Biochem. J. (1988) 253, 827–833 (Printed in Great Britain)

AlF_4^- reversibly inhibits 'P'-type cation-transport ATPases, possibly by interacting with the phosphate-binding site of the ATPase

Ludwig MISSIAEN,*†‡ Frank WUYTACK,* Humbert DE SMEDT,* Matty VROLIX* and Rik CASTEELS*

* Department of Physiology and the † Department of Obstetrics and Gynecology, Catholic University of Leuven, B-3000 Leuven, Belgium

The only known cellular action of AlF_4^- is to stimulate the G-proteins. The aim of the present work is to demonstrate that AlF_4^- also inhibits 'P'-type cation-transport ATPases. NaF plus $AlCl_3$ completely and reversibly inhibits the activity of the purified $(Na^+ + K^+)$-ATPase (Na^+- and K^+-activated ATPase) and of the purified plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase (Ca^{2+}-stimulated and Mg^{2+}-dependent ATPase). It partially inhibits the activity of the sarcoplasmic-reticulum $(Ca^{2+} + Mg^{2+})$-ATPase, whereas it does not affect the mitochondrial H^+-transporting ATPase. The inhibitory substances are neither F^- nor Al^{3+} but rather fluoroaluminate complexes. Because AlF_4^- still inhibits the ATPase in the presence of guanosine 5′-[β-thio]diphosphate, and because guanosine 5′-[βγ-imido]triphosphate does not inhibit the ATPase, it is unlikely that the inhibition could be due to the activation of an unknown G-protein. The time course of inhibition and the concentrations of NaF and $AlCl_3$ required for this inhibition differ for the different ATPases. AlF_4^- inhibits the $(Na^+ + K^+)$-ATPase and the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase non-competitively with respect to ATP and to their respective cationic substrates, Na^+ and Ca^{2+}. AlF_4^- probably binds to the phosphate-binding site of the ATPase, as the K_i for inhibition of the $(Na^+ + K^+)$-ATPase and of the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase is shifted in the presence of respectively 5 and 50 mM-P_i to higher concentrations of NaF. Moreover, AlF_4^- inhibits the K^+-activated *p*-nitrophenylphosphatase of the $(Na^+ + K^+)$-ATPase competitively with respect to *p*-nitrophenyl phosphate. This AlF_4^--induced inhibition of 'P'-type cation-transport ATPases warns us against explaining all the effects of AlF_4^- on intact cells by an activation of G-proteins.

INTRODUCTION

NaF, in the presence of $AlCl_3$, is known to be a potent modulator of G_s, G_i and transducin [1–7]. The active compound of this combination is thought to be AlF_4^- [1,3,7,8]. The observed effects of AlF_4^- on microsomes (microsomal fractions) [9–12] or on intact cells [13–16] are therefore ascribed to a stimulation of G-proteins. Because the structure of AlF_4^- is very similar to that of a phosphate group, PO_4^{3-} [17,18], we examined whether ATPases of the 'P'-class, which form a covalent phosphorylated intermediate [19], are affected by AlF_4^-. We therefore studied the effect of AlF_4^- on the $(Na^+ + K^+)$-ATPase and on the $(Ca^{2+} + Mg^{2+})$-ATPases of the plasma membrane and of the sarcoplasmic reticulum. We further investigated whether AlF_4^- also affects 'F'-type ATPases [19], by studying its effect on the mitochondrial H^+-ATPase. It could be shown that AlF_4^- reversibly and non-competitively inhibits 'P'-type cation-transport ATPases, possibly by interacting with the phosphate-binding site of the ATPase.

EXPERIMENTAL

Membrane preparation and purification of the ATPase

The technique for purifying the $(Na^+ + K^+)$-ATPase from pig kidney, for isolating the plasmalemmal $(Ca^2 + Mg^{2+})$-ATPase from stomach smooth muscle and for obtaining a sarcoplasmic-reticulum fraction from rabbit skeletal pectoralis major muscles, have all been described [20–22]. Rat liver mitochondria were prepared from fresh rat liver homogenized in 5 vol. of an ice-cold medium containing 100 mM-KCl, 5 mM-$MgCl_2$ and 50 mM-Tris/HCl, pH 7.2. The material was gently minced with a pair of scissors, then homogenized in a Potter homogenizer and centrifuged in a Sorvall SS34 rotor for 10 min at 1000 rev/min ($r_{av.}$ 8.26 cm). The supernatant was further centrifuged in a SS34 rotor for 15 min at 3000 rev./min ($r_{av.}$ 8.26 cm) and the pellet resuspended in 0.25 M-sucrose.

