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Choleratoxin ADP-ribosylates transducin only when it is bound to photoexcited rhodopsin and depleted of its nucleotide

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The sensitivity of transducin (T) to choleratoxin (CT) in retinal cells depends on illumination and on the presence of GTP or analogs Low concentrations of GPP-NH-P or GPP-CH_2-P increase ADP-ribosylation while GTPγS inhibits it We show that GTP analogs permanently activate an ADP-ribosylating factor (ARF) which mediates CT action on retinal cell membranes when transducin-depleted membranes were pre-activated by GTP analogs, re-added transducin became sensitive to CT in the absence of nucleotide, and presence of photoexcited rhodopsin (R^*) Any subsequent G-nucleotide addition (even GDP) decreased ADP-ribosylation Thus nucleotide-free transducin molecule in R^*–T_{empty} complex is the CT substrate

Transducin, Rhodopsin, Choleratoxin, ADP-ribosylation factor (ARF)

1 INTRODUCTION

Bacterial toxins modify the α subunit of heterotrimeric G-proteins by catalyzing site specific ADP-ribosylation [1] In a large variety of cells choleratoxin (CT) catalyzes ADP-ribosylation of Gsα on an arginine which is located close to the GTP binding site [2]. This leads to a reduced GTPase rate and an enhancement of Gs-induced cyclase activity The process depends on ADP-ribosylation factors (ARF) generally present in the cell [3,4], but does not seem to require other cofactors.

G_o and the G_i proteins which are sensitive to ADP-ribosylation by pertussis toxin (PT) were generally considered insensitive to CT, although an arginine equivalent to that modified by CT in Gsα is found in all of them But in HL60 cells, a PT-sensitive G_i that couples a chemotactic receptor to a phospholipase C was found to be sensitive to CT, provided the receptor was activated by its agonist, fMLP [5,6]. This effect was inhibited by GTP or GTPγS

Transducin (T) in retinal cells is sensitive to both PT and CT [7–9], but the conditions of ADP-ribosylation by CT are poorly understood Continuous illumination seems necessary and guanine nucleotides have strange effects GTP decreases a little the ADP-ribosylation rate, the non-hydrolyzable analog GPP-NH-P is stimulatory while surprisingly GTPγS seems strongly inhibitory. It was hence suggested that the substrate for CT would be a transient complex of $T\alpha_{GPP\text{-}NH\text{-}P}$–$T\beta\gamma$ with a photointermediate of rhodopsin [9]. As we had characterized the transitory complex between photoexcited rhodopsin (R^*) and transducin, and demonstrated its stabilisation upon removal of guanine nucleotides [10], we suspected that the CT substrate might rather be this stable R^*–T_{empty} complex, and that the strange dependence of CT action on GTP analogs could be partly due to the action of nucleotides on ADP-ribosylating factors present in the cell. To check this hypothesis we pre-activated ARF by GTP analogs in transducin-depleted retinal membrane, reconstituted the system by adding back transducin, and ADP-ribosylated with CT under transient or continuous illumination, with or without GTP analogs Our data suggest that the G-protein is a substrate for CT-catalyzed ADP-ribosylation only when it is bound to an activated receptor and devoid of its nucleotide.

2 MATERIALS AND METHODS

2.1 *Buffers*

Iso buffer Tris-HCl 20 mM, pH 7 5, NaCl 120 mM, $MgCl_2$ 0 1 mM, *Hypo buffer* Tris-HCl 5 mM, pH 7 5, $MgCl_2$ 0 1 mM, *ADP-ribosylation buffer* NaH_2PO_4 200 mM, arginine 12 mM, thymidine 1 mM, $MgCl_2$ 4 mM, DTT 10 mM, pH 7 0

2.2 *ROS membrane preparations*

Cattle retinal rod outer segment membranes (ROS) were prepared under dim red light as described [11] and stored at −80°C Thawed pellets were resuspended in buffer and homogenized in a teflon-glass homogenizer All procedures were conducted at 4°C and under dim red light except when specified

Photoactivation was achieved by 1 min illumination with orange light (λ > 540 nm, Kodak Wratten 21 filter), and photoregeneration of R^* (during long incubations with CT) by continuous illumination with blue light (λ_{max} ≈ 460 nm, Kodak Wratten 48 filter)

