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cGMP Phosphodiesterase Dependent Light-Induced Scattering Changes in Suspensions of Retinal Disc Membranes

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ABSTRACT: Light-induced GTP-dependent scattering changes are studied in suspensions of retinal disc membranes to which one or both of the purified proteins involved in the phototransduction mechanism (G-protein and cGMP phosphodiesterase) are reassociated; a scattering change which depends on the presence of both G-protein (G) and inhibited cGMP phosphodiesterase (PDE) and on an ATPase-dependent process, previously described in Bennett [(1986) *Eur. J. Biochem. 157*, 487-495] is compared to the signal observed in the absence of PDE or of ATP and to PDE activity. The same signal can also be induced either in the dark or in the light by addition of preactivated G in the presence of inhibited PDE. This PDE-dependent scattering change is composed of two components (fast and slow); the variation of the amplitude and kinetics of both components with PDE or G concentration is similar to the variation of the active PDE state with two activator G_{GTP} molecules (G with GTP bound), calculated with dissociation constants previously reported for the interaction between G_{GTP} and PDE [Bennett, N., & Clerc, A. (1989) *Biochemistry 28*, 7418-7424]. The two components are therefore proposed to be associated with processes which depend on the formation of the active PDE state with two activators.

Absorption of a photon by the photosensitive protein rhodopsin in retinal discs leads to the closing of sodium channels in the outer membrane of the rod. The channels are kept open in the dark by direct binding of cGMP, the concentration of which is rapidly reduced upon excitation of rhodopsin. Excited rhodopsin catalyzes the exchange of GTP for bound GDP on a GTP-binding protein (transducin or G);[1] this results in the formation of $G_{\alpha GTP}$ (α subunit of G with GTP bound) which is the activator of the rod cGMP phosphodiesterase (PDE) [reviewed by Pugh and Lamb (1990)]. Both G and PDE are peripheral proteins which can be easily dissociated from the membrane at low ionic strength and can reassociate to the membrane at moderate ionic strength (Kühn, 1985). Light-induced scattering changes in rod suspensions, first described by Hofmann et al. (1976) and by Bignetti et al. (1980), have been shown to be associated with the first steps of the light-induced enzymatic cascade: "P-signal" (Hofmann et al., 1976) or "binding signal" (Kühn et al., 1981) associated with the formation of a complex between excited rhodopsin and G in the absence of GTP; "dissociation signal" associated with the activation of G in the presence of GTP (Kühn et al., 1981) and, more precisely, with the dissociation of $G_{\alpha GTP}$ from the membrane (Bruckert et al., 1988). Several scattering changes were later described (Lewis et al., 1984; Caretta & Stein, 1985, 1986; Kamps et al., 1985; Bennett, 1986), which present a number of similarities, and in particular were all observed under conditions which correspond to the activation of PDE. The relation between these signals and PDE activation was, however, later questioned by results obtained with preparation of high structural integrity (Wagner et al., 1987).

We report here a further study of a light-scattering change (termed PAS for phosphodiesterase activation signal) which was previously shown to be specifically dependent on the presence of G, GTP, and PDE and in addition on a previous ATP-dependent process which was interpreted as swelling of the discs or vesicles (Bennett, 1986). In the present study, reconstituted systems of well-controlled protein and nucleotide composition were used. The results are compared to previous measurements of PDE activity under similar conditions (Bennett & Clerc, 1989).

Experimental Procedures

Dark or bleached rod outer segments (ROS) are prepared from fresh bovine eyes according to the method of Kühn (1985) and kept in liquid nitrogen. ROS fragments composed

[1] Abbreviations: ROS, rod outer segment; R*, photoexcited rhodopsin; G, retinal G-protein (transducin); G_{GDP} or G_{GTP}, G-protein with GDP or GTP bound; G*, activated G (G_{GTP} or $G_{GTP\gamma S}$); GTPγS, guanosine 5'-*O*-thiotriphosphate; PDE, cGMP phosphodiesterase.

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of discs stacks are obtained by suspension of the frozen ROS pellets in isotonic buffer. PDE and G are prepared from bleached ROS, after several isotonic washes which remove the soluble proteins. PDE is first extracted with low salt buffer, and then G is extracted with low salt buffer with added GTP which dissociates R*–G complexes. PDE is further purified on a DE52-Sephadex G100 column (Baehr et al., 1979), and the membranes are washed several more times to eliminate contaminant proteins; the membranes obtained after such extensive washing consist of vesicles. When required, G is preactivated with GTPγS by incubating G_{GDP} with bleached membranes (R*/G = 0.25–1) and GTPγS (5–10-fold excess) for 15–30 min in an ice bath (low salt buffer); $G_{GTP\gamma S}$, containing its three subunits ($\beta\gamma$ and αGTPγS) is obtained in the supernatant after centrifugation of the suspension; it is then separated from free GTPγS by filtration on a Pharmacia NAP10 column. Proteins are concentrated with Millipore immersible CX-30 ultrafiltration units and with Amicon centricon-30 (for G) or -100 (for PDE) microconcentrators. All experiments are carried out within two or three days following extraction of the proteins.

G and PDE concentrations are measured by the method of Bradford (1976); rhodopsin concentration is measured by absorbance at 500 nm.

PDE activity is measured with a pH-meter according to Liebman and Evanczuk (1981) as previously described (Bennett & Clerc, 1989). Trypsin activation is performed by a 15-min incubation at 20 °C with TPCK trypsin (75 μg/mL, 3 μg/pmol of PDE), and proteolysis is stopped by addition of soybean trypsin inhibitor (5 μg/μg of trypsin).

Light-scattering changes are measured as transmittance chances as previously described (Kühn et al., 1981) using a modified DURRUM D117 spectrophotometer.

All experiments are carried out in a buffer containing 120 mM KCl, 1 mM $MgCl_2$, and 1 mM MOPS (pH changes) or 10 mM MOPS (scattering changes), pH 7.2, at 20 °C.

Calculations of PDE activity and of the proportion of G_{free}, G_{bound}, G·PDE, and $(G)_2$PDE are done according to a model [model 2, described in the supplementary material of Bennett and Clerc (1989)] in which two molecules of activator G_{GTP} are involved per molecule of PDE:

$$G + PDE \underset{K_1}{\rightleftharpoons} G\cdot PDE \quad (V_{max1}, \text{low activity})$$

$$G + G\cdot PDE \underset{K_2}{\rightleftharpoons} (G)_2PDE \quad (V_{max2}, \text{high activity})$$

which lead to the equation $X^3 + AX^2 + BX + C = 0$ where $X = [G_{free}]$, $A = 2[PDE_{total}] - [G_{total}] + K_2$, $B = K_2([PDE_{total}] - [G_{total}] + K_1)$, and $C = -K_1K_2\,[G_{total}]$. Resolution of this equation gives

$$[(G)_2PDE] = X^2[PDE_{total}]/(X^2 + K_2X + K_1K_2)$$

$$[G\cdot PDE] = [PDE_{total}]XK_2/(X^2 + K_2X + K_1K_2)$$

PDE activity is calculated from the concentrations of G·PDE and $(G)_2$PDE:

$$V/V_{maxT} = ([(G)_2PDE] + k[G\cdot PDE])/[PDE_{total}] \text{ or } [G_{total}]$$

with V_{maxT} = activity of trypsin-activated enzyme, and $k = V_{max1}/V_{max2}$ (V_{max} of the two active states G·PDE and $(G)_2$PDE).