Preincubation of the ATPase

The ATPase was preincubated for a 10 min period (unless otherwise indicated) at 37 °C in a medium containing 0.25 M-sucrose and 30 mM-imidazole, pH 6.8, and supplemented with NaF plus $AlCl_3$. The enzyme concentration in this preincubation medium was 10 μg/ml for the purified $(Na^+ + K^+)$-ATPase and 40 μg/ml for the purified plasmalemmal $(Ca^2 + Mg^{2+})$-ATPase. The protein concentration of the sarcoplasmic-reticulum fraction and of the mitochondrial fraction in the preincubation medium was respectively 10 and 5 mg/ml. As a control, the ATPase was also preincubated for the same period of time at 37 °C in a medium containing

Abbreviations used: $(Ca^{2+} + Mg^2)$-ATPase, Ca^{2+}-stimulated and Mg^{2+}-dependent adenosine triphosphatase; $(Na^+ + K^+)$-ATPase, Na^+- and K^+-activated adenosine triphosphatase; mitochondrial H^+-ATPase, mitochondrial H^+-transport adenosine triphosphatase; GDP[S], guanosine 5′-[β-thio]diphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; pNPP, *p*-nitrophenyl phosphate; G_s and G_i, guanine nucleotide-dependent stimulatory and inhibitory proteins.

‡ To whom correspondence and reprint requests should be sent, at the following address: K.U. Leuven, Laboratory of Physiology, Herestraat 49, B-3000 Leuven, Belgium.

only 0.25 M-sucrose and 30 mM-imidazole. After this preincubation, 10 μl of the ATPase was transferred to 990 μl of assay medium.

Enzyme assays

The ATPase activities were measured at 37 °C with an NADH-coupled enzyme assay [23] in a medium that, unless otherwise indicated, had the following composition: 100 mM-KCl, 30 mM-imidazole/HCl, pH 6.8, 5.7 mM-$MgCl_2$, 5 mM-ATP, 1 mM-EGTA, 1.5 mM-phosphoenolpyruvate, 0.26 mM-NADH, 5 mM-NaN_3, 0.1 mM-ouabain, pyruvate kinase (40 units/ml) and lactate dehydrogenase (36 units/ml). The $(Ca^{2+}+Mg^{2+})$-ATPase activity was measured by comparing the rate of ATP hydrolysis in this assay medium and that obtained after addition of 0.87 mM-$CaCl_2$, corresponding to a free Ca^{2+} concentration of 10 μM. For measuring the maximum activity of the plasmalemmal $(Ca^{2+}+Mg^{2+})$-ATPase, calmodulin was added at a saturating concentration of 0.6 μM. The $(Na^{+}+K^{+})$-ATPase activity was calculated from the inhibition of the rate of ATP hydrolysis by 0.1 mM-ouabain in the assay medium described above, except that the Na^{+} concentration was increased to 91 mM and the K^{+}-concentration decreased to 11 mM. Mitochondrial H^{+}-ATPase activity was measured from the inhibition of the rate of ATP hydrolysis by 5 mM-NaN_3, in the assay medium described above, but supplemented with 10 μM-dinitrophenol. All enzyme activities were measured for a 2 min period and were found to be linear with time and proportional to the protein concentration in that interval. Proteins were measured by the method of Lowry *et al.* [24], with serum albumin as a standard.

p-Nitrophenylphosphatase was measured spectrophotometrically at 420 nm by determining the amount of *p*-nitrophenol released/min at 37 °C. The K^{+}-activated *p*-nitrophenylphosphatase activity of the $(Na^{+}+K^{+})$-ATPase [25] was measured in an assay medium containing 50 mM-Tris/HCl, pH 7.4, 3 mM-$MgCl_2$ and the indicated concentration of pNPP, with or without 10 mM-KCl. The ATP-dependent Ca^{2+}-activated *p*-nitrophenylphosphatase of the plasmalemmal $(Ca^{2+}+Mg^{2+})$-ATPase [26] was measured in a medium containing 10 mM-$MgCl_2$, 0.1 mM-$[Ca^{2+}]$, 0.5 mM-EGTA, 150 mM-Tris/HCl, pH 7.4, 50 mM-KCl, and the indicated concentration of pNPP, with or without 0.6 mM-ATP.

Materials

Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, p[NH]ppG, GDP[S], ATP and pNPP were obtained from Boehringer, Mannheim, Germany; NADH was from Sigma Chemical Co., St. Louis, MO, U.S.A.; deferoxamine was from Ciba–Geigy, Basel, Switzerland; ouabain, NaF, BeF_2, $AlCl_3$ and dinitrophenol from Merck, Darmstadt, Germany. Calmodulin was prepared from bovine brain as described by Gopalakrishna & Anderson [27].

