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Fluoride complexes of aluminium or beryllium act on G-proteins as reversibly bound analogues of the γ phosphate of GTP

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Fluoride activation of G proteins requires the presence of aluminium or beryllium and it has been suggested that AlF_4^- acts as an analogue of the γ-phosphate of GTP in the nucleotide site. We have investigated the action of AlF_4^- or of BeF_3^- on transducin (T), the G protein of the retinal rods, either indirectly through the activation of cGMP phosphodiesterase, or more directly through their effects on the conformation of transducin itself. In the presence of AlF_4^- or BeF_3^-, purified T_α subunit of transducin activates purified cyclic GMP phosphodiesterase (PDE) in the absence of photoactivated rhodopsin. Activation is totally reversed by elution of fluoride or partially reversed by addition of excess $T_{\beta\gamma}$. Activation requires that GDP or a suitable analogue be bound to T_α: T_α-GDP and T_α-GDPαS are activable by fluorides, but not T_α-GDPβS, nor T_α that has released its nucleotide upon binding to photoexcited rhodopsin. Analysis of previous works on other G proteins and with other nucleotide analogues confirm that in all cases fluoride activation requires that a GDP unsubstituted at its β phosphate be bound in T_α. By contrast with alumino-fluoride complexes, which can adopt various coordination geometries, all beryllium fluoride complexes are tetracoordinated, with a Be–F bond length of 1.55 Å, and strictly isomorphous to a phosphate group. Our study confirms that fluoride activation of transducin results from a reversible binding of the metal–fluoride complex in the nucleotide site of T_α, next to the β phosphate of GDP, as an analogue of the γ phosphate. This model explains all the effects of fluorides on G proteins and some effects on other enzymes: ATPases, phosphatases.

Key words: transducin/cGMP-phosphodiesterase/adenylate cyclase/phosphatase/vanadate

Introduction

Fluoride anions, generally introduced as KF or NaF solutions have long been known to influence the activity of a variety of enzymatic systems: phosphatases, phosphorylases and kinases are inhibited (Hewitt and Nicholas, 1963). Rall and Sutherland (1958) first pointed out the fluoride activation of adenylate cyclase. Fluorides were later found to act on Gs, the GTP binding protein (or G-protein) that couples hormone sensitive membrane receptors to adenylate cyclase (Howlett *et al.*, 1979). Similar effects have been observed on other systems involving G-proteins: retinal cGMP phosphodiesterase with transducin (T), hormonal inhibited adenylate cyclase with Gi and hormonal stimulated phospholipases C (Sitaramayya *et al.*, 1977; Katada *et al.*, 1984; Guillon *et al.*, 1986). Fluoride activation is indeed often used as evidence for involvement of a G protein in a system. Fluorides act on the G_α subunit, which carries the guanine nucleotide site (Northup *et al.*, 1983). Sensitivity is rather low: millimolars of F^- anions are needed for activation. Non-Michaelian dose–response curves exclude the existence of a single binding site for one fluoride anion. Furthermore, sensitivity to fluoride depends, in a puzzling way, on the type of guanoside nucleotide or analogue bound: G-proteins with GDP or guanosine 5′-(α, β-imidodiphosphate) (GP-CH2-P) bound are activable by fluoride, but not G-proteins with GMP, guanosine-5′-O-(2-thiodiphosphate) (GDPβS) or guanosine-5′-O-(2-thiotriphosphate) (GTPβS) bound (Eckstein *et al.*, 1979; Downs *et al.*, 1980).

A breakthrough came a few years ago as Sternweis and Gilman (1982) demonstrated that fluoride activation of adenylate cyclase requires the presence of trace amounts of aluminium. This had long been overlooked because at the concentration used, NaF or KF solutions etch aluminium from the glassware. Micromolar amounts of aluminium are sufficient. In millimolar fluoride, this aluminium is complexed mainly as AlF_4^-, which was hence suggested to be the active species. Beryllium was the only other metal ion found effective when complexed with fluoride.

We have investigated the molecular mechanism of fluoride activation on the retinal transducin cascade, the easiest G protein system to study (Kühn, 1981; Chabre, 1985; Stryer, 1986). In a preliminary report (Bigay *et al.*, 1985), we demonstrated that alumino-fluoride ions activate stoichiometric amounts of transducin, with an affinity in the micromolar range. We observed that this activation requires the presence of GDP or a suitable analogue in the nucleotide site of T_α: the effect was suppressed when GDP had been released upon the interaction of transducin with activated rhodopsin, or when GDPβS was substituted to GDP. Noticing structural analogies between an AlF_4^- ion and a phosphate group we suggested that AlF_4^- acts as a high affinity analogue of phosphate: it would bind next to the β phosphate of GDP in the nucleotide site of T_α, and mimic a γ-phosphate, hence confering on the protein its 'active' conformation normally observed with GTP, or GTPγS bound.

We present here a complete set of evidences in favour of this model, and we analyse its specific properties related to the non-covalent binding of the metal–fluoride complex to the GDP moiety. Using purified transducin subunits and various nucleotide analogues, we demonstrate that: (i) activation of T_α-GDP by AlF_4^- does not require the presence of $T_{\beta\gamma}$, nor the catalysis by activated receptors; (ii) activation by AlF_4^- requires the presence on T_α of GDP or of a GDP analogue that is unsubstituted on its β phosphate; (iii) the binding of AlF_4^- and subsequent activation are fully reversed upon dilution of AlF_4^- and partially reversed upon addition of $T_{\beta\gamma}$; (iv) beryllium fluoride complexes, which are always tetracoordinated and strictly isomorphous to phosphate act exactly like alumino-fluoride complexes. Analysing the extensive literature on fluoride effects on other G proteins, our model provides a rationale for all previous observations, some of which had remained very puzzling until now. We also observe that vanadate, a known phosphate

