A novel magnesium-dependent mechanism for the activation of transducin by fluoride

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Activation of transducin-GDP by NaF is mainly mediated by aluminofluoride or beryllofluoride complexes acting as GTP γ-phosphate analogs. In millimolar magnesium, NaF at concentrations above 3 mM is active even in the absence of aluminium or beryllium. This activation has a Hill coefficient of 3 with respect to F^-, and its rate is linear with respect to Mg^{2+} concentrations above 2 mM. Upon fluoride dilution, inactivation rate is hundreds of times faster than for aluminofluoride-activated TαGDP. We propose that at high NaF concentrations, 3 hydrogen-bonded fluorides in the γ-phosphate site of TαGDP entrap a magnesium counterion and this induces the transconformation to the TαGTP form.

Fluoride; Magnesium; Transducin; G-protein

1. INTRODUCTION

Fluoride activation of transducin and other G-proteins is usually due to traces of aluminium forming, in the presence of millimolar NaF or KF, complexes such as AlF_4^- and $AlF_3(OH)^-$ [1]. Beryllium, when added as $BeCl_2$, also forms complexes such as BeF_3^-. These complexes (abbreviated AlFx and BeFx) bind with micromolar affinities in the nucleotide site of the α subunit of transducin (Tα), next to the bound GDP and simulate the presence of the γ-phosphate of a GTP [2,3]. This induces, as for a GDP/GTP exchange, the dissociation of the 'activated' Tα subunit from T$\beta\gamma$ and, in media of physiological ionic strength, its subsequent solubilization from the membrane. Activation also induces a conformational change of Tα which results in a 70% increase in its intrinsic tryptophan fluorescence [4,5]. While monitoring by this technique the kinetics of transducin activation, we observed that, at concentrations above 3 mM, NaF without added $AlCl_3$ or $BeCl_2$ induced an activation that could not be due to aluminium contamination. But our media always contained 2 mM magnesium, a regular component of the cytoplasmic milieu, which is required for the functional structure of transducin [6,7]. We therefore searched for a possible implication of magnesium in the fluoride-dependent activation. Our experiments suggest that although magnesium does not form stable complexes with fluoride, it can cooperate with fluoride to

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activate transducin, albeit at a much lower affinity than Al or Be.

2. MATERIALS AND METHODS

All salts were of the highest available purity, with known aluminium contaminations in the ppm range. But aluminium contamination is not quoted on any available commercial NaF sample. Our NaF batch from Merck (Suprapur grade) was analyzed by atomic absorption spectroscopy and showed a contamination of 3×10^{-5} mole of aluminium per mole of NaF. Solutions were made and stored in plastic containers. TαGDP was solubilized from illuminated bovine rod outer segments (ROS) membranes by 200 μM GTP in KCl 120 mM, $MgCl_2$ 1 mM, Tris-HCl 20 mM, pH 7.5, separated from excess nucleotide on Sephadex G-25, and purified on a Pharmacia FPLC Polyanion-SI column by elution with a 0-660 mM Na_2SO_4 gradient in $MgSO_4$ 10 mM, β-mercaptoethanol 5 mM, PMSF 0.1 mM, Tris-HCl 20 mM, pH 7.5, as described previously [8]. For fluorescence measurements, the protein solution was diluted 40-200 times, down to 50 nM, in a 10X10 mm cuvette containing 1.6 ml standard 'TKM buffer': Tris-HCl 20 mM, pH 7.5, KCl 120 mM, $MgCl_2$ 2 mM, DTT 1 mM, and appropriate amounts of NaF, $AlCl_3$ or $BeCl_2$. Measurements were performed at 25C, with stirring, in a Shimadzu RF 5000 fluorimeter with excitation at 284 nm and emission at 340 nm (bandwidths 1.5 and 30 mm, respectively).