RESULTS

The inhibitory effect of NaF plus $AlCl_3$ on the various 'P'-type cation-transport ATPases, and the absence of any effect on a 'F'-type ATPase are illustrated in Fig. 1. A preincubation of the purified $(Na^{+}+K^{+})$-ATPase and of the purified plasmalemmal $(Ca^{2+}+Mg^{2+})$-ATPase for 10 min at 37 °C in a solution containing 0.25 M-sucrose and 30 mM-imidazole and supplemented with 10 mM-NaF and 10 μM-$AlCl_3$ results in a complete inhibition of these enzymes (Fig. 1, columns a and b). As, under our experimental conditions, 10 μl of ATPase was transferred to 990 μl of assay medium, the concentrations of F^{-} and Al^{3+} in the assay medium are expected to be 100 times lower than in the preincubation medium. The complete inhibition which occurred during the preincubation period is still maintained during the ATPase assay, because the enzyme remains completely inhibited for 25 min after adding the ATPase to the assay medium. This finding suggests that the binding of the inhibitory substance to the ATPase must be stable under our assay conditions. A similar preincubation for 10 min of a sarcoplasmic-reticulum fraction from rabbit pectoralis major skeletal muscle inhibits the $(Ca^{2+}+Mg^{2+})$-ATPase of the sarcoplasmic reticulum only by 36 ± 3 % ($n = 4$) (Fig. 1, column c). As the preincubation medium was not supplemented with K^{+} or Mg^{2+} ions, it can be concluded that millimolar concentrations of K^{+} or Mg^{2+} are not necessary for these inhibitions. These findings do not, however, exclude that contaminant trace amounts of some bivalent metal ions might be a cofactor in the reaction. The F_0F_1-type ATPase of rat liver mitochondria is not modified at all by a preincubation with 10 mM-NaF and 10 μM-$AlCl_3$ (Fig. 1, column d). This finding also allowed us to exclude any aspecific effect of these ions on the ATPase assay.

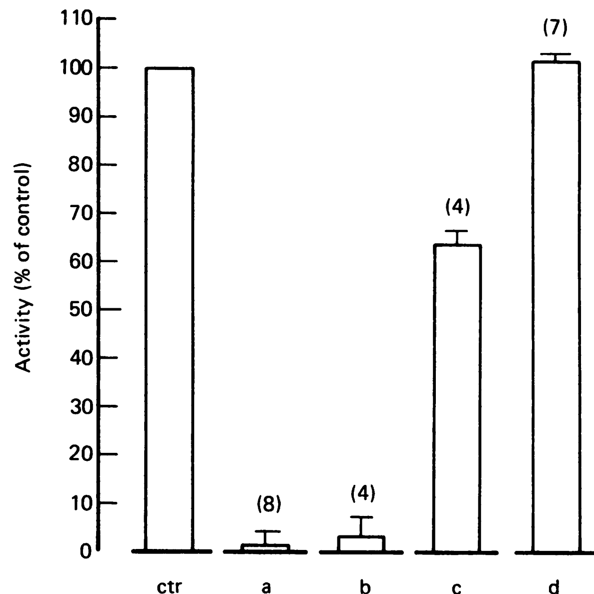

Fig. 1. Effect of NaF and $AlCl_3$ on the different ATPases

The purified $(Na^{+}+K^{+})$-ATPase (*a*), the purified plasmalemmal $(Ca^{2+}+Mg^{2+})$-ATPase (*b*), the sarcoplasmic reticulum (*c*) and the mitochondria (*d*) were preincubated for 10 min at 37 °C in a medium containing 0.25 M-sucrose, 30 mM-imidazole, 10 mM-NaF and 10 μM-$AlCl_3$. The ATPase activities [$(Na^{+}+K^{+})$-ATPase in column a, $(Ca^{2+}+Mg^{2+})$-ATPase in columns b and c and H^{+}-ATPase in column d] were measured as described in the Experimental section and are expressed as a percentage of the activities obtained after preincubating in the same medium without NaF and $AlCl_3$ (control, ctr). The results are presented as means ± S.E.M., the numbers of observations being given in parentheses.

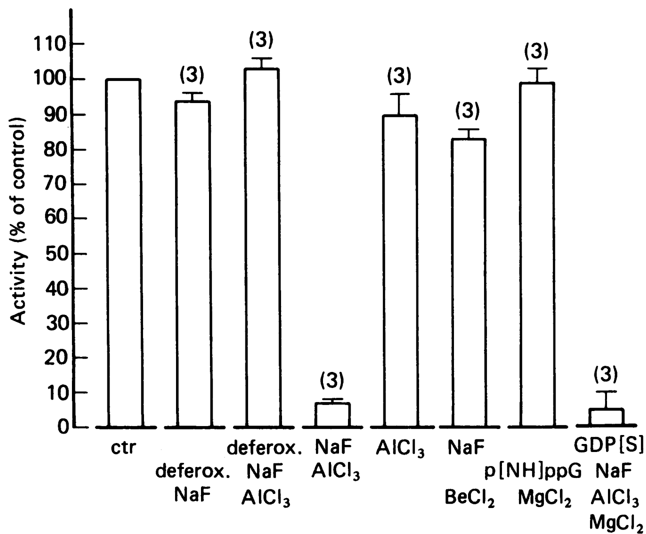

Fig. 2. Effect of NaF, $AlCl_3$, $BeCl_2$, deferoxamine, p[NH]ppG plus $MgCl_2$ and GDP[S] plus $MgCl_2$ on the $(Na^+ + K^+)$-ATPase

The purified $(Na^+ + K^+)$-ATPase was preincubated for 10 min at 37 °C in a medium containing 0.25 M-sucrose and 30 mM-imidazole, and supplemented with the compounds listed beneath the columns. In the last experiment, the $(Na^+ + K^+)$-ATPase was first exposed for 10 min to GDP[S] plus $MgCl_2$, before adding it to the preincubation medium containing NaF plus $AlCl_3$. The following concentrations were used: NaF: 1 mM, $AlCl_3$: 10 μM, $BeCl_2$: 10 μM, deferoxamine (deferox.): 0.5 mM, p[NH]ppG: 100 μM in 1 mM-$MgCl_2$ and GDP[S]: 100 μM in 1 mM-$MgCl_2$. The ATPase activities remaining after this preincubation are expressed as a percentage of the activity, obtained after preincubation without these compounds (control, ctr). The results are expressed as means ± S.E.M. for the numbers of observations given in parentheses.