The values taken for the dissociation constants (K_1 = 100 nM and K_2 = 600 nM) and for k (k = 0.05–0.10) are those estimated from measurements of PDE activity in Bennett and Clerc (1989).

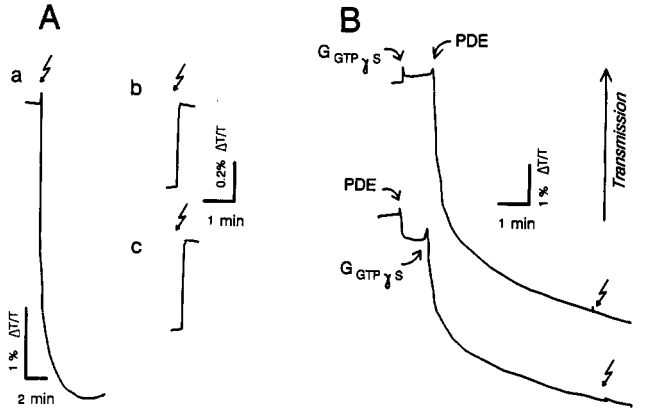

FIGURE 1: PDE-dependent light-scattering signals in suspensions of washed disc membranes (dark) with added purified proteins. Membranes are incubated for about 15 min in the dark in the presence of 500 μM ATP until saturation of the ATP-induced transmittance increase (swelling) (Bennett, 1986). (A) Light-induced signals in the presence of dark membranes (5 μM rhodopsin) and G (0.4 μM) and 200 μM GTP: (a) 30 nM PDE (PAS); (b) no added PDE (dissociation signal); (c) 30 nM PDE, preactivated before reconstitution by incubation with trypsin, which was inhibited by excess trypsin inhibitor before mixing the PDE preparation with the membranes (dissociation signal). Trypsin-activated PDE was checked to be fully active in a control experiment. Flashes ($R^*/R = 10^{-3}$) are indicated by an arrow. (B) Protein-induced signals in the presence of dark membranes (7 μM rhodopsin). Addition of $G_{GTP\gamma S}$ (200 nM) in the presence of PDE (70 nM) or of PDE (70 nM) in the presence of $G_{GTP\gamma S}$ (200 nM) induces a light-scattering signal similar to the PAS. A flash ($R^*/R = 10^{-3}$, arrow) applied after completion of the signal does not induce any further scattering change. $G_{GTP\gamma S}$ was separated from free GTPγS as described in Experimental Procedures; the concentration of contaminant free GTPγS in the membrane suspension was estimated as less than 0.4 μM, a concentration which does not induce any signal when it is added in the absence of G.

GTP and GTPγS (solutions) were purchased from Boehringer; cGMP was from Sigma.

Results

Requirement of Inhibited PDE. We have previously reported (Bennett, 1986) that the light-induced scattering change PAS requires the same conditions (presence of G and GTP) and presents the same GTP and light sensitivity as the dissociation signal associated with activation of G (formation of $G_{\alpha GTP}$) (Kühn et al., 1981; Bennett, 1982; Bennett & Dupont, 1985); it requires however two additional conditions: (a) the presence of PDE and (b) previous swelling of the discs either by an ATP-dependent process or by the presence of external permeant ions (ammonium acetate) or by addition of two ionophores allowing the influx of external impermeant anion and cation or even by mechanical treatments (extensive washing, sonication) which destroy the flat structure of the discs. If the swelling is inhibited (absence of ATP, inhibition of ATPase activity by vanadate, addition of sucrose outside the vesicles, replacement of chloride by certain anions such as SO_4, NO_3, or large impermeant anions), the signal induced by a flash even in the presence of PDE is a normal dissociation signal (Bennett, 1986).

Light-induced scattering signals observed in the presence of GTP and after swelling of the discs in the dark in the presence of ATP are shown in Figure 1A. A flash induces a PAS after swelling only if inhibited PDE (a) is present. If PDE is activated before reconstitution by incubation with trypsin, which degrades the inhibitory subunit (Hurley & Stryer, 1982), again the signal induced by the flash is a dissociation signal (c), identical to the signal observed in the absence of PDE (b) or when swelling is inhibited. Addition

of G preactivated with GTPγS (instead of G, GTP, and light) itself induces in the dark a scattering signal similar to the PAS only if PDE is already present in the suspension (Figure 1B); no signal is elicited by the flash after $G_{GTP\gamma S}$ and PDE addition, consistent with the two proposed hypotheses that (i) the dissociation signal is associated with activation of G (therefore cannot be observed if G has been preactivated), and (ii) the PAS has been induced in the dark by addition of $G_{GTP\gamma S}$ in the presence of PDE or by addition of PDE in the presence of $G_{GTP\gamma S}$. Addition of GTPγS alone at concentrations higher than the concentration of contaminant free GTPγS from the $G_{GTP\gamma S}$ preparation has no effect; this indicates that the amount of excited rhodopsin in the dark membranes is negligible, probably due to the presence of contaminant rhodopsin kinase which inactivates traces of excited rhodopsin (if any) during incubation with ATP. These results indicate that the PAS is associated with the interaction between activated G and inhibited PDE or with a subsequent process.

Note that the presence of 500 μM ATP (concentration used for swelling the discs in the light-scattering experiments) does not induce rapid reversal of the PAS and dissociation signal. These are observed, however, when rod fragments containing soluble proteins are used or when the suspension is supplemented with rhodopsin kinase and arrestin which are involved in the inactivation of excited rhodopsin (Bennett & Sitaramayya, 1988).

Comparison of the PAS with the Dissociation Signal (Formation of PDE Activator) and with cGMP Hydrolysis (Result of PDE Activation) under the Same Conditions. In a previous study of PDE activity (Bennett & Clerc, 1989), we have determined two dissociation constants (K_1 = 100 ± 50 nM and K_2 = 600 ± 100 nM) for $G_{\alpha GTP}$ binding to PDE, from the variation of activity measured as a function of PDE or G concentrations (with [G] or [PDE] constant, respectively). PDE can thus exist as two activated states: one state with one activator, having a low activity (0.05–0.10 that of trypsin-activated PDE), which is predominant when [PDE] ≫ [G], and one state with two activators, having a high activity (similar to that of trypsin-activated PDE), which is predominant when [G] ≫ [PDE]. In this study, the two active states are referred to as G·PDE and $(G)_2$PDE for simplicity, although we do not exclude the possibility that each complex dissociates totally or partially to G·PDEγ and PDEαβ or PDEαβγ, since PDE has been shown to possess two γ subunits per αβ subunit (Deterre et al., 1988).