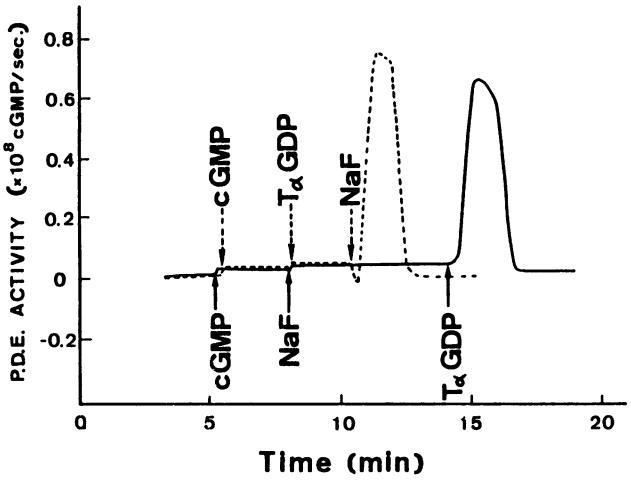

Fig. 1. Activation of retinal PDE by purified T_α-GDP and AlF_4^- in a reconstituted system (see Materials and methods). The curves are the first derivative of the pH recordings, a direct live recording of the PDE activity. The successive additions are: 0.84 μmol cGMP; 120 nmol T_α-GDP; AlF_4^- = NaF to 10 mM + $AlCl_3$ to 30 μM. Final volume 2 ml. (——) AlF_4^- added before T_α-GDP. (-------) AlF_4^- added after T_α-GDP. The final inactivation results in both cases from the exhaustion of cGMP.

analogue, acts, although less efficiently, through an analogous process.

Results

Fluoride effects are most easily observed on the activity of the effector enzymes of G-proteins: adenylate cyclase, phospholipase C or here cGMP phosphodiesterase. This is, however, an indirect, or secondary effect: the substrate of fluoride is the G protein itself: upon the action of AlF_4^-, transducin is 'activated' as if it had exchanged its GDP for GTP or GTPγS. We have two other ways to monitor this activation of transducin directly: one is to rely on the dissociation of activated T_α from $T_{\beta\gamma}$ and subsequent release of T_α from the disc membrane at physiological ionic strength (Kühn, 1981). Alternatively one can use the proteolytic sensitivity to trypsin as an assay for the active conformation of T_α: Fung and Nash (1983) have shown that in its activated form (with GPP-NH-P bound) T_α has a proteolytic sensitivity markedly different from that of T_α-GDP; the resistance of a 32 kd fragment of T_α to further proteolysis is characteristic of the activated conformation (see legend of Figure 2). Stein *et al.* (1985) had already noticed that 'fluoride' activation induced on T_α the same change in proteolytic sensitivity as that seen with nonhydrolyzable GTP analogues. We have used alternatively or concommitently these three approaches to study the action of AlF_4^-.

Purified T_α-GDP is activated by AlF_4^- in the absence of $T_{\beta\gamma}$

In our preliminary report (Bigay *et al.*, 1985), we documented the one to one stoichiometric activation of transducin by AlF_4^-, and suggested that the binding and activation site was on the T_α subunit. Most of this work had, however, been done by the solubilization assay, or the PDE assay, on ROS membranes containing the holoenzyme $T_\alpha - T_{\beta\gamma}$. Here we demonstrate that purified T_α-GDP, separated from $T_{\beta\gamma}$, is activated by AlF_4^-. First, PDE activation was measured on an artificially reconstituted system with pure T_α and pure PDE, (see Material and methods). The activity induced by AlF_4^- on this system (Figure 1) is comparable to that observed on the native membrane containing the same molar amount of PDE and of transducin holoenzyme. Then

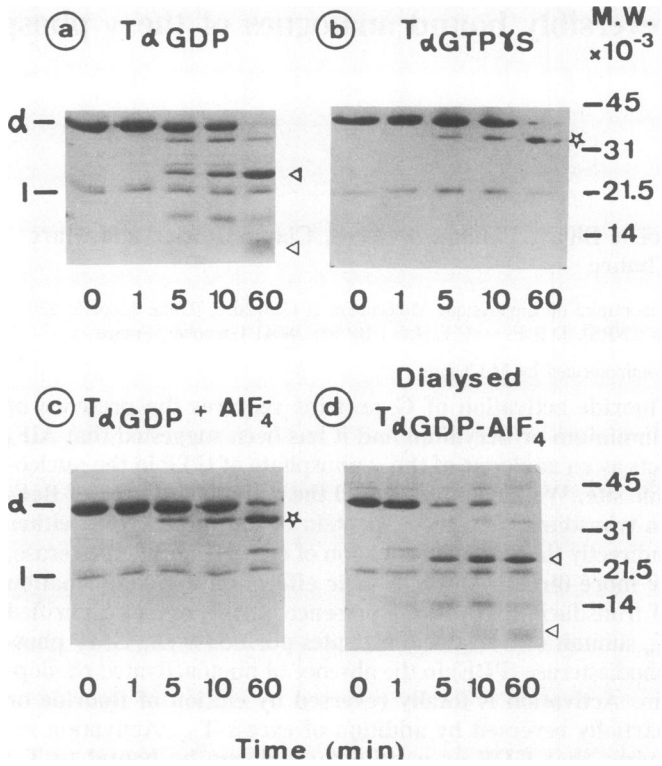

Fig. 2. Limited proteolysis studies of the conformation of T_α. (see Materials and methods, 5 μg T_α per well, 16% acrylamide SDS−PAGE.) When activated by GTPγS **(b)**, T_α is rapidly cleaved by trypsin into a 38 kd and then a 32 kd fragment (☆) that resists further proteolysis (Fung and Nash, 1983). By contrast, when T_α is in the inactive conformation T_α-GDP **(a)**, the 32 kd fragment is further cleaved in fragments of 23 kd and 12 kd (◁); **(c)** effect of AlF_4^- ($AlCl_3$ 30 μM + NaF 10 mM) on purified T_α-GDP. The pattern is nearly identical to that observed in **(b)** for T_α-GTPγS: the 23 kd fragment appears only weakly after 60 min proteolysis; **(d)** upon elution of AlF_4^-, the pattern reverses to that observed in **(a)** for inactive T_α-GDP.