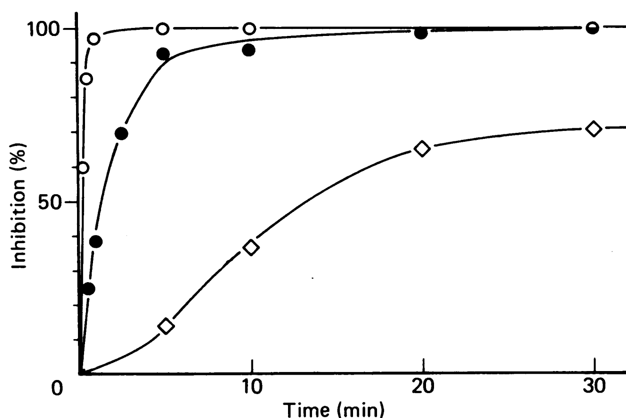

Fig. 3. Time course of inhibition of the different ATPases by NaF plus $AlCl_3$

The percentage inhibition by 1 mM-NaF and 10 μM-$AlCl_3$ of the $(Na^+ + K^+)$-ATPase (○), the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase (●) and the sarcoplasmic-reticulum $(Ca^{2+} + Mg^{2+})$-ATPase (◇) after preincubation of the ATPase for a variable period of time at 37 °C in a medium containing 0.25 M-sucrose and 30 mM-imidazole is represented. The horizontal axis represents the preincubation time (in min) and the vertical axis the percentage inhibition.

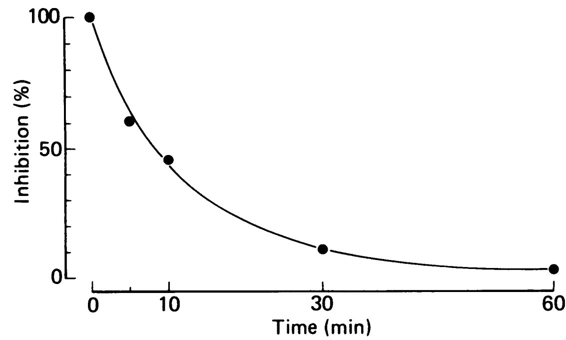

Fig. 4. Reversion of the NaF plus $AlCl_3$-induced inhibition of the $(Na^+ + K^+)$-ATPase by deferoxamine

The purified $(Na^+ + K^+)$-ATPase was first preincubated for 10 min at 37 °C in a medium containing 0.25 M-sucrose, 30 mM-imidazole, 1 mM-NaF and 10 μM-$AlCl_3$. This procedure completely inhibited the enzyme. After this 10 min preincubation, 0.5 mM-deferoxamine was added to the preincubation medium, and the ATPase was subsequently preincubated for various periods of time in the medium containing sucrose, imidazole, NaF, $AlCl_3$ and deferoxamine. The horizontal axis represents the time (in min) of preincubation; time '0' is the moment of adding deferoxamine. The vertical axis represents the percentage inhibition, normalized to the inhibition of the ATPase, observed immediately before adding deferoxamine.

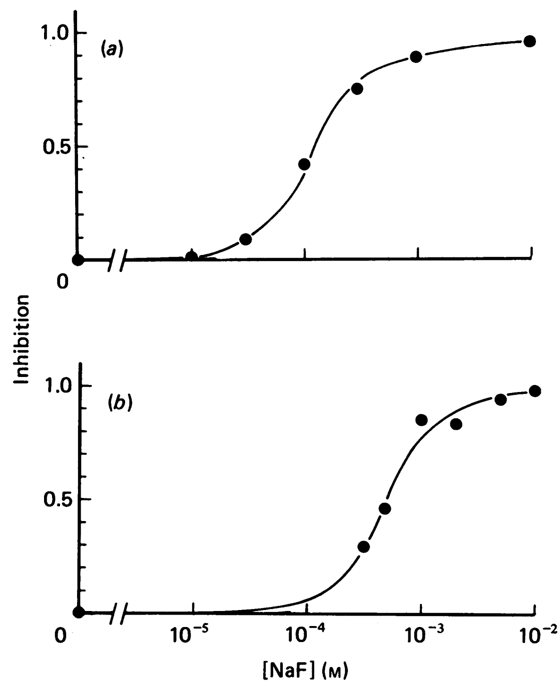

Fig. 5. Inhibition of the $(Na^+ + K^+)$-ATPase activity and the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase activity by NaF in the presence of a constant $AlCl_3$ concentration

The purified $(Na^+ + K^+)$-ATPase (*a*) and the purified plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase (*b*) were preincubated with the different concentrations of NaF for 10 min at 37 °C in a medium containing 0.25 M-sucrose and 30 mM-imidazole, supplemented with 10 μM-$AlCl_3$. The curves represent the inhibition normalized to that observed at 10 mM-NaF.