Figure 2A shows examples of the light-scattering changes observed under conditions where either of the two activated states (PDE activated by two activators $G_{\alpha GTP}$ molecules, [G] ≫ [PDE]; or only one activator, [PDE] ≫ [G]) is predominant. The dissociation signals observed in the absence of PDE for the same G concentrations are drawn on the same figure for comparison of the kinetics. At high G concentration (Figure 2A(a)) and with [G] ≫ [PDE] (two molecules of G involved in the activation of a single PDE), the PAS is preceded by a partial dissociation signal and is clearly slower than the dissociation signal. At low G concentration (Figure 2A(b)), the dissociation signal amplitude is smaller, and its duration is shorter; with [G] < [PDE] (only one G involved per PDE at the steady state), the PAS is also preceded by a partial dissociation signal at the lower PDE concentrations (not shown), which is masked when [PDE] increases; the signal then apparently consists of two phases, with the kinetics of the rapid phase appearing similar to that of the dissociation signal, while that of the slow phase is markedly slower. Note that the existence of a fast phase of the PAS in the first case

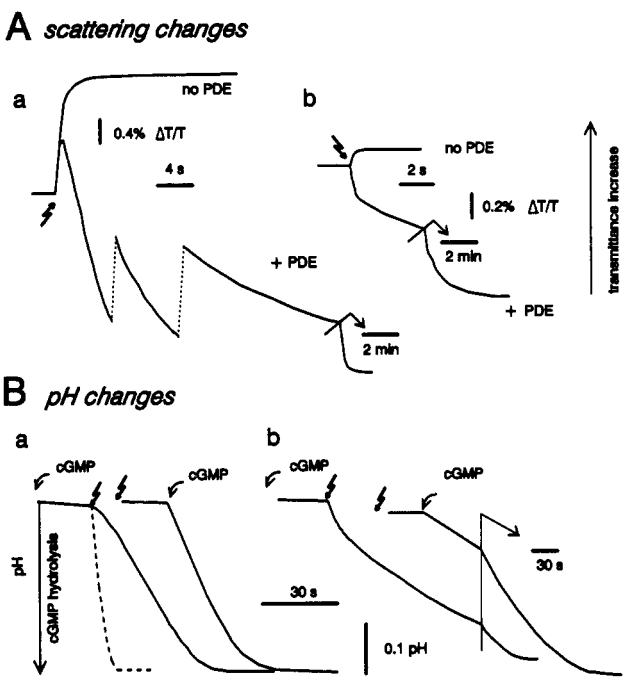

FIGURE 2: Light-induced scattering changes and measurement of PDE activity in suspensions of washed disc membranes (dark) with different proportions of G and PDE. (A) Light-induced scattering changes observed in the presence (PAS) or in the absence (dissociation signal) of PDE after ATP-induced swelling for two different concentrations of G and PDE. Flashes ($R^*/R = 10^{-3}$) are indicated by arrows. Note that the end of the PAS is shown at a different time scale (change indicated by a broken arrow): (a) 1.5 μM G ± 100 nM PDE (condition in which 67% of PDE is in the $(G)_2$PDE state; from Bennett and Clerc (1989); see Figure 4A); (b) 100 nM G ± 350 nM PDE (condition in which 69% of PDE is in the G·PDE state; see Figure 4B). Concentrations: rhodopsin, 7.5 μM; ATP, 500 μM; GTP, 200 μM. (B) Light-induced and cGMP-induced PDE activity measured under the same conditions: (a) 1.5 μM G, 100 nM PDE; (b) 100 nM G, 350 nM PDE. The derivative of the light-induced pH trace (a), which represents the rate of PDE activation, is indicated by the dotted line on the pH trace. Concentrations: rhodopsin, 7.5 μM; cGMP, 500 μM; ATP, 500 μM; GTP, 200 μM.

(Figure 2A(a)) could be masked by the dissociation signal as long as its amplitude is smaller than that of the dissociation signal.

The onset of light-induced PDE activity under identical conditions has been further investigated in order to detect the possible existence of initial rates which would be distinct from the steady-state rates; examples are shown in Figure 2B. At high G concentration (Figure 2B(a), same conditions as in Figure 2A(a)), the onset of light-induced PDE activity is sigmoid, the maximal activity being reached at approximately the same time as the dissociation signal reaches its maximal amplitude (Figure 2A(a)). The dotted line in Figure 2B(a) is the derivative of the pH variation, i.e., the variation of the rate of cGMP hydrolysis as a function of time: it represents the rate with which maximal activity is reached, or the "activation" of PDE. For a given concentration of G and PDE, the activation of PDE is expected to depend on the rate of formation of the activator $G_{\alpha GTP}$ and on its rate of interaction with inhibited PDE. The derivative of the pH trace associated with cGMP hydrolysis (Figure 2B(a)) is very similar to the dissociation signal (Figure 2A(a)) which represents the increase of the amount of activator $G_{\alpha GTP}$ as a function of the time after the flash. This indicates that interaction with PDE is fast compared to the formation of the activator [see also the comparison between dissociation signal and onset of PDE activity (Bennett, 1982)]. Note that when PDE is flash activated before the addition of cGMP, the sigmoid start is not

observed, consistent with the fact that the rate-limiting step (formation of G_{GTP}) is completed before cGMP addition. At low G concentration, and with [PDE] ≫ [G] (Figure 2B(b)), when hydrolysis is induced by the flash, the sigmoid start is hardly detectable; it is followed by a short burst of higher rate, which precedes the steady-state activity. The onset of cGMP hydrolysis is again different when hydrolysis is induced by cGMP (PDE activated before addition of cGMP): neither the sigmoid start nor the initial burst is observed. The existence of an initial burst of activity is at first sight strikingly similar to the existence of a fast phase in the scattering signal. However, the duration of the burst of activity is longer than that of the first phase of the PAS under the same conditions (note the different time scale in parts A and B of Figure 2), and the difference between the higher and lower activity (factor of 2) is much less marked than that between the fast and slow phase of the PAS (factor of 10). This suggests that the fast phase of the PAS is not directly related to PDE activity; given its fast kinetics, it could be related to the interaction between G_{GTP} and PDE. A possible explanation for the light-induced initial burst of PDE activity under conditions where [PDE] ≫ [G] could be that G_{GTP} first interacts with PDE as a dimer (Phillips et al., 1989; Vaillancourt et al., 1990), thus giving rise initially to a small number of fully activated PDE with two activators per molecule; alternatively, the concentration of activated G could locally be sufficient to form $(G)_2PDE$ molecules before dilution of the proteins by diffusion in the membrane. Since the affinity of one of the binding sites is lower than that of the other (Bennett & Clerc, 1989), and since the number of PDE molecules is much larger than that of activators, one activator would then dissociate and rebind to an inactive PDE, thus giving rise to a larger number of PDEs of low activity, having only one activator per molecule. Note that this situation is likely to occur under very low levels of excitation which lead to activation of much less than 10% of the G ([G*] ≪ [PDE]); such a mechanism would allow fast detection of very low excitation with high sensitivity in spite of the reduced steady-state activity of PDE with a single activator.