proteolytic tests on purified T_α in solution demonstrated that, on addition of AlF_4^-, the proteolytic pattern of pure T_α-GDP in solution converts to that observed for T_α-GTPγS (Figure 2). This proves that the change of conformation induced by AlF_4^- occurs on purified T_α-GDP in the absence of $T_{\beta\gamma}$ and of ROS membrane with photoexcited rhodopsins. The solubilization test cannot be used on purified T_α, as it requires the use of native membranes with $T_{\beta\gamma}$.

Activation of T_α by AlF_4^- is fully reversed upon dilution of fluoride

Aliquots of a solution of AlF_4^--activated transducin were diluted in a medium containing or not the same amount of AlF_4^-, and reconcentrated (see Materials and methods). The conformation of T_α was assessed by the proteolytic test. After elution of AlF_4^-, T_α regained the proteolytic sensitivity of inactive T_α-GDP (Figure 2). The conformation of active T_α-GDP-AlF_4^- was preserved, if AlF_4^- was maintained in the dilution medium, or recovered if AlF_4^- was readded after its elution. The activation of T_α-GDP by AlF_4^- results therefore from a binding equilibrium:

$$T_\alpha\text{-GDP} + AlF_4^- \rightleftharpoons T_\alpha\text{-GDP-}AlF_4^- \qquad (1)$$

Activation of T_α-GDP by AlF_4^- is partially reversed by addition of $T_{\beta\gamma}$

The reversibility of AlF_4^- binding, combined with the binding

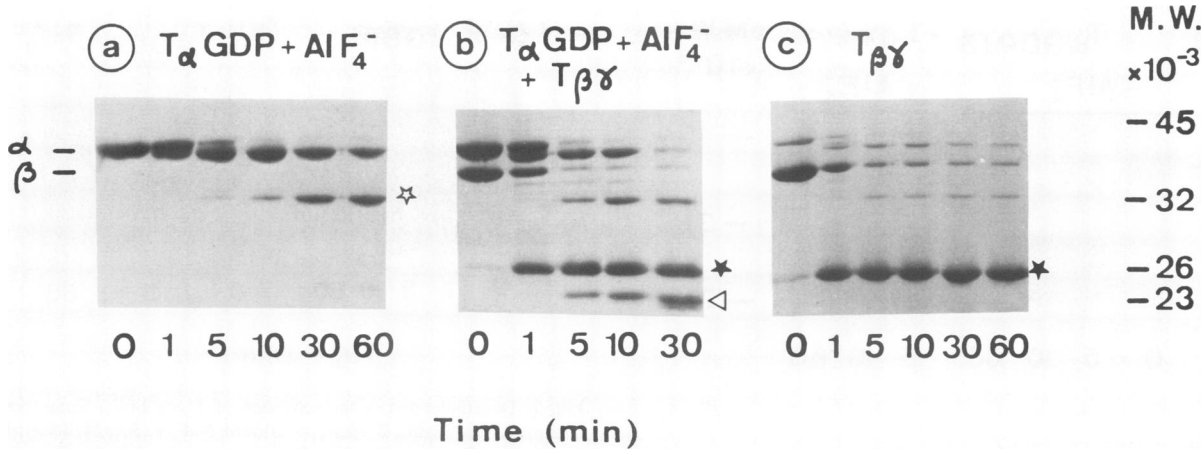

Fig. 3. Reversion, by addition of $T_{\beta\gamma}$, of the effect of AlF_4^- on T_α-GDP. Same methods and notations as in Figure 2, 5 μg T_α per well, 13% acrylamide SDS−PAGE; **(a)** control proteolysis of AlF_4^--activated T_α-GDP; **(b)** in the presence of $T_{\beta\gamma}$ (10 μg per well) aliquots of the same solution are cleaved down to the 23 kd fragment, as for inactivated T_α-GDP in Figure 1a; **(c)** control proteolysis of partially purified $T_{\beta\gamma}$ (10 μg per well).

equilibrium of T_α-GDP with $T_{\beta\gamma}$:

$$T_\alpha\text{-GDP} + T_{\beta\gamma} \rightleftharpoons T_\alpha\text{-GDP-}T_{\beta\gamma} \quad (2)$$

should result in a partial reversion by $T_{\beta\gamma}$ of the AlF_4^- induced activation of T_α:

$$T_\alpha\text{-GDP-}AlF_4^- + T_{\beta\gamma} \rightleftharpoons T_\alpha\text{-GDP-}T_{\beta\gamma} + AlF_4^- \quad (3)$$