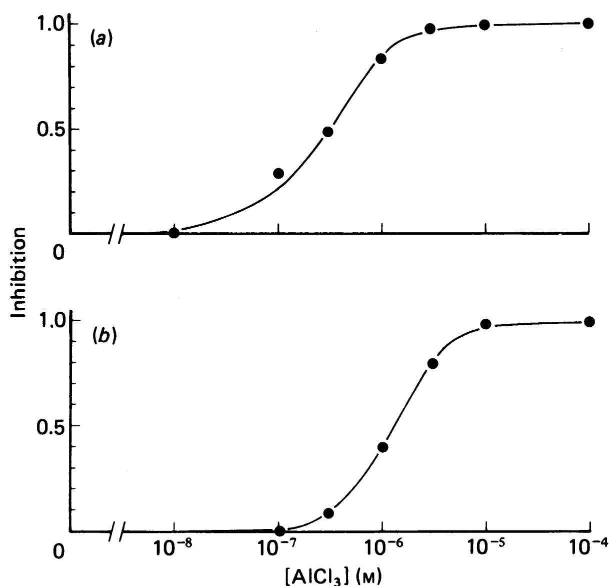

Fig. 6. Inhibition of the $(Na^+ + K^+)$-ATPase activity and the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase activity by $AlCl_3$ in the presence of a constant NaF concentration

The experiments were performed as described in the legend of Fig. 5, with the indicated concentrations of $AlCl_3$. For the $(Na^+ + K^+)$-ATPase (*a*) the NaF-concentration was 0.3 mM, and for the $(Ca^{2+} + Mg^{2+})$-ATPase (*b*) the NaF concentration was 1 mM.

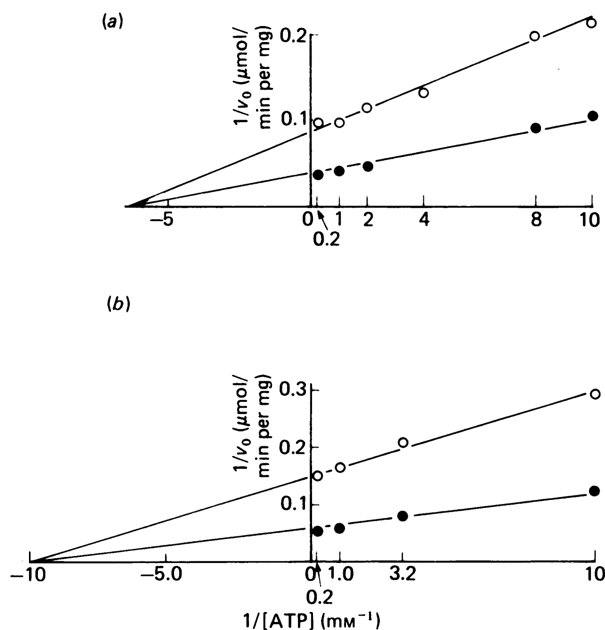

Fig. 7. Lineweaver–Burk analysis of the NaF plus $AlCl_3$-induced inhibition of the $(Na^+ + K^+)$-ATPase and the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase with respect to ATP

The $(Na^+ + K^+)$-ATPase (*a*) was preincubated for 10 min at 37 °C in a medium with 30 mM-imidazole and 0.25 M-sucrose and without (●) or with 10 µM-$AlCl_3$ plus 0.2 mM-NaF (○). The plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase (*b*) was similarly preincubated without (●) or with 10 µM-$AlCl_3$ plus 1 mM-NaF (○). The ATPase activities were measured in media with different concentrations of ATP. The results are represented as Lineweaver–Burk plots.

Fluoroaluminate complexes rather than F^- or Al^{3+} alone are involved in these inhibitions, as is illustrated in Fig. 2. The addition of 0.5 mM-deferoxamine, a chelator of aluminum [14], during the preincubation, completely prevents the inhibition of the $(Na^+ + K^+)$-ATPase by NaF plus $AlCl_3$. This finding indicates that NaF alone does not inhibit the ATPase. According to published values for stability constants [28], several fluoroaluminate complexes are formed under our working conditions (10 mM-NaF and 10 µM-$AlCl_3$). AlF_4^- is the most stable species, and the free Al^{3+} concentration is as low as 10 pM. Even at 10 µM-$AlCl_3$, the ATPase is not significantly affected by $AlCl_3$ in the absence of NaF. Although $BeCl_2$ promotes activation of G-proteins by NaF [1], it is not a good substitute for $AlCl_3$ for the inhibition of the ATPase. $BeCl_2$ plus NaF inhibits the $(Na^+ + K^+)$-ATPase only by 17 ± 3% ($n = 3$). A preincubation of the $(Na^+ + K^+)$-ATPase with 100 µM-p[NH]ppG in the presence of 1 mM-$MgCl_2$ does not inhibit its activity, although p[NH]ppG is known to activate the G-proteins irreversibly. If the $(Na^+ + K^+)$-ATPase is exposed to 100 µM-GDP[S] in the presence of 1 mM-$MgCl_2$, AlF_4^- still completely inhibits the ATPase, although it has been described that GDP[S] prevents the stimulation of G-proteins by AlF_4^- [8].