Dependence of the Fast Phase of the PAS on the Presence of ATP and on ATP-Dependent Swelling. The PAS was previously studied under conditions where G ≫ PDE (corresponding to the native ratio of the two proteins), and therefore where the fast phase is either inexistent or masked. The question therefore arises whether this fast phase presents the same dependence as the slow phase with respect to ATP-dependent swelling. Figure 3A shows the effect of PDE addition to a suspension of ROS fragments on the light-induced scattering changes observed either in the absence of ATP (a), in the presence of ATP added after incubation with vanadate (b), and after ATP-induced swelling (c). In the absence of ATP, the amplitude of the dissociation signal decreases with increasing concentration of PDE until complete disappearance (for about 7 times the concentration of G under the experimental conditions used), but the polarity of the signal is not inverted; identical signals are observed when ATP is added after incubation with vanadate, which inhibits ATP-dependent swelling (Bennett, 1986). The fast phase of opposite polarity is only observed for [PDE] > [G] in (c), after ATP-dependent swelling. Note that in this experiment, we use ROS fragments supplemented with PDE instead of a fully reconstituted system because as mentioned above (see the first paragraph of the Results), when washed membrane vesicles are used as membrane support, in the presence of PDE, a partial PAS is most of the times (even in the absence of ATP) superimposed to the dissociation signal, whose amplitude cannot therefore be correctly measured.

The reduction of dissociation signal amplitude as a function of PDE concentration precisely matches the reduction of remaining $[G_{free}]$ (Figure 3B), calculated using the constants estimated in Bennett and Clerc (1989), consistently with the hypothesis that the dissociation signal is associated with dissociation of $G_{\alpha GTP}$ from the membrane (Bruckert et al., 1988) and that $G_{\alpha GTP}$ rebinds to the membrane while activating membrane-bound PDE. The amplitude of the fast phase of opposite polarity, calculated by addition of the fast phase of the PAS measured in (c) and of the corresponding dissociation signal measured in (b), is plotted in Figure 3C as a function of PDE concentration; its variation is clearly distinct from that of the remaining free G (Figure 3B), and follows reasonably well that of the $[(G)_2PDE]/[G_{total}]$ ratio, with an initial increase up to 100–700 nM PDE followed by a decrease with further increase of PDE concentration. The variation of $[G{\cdot}PDE]/[G_{total}]$ and of $[G_{bound}] = (2[(G)_2PDE] + [G{\cdot}PDE])/[G_{total}]$ is also shown for comparison. The results suggest that if (and only if) ATP-induced swelling is permitted, formation of the activated PDE state with two activators ($(G)_2PDE$) is associated with a fast decrease of transmittance (scattering increase), whose kinetics are similar to those of the formation of G_{GTP}.

If this hypothesis is correct, this fast signal should exist independently of the concentration of PDE. The variation of the $[(G)_2PDE/[G_{total}]$ ratio as a function of $[G_{total}]$ [for constant [PDE] = 100 nM, as in the example shown in Figure 2A(a)] is shown in Figure 3D; whatever the concentration of G, the $[(G)_2PDE]/[G_{total}]$ ratio does not exceed 6.7% (maximal value, for [G] = 400 nM). Since the amplitude of the dissociation signal is only slightly reduced by the presence of PDE when [G] ≫ [PDE] (Figure 3B) and is proportional to the amount of G (see Figure 5), the amplitude of the fast phase corresponding to the formation of $(G)_2PDE$ is expected to be smaller than that of the dissociation signal under these conditions and the fast phase is expected to be completely masked, as indeed observed.

Variation of the Amplitude and Kinetics of the PAS as a Function of Total G or PDE Concentration. The amplitude and kinetics of the signals have been measured in fully reconstituted systems at varying G concentrations (constant [PDE]) or at varying PDE concentrations (constant [G]). The experimental points obtained for varying G concentration (constant [PDE]), conditions under which only a slow phase is observed, are plotted in Figure 4A (symbols). The variations of amplitude and kinetics of the fast and slow phase of the signal obtained for varying [PDE] (constant [G]) are shown in Figure 4B. The theoretical variations of steady-state PDE activity and of the proportions of the two active PDE states (with one or two activators) under the same conditions, calculated from the dissociation constants between G and PDE and from the V_{max} of the two active PDE states [from Bennett and Clerc (1989)] are also drawn in the figure. The scale of the theoretical curves relative to the transmittance changes of the left- and right-hand scales has been imposed to fit the results in Figure 4A, and the ratio has been kept unchanged in Figure 4B.

Comparison of the theoretical curves and experimental data shows that the variation of both the amplitude and initial rate of the slow phase of the PAS are very close to that of the activated PDE state with two activators in both parts A and B of Figure 4 (and clearly distinct from the variation of remaining free G or total bound G predicted in parts B and D