To search for this effect, aliquots of pure AlF_4^--activated T_α-GDP were proteolyzed in the absence or presence of added $T_{\beta\gamma}$ (2 $T_{\beta\gamma}$ per T_α). As seen in Figure 3, this addition induces a significant change in the proteolytic pattern of T_α, which essentially recovers the characteristics of the inactive conformation T_α-GDP. But no quantitation of the equilibrium (3) can be derived from these data as this approach does not measure the relative amounts of T_α-GDP-$T_{\beta\gamma}$ versus T_α-GDP-AlF_4^- at equilibrium: the proteolytic attack of $T_{\beta\gamma}$ produces rapidly a stable 26 kd fragment. Fung and Nash (1983) noticed that the cleavage of $T_{\beta\gamma}$ does not reduce the ability of transducin to bind and hydrolyse GTP. As $T_{\beta\gamma}$ must associate to T_α to allow the binding of transducin to R^* and the loading of GTP, this indicates that cleaved $T_{\beta\gamma}$ keeps the capacity of binding T_α and even the 38 kd fragment of T_α. Upon release of AlF_4^- [equilibrium (1)], T_α or its 38 kd fragment reverses to the T_α-GDP conformation, that is stabilized by binding to $T_{\beta\gamma}$ or its 26 kd fragment [equilibrium (2)]. There, T_α is cleaved by trypsin down to the 23 kd fragment that dissociates from $T_{\beta\gamma}$. $T_{\beta\gamma}$ can then bind another T_α 38 kd fragment. Therefore, even if at equilibrium only a small proportion of T_α is maintained in the inactive conformation of T_α-GDP bound to $T_{\beta\gamma}$, this bound T_α will be proteolysed and the equilibrium (3) shifted towards the right. But PDE activity assays indicate that this reversion by addition of $T_{\beta\gamma}$, of AlF_4^--induced activation, is only limited: in the reconstituted system, with AlF_4^- present, when the PDE was activated submaximally by suitable amounts of purified T_αGDP, addition of an excess of $T_{\beta\gamma}$ did not induce a significant and reproducible reduction of the PDE activity. By contrast with that of T_α-GDP-AlF_4^-, the proteolytic pattern of T_α-GTPγS is not modified by addition of even a large excess of $T_{\beta\gamma}$ (not shown): no equilibrium equivalent to (1) can exist for T_α-GTPγS.

Activation by AlF_4^- requires that T_α has a GDP bound. GDPαS is a suitable analogue, but not GDPβS

Except when transducin interacts with photoexcited rhodopsin (R^*), the nucleotide bound in T_α is not exchangeable, be it

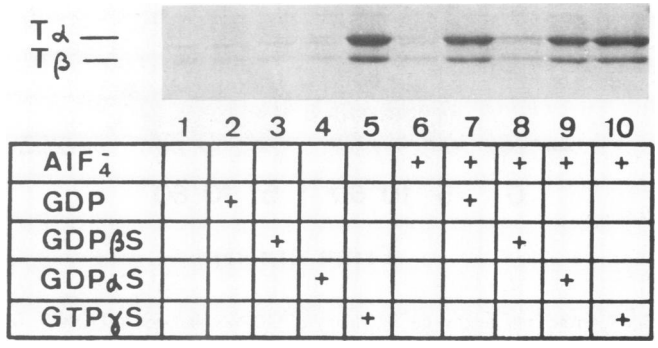

Fig. 4. Demonstration, by the T_α solubilization assay, of the requirement for GDP or GDPαS for alumino-fluoride activation of T_α. Solubilization of T_α from native ROS membranes is used as an activation test. Extractions were performed on aliquots (0.4 mg rhodopsin per sample) of a ROS membrane suspension (2.5 mg rhodopsin/ml) in medium salt buffer (see Materials and methods). Here the membrane suspension has been illuminated and then incubated under various conditions as listed in the grid below. AlF_4^-: 30 μM $AlCl_3$ + 10 mM NaF; GDP, GDPαS, GDPβS and GTPγS: 100 μM. After sedimentation of the membranes, the concentration of T_α in the supernatants are assessed by SDS−PAGE (10% acrylamide). In the presence of GTPγS the complete pool of T_α is activated and solubilized, irrespective of the presence or not of AlF_4^-; this is shown as a control (5 and 10). T_α with an empty site (1) or with GDP, GDPαS or GDPβS (2 and 4) is not activated when AlF_4^- is absent. AlF_4^- do not activate T_α when the guanoside site is empty (5) or contains GDPβS (8). Activation by AlF_4^- requires the presence in the site of GDP (7) or GDPαS (9).

GDP, GTP or one of their analogues: the site does not empty even at vanishing concentration of free nucleotides. To empty the site one must let transducin bind to R^*: the site opens and the nucleotide becomes rapidly exchangeable (Bennett and Dupont, 1985). If the total nucleotide concentration is lower than 10^{-5} M, the site remains empty. But then the binding affinity of T_α for R^* becomes very high, the R^*-T_α complex seems undissociable.

We previously noted (Bigay *et al.*, 1985) that, in the absence of added nucleotide, illumination of ROS membranes reversed the AlF_4^- induced solubilization of T_α. The interpretation was that upon illumination, T_α binds to R^*, the nucleotide site opens, GDP is released and AlF_4^- becomes ineffective, as it requires the presence of GDP in the site. Addition of 100 μM GDP restored the effect of AlF_4^-. GDPβS was not a good substitute for GDP,