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2 3 *Elution of transducin pre-activation of ARF and membrane reconstitution*

Crude ROS (50 μM rhodopsin) were sedimented in Iso buffer supplemented with 3 mM $MgCl_2$ to eliminate soluble proteins while keeping transducin Transducin was eluted by washing the ROS in Hypo buffer, the membrane pellet was resuspended in Iso buffer, and incubated 10 min at 37°C with 10 μM GTPγS (or no nucleotide for control) to stimulate the membrane bound ARF, unbound GTPγS was removed by sedimentation and resuspension in Iso buffer The transducin extracts were added back, under isotonic conditions, to the processed membrane pellet and the suspension was illuminated for 1 min with orange light to induce R*–T complex formation An additional washing in Hypo buffer removed the cGMP phosphodiesterase and insured complete depletion of GDP from the R*-bound transducin The final pellet was subjected to ADP-ribosylation

2 4 *ADP-ribosylation by choleratoxin*

Choleratoxin from Sigma (0 5 mg/ml) was first activated for 15 min at 37°C in Tris-HCl 50 mM, pH 7 5, DTT 50 mM Crude or processed ROS membranes (70 μM rhodopsin) were incubated for 150 min at 30°C with 75 μg/ml CT, 500 μM ^{32}P NAD from N E N (20–50 μCi/μmol), with guanine nucleotides as specified, in the dark or in blue light The samples were then supplemented with 100 μM GTPγS, diluted twice with H_2O and boiled for 5 min in the presence of denaturing buffer for SDS PAGE analysis (Laemmli gels with 10% acrylamide)

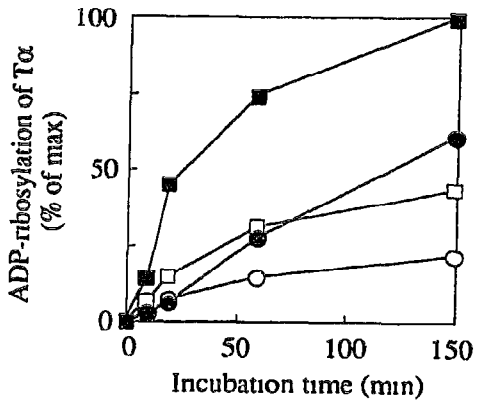

Fig 2 Effect of extraction and reconstitution procedure on the ADP-ribosylation of Tα in the processed ROS membranes Transducin was extracted from crude ROS membrane and added back after further washing of the membranes, as described in Methods, without the step of incubation with GTPγS The processed membranes (○ ●) and crude photo-activated ROS membranes (□, ■) were incubated for various times with CT and ^{32}P NAD in blue light with (●, ■) or without (○ □) GPP-NH-P (100 μM) Densitometry of autoradiograms of ^{32}P labelled Tα on SDS gels The loss of soluble ARF in the extraction procedure decreases by 40% the ADP-ribosylation rate

3 RESULTS AND DISCUSSION

Transducin is the major substrate for CT-catalyzed ADP ribosylation in crude ROS suspensions. This process depends on illumination and on the presence of guanine nucleotides which influence the interaction of transducin with R*, suggesting that this interaction determines transducin sensitivity to CT. But the light insensitive ADP-ribosylation of other proteins in the ROS depends also on the presence of GTP analogs (Fig 1) This suggests that beside their action on transducin, the GTP analogs influence the action of CT, probably by activating an ADP-ribosylating factor (ARF) present in the preparation