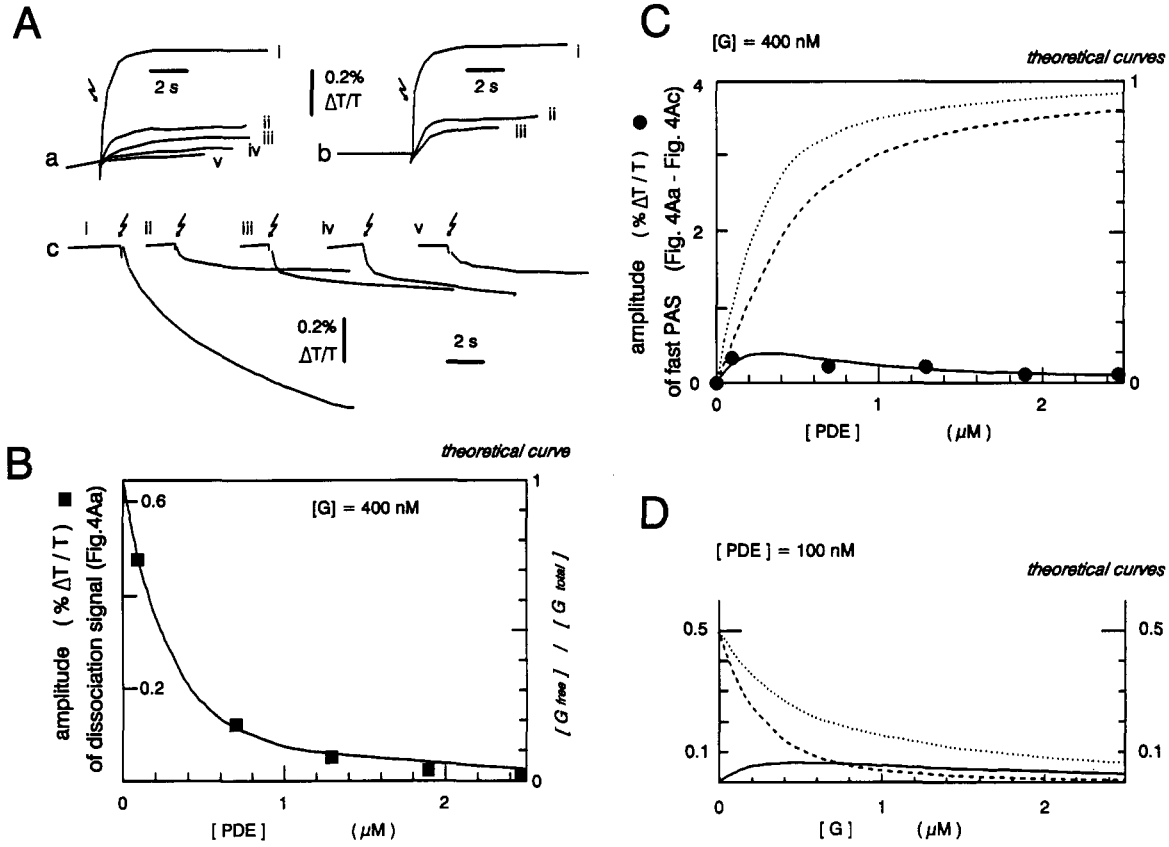

FIGURE 3: The fast phase of the PAS: swelling dependence and variation with PDE concentration. (A) Light-induced scattering changes in suspension rod fragments (disc stacks) supplemented with increasing amounts of PDE: (a) no ATP; (b) addition of 500 μM ATP after 5 min of incubation with 40 μM vanadate (no ATP-induced swelling); (c) 500 μM ATP. Several flashes ($R^*/R = 10^{-3}$) were applied sequentially at 10-min intervals to the same suspension after successive additions of 600 nM PDE: the PDE concentration before each flash was (i) 100 nM (no addition, estimated concentration), (ii) 700 nM, (iii) 1.3 μM, (iv) 1.9 μM, or (v) 2.5 μM. Hydroxylamine (5 mM) was present in order to accelerate the decay of R* between flashes. In control experiments in the presence of hydroxylamine where no PDE was added, the successive flashes produced only slightly reduced signals (about 3–5% reduction per flash). Concentrations: rhodopsin, 10 μM; [G] [measured from binding signals as described in Bennett and Dupont (1985)], 400 nM; estimated [PDE] in the ROS fragments, 100 nM (1% of [rhodopsin]); GTP, 500 μM. (B) Comparison of the variation of the amplitude of the dissociation signal as a function of the concentration of PDE (from part A(a)) with the theoretical variation of remaining $[G_{free}]/[G_{total}] = 1 - 2\{\%(G)_2PDE\} - \%G{\cdot}PDE$, using the two dissociation constants (K_1 = 100 nM and K_2 = 600 nM) previously estimated for the interaction between G and PDE (Bennett & Clerc, 1989). The scale of the theoretical curve was chosen to give the best fit with the data. (C) Comparison of the variation of the amplitude of the fast phase (obtained by adding the amplitude of the signal in (b) to that in (a) for each PDE concentration) with the theoretical variation of bound G ($[G{\cdot}PDE]/[G_{total}]$, broken line; $[(G)_2PDE]/[G_{total}]$, full line; and total bound G = $(2[(G)_2PDE] + [G{\cdot}PDE])/[G_{total}]$, dotted line). Only the variation of $[(G)_2PDE]/[G_{total}]$ first increases (6.7% at 100 nM PDE; maximum 10% at 400 nM PDE) and then decreases with increasing PDE concentration and is qualitatively similar to that of the fast phase. The scale of the theoretical curves was chosen to give the best fit with the data. (D) Calculation of the proportion of bound G [$G{\cdot}PDE$, $(G)_2PDE$, and total bound G have the same representation as in (C)] with increasing G concentrations ([PDE] constant = 100 nM). Under the conditions of Figure 2A(a) (1.5 μM G, 100 nM PDE), the calculated proportion of $(G)_2PDE$ is 3.4% and 6.7% under the conditions of trace i in part A(c) (100 nM PDE, 400 nM G). Since the example shown in part A(c) (trace i) corresponds to the maximum proportion of $(G)_2PDE$ with 100 nM PDE and varying [G], and since the fast phase is not resolved from the dissociation signal under these conditions, it can be suggested that if the fast phase is associated with the $(G)_2PDE$ state, its amplitude is likely to be smaller than that of the dissociation signal for whatever [G] > 400 nM.

of Figure 3). The variation of PDE activity is similar, although significantly different at high PDE concentrations (Figure 4B, slow component). PDE activity indeed consists of two components: V_{max1} of active PDE with a single activator and V_{max2} of active PDE with two activators, with $V_{max1} \sim 0.05$–0.1 V_{max2}, from Bennett and Clerc (1989). The active PDE with only one activator, which is predominant at high PDE concentration ([G] ≪ [PDE]) gives rise to an almost inexistent scattering signal at the steady state, with an amplitude and initial rate inferior to 1% that of the signal associated with the active PDE state with two activator G_{GTP} molecules. This indicates that the efficiency of the two active PDE states with respect to the process giving rise to the light-scattering signal is not equivalent to their efficiency in their ability to hydrolyze cGMP, the state with one activator being significantly more efficient for cGMP hydrolysis than for the PAS.

The variations of the amplitude and kinetics of the fast component as a function of PDE concentration (constant [G]) are shown in Figure 4B, above those of the slow component, revealing at least a factor of 10 between the initial rates of the two components, whatever the PDE concentration. Note that the variation of the initial rate of the fast component as a function of PDE concentration also behaves like the variation of $(G)_2PDE$, first increasing and then decreasing with increasing [PDE].

The fact that the initial rate of both components of the scattering signal varies in the same manner as the $(G)_2PDE$ state suggests that the processes revealed by this signal depend on the concentration of $(G)_2PDE$ ($V_i = k[(G)_2PDE]$); this would indicate that the two components are in fact associated with kinetically distinct processes which both occur after formation of $(G)_2PDE$.

It can be noted that, contrary to that of the PAS, the amplitude of the dissociation signal (Figure 5) increases linearly