The time course of the inhibitory effect of 1 mM-NaF plus 10 µM-$AlCl_3$ on the three 'P'-type cation-transport ATPases is illustrated in Fig. 3. The $(Na^+ + K^+)$-ATPase was completely inhibited within 2 min, the inhibition of the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase occurring at a lower rate. The inhibition of the sarcoplasmic-reticulum $(Ca^{2+} + Mg^{2+})$-ATPase proceeded at the lowest rate, and even after 30 min its inhibition had not yet reached a steady-state level.

The inhibition of the $(Na^+ + K^+)$-ATPase induced by a preincubation for 10 min with 1 mM-NaF plus 10 µM-$AlCl_3$ was reversed by the subsequent addition of 0.5 mM-deferoxamine (Fig. 4). The $(Na^+ + K^+)$-ATPase was first preincubated for 10 min at 37 °C in a solution containing 0.25 M-sucrose, 30 mM-imidazole, 1 mM-NaF and 10 µM-$AlCl_3$. After 10 min, the enzyme was completely inhibited. The subsequent addition of 0.5 mM-deferoxamine to the same preincubation medium slowly reversed this inhibition. This recovery of enzyme activity proceeded more slowly than the onset of the inhibition. The reversal of the inhibition was much slower for the purified plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase than for the $(Na^+ + K^+)$-ATPase. We have not performed kinetic measurements on this $(Ca^{2+} + Mg^{2+})$-ATPase, because a preincubation of several hours would be required, during which some loss of enzyme activity would occur.

The concentration-dependency of the inhibition of the $(Na^+ + K^+)$-ATPase and of the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase by NaF and $AlCl_3$ is illustrated in Figs. 5 and 6. We have not performed these experiments on the $(Ca^{2+} + Mg^{2+})$-ATPase of the sarcoplasmic reticulum, because NaF plus $AlCl_3$ does not induce a steady-state inactivation of this enzyme after 10 min preincubation. In the presence of 10 µM-$AlCl_3$, NaF inhibits the $(Na^+ + K^+)$-ATPase and the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase in a concentration-dependent manner (Fig. 5), with a K_i for the $(Na^+ + K^+)$-ATPase of 1.3×10^{-4} M and for the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase of 5.0×10^{-4} M. Fig. 6 illustrates the concentration of $AlCl_3$ required for this inhibition at a constant NaF concentration of 0.3 mM for the

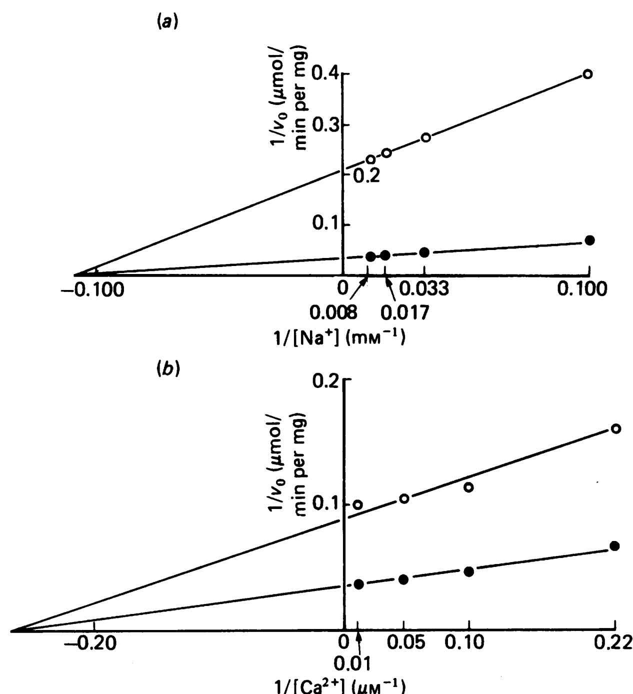

Fig. 8. Lineweaver–Burk analysis of the NaF-plus-$AlCl_3$-induced inhibition of the $(Na^+ + K^+)$-ATPase and the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase with respect to respectively $[Na^+]$ and $[Ca^{2+}]$

The $(Na^+ + K^+)$-ATPase (*a*) was preincubated for 10 min at 37 °C in a medium without (●) or with 10 μM-$AlCl_3$ plus 0.2 mM-NaF (○). The plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase (*b*) was similarly preincubated without (●) or with 10 μM-$AlCl_3$ plus 1 mM-NaF (○). The ATPase activities were measured in assay media with different concentrations of Na^+ or Ca^{2+} respectively. The results are represented as Lineweaver–Burk plots.

