Fluoroaluminates activate transducin-GDP by mimicking the γ-phosphate of GTP in its binding site

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Fluoride activation of the cGMP cascade of vision requires the presence of aluminum, and is shown to be mediated by the binding of one AlF_4^- to the GDP/GTP-binding subunit of transducin. The presence of GDP in the site is required: AlF_4^- is ineffective when the site is empty or when GDPβS is substituted for GDP. This sensitivity to the sulfur of GDPβS suggests that AlF_4^- is in contact with the GDP. Striking structural similarities between AlF_4^- and PO_4^{3-} lead us to propose that AlF_4^- mimics the role of the γ-phosphate of GTP.

Fluoride *Aluminum* *Phosphate* *Transducin* *G-protein* *Adenylate cyclase*

1. INTRODUCTION

Fluorides have long been known to influence the activity of hormone-sensitive adenylate cyclase systems [1] and of the analogous cGMP phosphodiesterase (PDE) system of retinal rod outer segments (ROS) [2]. The target of F^- is the α-subunit of the GTP-binding proteins, called G/F, N_s, N_i or G_s, G_i in hormonal systems, and transducin (T) in the retina [3,4]. These proteins activate the cyclase, or PDE, when loaded with GTP by a GTP/GDP exchange catalysed by liganded hormone receptor, or light-activated rhodopsin. They become inactive when the GTP is hydrolysed to GDP. In a normal activation-deactivation cycle [5], with nucleotides in the medium, the site on T_α is virtually always occupied: it is occluded, and the nucleotide is non-exchangeable until T_α binds to R* [6]. This binding causes the site to open and allows a fast exchange of the bound GDP for GTP; then $T_\alpha \cdot$GTP quickly dissociates from $T_{\beta\gamma}$ and from R*, with the GTP again occluded in the site where it will eventually be hydrolysed to GDP. $T_\alpha \cdot$GTP leaves the membrane and activates PDE by binding to its inhibitor subunit. The nucleotide site may be emptied by removing all guanyl nucleotides in the presence of R*. In this case T_α-$T_{\beta\gamma}$ will not dissociate from R* until its decay to opsin.

Fluorides in the millimolar range confer upon the GDP-bound protein structural and functional properties of the GTP-bound one [4,8]. The requirement of a trace amount of aluminum was recently demonstrated for fluoride activation of hormonal systems and it was suggested that AlF_4^- was indeed the active species [9]. We demonstrate that in the retinal system one AlF_4^- activates with high affinity one $T_\alpha \cdot$GDP molecule. The presence of GDP is required: AlF_4^- has no effects on T_α when the nucleotide site is empty or contains GDPβS, even though $T_\alpha \cdot$GDPβS is functionally and structurally similar to $T_\alpha \cdot$GDP. The sensitivity of AlF_4^- to the sulfur of GDPβS suggests that AlF_4^- binds close to the GDP. Striking structural similarities between AlF_4^- and PO_4^{3-} lead us to propose that AlF_4^- interacts with the GDP in the nucleotide site, where it mimics the role of the γ-phosphate of GTP.

Abbreviations: GDPβS, guanosine 5′-*O*-(3-thiodiphosphate); R*, photoexcited rhodopsin

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2. MATERIALS AND METHODS

These studies were conducted with purified bovine ROS membranes [10], in which the native content of transducin (~1/10 rhodopsins) and PDE was preserved, or alternatively with reconstituted systems: purified transducin subunits, in various states [11], and partially purified PDE were added back to ROS membranes that had been washed free of all peripheral proteins and in which rhodopsin was virtually the only protein component left. $T_\alpha \cdot GDP$ (or permanently activated $T_\alpha \cdot GTP\gamma S$) and $T_{\beta\gamma}$ were purified separately or as an associated $T_\alpha \cdot GDP\text{-}T_{\beta\gamma}$ complex [11]. In native or reconstituted systems, functionality was assessed by monitoring cGMP hydrolysis by the pH-metric method [12]. As characterized by Kühn [7,10], inactive $T_\alpha \cdot GDP$ and active $T_\alpha \cdot GTP$ (or $T_\alpha \cdot GTP\gamma S$) were also differentiated by their binding affinities: $T_\alpha \cdot GDP$ binds strongly to $T_{\beta\gamma}$ and the complex binds to ROS membranes in the dark at moderate ($\sim 10^{-1}$ M) ionic strength and binds strongly to R* upon illumination; in contrast, $T_\alpha \cdot GTP\gamma S$ dissociates from $T_{\beta\gamma}$ and does not bind to ROS membranes in the dark or to R*. Activation of the system by GTP or $GTP\gamma S$ is therefore always correlated with separation of $T\alpha$ from the membrane and its solubilization in moderate ionic strength media [7,10]. Structural differences between $T_\alpha \cdot GDP$ and $T_\alpha \cdot GTP\gamma S$ were also observed through their different sensitivity to proteolytic enzymes [13].

