rapid turn-off of the light response as mentioned above. In the bovine PDE, reassociation of the membrane-bound Pγ at the inhibitory site on Pαβ may result in an 80% reduction in the catalytic activity of Pαβ.

In conclusion, it seems reasonable to speculate that these differences between the bovine and frog in the regulation of PDE by its γ subunits may be a reflection of, and adaptation to, the environmental and behavioural differences between the two species (diurnal for bovine versus predominantely nocturnal for frog).

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