$(Na^+ + K^+)$-ATPase and of 1 mM for the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase. The K_i for inhibition by $AlCl_3$ of the $(Na^+ + K^+)$-ATPase is 3.2×10^{-7} M, and that for the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase is 1.3×10^{-6} M.

The effect of NaF and $AlCl_3$ on the [ATP]-dependency of the $(Na^+ + K^+)$-ATPase and the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase is represented in Fig. 7. NaF plus $AlCl_3$ lowers the V_{max} for ATP, without affecting the K_m value, as illustrated in the double-reciprocal plots. This suggests that the type of inhibition is non-competitive with respect to ATP. The double-reciprocal plots in Fig. 8 show that NaF plus $AlCl_3$ also inhibits the two ATPases non-competitively with respect to their cationic substrates, Na^+ and Ca^{2+} respectively.

The non-competitive inhibitory effect of AlF_4^- on the ATPase could be explained by the binding of AlF_4^- to the phosphate-binding site of the ATPase. Fig. 9(*a*) illustrates that, when 5 mM-P_i is present during the preincubation, the K_i for the inhibition of the $(Na^+ + K^+)$-ATPase by AlF_4^- is shifted to higher concentrations of NaF. The K_i for inhibition of the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase by AlF_4^- is, in the presence of 50 mM-P_i, similarly shifted to higher concentrations of NaF (Fig. 9*b*). In the absence of NaF, neither concentration of P_i affects the activities of the ATPases. A second argument in favour of the hypothesis that

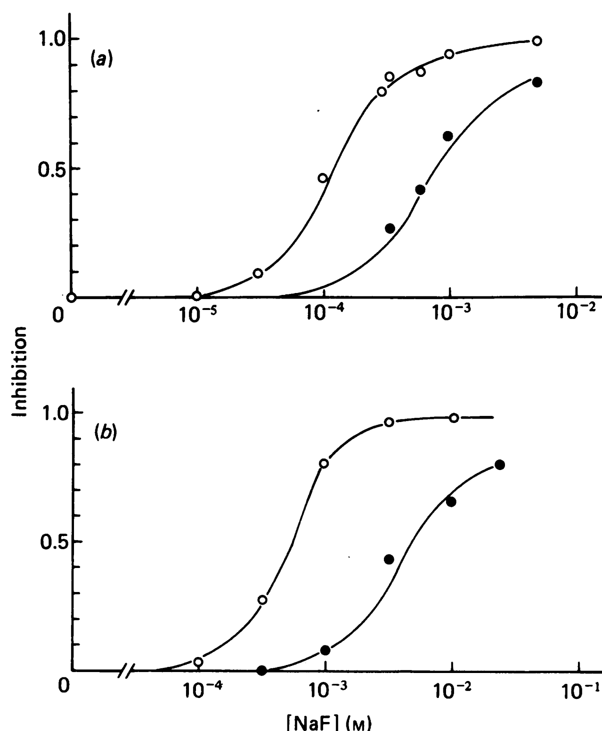

Fig. 9. Effect of P_i on the NaF-induced inhibition of the $(Na^+ + K^+)$-ATPase and the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase in the presence of 10 μM-$AlCl_3$

The purified $(Na^+ + K^+)$-ATPase (*a*) was preincubated with the different concentrations of NaF for 10 min at 37 °C in a medium containing 0.25 M-sucrose, 30 mM-imidazole, 10 μM-$AlCl_3$, with (●) or without (○) 5 mM-P_i. The purified plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase (*b*) was preincubated with the different concentrations of NaF for 10 min at 37 °C in a medium containing 0.25 M-sucrose, 30 mM-imidazole and 10 μM-$AlCl_3$, with (●) and without (○) 50 mM-P_i. The curves represent the inhibition normalized to that inhibition observed at the maximal concentration of NaF plus 10 μM-$AlCl_3$ in the absence of P_i.

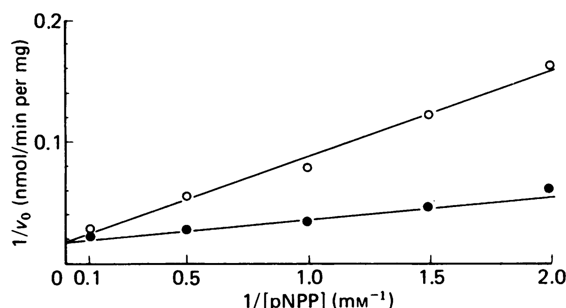

Fig. 10. Lineweaver–Burk analysis of the NaF-plus-$AlCl_3$-induced inhibition of the K^+-activated *p*-nitrophenylphosphatase activity by the $(Na^+ + K^+)$-ATPase with respect to pNNP

The purified $(Na^+ + K^+)$-ATPase was preincubated for 10 min at 37 °C in a medium containing 0.25 M-sucrose and 30 mM-imidazole, without (●) or with 100 μM-NaF plus 10 μM-$AlCl_3$ (○). The K^+-activated *p*-nitrophenylphosphatase activity was subsequently measured at different concentrations of pNPP. The results are represented as a Lineweaver–Burk plot.