3. RESULTS AND DISCUSSION

In the presence of NaF and $AlCl_3$, it was first observed that purified $T_\alpha \cdot GDP$ acquired the capacity to activate PDE in reconstituted systems in the dark with an efficiency comparable to that of $T_\alpha \cdot GTP\gamma S$. Under the same conditions, in dark native membranes, $T_\alpha \cdot GDP$ dissociates quantitatively from $T_{\beta\gamma}$ and the membrane. The requirement for aluminum can only be demonstrated when working in clean plastic vessels: as pointed out in [9], common glassware releases sufficient amounts of aluminum to elicit activation in the presence of NaF. The quantitative requirements for $AlCl_3$ and NaF are shown in fig.1. Maximum efficiency is achieved at an NaF concentration

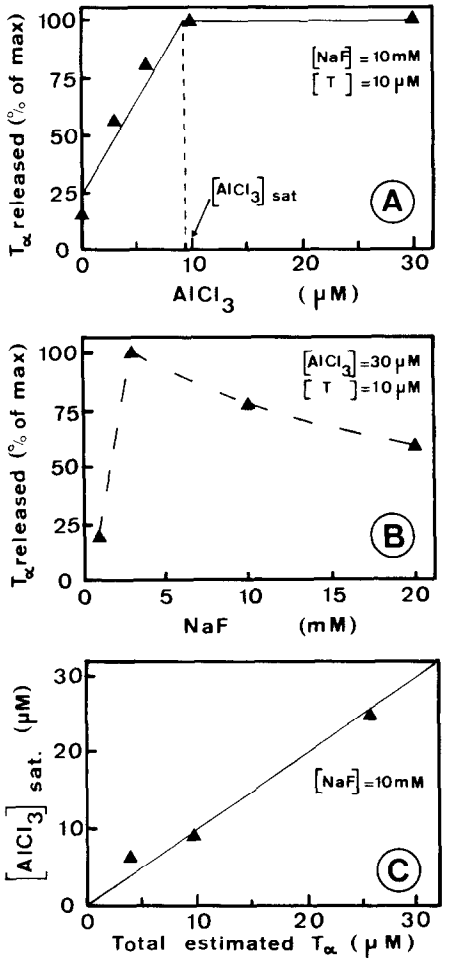

Fig.1. Demonstration of the activation of T_α by a stoichiometric amount of AlF_4^-. The activation of T_α was monitored through its solubilization from dark ROS membranes in moderate ionic strength medium [7,10]. Buffer composition (in mM): Hepes, 5 (pH 7.5); NaCl, 120; $MgCl_2$, 0.5; DTT, 1. After washing once in this medium, the ROS membrane pellet was resuspended, and NaF and $AlCl_3$ were added sequentially. The suspension was incubated for 6 min at 20°C and centrifuged. Total transducin concentration was estimated as 1/10 that of rhodopsin. T_α extracted in the supernatant was determined by densitometry on Coomassie blue-stained SDS-PAGE (10% acrylamide [19]). Similar results were obtained by the assay of Bradford [20] of the proteins in the supernatant. (A) Requirement for $AlCl_3$ at 10 mM NaF. (B) Requirement for NaF at 30 μM $AlCl_3$. (C) Concentration of $AlCl_3$ required to saturate the release of T_α (as shown in A for 10 μM T_α), at various total transducin concentrations. Assuming that all of the aluminum is complexed into AlF_4^- [14,15], the straight line fit demonstrates a stoichiometry of one AlF_4^- per T_α.

where the predominant fluoroaluminate complex ion is AlF_4^- [14,15]. Dose-activation curves were run at various T_α concentrations. Full activation was always obtained with total AlF_4^- concentrations corresponding to one AlF_4^- per T_α (fig.1C). This demonstrates a stoichiometry of one AlF_4^- bound per T_α and a binding constant of the order of 1 μM or higher. AlF_4^--activated $T_\alpha \cdot GDP$ has the proteolytic sensitivity characteristics of $T_\alpha \cdot GTP\gamma S$ [4].