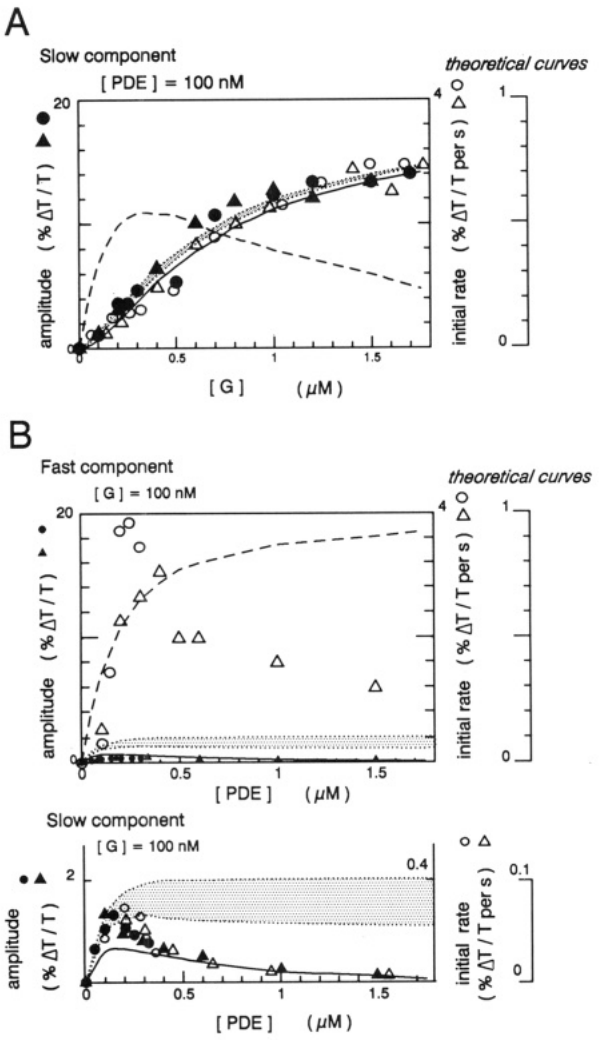

FIGURE 4: Variation of the amplitude (filled symbols) and initial rate (open symbols) of the PAS as a function of total G or PDE concentrations. (A) Conditions: 100 nM PDE, varying G concentration (slow component), two different experiments. Rhodopsin concentration, 5 μM, R*/R = 10^{-3}; GTP concentration, 200 μM. The curves are theoretical curves computed from the model described in Experimental Procedures; the full line corresponds to the variation of the $[(G)_2PDE]/[PDE_{total}]$ ratio, the broken line to that of the $[G{\cdot}PDE]/[PDE_{total}]$ ratio, and the dotted area corresponds to the estimated PDE activity, the minimum and maximum values being calculated as $\{[(G)_2PDE] + 0.05[G{\cdot}PDE]\}/[PDE_{total}]$ and $\{[(G)_2PDE] + 0.1\text{-}[G{\cdot}PDE]\}/[PDE_{total}]$, respectively, from Bennett and Clerc (1989). The scale of the theoretical curves was chosen to give the best fit with the data. (B) Conditions: 100 nM G, varying PDE concentration, fast and slow components (two experiments, with the same protein preparations as in (A), same symbols). The slow component is shown at an enlarged scale. Minimum and maximum PDE activity are calculated as $\{[(G)_2PDE] + 0.05[G{\cdot}PDE]\}/[G_{total}]$ and $\{[(G)_2PDE] + 0.1[G{\cdot}PDE]\}/[G_{total}]$, respectively, from Bennett and Clerc (1989). In this set of measurements, the maximal amount of complexes that can be formed is limited by the fixed concentration of G (100 nM); the proportions of $(G)_2PDE$, G·PDE, and PDE activity are therefore given as fractions of the total G concentration instead of total [PDE] as in (A), where the limiting component is PDE (same representation as in (A); full line, broken line, and dotted area, respectively). The correspondence between the scale of the theoretical curves and the scale of the transmittance changes is imposed from (A) independently of the data in (B).

with G concentration in the range studied, without saturation, consistent with the hypothesis that it is directly related to activation of G (or the related dissociation of G_{GTP} from the membrane). The variation of the initial rate of the transmittance change is also different from that of the PAS: it appears constant over the whole concentration range studied, consistent with the previously proposed hypothesis that the rate-limiting step is a conformation change of the G-protein (Bennett & Dupont, 1985).

Discussion

The results presented here suggest the existence of two distinct light-scattering components associated with the presence of inhibited PDE and dependent on previous ATP-dependent swelling of the discs: (1) A fast component, which under our experimental conditions is only observed when the concentration of PDE is much larger than that of G. Its kinetics appear similar to (although slightly slower than at low PDE concentration) those of the dissociation signal associated with the formation of the PDE activator G_{GTP} (Figure 2A) and of the activation of PDE (measured from the derivative of the pH change associated with cGMP hydrolysis (Figure 2B)). From its kinetic properties, this signal is proposed to be associated with a process related to or occurring shortly after interaction between G_{GTP} and PDE and which depends more precisely (from its variation as a function of PDE concentrations; Figures 3C and 4B) on the formation of $(G)_2PDE$; although because of its small amplitude we can only observe it under conditions where the dissociation signal is sufficiently small, theoretical calculations suggest that this component may be present under all conditions studied.

(2) A slow component (previously termed PAS for PDE activation signal), which is in fact markedly slower than PDE activation. The variation of both its amplitude and kinetics as a function of G or PDE concentration (constant PDE or G, respectively) closely follow the theoretical variation of the activated state of PDE with two activator G_{GTP} molecules (Bennett & Clerc, 1989); the active state of PDE with only one activator, whose activity is only 0.05–0.10 that of the active state with two activators, is even less efficient in inducing the process giving rise to the scattering signal (in the absence of cGMP). The underlying process is therefore also proposed to be associated with or depend on the formation of $(G)_2PDE$.

The nature of the processes associated with these PDE-dependent light-scattering changes still remains unclear. Lewis et al. (1984) (G^+ signal) and later Caretta and Stein (1985, 1986) described a light-scattering signal which appears identical to our slow component and proposed that it may be related to aggregation phenomena; aggregation was recently determined to occur between activated PDE and bleached rhodopsin (Caretta et al., 1990). Our signal, however, does not require the presence of bleached rhodopsin since it can be induced in the dark by G preactivated with GTPγS (Figures 1 and 6). If activated PDE aggregates with bleached rhodopsin but not with rhodopsin, as reported by Caretta et al. (1990), the light-scattering signal that we observe is therefore associated with a step preceding this aggregation and which involves interaction between G_{GTP} and PDE. Caretta and Stein (1986) also report that a striking change in the binding of PDE to the membrane occurs in parallel to their scattering change; although they do not propose that the scattering change may be directly associated with the increased binding itself, this appears as a possible hypothesis, which would deserve further investigation. In a recent report (Bennett et al., 1989), it was suggested that the scattering signal PAS could be related to direct activation of the cGMP-dependent channels in the absence of cGMP by activated PDE; although we have not as yet been able to demonstrate this hypothesis, it should be kept in mind that the occurrence of the PAS requires swelling of the discs and that its amplitude is proportional to the extent of swelling, both processes being dependent of the ionic composition of the external medium. While the ATP-dependent

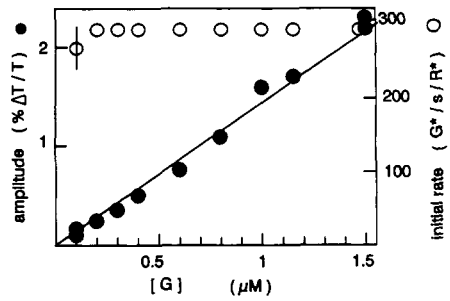

FIGURE 5: Variation of the amplitude and initial rate of the dissociation signal as a function of G concentration. Rhodopsin concentration, 5 μM; $R^*/R = 10^{-3}$ ($R^* = 5$ nM); GTP concentration, 200 μM. The initial rate in $G^*/(s \cdot R^*)$ is calculated supposing that the dissociation signal corresponds to activation of all the G present. At 1 μM G, the rhodopsin concentration can be lowered to 1 μM without reduction of the amplitude or initial rate of the signal; below this value, the amplitude decreases and the initial rate is slowed down.

swelling was also described by Uhl et al. (1979), Parker et al. (1987) report an ATP-dependent light-scattering change associated with filament formation. Their ATP and membrane concentration dependence is, however, very different from that in our experiments: half-saturation at 400 μM ATP (compared to about 100 μM) and optimal effect for 100 μM rhodopsin (while all our experiments are carried out at 2–10 μM rhodopsin); the existence of a relation between their G^+ signal [described by Lewis et al. (1984), apparently identical to the PAS] and their ATP-dependent filament formation is not observed, the amplitude of the G^+ signal being on the contrary smaller in preparations which show better filament growth. This seems to indicate that the origin of the light-induced signal is not likely to be an interaction between the light-activated enzymes and the cytoskeletal structure.