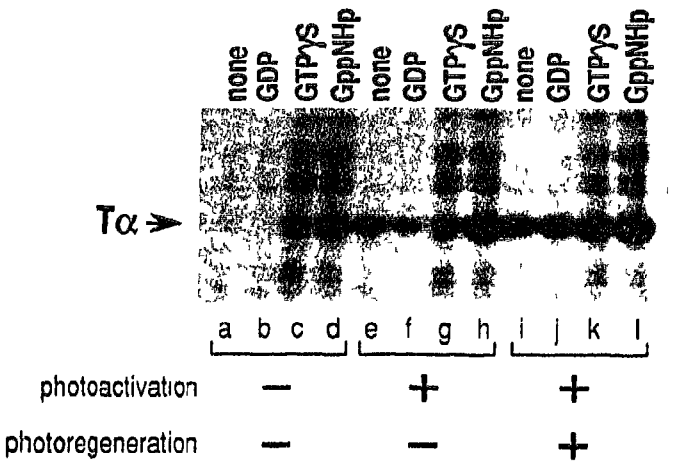

Fig 1 Influence of light and of nucleotides on the CT-catalyzed ADP-ribosylation of crude ROS membranes Autoradiogram of SDS gel Photoactivation orange light (λ > 540 nm) for 1 min, before incubation with CT and ^{32}P NAD, photoregeneration blue light (λ_{max} = 460 nm) during incubation with CT. GDP 100 μM, GTPγS 10 μM, GPP-NH-P 100 μM

Pre-illumination of crude ROS before the incubation with CT in the absence of nucleotides induced significant ADP-ribosylation of Tα, as compared to the labelling of all other substrates (Fig 1e), GDP decreased the labelling of Tα (Fig. 1f) Since photoactivation of rhodopsin in the absence of nucleotides induces the formation of stable $R^*–T_{empty}$ complexes [10] and addition of more than 20 μM GDP causes partial dissociation of these into free R* and inactive $T\alpha_{GDP}–T\beta\gamma$, the specific substrate of CT might well be GDP-depleted and R*-bound Tα The puzzling observation already made by other authors [7,9] that GPP-NH-P increases while GTPγS decreases the labelling of Tα, although both increase that of other substrates (Fig 1g,h), could be explained by the relative efficiencies of these analogs in dissociating $R^*–T_{empty}$ complexes submicromolar con-

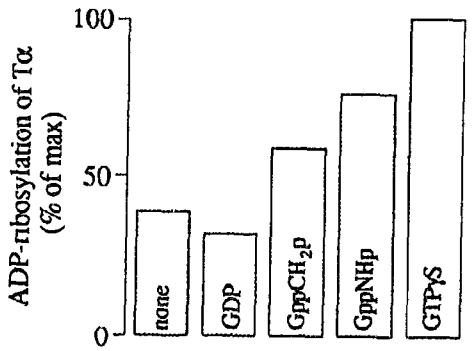

Fig 3 Effect of nucleotides at the pre-activation step. ROS membranes were depleted of transducin (see Methods), and incubated with various nucleotides (10 μM), transducin was added back, the reconstituted membranes were illuminated, washed of nucleotides and incubated with CT and ^{32}P NAD in the dark Densitometry of autoradiograms of ^{32}P labelled Tα on SDS gels

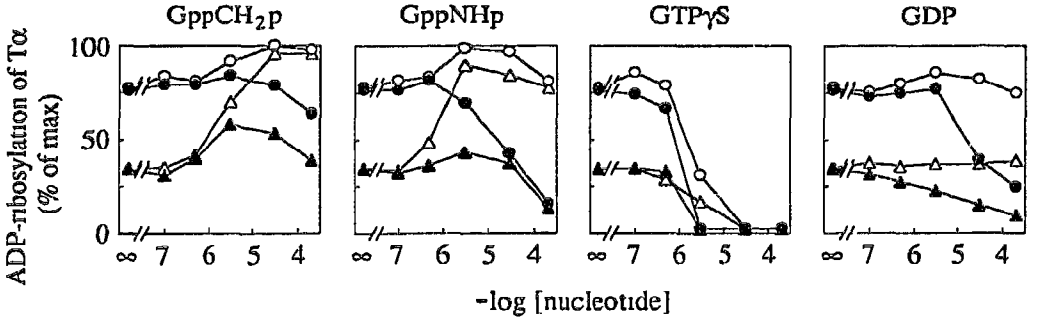

Fig 4 Effect of nucleotides on pre-activated ROS membranes ROS membranes were depleted of transducin and incubated with 10 μM GTPγS (○,●), or without nucleotide for control (△,▲) Transducin was added back, the reconstituted membranes were illuminated, washed of nucleotides and incubated with CT, [^{32}P]NAD and various quantities of nucleotides, as indicated, in the dark (●,▲) or under continuous illumination with blue light (○,△)

centrations of GTPγS rapidly dissociate activated $T\alpha_{GTP\gamma S}$ from R^* thus rendering it insensitive to CT, GPP-NH-P is hundred times less efficient [12] and its stimulation of ARF could more than compensate for a slow dissociation of $T\alpha_{GPP\text{-}NH\text{-}P}$ from R^*

