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EFFECTS OF FLUORIDE ON RETINAL ROD OUTER SEGMENT cGMP PHOSPHODIESTERASE AND G-PROTEIN

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SUMMARY : The effects of fluoride on ROS phosphodiesterase and G-protein have been studied using membrane-free extracts. When G-protein was present NaF, at millimolar concentrations, stimulated PDE activity however, in a G-protein free extract, cGMP hydrolysis was inhibited by high fluoride concentrations. Fluoride was also found to profoundly inhibit the ability of G-protein to bind a GTP analogue, GTPγS, both in the presence and absence of rhodopsin. Aluminium greatly modified these effects of fluoride on PDE and G-protein. The possibility that fluoride activates PDE through its effect on G-protein is discussed.

Since its effects on adenylate cyclase activity were first described, fluoride has served as a convenient activator of this enzyme in the absence of specific hormone (1). Reconstitution studies have shown that fluoride acts on the guanine-nucleotide binding regulatory component (G-protein) of adenylate cyclase (2,3). Although we have previously shown that, in an analogous system, fluoride is also capable of activating ROS[1] cGMP phosphodiesterase in the absence of bleached rhodopsin (4), there is no information on the role of ROS G-protein in this activation. Here we report that fluoride not only activates PDE but also acts on ROS G-protein : fluoride was found to inhibit formation of what is considered to be the active form of G-protein (i.e. G-protein-bound GTP). We suggest that fluoride activates PDE by preventing an inhibitory effect that inactive G-protein exerts on PDE.

MATERIALS AND METHODS

Materials - [^{3}H]cGMP, [^{14}C]guanosine and [^{35}S]GTPγS were purchased from New England Nuclear ; cGMP and snake venom (Crotalus atrox, V-700) from

[1] The abbreviations used are : ROS, rod outer segment ; PDE, 3':5'-cyclic nucleotide phosphodiesterase ; cGMP, guanosine-3':5'-cyclic monophosphate ; GTP, guanosine triphosphate ; GTPγS, guanosine-5'-[γ-thio]triphosphate ; EDTA, ethylenediaminetetraacetic acid.

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Sigma ; GTPγS from Boehringer Mannheim ; QAE Sephadex A-25 from Pharmacia ; NaF (analytical grade) from Merck ; $AlCl_3$ from EGA-Chemie ; and nitrocellulose filters (HAWP) from Millipore.

ROS preparation and extraction - Retinal ROS were prepared under dim red light from dark-adapted bovine eyes using a standard flotation technique (5) then suspended in 66 mM (i.e. isotonic) $NaPO_4$ buffer, pH 7.0 and either exposed to a 5 min bleaching (250 W light source at 30 cm) at 0°C, or kept under dim red light (darkness). After centrifugation (60,000 g ; 15 min), the ROS pellets were resuspended (2 mg rhodopsin ml^{-1}) in 10 mM Tris-HCl, pH 7.5 buffer containing 1 mM EDTA and 1 mM dithioerythritol, then centrifuged (240,000 g ; 30 min). The supernatant from illuminated ROS is hereafter referred to as "LTE" ("light tris-extract") and that from dark-kept ROS as "DTE" ("dark tris-extract"). LTE protein concentration, as determined by the Coomassie blue binding method (6) was typically 60 % lower than that of DTE, due to the absence of G-protein (7). Therefore, in order to keep the amount of PDE catalytic unit constant when measuring cGMP hydrolysis, DTE incubations contained 2.5-fold the protein content of LTE incubations.

ROS G-protein purification - ROS were bleached as described above, then extracted with 10 mM Tris-HCl, pH 7.5 buffer containing 1 mM EDTA and 1 mM dithioerythritol followed by centrifugation (240,000 g ; 30 min). The process was twice repeated, then the pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.5 containing 1 mM dithioerythritol, 0.5 mM $MgSO_4$ and 1 mM GTP and incubated (30°C, 15 min) before centrifugation (240,000 g, 30 min). The G-protein containing supernatant was concentrated, and excess GTP eliminated, by chromatography on a DE-52 column (2.5 cm x 0.5 cm diameter) as described by Baehr et al. (8).