3. RESULTS

Upon activation of purified TαGDP subunits of transducin, large variations of tryptophan fluorescence are observed, which allow precise estimates of kinetic constants in the range from 1 to 10^{-4} s^{-1} (Fig. 1). All incubations and measurements of Fig. 1 were performed in our standard TKM buffer (containing 2 mM $MgCl_2$). Traces a and b illustrate the requirement of micromolar, beryllium or aluminium for activation of TαGDP by 1 mM NaF. In both cases the fluorescence change goes to saturation and is not increased by further addition of fluoride. The kinetics of this activation
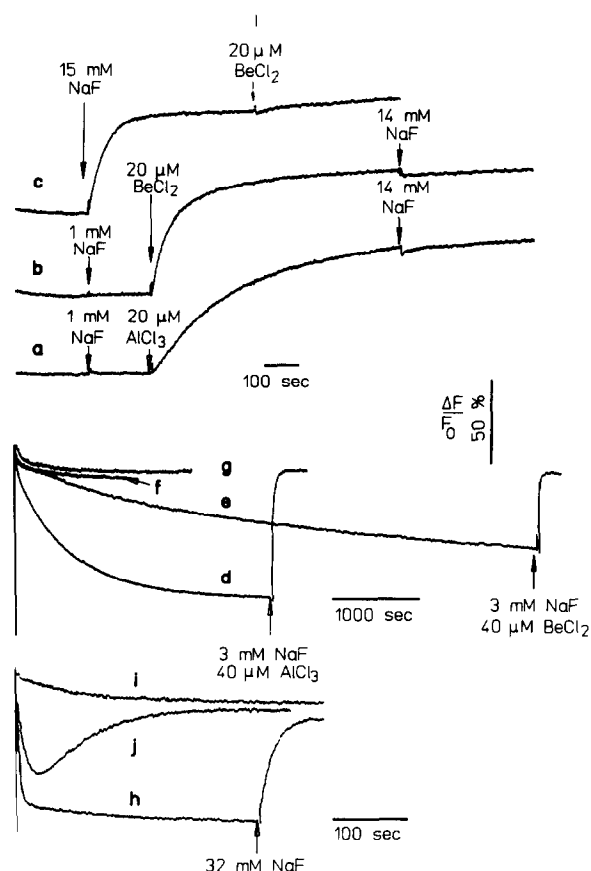
Fig. 1. Kinetics of fluorescences changes correlated with transducin activation or deactivation. (a, b, c) Activation kinetics; 50 nM solutions pf TαGDP in TKM (Tris, KCl, MgCl₂) buffer are activated by injections of NaF, AlCl₃ or BeCl₂ to the indicated concentrations. (d,e,f,g) Deactivation kinetics and controls; concentrated solutions of TαGDP that have been activated at saturation by incubating for 25 min at 25C in TKM buffer with 3 mM NaF and 40 μM AlCl₃ (d), or 3 mM NaF and 40 μM BeCl₂ (e) are diluted 200-fold (50 nM final) in TKM buffer or (f,g) diluted in their respective incubation medium for controls. (h,i) Deactivation kinetics and control for transducin activated by incubation (25 min, 25C) in TKM buffer with 32 mM NaF; (h) dilution (100-fold, 50 mM final) in TKM buffer or (i) in TKM bufer and 32 mM NaF. (j) Evolution of transducin upon its dilution (100-fold, 50 nM final) from TKM buffer with 32 mM NaF alone into TKM buffer with 1 mM NaF and 10 μM AlCl₃.

depend linearly on the concentration of Al or Be and on a complex manner on that of fluoride: this reflects the dependence on fluoride concentration of the formation of the activating complexes AlFx and BeFx [9,10]. The difference in kinetics between traces a and b, for the same addition of AlCl₃ and BeCl₂, respectively, illustrate these different dependences and possibly different affinities of AlFx and BeFx complexes for the γ-phosphate site. This will be analyzed in detail elsewhere, but we observed that a fluorescence increase of comparable kinetics could be obtained upon addition of 15 mM NaF alone; the fluorescence was not enhanced by further addition of BeCl₂ (Fig. 1c) or AlCl₃ (not shown). Aluminium contamination from all the salts present in this concentrated NaF solution was estimated

to be less than 0.47 μM. This was too low, by more than a factor of 10, to account for the observed activation kinetics. The same activation by 15 mM of NaF alone was observed after 1 mM EDTA + 1 mM MgCl₂ had been added to the bufer to chelate aluminium while keeping free magnesium constant. This strongly suggested that aluminium was not involved in this activation.