Increase of labelling upon continuous illumination during the long incubation with CT (Fig 1i,j,k,l) was also expected Blue light regenerates R^* from its first decay product Meta III rhodopsin In the absence of nucleotide this has little effect, as the R^*–T_{empty} complexes are stable, but in the presence of GDP or GTPγS they dissociate, and the released R^* decay to inactive products Thus photoregeneration helps maintain a large pool of R^* which favors the formation of new R^*–T complexes, hence the increase in labelling With GPP-NH-P, ADP-ribosylation was already saturated without photo-regeneration (Fig 1h) and could not be further enhanced

To distinguish the effect of GTP analogs on transducin from that on the putative ARF, transducin was eluted from ROS membranes on which the remaining ARF could be 'pre-activated' by GTP analogs The eluted transducin was then added back, the membranes were illuminated to induce the binding of T to R^*, and washed to remove all the nucleotides and stabilize the R^*–T_{empty} complexes Effects of nucleotides on these complexes were then tested by adding them together with CT during the ADP ribosylation step We first checked that the multiple washings of this procedure did not extract all the ARF which is partially soluble [13] In reconstituted, but not 'pre-activated' membranes, CT action on transducin was more than 60% of that measured in native ROS membranes, and was still sensitive to the addition of GPP-NH-P during the ADP-ribosylation step (Fig 2) We then observed that ARF pre-activation by GTP analogs in transducin-depleted membranes increased the CT dependent ADP-ribosylation of the transducin that was re-added to the membranes after washing out the GTP analogs (Fig 3) incubating transducin-depleted membranes for 10 min at 37°C with 10 μM GTPγS or GPP-NH-P notably increased the later CT dependent labelling, demonstrating the 'pre-activation' of membrane bound ARF

On such pre-activated and reconstituted ROS membranes, addition of any one of the GTP analogs, or even of GDP, during the incubation with CT never increased, and at sufficient concentrations depressed the high level of ADP-ribosylation of transducin observed in the absence of nucleotides (Fig 4) The order of effectiveness of the GTP analogs in inhibiting ADP ribosylation of transducin in these reconstituted systems parallels that previously observed [12] for eluting transducin from illuminated disc membranes. This confirms that dissociating transducin from R^*–T_{empty} complexes into a stable 'active' TαGTP form, with GTP analogs, as well as into the inactive $T\alpha_{GDP}$–T$\beta\gamma$ form, with GDP, suppresses its sensitivity to the toxin

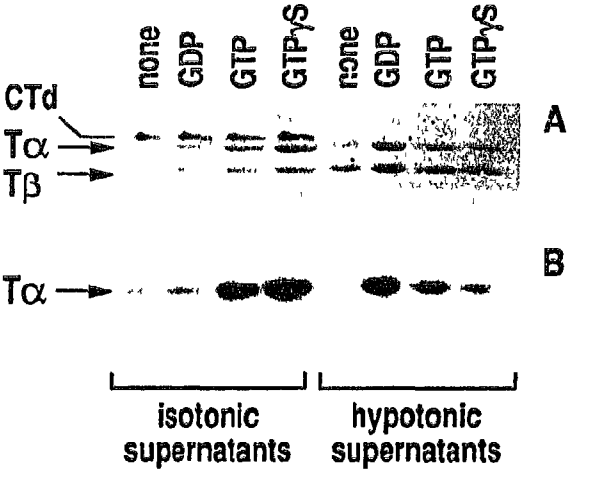

Fig 5 Solubilisation pattern of ADP-ribosylated transducin Transducin in pre-activated and reconstituted ROS membranes was ADP-ribosylated in the dark by 150 min incubation at 30°C with CT and [^{32}P]NAD, without nucleotide The reaction was blocked at 4°C Four aliquots were complemented with GDP (200 μM), GTP (200 μM), GTPγS (50 μM) or no nucleotide, and sedimented in the isotonic reaction buffer The pellets were resuspended in Hypo buffer, complemented with the same nucleotide as before, and sedimented again Both isotonic and hypotonic supernatants were analyzed for transducin content (stained gel, A) and for ^{32}P labelling (autoradiogram, B) CTd=cholera toxin A_1 subunit dimer.