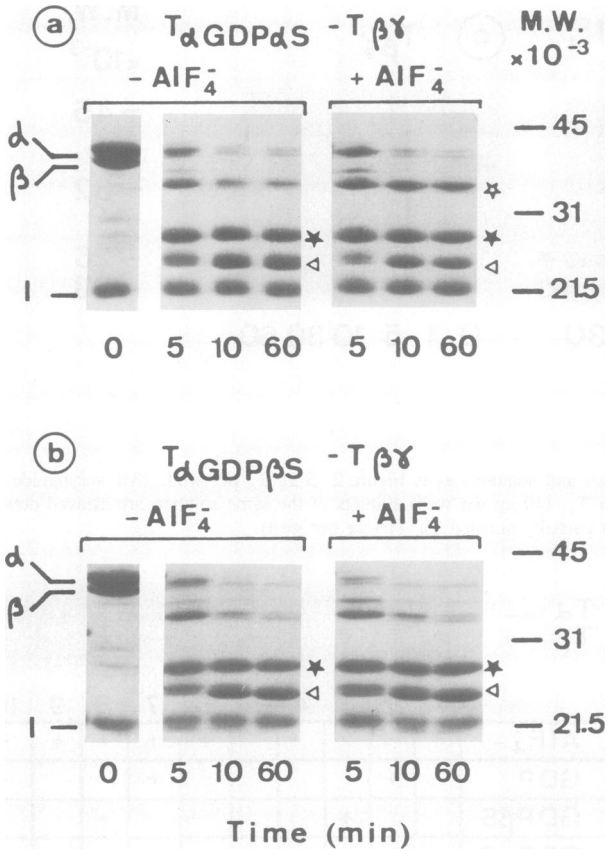

Fig. 5. Proteolytic test of the efficiency of GDPαS, but not of GDPβS, for alumino-fluoride activation of T_α. Same methods and notations as in Figure 2, 13% acrylamide SDS−PAGE, 5 μg T_α per well. The assays have been performed on the holoenzyme T_α-GDP-$T_{\beta\gamma}$. **(a)** with GDPαS, in the presence of AlF_4^- the 32 kd fragment of T_α is stabilized, but not totally, due to the presence of $T_{\beta\gamma}$, as in Figure 3. **(b)** with GDPβS, AlF_4^- has no effect, the cleavage pattern remains that of T_α-GDP.

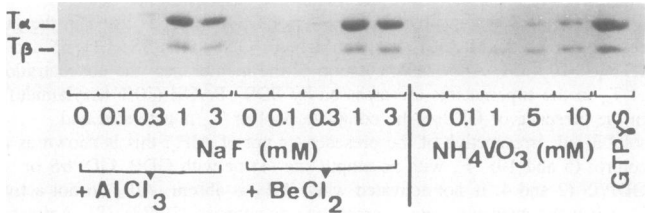

Fig. 6. Comparison of the activation of transducin by aluminium fluoride, beryllium fluoride and vanadate. Same methods and notations as in Figure 4. Aliquots of a membrane suspension (2.5 mg rhodopsin/ml) were incubated in the presence of $AlCl_3$ (30 μM) or $BeCl_2$ (30 μM) and the indicated concentrations of NaF, or in the presence of the indicated concentrations of NH_4VO_3 without NaF. A control aliquot is illuminated and incubated in the presence of 100 μM GTPγS to solubilize the complete pool of T_α. Micromolar amounts of aluminium or beryllium are both maximally active when the NaF concentration is between 1 and 3 mM. Vanadate solubilizes T_α, but only partially, even when added at 10 mM concentration.

although this analogue binds properly to the site. By contrast, GDPαS is now found to behave as a good substitute of GDP for AlF_4^- activation. This was first observed by the solubilization test: Addition of GDPαS to a suspension of illuminated ROS membranes restores the AlF_4^--induced solubilization of T_α (Figure 4), as does GDP and unlike GDPβS. T_α-GDPβS as well

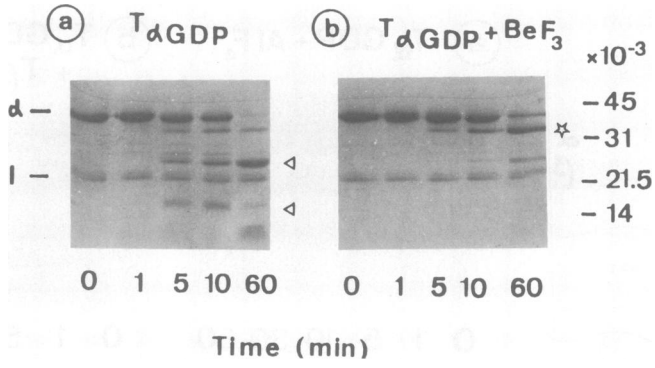

Fig. 7. Proteolytic test of the activation of T_α by BeF_3^-. Same methods and notations as in Figure 2. BeF_3^- = 10 mM NaF + 30 μM $BeCl_2$; the 32 kd fragment of T_α is stabilized by BeF_3^- as it was by AlF_4^- in Figure 2c.

as T_α-GDPαS have the proteolytic sensitivity of T_α-GDP (Figure 5). In the presence of AlF_4^- however, only T_α-GDPαS shows the proteolytic sensitivity of activated transducin, like T_α-GDP and unlike T_α-GDPβS (Figure 5). The substitution of a sulphur on the β phosphate of GDP hinders the AlF_4^- activation of T_α.

Beryllium−fluoride complexes activate T_α-GDP by a similar process and with a comparable affinity

Among many metals tested in the presence of fluoride, Sternweis and Gilman (1982) found that only beryllium could substitute for aluminium for the activation of adenylate cyclase by Gs. We first tried the solubilization assay with $BeCl_2$. Micromolar amounts of $BeCl_2$, added to the NaF solution, induced the solubilization of T_α from ROS membrane (Figure 6); the sensitivity with respect to the NaF concentration is close to that observed for aluminium. The known beryllium fluoride complexes equilibria (Mesmer and Baes, 1969; Everest, 1973) suggest then that the active species is BeF_3^-. As for AlF_4^-, the effect is reversed upon illumination in the absence of added GDP (not shown). Proteolytic studies confirm that BeF_3^- has the same conformational effect on T_α as AlF_4^- (Figure 7).

Vanadate, at millimolar concentration, also dissociates T_α from $T_{\beta\gamma}$

Vanadate acts in other enzymatic systems as an analogue of phosphate (Mansour *et al.*, 1979). Del Priore and Lewis (1985) have shown that vanadate activates the PDE in dark adapted ROS membrane, but only when added at millimolar concentration, close to the limit where it becomes inhibitory. We found that vanadate does solubilize T_α-GDP, but only partially, and also only when applied at millimolar concentration (Figure 7). Illumination reverses the effect, again suggesting that GDP is required in the site to allow activation (not shown). The activation by vanadate was not detectable, however, by the proteolytic technique. This is probably due to the limited efficiency of this analogue.

Discussion

Transducin is a member of the family of closely related G-proteins. Much work has been performed in the past on fluoride activation of the G protein system controlling adenylate cyclase activity. This will be discussed here together with our own results on transducin.