Deactivation kinetics were measured upon diluting into NaF-free TKM buffer, concentrated solutions of Tα that had previously been activated at saturation by 25 min incubation with 3 mM NaF and 40 μM AlCl₃ (d) or BeCl₂ (e), or with 32 mM NaF alone (h). For controls, dilutions were also made into the solutions used for activation. Transducin that had been activated by AlFx or BeFx became deactivated with half-times of 500 and 5000 s, respectively (f.g). Deactivation took less than 5 s, that is a 2–3 orders of magnitude shorter half-time, for transducin activated by high concentration of NaF without added AlCl₃ (h). This difference in deactivation kinetics suggests that the activated state induced by NaF without added AlCl₃ is in some ways different from that induced by AlFx or BeFx. All deactivations could be fully reversed by readdition of the original activating salts (d,e,h). In another experiment, transducin that had been activated in 32 mM NaF without added AlCl₃ was diluted 100-fold into a cuvette with TKM buffer containing 1 mM NaF and 10 μM AlCl₃. This NaF concentration would be low to induce activation without added AlCl₃ (see Fig. 1 1a and b). A biphasic trace (j) was then observed, with a fast decaying phase, as in (h), followed by a rising phase that parallels that observed when injecting inactivated TαGDP into the same medium. Thus, whatever had activated transducin in concentrated NaF was released before activation by AlFx took place. This further proves that AlFx formed from contaminant, aluminium in the concentrated NaF solution was not responsible for the previous activation.

Another test for the activation of transducin by high concentrations of NaF without added AlCl₃, was the solubilisation of Tα from ROS membrane in TKM buffer (Fig. 2a). Full solubilisation was, however, not reached even with our maximal concentration of 32 mM NaF (pF 1.5). This apparently conflicts with the saturated fluorescence changes induced by 32 mM NaF on purified TαGDP (Fig. 1h). But the solubilisation test is made on the membrane-bound holoenzyme. TαGDP-Tβγ and Tβγ probably affects the equilibrium activation of Tα by aluminium-free NaF as it affects its activation by AlFx [3]. Solubilization by NaF without added AlC3 required the presence of GDP in the nucleotide site of Tα, and was inhibited upon substitution with GDPβS (Fig. 2b). This suggests that, as for AlFx or BeFx, the activator in this case interacts with the β phosphate of GDP in the Tα nucleotide site.
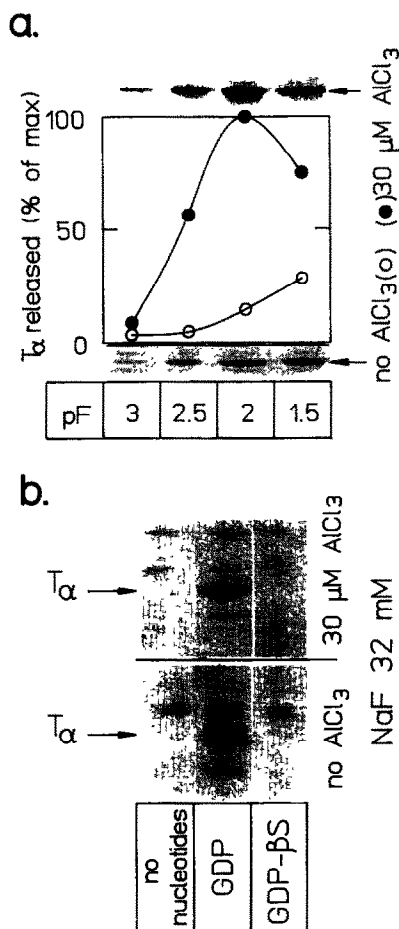
Magnesium being the only divalent metal present in