cGMP phosphodiesterase assay - PDE activity was assayed by modifying the method already described (9). The incubation mixture (250 µl final volume) contained 50 mM Tris-HCl buffer, pH 8.0, 5.0 mM [^{3}H]cGMP (150,000 dpm), 1 mM $MgSO_4$, 0.5 mM dithioerythritol, 10 µg DTE protein or 4 µg LTE protein and NaF, $AlCl_3$ or G-protein as described. Incubations (30°C, performed in triplicate) were started (after 3 min preincubation) by substrate addition and terminated (after 3 min) by boiling. After cooling to 4°C, 50 µl of snake venom (0.5 mg ml^{-1}) were added and the mixture was incubated at 30°C for a further 20 min. After the addition of 700 µl of [^{14}C]guanosine (30 µg ml^{-1} in 50 mM NH_4-formate buffer, pH 7.5, 20 000 dpm 700 μl^{-1}) to calculate the recovery of the QAE Sephadex step, 1 ml of a rapidly stirring slurry of QAE Sephadex A-25 (50 % v|v in 50 mM NH_4-formate buffer, pH 7.5) was added. After centrifugation, a 1 ml aliquot of the supernatant was dissolved in Biofluor (NEN) emulsifier cocktail and counted for ^{3}H and ^{14}C.

GTPγS binding assay - Binding of [^{35}S]GTPγS to ROS G-protein was determined by adapting the method of Northup et al. (10) essentially as previously described (11). The incubation mixture (100 µl final volume) contained 20 mM Tris-HCl buffer, pH 8.0, 0.05 M NaCl, 4 mM $MgSO_4$, 5 µM [^{35}S]GTPγS (300,000 dpm), 8 µg of DTE protein and rhodopsin, NaF and $AlCl_3$ as described. Incubations (carried out under dim red light at 30°C) were started by GTPγS addition and terminated by the addition of 1 ml of ice-cold 20 mM Tris-HCl buffer, pH 7.5 containing 2 mM $MgSO_4$ followed by nitrocellulose filtration and washing as in (11). After drying, filters were suspended in Biofluor emulsifier cocktail and counted for radioactivity.

RESULTS

It has recently been reported (12) that Al^{3+} (or Be^{3+}) is an essential requirement for NaF stimulation of adenylate cyclase. As shown in figure 1, the addition of excess (0.1 mM) Al^{3+} significantly increased DTE PDE activation at low NaF concentrations. High NaF concentrations inhibited PDE.

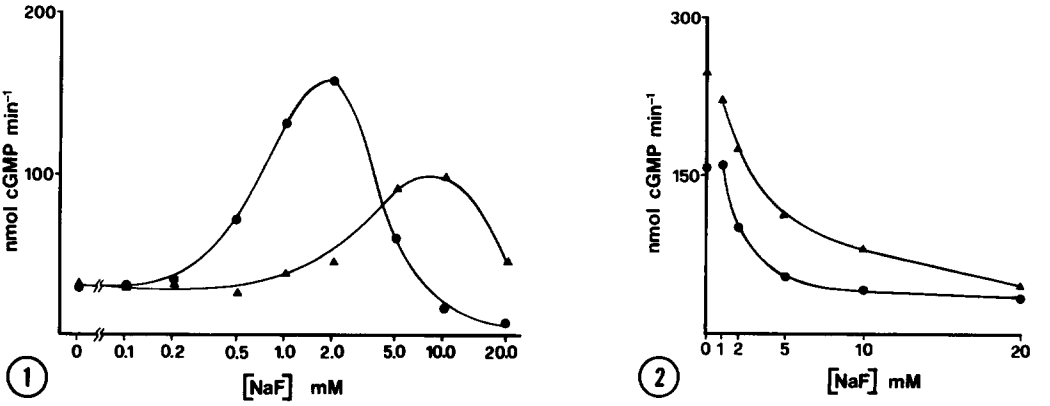


Fig. 1 - Effects of sodium fluoride concentration on DTE PDE activity (nmol cGMP hyrolysed min^{-1} 10 µg $protein^{-1}$) ; ● , 0.1 mM $AlCl_3$; ▲ , no $AlCl_3$ added.