When reconstituted ROS membranes were subjected to the same processing but without the addition of GTPγS during the 'pre-activation' step, the incubation with CT and no nucleotide resulted in a lower level of ADP-ribosylation; addition of GPP-CH_2-P or GPP-NH-P during the incubation with CT had biphasic effects low concentrations increased the ADP-ribosylation yield, while higher ones decreased it (Fig 4), GTPγS as well as GDP were again only inhibitory. The biphasic patterns obtained with GPP-NH-P and GPP-CH_2-P are then clearly due to two effects of these GTP analogs, on ARF, which they already activate at low concentrations, and on the receptor-bound transducin, which they activate and dissociate from R*, but only at higher concentrations. The high efficiency of GTPγS in dissociating transducin from R* always overcomes its stimulatory effect on ARF In the intermediate range of GTP analog concentrations (1–100 μM), GPP-NH-P is overall stimulatory while GTPγS is inhibitory, in accord with the effects of these analogs on the ADP-ribosylation of crude ROS membranes As for GDP, it does not activate ARF and can therefore only be inhibitory at high concentrations by causing the dissociation of inactive transducin from R*

Photoregenerating R* does not change the level of ADP-ribosylation observed in the absence, or with very low levels of GTP analogs during the incubation with CT. This was expected since light does not affect ARF But the photoregeneration increases the effect of GPP-NH-P or GPP-CH_2-P and raises the level at which GTPγS and GDP becomes inhibitory (Fig. 4). This reflects the photoregeneration of R* and of R^*–T_{empty} complexes, counteracting their dissociation induced by the higher concentrations of GTP analogs

Does ADP-ribosylation by itself modify the activation state of Tα and its interaction with R*? Once ADP-ribosylation process completed, isotonic or hypotonic washings will not elute labelled Tα from the reconstituted membranes if there are no nucleotides in the buffer (Fig 5) With GTP or an analog added, Tα dissociates from Tβγ and elutes in isotonic buffer, Tβγ elutes only in a subsequent hypotonic wash. With GDP added, Tα will elute only in hypotonic buffer, together with Tβγ. These elution patterns are identical to that of unmodified transducin in native illuminated ROS membranes [11] Thus the ADP-ribosylation of Tα in a R^*–T_{empty} complex does not markedly modify the interaction of transducin with R* $^{ADPr}T\alpha$ is not 'activated' by the modification itself, only by the later binding of a GTP or GTP analog, which will induce the release of active $^{ADPr}T\alpha_{GTP}$ The enhanced activity of $^{ADPr}T\alpha_{GTP}$, compared to that of unmodified $T\alpha_{GTP}$, is due to a slower hydrolysis of GTP in the modified Tα rather than to the modification itself If GDP is first presented to the R^*-$^{ADPr}T_{empty}$ complex, it will also bind, and release from R* inactive $^{ADPr}T\alpha_{GDP}$–Tβγ that will, however, remain membrane bound in isotonic buffer

In conclusion, we have demonstrated that the G-protein transducin is the substrate of Choleratoxin only if it is bound to photoexcited rhodopsin (equivalent to an agonist-activated receptor) and if its nucleotide site is depleted Neither inactive $T\alpha_{GDP}$–Tβγ, nor active $T\alpha_{GTP}$ are CT substrates We might speculate that the arginine residue modified by CT is involved in the binding of GDP as well as GTP, and is accessible to the toxin only when the nucleotide site is empty It is noteworthy that Gs, the only G-protein which seems sensitive to CT without requiring agonist-activation of its coupled receptor, has an unusually high rate of spontaneous GDP/GTP exchange This implies that its nucleotide site transiently empties at a high frequency, and this might be the cause of its sensitivity to choleratoxin

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