Fig. 2. Activation of transducin, monitored through the solubilization of Tα from ROS membranes. The membranes were illuminated, washed free of soluble proteins by sedimentation in 5 mM Tris HCl, pH 7.5, and resuspended in TKM buffer with 100 μM GDP and the indicated concentrations of NaF and AlCl₃ (a) or (b) resuspended in TKM buffer with 32 mM NaF alone or with 100 μM GDP or 100 μM GDPβS. The suspensions were incubated for 6 min at 20C and sedimented. The solubilized Tα was assayed in the supernatants by densitometry of Coomassie blue-stained SDS PAGE.
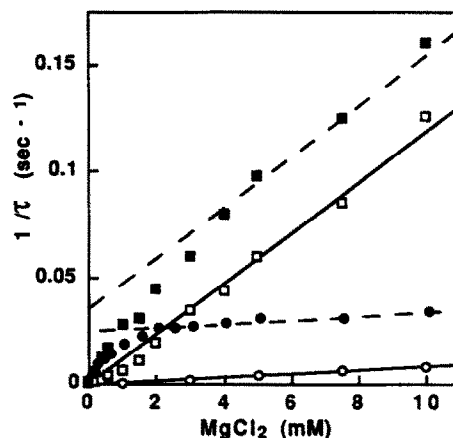

Fig. 3. Dependence on magnesium of NaF-induced activation of transducin. The activation rates are deduced from the initial slopes of the fluorescence kinetics curves observed after injection of 50 nM TαGDP in a buffer containing 120 mM KCl, 20 mM Tris-HCl, pH 7.5, the indicated amount of MgCl₂ and (¼) 3 mM NaF, (●) 3 mM NaF + 10 μM BeCl₂ (□) 15 mM NaF, (■) 15 mM NaF + 10 μM BeCl₂.

contribution of NaF in the absence of AlC₃ or BeCl₂, the activation rate due to AlFx or BeFx appears insensitive to magnesium concentrations above 2 mM.

Fluoride anions do not combine with magnesium cations to form stable complexes, as they do with aluminium or beryllium: the two ions remain dissociated in solution. The contributions of fluoride and magnesium to the activation process can therefore be analyzed separately. The activation dose-response curve with respect to fluoride, at a fixed 2 mM magnesium concentration, shows a clear cooperativity, with a Hill coefficient of 3 (Fig. 4). This suggests that at least 3 fluoride anions are implicated in the activation process.
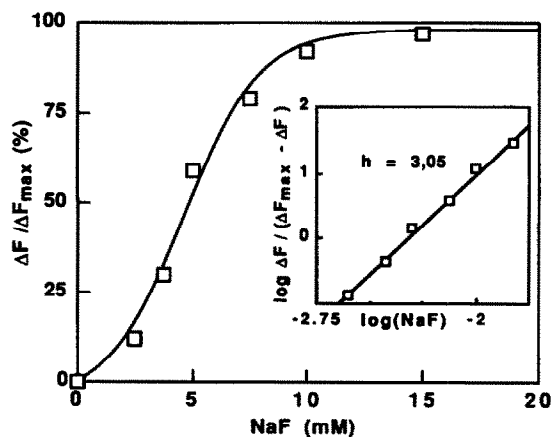
the buffer, we investigated its possible influence on the kinetics of activation by NaF (Fig. 3). Without AlCl₃ or BeCl₂ the activation kinetics varied linearly with magnesium in the range from 2 to 10 mM, at 3 mM NaF, where this activation was slow and partial, as well as at 15 mM NaF, where it was fast and saturated. This strongly suggests that a low-affinity binding of one Mg^{2+} is required for activation. Linearity is lost below 2 mM magnesium, but this is probably due to another role of magnesium which binds with better than 10^{-4} M affinity to GDP in the nucleotide site, in a fluoride-independent way, and is required for the proper functioning of the protein [6,7]. In the presence of AlCl₃ or BeCl₂, at both 3 and 15 mM NaF concentrations, the activation kinetics plots parrallel the corresponding ones without these additions. Thus, if one subtracts the