Fig. 2 - Effects of sodium fluoride concentration on LTE PDE activity (nmol cGMP hydrolysed min^{-1} 4 µg $protein^{-1}$) ; ● , 0.1 mM $AlCl_3$; ▲ , no $AlCl_3$ added.

This appears to be a direct effect on the PDE catalytic unit since LTE (i.e. devoid of G-protein) PDE activity was found to be inhibited, more profoundly in the presence of Al^{3+}, by NaF (Fig. 2).

From figure 3, it can be seen that LTE exhibits considerably higher PDE activity than DTE. Addition of 1 mM NaF and 0.1 mM $AlCl_3$ activated DTE to

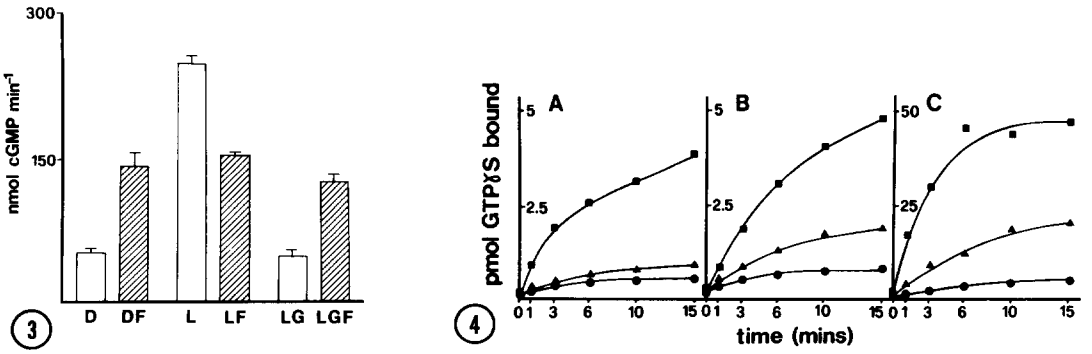


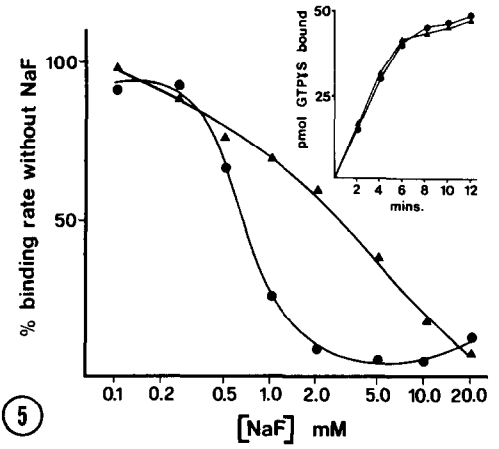
Fig. 3 - Effects of sodium fluoride on ROS PDE activity (nmol cGMP hydrolysed min^{-1}) in the presence or absence of G-protein. Incubations contained : D, 10 µg DTE protein ; DF, 10 µg DTE protein, 1 mM NaF and 0.1 mM $AlCl_3$; L, 4 µg LTE protein ; LF, 4 µg LTE protein, 1 mM NaF and 0.1 mM $AlCl_3$; LG, 4 µg LTE protein, 6 µg G-protein ; LGF, 4 µg LTE protein, 6 µg G-protein, 1 mM NaF and 0.1 mM $AlCl_3$. All incubations contained 30 mM NaCl. Results from a single, representative experiment are shown. Error bars represent SEMs of triplicate determinations.

Fig. 4 - Effects of sodium fluoride on DTE GTPγS binding : A, no rhodopsin added ; B, 0.3 µg rhodopsin added ; C, 0.3 µg bleached rhodopsin added (note different ordinate scale). ■ , no NaF/$AlCl_3$; ▲ , 10 mM NaF, no $AlCl_3$; ● , 2 mM NaF, 0.1 mM $AlCl_3$.

about 60 % of the LTE level, whereas the same additions inhibited LTE by about 35 % (i.e. to about the DTE-NaF level). Recombination of G-protein with LTE inhibited PDE activity to a level similar to that of untreated DTE. Addition of NaF and $AlCl_3$ to recombined LTE and G-protein reactivated PDE.