The γ phosphate-analogue model of AlF_4^- accounts for the nucleotide dependence of the fluoride effect on G-proteins

Our main argument is that GDP, or a GDP analogue that is not

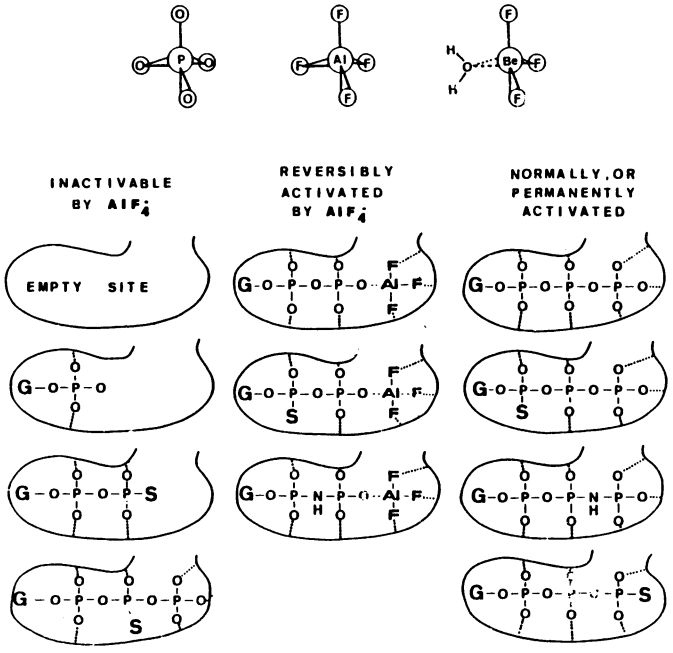

Fig. 8. Structural analogies between AlF_4^-, $BeF_3(OH_2)^-$ and a phosphate group; models for the insertion of AlF_4^- into the nucleotide site of T_α and interpretation of the effects of the various nucleotide analogues.

modified at its β phosphate, is required to be bound into the nucleotide site of G_α to allow its activation by AlF_4^-. As already stated, isolated G-proteins normally keep a GDP molecule permanently bound. It is not extractable nor exchangeable except when the G-protein is interacting with a liganded receptor (or photoexcited rhodopsin), or unless one has artificially perturbed the protein structure, for example by adding 1 M ammonium sulphate and 20% glycerol in the medium (Ferguson *et al.*, 1986).

Eckstein *et al.* (1979) first pointed out that fluoride is ineffective when GDP is replaced by GDPβS. This correlates with our result on transducin. On the other hand, Downs *et al.* (1980) observed activation by fluoride of G proteins loaded with GP-NH-P, an analogue of GDP that is unmodified at the β phosphate. We get the same result with GDPαS in transducin. It was claimed by Eckstein *et al.* (1979), that fluoride could activate G_α which had its GDP site emptied by washing the membranes with EDTA in the presence of receptors and of an agonist. The evidence that this procedure does produce empty G_α was, however, only indirect. Our experience with transducin is that under these conditions, if R^* is in excess over T, empty T_α does not dissociate from R^* or, if T is in excess over R^*, most T_α keep their GDP bound. Membrane systems used for adenylate cyclase studies have an excess of G-proteins over receptors. It is therefore unlikely that the agonist plus EDTA treatment quantitatively empties the nucleotide sites of all the G-proteins. Downs *et al.* (1980) have suggested that a small residual pool of endogenous GDP bound to various sites on the membrane, and not larger than the number of G proteins, could account for the fluoride stimulation in these experiments.

Cassel and Selinger (1978) have demonstrated that liganded receptors could also catalyse the exchange of GDP for GMP, provided the concentration of GMP was high enough to compensate for its low affinity. G_α-GMP dissociates from the receptor, but retains the capacity to exchange the weakly bound GMP for a guanoside diphosphate or triphosphate even when the receptors have been blocked by an antagonist. By contrast with GDP, GTP and their analogues, GMP is therefore not locked into its site in G_α. This capability of exchange for another nucleotide implies that GMP can be released:

$$G_\alpha\text{-GMP} + GDP \rightleftharpoons \text{empty } G_\alpha + GMP + GDP \rightleftharpoons G_\alpha\text{-GDP} + GMP$$

Preincubation of membranes with GMP in the presence of hormone, followed by a washing out of GMP in the presence of antagonist should therefore produce empty G_α. Downs *et al.* (1980) have performed many experiments based on this procedure. From their data (their Figures 3 and 5 and table III) we deduce that neither G_α GMP nor empty G_α are activable by fluoride. This correlates with our observation that AlF_4^- does not activate transducin which has been depleted of its GDP by washing in the presence of an excess of R^*.

A last case is that of GTPβS: this analogue elicits only a very weak activation which is not further increased by fluoride (Eckstein *et al.*, 1979): A γ-phosphate being already present, AlF_4^- cannot enter the site.

Figure 8 sums up all these results. The nucleotide binding site is schematized with the nucleotide part locked into the site, as it is when the G protein is not coupled to liganded receptors. The Mg^{2+} ion that interacts with the β-phosphate of the guanoside into the site (Yamanaka *et al.*, 1985) is not visualized. AlF_4^- mimics the role of the γ-phosphate only if the β-phosphate is present and is unsubstituted on its oxygens. As drawn, it is unlikely that the whole AlF_4^- complex binds with its four fluoride atoms. What the high concentration of F^- anion in the solution does, is to induce the formation of a soluble tetracoordinated state of aluminium (or beryllium), which has the same geometry, size and coordinance as a phosphate. Most probably, when entering the site, one of the fluoride atoms is released and the tetracoordinated aluminium binds to the oxygen on the β-phosphate. If the β phosphate is absent (empty site or GMP), has its oxygen substituted (GDPβS) or is already bound to a γ-phosphate (GTPβS), aluminium cannot bind, and fluorides have no effects. Recent studies using GTPβS (Yamanaka *et al.*, 1985) demonstrate that the two 'lateral' (Pro R and Pros S) oxygens of the β-phosphate are both involved in interactions with the protein or with the Mg^{2+} ion that is also bound to this site. Therefore in the case of GDPβS, the sulphur atom occupies the axial position of the oxygen normally involved in the $P_\beta-O-P_\gamma$ bond. It is very plausible that a large sulphur atom in such a position hinders the binding of the alumino-fluoride complex.