Fig. 4. Activation of transducin in TKM buffer (with 2 mM MgCl₂ by NaF without added AlCl₃ or BeCl₂. Dose-response curve with respect to fluoride concentration and corresponding Hill plot.
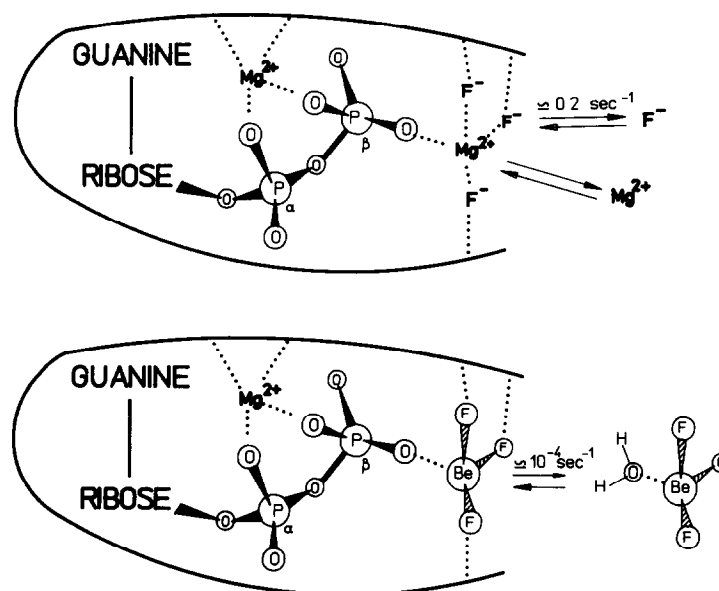
Fig. 5. Models for insertion of fluorometallic complexes in the nucleotide site of TαGDP. Preformed AlF$_x$ or BeF$_x$ tetrahedral complexes interact by a bimolecular reaction with the terminal oxygen of the GDP β-phosphate. Fluoride and magnesium ions, which do not form stable complexes in solution, can also bind separately in the site to form a γ-phosphate analogue. The dotted lines represent hydrogen bonds or weak ionic bonds.

4. DISCUSSION

That at high NaF concentrations some activation of G-proteins might not be attributable to aluminium contamination, had already been suspected by Higashijima et al. [4], who even mentioned magnesium as a possible candidate. Our kinetic data demonstrate that, at NaF concentrations above 5 mM, activation can indeed be induced by the binding of one magnesium cation and 3 fluoride anions to TαGDP. The activation process is sensitive to the presence of GDP and particularly to its terminal oxygen on the β-phosphate; it is very quickly reversed upon depletion of fluoride in the medium. All this concurs with the model shown on Fig. 4. The Mg--F ionic bonding length (1.72 A as measured in MgF$_2$ crystals) is close enough to the P–O bond length (1.56–1.6 A) to make the model sterically plausible. The steric analogy with our previous model for aluminofluoride or beryllofluoride activation is evident, but the two activation processes are dynamically very different: instead of a preformed tetrahedral complex binding with high affinity (low off-rate) in the γ-phosphate site of transducin, one assumes here the hydrogen-bonding, most probably to the donor groups that normally bind the γ-phosphate oxygens, of 3 fluoride anions which in turn entrap a magnesium counterion (Fig. 5). The affinities are low (high off-rate), both for the fluorides, as indicated by the fast in-activation rate in low fluoride, and for the magnesium (data not shown).

This new model complements our previous proposal for the activation by aluminofluoride or beryllofluoride complexes. Aluminofluoride complexes probably re-

main the predominant vehicle for NaF activation of G-proteins if no special care is taken to eliminate aluminium from solutions, when magnesium and fluoride concentrations do not exceed the millimolar range. It remains to investigate whether the validity of this fluoride + magnesium model extends to the broad range of nucleotide- or phosphate-binding proteins (ATPases, phosphatases) which are sensitive to aluminofluorides [10].

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