As observed by Stein et al. [4] illumination appeared to cancel the effects of AlF_4^-, on PDE activation as well as on the binding properties of T_α. In contrast to the suggestion of Stein et al., this is not due to the blocking of access to the 'fluoride site' by the previous binding of R* to T_α: when AlF_4^- is applied first in the dark, solubilization of T_α and activation of PDE are reversed upon strong illumination; T_α appears to bind quantitatively to R* in the presence of AlF_4^- as it does in the absence of AlF_4^-. The presence of $T_{\beta\gamma}$ is required for this rebinding to take place, as it is for the binding of $T_\alpha \cdot GDP$ to R* [7]. We noticed, however, that the deactivation by strong illumination of AlF_4^--activated T_α is a slow process, which appears to require a few minutes to develop. A significant observation was then that a few micromolar GDP, which does not interfere with the binding of T_α to R* in the absence of AlF_4^-, or activate the system in the absence of AlF_4^-, suffices in the presence of AlF_4^- to prevent or reverse the rebinding of T_α to R* and to preserve or restore the PDE activation (fig.2). Our interpretation is that AlF_4^--activated $T_\alpha \cdot GDP$ (as does probably $T_\alpha \cdot GTP$) still has a low affinity for $T_{\beta\gamma}$ and R*. A very small proportion of AlF_4^--activated $T_\alpha \cdot GDP$ binds back to $T_{\beta\gamma}$ and to R*, then the site opens, the bound GDP is released and, in the absence of nucleotide in the medium it is not replaced, as the affinity of the site is in the 10^{-5} M range [6]. With no nucleotide in the site, T_α cannot be activated by AlF_4^-, the binding of T_α to R* becomes irreversible, and progressively all of the T_α becomes bound. A few micromolar GDP in the medium suffices to keep a GDP in the open site of an R*-bound T_α and to prevent this phenomenon. AlF_4^- alone, without nucleotide in the site, is therefore unable to activate T_α: binding of a GDP molecule and of an AlF_4^- is concurrently required. Indications that the AlF_4^--binding site is in close proximity to the

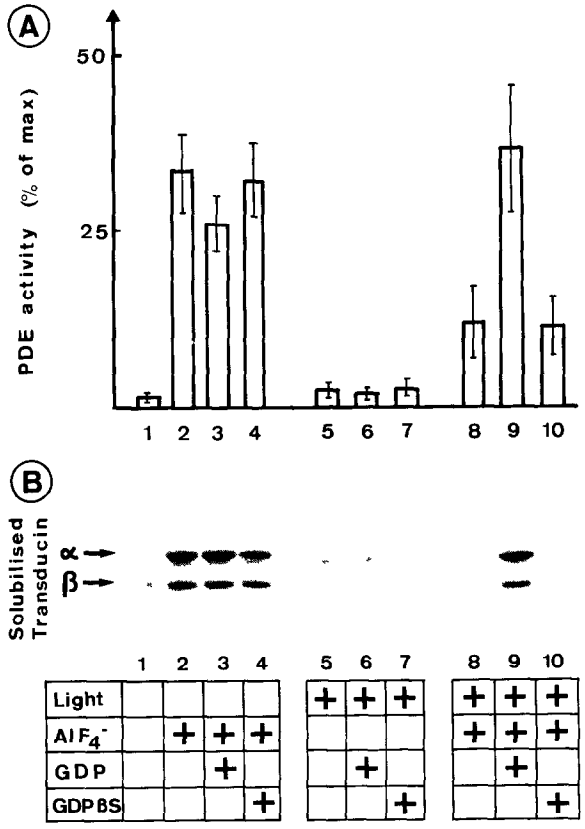

Fig.2. Demonstration of the requirement for the presence of GDP in the site for AlF_4^- activation. Measurements are performed on aliquots of the same ROS membrane preparation. Incubation conditions are shown in the grid below. Light: flash photoexciting ~90% of the rhodopsin. AlF_4^-: 30 μM $AlCl_3$ + 10 mM NaF. GDP and GDPβS: 100 μM. (A) PDE activity measured by the pH-metric method [12], normalized to the maximal activity observed upon illumination in the presence of GTPγS. Buffer composition (in mM): Hepes, 10 (pH 7.5); KCl, 120; $MgCl_2$, 2; cGMP, 0.4. ROS membrane concentration: 12 μM rhodopsin. (B) Solubilization of T_α, observed on SDS-PAGE (same procedure as in fig.1). In the dark (lanes 1–4), T_α has retained its native GDP occluded in the site [6]. AlF_4^- alone activates the system; added GDP and GDPβS have no effects. Upon illumination in the absence of AlF_4^- (lanes 5–7), T_α binds to R*, and added GDP and GDPβS do not activate the system. Upon illumination and in the presence of AlF_4^- (lanes 8–10), in the absence of added GDP (lane 8), T_α remains bound to R* and inactive. The nucleotide site is open, its dissociation constant being ~20 μM [6], most of the GDP has been released, and under this condition, AlF_4^--induced activation is quenched (cf. lanes 8 and 2). Addition of GDP restores AlF_4^--induced activation (lane 9) but GDPβS is ineffective.