The efficiency of beryllium strengthens the hypothesis that fluorides act by forming tetrahedral metal-ion complexes

Beryllium is the only metal ion that seems able to replace aluminium. The fluoride concentration dependence of the beryllium activating efficiency suggests that the active species is BeF_3 (Mesmer and Baes, 1969). But, as drawn for BeF_3^- in Figure 8, all beryllium complexes are tetracoordinated: $BeF(OH_2)_3^+$, $BeF_2(OH_2)_2$, $BeF_3(OH_2)^-$ as well as BeF_4^{2-}. The Be−F bond length is 1.55 Å, close to the Al−F bond length and exactly equal to the P−O bond length in PO_4H_3. Unlike aluminium, beryllium does not expand its coordination number above 4. Therefore the fact that beryllium fluoride is active reinforces the conviction that aluminium also acts through its tetrahedral phosphate-like complex, AlF_4^-. Why are only aluminium and beryllium active? A scan through inorganic chemistry handbooks revealed no other ion, whose fluoride complexes have the same size, are tetrahedral and water soluble at reasonable pH.

The analogies of AlF_4^- or BeF_3^- with PO_4^{3-} have their limits, however: As required for the deactivation of G proteins, after

hydrolysis of GTP the γ-phosphate has a low affinity for GDP in the site and is released. We have already checked (Bigay *et al.*, 1985) that phosphate ions, even added at millimolar concentration, are not activators of T_α-GDP. By contrast, fluoride complexes show high affinity for binding to GDP in the site. Vanadate, a more classical phosphate analogue, has a much lower affinity, in the millimolar range, probably not much higher than that of free phosphate.

The model provides new insights into the mechanism of action of GTP in G proteins

GDP complemented by AlF_4^- may be considered as a new type of non hydrolyzable, but reversibly bound GTP analogue, different from the classical non hydrolyzable analogues GTPγS or GPP-NH-P. Pure T_α-GDP is not activated by GTPγS in the absence of R^*, but activation by AlF_4^- or BeF_3^- bypasses this requirement for a catalysis of the nucleotide exchange by the activated receptor, or, for other G proteins, the liganded hormone receptor. GTPγS cannot be extracted from T_α, nor exchanged in the absence of activated receptors, but deactivation of T_α-GDP-AlF_4^- is possible by simple elution of fluoride: this is equivalent to a controllable GTPase step. The use of AlF_4^- opens new types of approach, for the study of the mechanism of activation by G proteins of their effector enzymes. The fact that the strictly tetracoordinated beryllium fluoride also induces the activation normally obained with GTP further indicates that, in the activation process of G proteins, the γ phosphate of GTP is never involved in a pentacoordinated intermediate state.

The model accounts for effects of fluoride on other types of enzymes

Besides the case of G proteins, the model has already been suggested to apply to a phosphatase and an ATPase: Lange *et al.* (1986) have recently demonstrated the requirement for aluminium, in the fluoride inhibition of hepatic microsomal glucose-G phosphatase. Although they did not realize the actual structural analogies, independently of our work they noticed that 'AlF_4^- functions as a high affinity analogue of $H_2PO_4^-$'. Robinson *et al.* (1986) recently demonstrated the aluminium, or beryllium requirement for fluoride inhibition of Na^+/K^+ dependent ATPase, and suggested the application of our model. In most cases, when fluorides have been reported to influence the function of an enzyme, the enzyme has a nucleotide cofactor, or is involved in a phosphate group transfer reaction. The phosphate-analogue model of AlF_4^- may therefore account for all these effects.

Conclusion

The idea that many effects of fluoride on various proteins result from the formation of metal−ion complexes that mimic the ubiquitous phosphate group, is very attractive. We have provided a convincing set of evidences for this hypothesis in the case of transducin and G proteins. Direct structural data on the exact location of the complexed aluminium or beryllium atom in the proximity of the β phosphate are however still lacking: this will probably have to wait for the resolution of the much needed crystallographic structure of transducin.

Materials and methods

Materials

GTPγS, GDPβS and GDP were from Boehringer. GDPαS was a kind gift from Dr F.Eckstein. Ultrapure NaF was from Riedel de Haen. NaF solutions were made and stored exclusively in plastic containers. All reactions were performed in plastic tubes.

Membrane preparation

Retinal rod outer segment (ROS) membranes were isolated under dim red light from dark-adapted bovine retinas as described by Kühn (1981), and stored as pellets at −80°C.

Protein extraction

ROS proteins, T_α-GDP, T_α-GTPγS, $T_{\beta\gamma}$ and PDE were extracted according to their specific properties of membranes attachment, as a function of ionic strength and rhodopsin illumination (Kühn, 1981; Deterre *et al.*, 1986). Medium salt buffer: 20 mM Hepes, pH 7.5, 120 mM KCl, 1 mM DTT; low salt buffer: 5 mM Hepes, pH 7.5, 1 mM DTT.

Protein purification

Proteins were purified by anion exchange chromatography on a Pharmacia f.p.l.c. polyanion SI column, as previously published (Deterre *et al.*, 1984). Elution of PDE and T_α was performed with a 0−300 mM Na_2SO_4 gradient in 20 mM Hepes, pH 7.5, 10 mM $MgSO_4$, 5 mM β-mercaptoethanol and 0.1 mM PMSF. Elution of $T_{\beta\gamma}$ with a 0−300 mM $MgCl_2$ gradient in 20 mM Hepes, pH 7.5, 5 mM β-mercaptoethanol and 0.1 mM PMSF.