Apart from Lewis et al. (1984) (rod fragments; G^+ signal) and Caretta and Stein (1985, 1986) (reconstituted systems), whose signals are strikingly similar to the slow component of our signal (although they do not add ATP, they disrupt the disc stacks by sonication or extensive low ionic wash which in our experiments has the same effect as ATP), several authors have previously described fast light-scattering signals using preparations of better preserved structure, which by definition contain native amounts of PDE, and may be suspected to show light-induced PDE-dependent scattering changes: Kamps et al. (1985, 1986) (intact permeabilized oriented rods; AT signal); Wagner et al. (1987, 1988) (rods or rod fragments; amplified P or P_A signal); Vuong et al. (1984) and Bruckert et al. (1988) (oriented rods; release signal). Several characteristics have been described which are common to both slow and fast signals: an effect of cGMP, which we also observe (not shown), was noted by Caretta and Stein (1985) (slow) and by Kamps and Hofmann (1986) and Wagner et al. (1988) (fast); the enhancement of the signal amplitude and acceleration of its kinetics by ATP was noted by Kamps and Hofmann (1986) (fast), while we proposed an explanation of such phenomenon (Bennett, 1986; this report), where the PAS (slow and fast components) is shown to be directly dependent on an ATP-dependent process. It can be noted that when rods of highly preserved structure are used, it is possible that the ATP-dependent process is at least partially conserved and addition of ATP may not be necessary for the signal to occur (Wagner et al., in fact, add ATP to their suspensions). It is important to note that in our experiments, the dissociation signal associated with activation of G is neither cGMP dependent (N. Bennett, unpublished results) nor increased or accelerated by ATP (see, for example, Figure 3A), suggesting that at least the fast signals described by Kamps

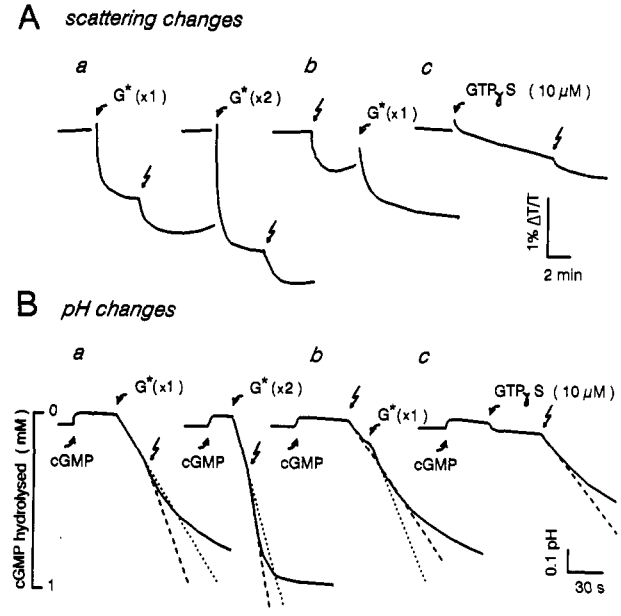

FIGURE 6: Additive effect of dark $G_{GTP\gamma S}$ addition and light in ROS fragments (disc stacks) for both the scattering signal PAS (A) and the PDE activity (B) [disscussion of Wagner et al. (1987)]. ROS fragments (2.8 μM rhodopsin) containing 190 nM G [measured from binding signals, as described in Bennett and Dupont (1985)], PDE (estimated concentration about 30 nM), and at least part of the rhodopsin inactivating system (rhodopsin kinase + arrestin) were incubated with 500 μM ATP for 15 min in order to allow swelling of the discs as well as inactivation of traces of excited rhodopsin. Preactivated G* ($G^*_{GTP\gamma S}$) at a final concentration of either 225 nM (noted as (x1)) or 450 nM (noted as (x2)) was then added in the dark; the estimated contaminant free GTPγS in the final suspension was less than 0.1 μM for 225 nM G* and less than 0.2 μM for 450 nM G*. The dark G*-induced scattering changes are shown in (A(a)) and the dark G*-induced PDE activity in (B(a)). Conditions: GTP, 100 μM; cGMP, 1 mM (B); flash intensity, $R^*/R = 2.10^{-3}$. Note that exact measurements of amplitudes or rate of cGMP hydrolysis are difficult due to reversal of light-induced GTP-dependent processes in relation with inactivation of R*. Addition of 10 μM free GTPγS in the dark (A(c) and B(c)) in the absence of G and GTP (a concentration equivalent to 50–100-fold of the amount of contaminant GTPγS in the experiment) does not induce any PDE activity and causes only a very slow scattering signal, which may be due to slow excitation of rhodopsin by the spectrophotometer beam. A flash applied after GTPγS addition induces PDE activity in (B(c)) similar to that in (B(b)), corresponding to activation by GTPγS of endogenous G; the light-induced scattering signal (A(c)) is slightly smaller than the signal in (A(a)) due to the partial dark signal.

et al. (1985) and by Wagner et al. (1987) resemble the fast component of the PAS more than the dissociation signal. Finally, the relation between the scattering change and PDE activity was suggested, although not demonstrated, by Caretta and Stein (1985, 1986) (slow) and by Kamps et al. (1985, 1986) (fast); it was not considered by Vuong et al. (1984), who simply assumed the signal to be associated with release of activated G from the membrane, and it was rejected by Bruckert et al. (1988) in view of the small amount of PDE compared to that of G and of the linear response upon increasing the concentration of G by a factor of 3 (but see Figure 4 and discussion below). Using rod outer segments of high structural integrity, Wagner et al. (1987) propose that their light-scattering signal is associated with activation of G rather than of PDE on the ground that addition of $G_{GTP\gamma S}$ to a suspension of rod fragments, containing the native amounts of G and PDE, increases the PDE activity but not the amplitude of light-induced scattering change. Repeating their experiments under similar conditions, we observed the same results (Figure 6), except that the signal that we observe under

these conditions is the slow component. Considering the data shown in Figures 1 and 4 and in Bennett and Clerc (1989), our interpretation is, however, different: at 2 μM rhodopsin, the native concentration of G in rod fragments (estimated as 200 nM) is not sufficient to induce maximal PDE activity (Bennett & Clerc, 1989) or a scattering change of maximal amplitude (Figure 4). Varying the concentration of G by a factor of 2 or 3 around this value produces a variation of about a factor of 2 or 3 in the resulting PDE activity or in the amplitude of the signal (Figure 4). Since addition of $G_{GTP\gamma S}$ (at a concentration of the same order as that present in the membranes) itself produces a partial scattering signal in the dark (as well as partial dark PDE activity), it is not surprising that a flash applied before or after addition of $G_{GTP\gamma S}$ induces signals of similar amplitude, which both correspond to the activation (or additional activation, if PDE is already partly activated by added $G_{GTP\gamma S}$) of PDE due to the activation of the endogenous G molecules by light and GTP. We find that when $G_{GTP\gamma S}$ is added before the flash, the total amplitude of the signal (dark signal induced by $G_{GTP\gamma S}$ addition plus light-induced signal) is indeed roughly proportional to the total PDE activity (dark activity induced by $G_{GTP\gamma S}$ plus light-induced activity). Note that under the conditions used it is not possible to distinguish between PDE activity and the proportion of active PDE state with two activators, which is the main contributor to PDE activity.