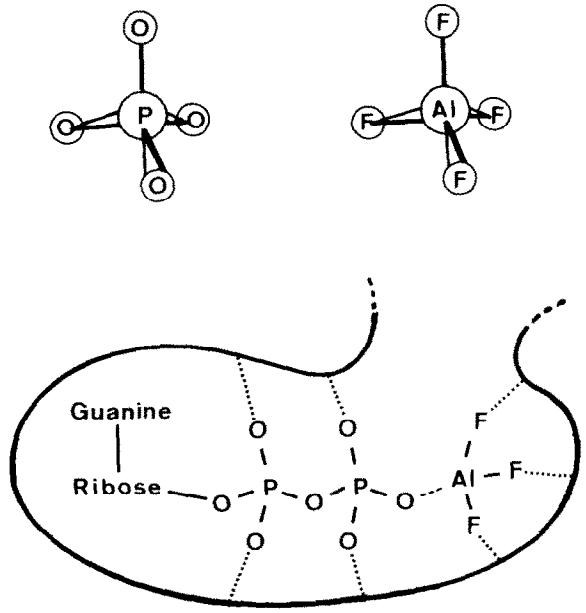

Fig.3. Structural analogies between PO_4^{3-} and AlF_4^- and schematic model proposed for the interaction of AlF_4^- with the GDP occluded in its site in T_α.

nucleotide-binding site are provided by the use of GDP analogs. GDPβS can be substituted for GDP by washing out the GDP of R*-bound T_α, adding GDPβS and letting R* decay to opsin. $T_\alpha \cdot$GDPβS seems to retain all of the characteristics of $T_\alpha \cdot$GDP: it binds to $T_{\beta\gamma}$ and to R*, and exchanges its nucleotide for GTP in the presence of R*, like $T_\alpha \cdot$GDP (T.M. Vuong and M. Chabre, unpublished). $T_\alpha \cdot$GDPβS also has the proteolytic sensitivity characteristics of $T_\alpha \cdot$GDP (not shown). However, $T_\alpha \cdot$GDPβS is not activated by AlF_4^-, as if the binding of the ion complex were directly hindered by the presence of the large sulfur atom on the β-phosphate. The observation that fluorides were inactive in the presence of GDPβS had previously been done on the hormone sensitive G-protein systems where, by contrast, fluoride activation was effective when the analog GP-CH_2-P had been substituted for GDP [16]. Our results and these previous findings strongly suggest that the fluoroaluminate complex binds in the nucleotide site, close to the β-phosphate of GDP.

It then struck us that the structure of the complex ion AlF_4^- is very similar to that of a phosphate group PO_4^{3-} [17,18]: both ions are tetrahedral, the central aluminum atom has the same size as a phosphorus atom; fluoride has a Van der Waals radius of 1.35 Å, like oxygen, and the Al–F bond length is 1.65–1.70 Å vs 1.55–1.60 Å for the average P–O bond length in PO_4^{3-}. We therefore propose that the activation of transducin (and of the analogous G_s and G_i proteins) by fluorides results from the binding of an AlF_4^- into the guanine nucleotide site, close to the β-phosphate of GDP (fig.3). F^- is known to be able to make hydrogen bonds which could substitute for that made by the oxygens of the phosphate group. The bound AlF_4^- simulates the presence of the bound γ-phosphate of GTP and therefore confers on the protein the structure of the active $T_\alpha \cdot$GTP state. The binding of AlF_4^- to GDP and the protein site must be tighter than that of a phosphate group, which is released from the protein after the hydrolysis of the γ P-O-P bond of GTP. This release is spontaneous, and we have checked that phosphate ions are not activators of the system, even at millimolar concentrations. The high electronegativity of F^- may account for this tight binding.

This model could also account for the effect of fluorides, or more likely, aluminofluoride complexes in other enzymatic systems involving phosphate groups, such as phosphatases.

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