Protein concentration and titration

Proteins were concentrated with a Centricon-10 Microconcentrator (Amicon) for small volumes (0−2 ml), and with an Immersible CX-10 Ultrafiltration Unit (Millipore) for larger volumes. Protein concentrations were titrated by the Coomassie blue staining method, using bovine serum albumin as standard.

cGMP-PDE assay

PDE activity was measured by the pH-metric method (Yee and Liebman, 1978). Reconstituted membrane assay system was obtained by adding purified PDE to dark adapted ROS membrane that had been extensively washed in distilled water. Membranes (1.25 μM final rhodopsin concentration) and PDE (120 nM final concentration) were incubated in 2 ml buffer (10 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 120 mM KCl) before the additions of T_α, cGMP, and AlF_4^-.

T_α solubilization assay

Activation of T_α in native ROS membranes was monitored through its solubilization from membrane suspensions (12 μM rhodopsin) in medium salt buffer after incubations under the indicated conditions. After sedimentation of the membrane pellet, the proteins in the supernatant were analysed by SDS−PAGE, as previously described (Bigay *et al.*, 1985).

Trypsin proteolysis assay

Transducin was incubated at 20°C in low salt buffer for indicated times, with TPCK (L-1 tosilamido-2-phenylethyl chloromethyl ketone)-treated trypsin from Worthington Bio (1 μg/20 μg transducin). Digestion was stopped by the addition of soybean trypsin inhibitor (from Sigma, 5 μg/μg trypsin). Tryptic fragments were analyzed by SDS−PAGE with 13 or 16% acrylamide.

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References

Bennett,N. and Dupont,Y. (1985) *J. Biol. Chem.*, **260**, 4156−4168.
Bigay,J., Deterre,P., Pfister,C. and Chabre,M. (1985) *FEBS Lett.*, **191**, 181−185.
Cassel,D. and Selinger,Z. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4155−4159.
Chabre,M. (1985) *A. Rev. Biophys. Biophys. Chem.*, **14**, 331−360.
Del Priore,L.V. and Lewis,A. (1985) *Biochem. Biophys. Acta*, **845**, 81−85.
Deterre,P., Bigay,J., Pfister,C. and Chabre,M. (1984) *FEBS Lett.*, **178**, 228−232.
Deterre,P., Bigay,J., Robert,M., Pfister,C., Kühn,H. and Chabre,M. (1986) *Proteins*, **1**, 188−193.
Downs,R.W., Spiegel,A.M., Singer,M., Reen,S. and Aurbach,G.D. (1980) *J. Biol. Chem.*, **255**, 949−954.
Eckstein,F., Cassel,D., Levkowitz,H., Lowe,M. and Selinger,Z. (1979) *J. Biol. Chem.*, **254**, 9829−9834.
Everest,D.A. (1973) In Bailar,J.C., Emeleus,H.J., Nyholm,R. and Trotman-Dickenson,A.F. (eds), *Comprehensive Inorganic Chemistry*. Pergamon Press, Oxford, Vol. 1, pp. 538−562.
Ferguson,K.M., Higashijima,T., Smigel,M.D. and Gilman,A. (1986) *J. Biol. Chem.*, **261**, 7393−7399.
Fung,B.K.K. and Nash,C.R. (1983) *J. Biol. Chem.*, **258**, 10503−10510.
Guillon,G., Mouillac,B. and Balestre,M.N. (1986) *FEBS Lett.*, **204**, 183−189.
Hewitt,E.J. and Nicholas,D.J.D. (1963) In Hochster,R.M. and Quastel,J.H. (eds), *Metabolic Inhibitors*. Academic Press, New York, Vol. 2, pp. 311−421.
Howlett,A.C., Sternweis,P.C., Macik,B.A., Van Arsdale,P.M. and Gilman,A.G. (1979) *J. Biol. Chem.*, **254**, 2287−2295.

Katada,T., Northup,J.K., Bokoch,G.M., Ui,M. and Gilman,A.G. (1984) *J. Biol. Chem.*, **259**, 3578–3585.
Kühn,H. (1981) In Miller,W.H. (ed.), *Current Topics in Membrane and Transport.* Academic Press, New York, Vol. 15, pp. 171–201.
Lange,A.J., Arion,W.J., Burchell,A. and Burchell,B. (1986) *J. Biol. Chem.*, **261**, 101–107.
Mansour,T.E., Choate,G. and Weng,L. (1979) In Atkinson,D.E. and Fox,C.F. (eds), *Modulation of Protein Function.* Academic Press, New York, pp. 1–11.
Mesmer,R.E. and Baes,C.F.,Jr (1969) *Inorg. Chem.*, **8**, 618–626.
Northup,J.K., Smigel,M.D., Sternweis,P.C. and Gilman,A.G. (1983) *J. Biol. Chem.*, **258**, 11369–11376.
Rall,T.W. and Sutherland,E.W. (1958) *J. Biol. Chem.*, **232**, 1065–1067.
Robinson,J.D., Davis,R.L. and Steinberg,M. (1986) *J. Bioenerg. Biomembranes*, **18**, 521–531.
Sitaramayya,A., Virmaux,N. and Mandel,P. (1977) *Exp. Eye Res.*, **25**, 163–169.
Stein,P.J., Halliday,K.R. and Rasenick,M.M. (1985) *J. Biol. Chem.*, **260**, 9081–9084.
Sternweis,P.C. and Gilman,A.G. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4888–4891.
Stryer,L. (1986) *A. Rev. Neurosci.*, **9**, 87–119.
Yamanaka,G., Eckstein,F. and Stryer,L. (1985) *Biochemistry*, **24**, 8094–8101.
Yee,R. and Liebman,P.A. (1978) *J. Biol. Chem.*, **252**, 8902–8909.

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