In figure 4, the effects of NaF on G-protein GTPγS binding are shown. In the presence of bleached rhodopsin (NaF absent) DTE maximally bound 6.3 pmol GTPγS μg^{-1} protein. Given that about 60 % of DTE protein content is G-protein, this gives a stoichiometry of about 10.5 pmol GTPγS μg^{-1} G-protein, or about 0.9 GTPγS per G-protein (assuming a m. wt. of 85 000 (13)). Both in the presence and absence of rhodopsin (bleached or not), NaF profoundly inhibited GTPγS binding. This inhibition was more pronounced in the presence of 0.1 mM Al^{3+}. As shown in figure 5, 0.1 mM Al^{3+} considerably enhanced the inhibition of GTPγS at low NaF concentrations.

NaF inhibits G-protein GTPγS binding, rather than promoting dissociation of the G-protein-bound GTPγS complex. As shown in figure 6, addition of



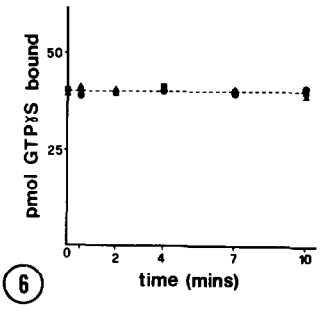


Fig. 5 - Effects of sodium fluoride concentration on DTE GTPγS binding. Incubations contained 0.1 μg bleached rhodopsin and were terminated after 3 min. Under these conditions, GTPγS binding proceeds linearly with respect to time for at least 4 min (see inset). Results are expressed as the percentage of the binding rates of non-fluoride containing blanks (~ 22 pmol GTPγS 3 min^{-1}, see inset). ● , 0.1 mM $AlCl_3$; ▲ , no $AlCl_3$.

Fig. 6 - Lack of effect of sodium fluoride on G-protein-bound GTPγS. Samples were preincubated for 3 min in the presence of 1 μg bleached rhodopsin then adjusted (after the 0 min sample was taken) to contain : ■ , no NaF/$AlCl_3$; ▲ ,10 mM NaF ; ● , 2 mM NaF, 0.1 mM $AlCl_3$.

fluoride to G-protein, after preincubation with GTPγS and bleached rhodopsin, did not decrease the level of bound GTPγS.

DISCUSSION

In this communication, we have shown that fluoride profoundly affects both ROS PDE and ROS G-protein. The stimulation of PDE activity at low NaF concentrations appears to be dependent on the presence of G-protein since no NaF-dependent stimulation of cGMP hydrolysis was observed with LTE (devoid of G-protein). In fact, high NaF concentrations were found to inhibit the PDE catalytic unit (Fig. 2). Furthermore, from figure 3, it seems that this activation consists of preventing an inhibitory effect that G-protein has on PDE. This speculation is supported by our observation that fluoride directly affects G-protein : both in the absence and in the presence of rhodopsin (bleached or not), fluoride inhibits G-protein GTPγS binding, i.e. it inhibits the formation of what is considered to be the G-protein form that activates PDE in the classical GTP-dependent light-stimulation of the enzyme. In the presence of excess Al^{3+}, the concentration of NaF required to give 50 % maximal PDE stimulation (about 0.7 mM) is very similar to that required to give 50 % GTPγS binding inhibition, therefore suggesting that the two events, PDE activation and G-protein inhibition by NaF, are related. Thus, in our experiments, the effect of NaF on G-protein appears to be obligatory for NaF activation (deinhibition) of PDE.

PDE inhibition by G-protein has already been reported. Baehr et al. (14) found that recombination of G-protein which they termed, at that time, 80 K protein : PDE in a 1-1 molar ratio gave 50 % inhibition of cGMP hydrolysis, whereas a 10:1 G-protein:PDE molar ratio only gave 85 % inhibition. Since, in DTE, G-protein was typically present in a greater than 7-fold molar excess over PDE, it is likely that G-protein is exerting a significant inhibitory effect on PDE, therefore NaF inhibition of G-protein would activate PDE. It would be interesting to know whether or not this inhibitory effect of G-protein has a physiological function. It is possible that a greater

understanding of the mechanism by which fluoride activates ROS PDE will lead to a greater understanding of the regulation of this enzyme **in vivo.**

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