In conclusion, it seems difficult to determine whether or not all of the above-cited signals are associated with the same process(es). Although some are described as "fast" (<1 s) (Vuong et al., 1984; Kamps et al., 1985; Wagner et al., 1987; Bruckert et al., 1988) and others are described as "slow" (≥1 min) (Lewis et al., 1984; Caretta & Stein, 1985, 1986; Bennett, 1986), the differences in kinetic properties of the signals may vary with the structural integrity of the cell, which determines the concentration of proteins and nucleotides, as well as the physical organization of the membranes: for example, using oriented rods, Bruckert et al. (1988) describe the existence of two signals associated with release of activated G from the membrane (a fast signal associated with dissociation from the membrane and a slow signal associated with a slow loss of protein from the rod), while using reconstituted systems, we can only observe a fast signal (dissociation signal, corresponding to dissociation of G from the vesicles). It should also be borne in mind that the method used (oriented or nonoriented rods, scattering angle) may allow detection of different phenomena; this has been well discussed, for example, in Uhl et al. (1990). Nevertheless, while we have determined with our system the precise conditions under which either the dissociation signal or the PAS (fast and slow or only slow component) is observed, this has not been determined with other preparations and other experimental setups; it is possible that, under certain conditions, only the fast component of the PAS is observed. Moreover, the PAS (fast and slow components) is not modified when the disc stack structure is destroyed and replaced by vesicles, suggesting that a better preserved structure is perhaps not an essential parameter. Indeed, apparently the same signal is also observed by the same groups in both isolated rods and intact retinas (fast signal; Kamps et al., 1985; Pepperberg et al., 1988) or even in reconstituted systems and intact retinas (slow signal; Caretta & Stein, 1985; Stein & Caretta, 1990). The fact that these signals occur in intact retinas is a capital indication that they are associated with a physiological process rather than with an in vitro artifact. Reconstitution experiments with purified proteins and well-controlled nucleotide and protein composition and concentrations appear, however, as a necessary step to establish the nature of the elements involved.

Registry No. GTP, 86-01-1; ATP, 56-65-5; cGMP phosphodiesterase, 9068-52-4.

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Rates of Spontaneous Exchange of Synthetic Radiolabeled Sterols between Lipid Vesicles†

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ABSTRACT: ^{14}C-Labeled sterols with structural variation in the polar function [3α-OH, 3-O$(CH_2)_2$O-$(CH_2)_2$O$(CH_2)_2$OH, 3α-NH_2, 3β-NH_2, and 3-OC(O)CHN=N] and at the 7 position (7-oxo, 7α-OH, and 7β-OH) were synthesized and incorporated into unilamellar vesicles for studies of the rates of transfer to an excess of acceptor vesicles. Cholesterol, cholestanol, and epicholesterol underwent full exchange in a single kinetic pool, and 90% of the 3α-triethoxycholesterol was exchangeable in one pool. Biphasic kinetics with full exchangeability were observed for cholesterylamines, which bear a positive charge at the 3 position; the slow phase reflects the high activation energy for inner-to-outer leaflet movement of the charged lipid. Biphasic kinetics were also found for cholesteryl diazoacetate, indicating that this photoaffinity probe and cholesterol have different mechanisms of transfer. Sterols that are more hydrophilic than cholesterol as estimated by reversed-phase high-performance chromatography (elution with acetonitrile–2-propanol, 4:1 v/v, with varying proportions of water) gave faster exchange rates than cholesterol, whereas sterols that are more hydrophobic gave slower exchange rates. However, the rates of [^{14}C]sterol desorption from the lipid–water interface are not correlated with the relative sterol hydrophobicity as estimated by the logarithm of the capacity factors using acetonitrile–2-propanol–water as the mobile phase. These studies suggest that the interaction of sterols with phospholipids provides the principal physical–chemical basis for determining the rates of spontaneous exchange of sterols between bilayers.

There has been considerable interest in recent years in the spontaneous intermembrane movement of unesterified cholesterol. Relatively rapid transfer or exchange of cholesterol has been observed between membrane structures such as vesicles, erythrocytes, lipoproteins, mycoplasmas, and mammalian cells in tissue culture [reviewed by Phillips et al. (1987), Dawidowicz (1987), and Bittman (1988)]. The rate of spontaneous transfer of cholesterol between membranes is a function of the degree of saturation of the phospholipid fatty acyl chains and the sphingomyelin content of the membrane (Phillips et al., 1987; Bittman, 1988) and also of the curvature of the surface from which desorption takes place (McLean & Phillips, 1984; Fugler et al., 1985). Studies of the rates of cholesterol movement between membranes composed of different phospholipids have indicated an inverse relationship between the unidirectional transfer or bidirectional exchange rate and the degree of molecular packing of cholesterol with phospholipids in the bilayer (Fugler et al., 1985; Phillips et al., 1987; Lund-Katz et al., 1988). In addition to the interactions experienced by cholesterol in the lipid matrix of the donor species, the rate of cholesterol release from donor vesicles and transfer to acceptor vesicles is sensitive to factors that modify the aqueous phase solubility of cholesterol, such as the presence of chaotropic salts (Clejan & Bittman, 1984a), bile salts (Vlahcevic et al., 1990), and polar water-miscible organic solvents (Bruckdorfer & Green, 1967; Quarfordt & Hilderman, 1970; Bruckdorfer & Sherry, 1984). A recent comparison of the rates of cholesterol and sitosterol exchange suggested that the rate of sterol exchange between vesicles decreases with increasing sterol hydrophobicity, since the presence of the 24α-ethyl group in sitosterol represents a constraint to the rate of intermembrane movement (Kan & Bittman, 1990, 1991). To evaluate further the effects of structural modifications in cholesterol on the movement of sterols between vesicles, we have synthesized various radiolabeled synthetic analogues of cholesterol. In this paper, we compare the effects of specific structural modifications in the sterol molecule on the rates of efflux from phospholipid vesicles. We have also used sterol transfer kinetics to determine whether the cholesterol photoaffinity probe cholesteryl diazoacetate (Middlemas & Raftery, 1987) behaves analogously to cholesterol with respect to exchange and thus with respect to interactions with donor phospholipids.

Materials and Methods

Chemicals. [4-^{14}C]Cholesterol (specific activity 57.5 mCi/mmol) and [9,10-^{3}H(N)]glycerol trioleate (specific activity 15.4 Ci/mmol) were obtained from Du Pont New England Nuclear (Boston, MA). [4-^{14}C]Epicholesterol (specific activity 57.5 mCi/mmol) was prepared as described previously (Yan & Bittman, 1990). Egg PC,[1] DPPC, DCP,

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[1] Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoyl-PC; DCP, dicetyl phosphoric acid; k', capacity factor.

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