AlF_4^- interacts with the phosphate-binding site of the ATPase is its competitive inhibition towards pNPP of the *p*-nitrophenylphosphatase activity by the two ATPases. The effect of 100 μM-NaF plus 10 μM-$AlCl_3$ on the [pNPP]-dependence of the K^+-activated *p*-nitrophenylphosphatase activity of the $(Na^+ + K^+)$-ATPase is represented in Fig. 10. The results are best fitted, up to 10 mM-pNPP, by a straight line in a Lineweaver–Burk plot. AlF_4^- inhibits the K^+-activated p-nitrophenylphosphatase activity of the $(Na^+ + K^+)$-ATPase competitively with respect to pNPP, indicating that AlF_4^- competes with pNPP for the same binding site on the ATPase. Also the ATP-dependent Ca^{2+}-activated *p*-nitrophenylphosphatase activity of the purified plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase is inhibited by AlF_4^-. A Lineweaver–Burk analysis of these inhibitions was not determined because it was not always possible to obtain steady-state inhibitions for the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase, especially at higher concentrations of pNPP. The AlF_4^--induced inhibition of the *p*-nitrophenylphosphatase is also slowed down and becomes incomplete at higher concentrations of pNPP.

DISCUSSION

NaF, in the presence of $AlCl_3$, is known to be a potent modulator of the G-proteins G_s, G_i and transducin [1–7]. It is unlikely that the inhibition of the 'P'-type ATPases by NaF plus $AlCl_3$ are due to an activation of G-proteins for the following reasons:

1. Preincubating the 'P'-type ATPases with 100 μM-p[NH]ppG in the presence of 1 mM-$MgCl_2$, which is known to activate G-proteins irreversibly, does not inhibit the 'P'-type ATPases.
2. AlF_4^- still inhibits the ATPase activity in the presence of 100 μM-GDP[S], a condition which completely abolishes the AlF_4^--induced stimulation of G-proteins [8].
3. The inhibition of the $(Na^+ + K^+)$-ATPase occurs at NaF-concentrations which are lower than those reported to stimulate the known G-proteins [4,5,8,10–12,15,29].

The lack of any inhibitory effect of NaF when Al^{3+} is complexed by deferoxamine, the lack of an effect of $AlCl_3$ in the absence of NaF, and the fact that both ions have to be present at the same time, suggest that fluoroaluminate complexes are involved in the observed inhibitions. The complexes of aluminium with fluoride are remarkably stable, and all species of $[AlF_n]^{3-n}$, with n varying between 1 and 6, are formed. Under our incubation conditions the predominant fluoroaluminate species would, according to published stability constants [28], be AlF_4^-. It was pointed out [8,15] that there are structural similarities between AlF_4^- and PO_4^{3-}. Both ions are tetrahedral and have a similar size. The findings that the inhibition is non-competitive towards ATP and the cationic substrates of the ATPase and that, in the presence of P_i, the K_i for inhibition is shifted to higher concentrations of AlF_4^-, suggest that AlF_4^- probably binds to the phosphate-binding site of the ATPase. This hypothesis is further substantiated by our observation that AlF_4^- inhibits the *p*-nitrophenylphosphatase activity of the $(Na^+ + K^+)$-ATPase in a competitive way with respect to pNPP.

The most important conclusion from the present study is that AlF_4^- inhibits the three 'P'-type cation-transport ATPases which we have studied, whereas it does not affect the single investigated 'F'-type ATPase. While this work was in progress, an inhibition of the $(Na^+ + K^+)$-ATPase by AlF_4^- was described [30]. Another 'P'-type ATPase, the $(K^+ + H^+)$-ATPase, has been reported to be completely inhibited by 0.5 mM-NaF [31]. However, the effect of Al^{3+} was not studied, and no precautions were taken to avoid complexation of F^- by contaminating Al^{3+}. It is therefore not unlikely that here also AlF_4^- might be responsible for the inhibition of the $(K^+ + H^+)$-ATPase.

The observation [14,15] that AlF_4^- increases the Ca^{2+} efflux from intact hepatocytes has led to the conclusion that an inhibition of the plasma-membrane Ca^{2+} pump by this substance in intact cells is unlikely. We have, however, obtained experimental evidence that AlF_4^- inhibits the Ca^{2+}-transport in intact smooth-muscle cells. However, the analysis of its effect on the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase is difficult because of the various ion-transport and exchange systems that function in these membranes. Further experiments are required to specify in detail the action of AlF_4^- on the 'P'-type cation-transport ATPases in intact tissues.

$BeCl_2$ is much less effective than $AlCl_3$ in inhibiting 'P'-type cation-transport ATPases, although it is a good substitute for $AlCl_3$ in the activation of G-proteins. For this reason, a comparison of the effects of $BeCl_2$ and $AlCl_3$ on intact cells might be advocated for differentiating the cellular effects which depend on activation of G-proteins from those induced by inhibition of 'P'-type cation-transport ATPases.

In conclusion, the complete inhibition of the $(Na^+ + K^+$-ATPase and of the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase and the partial inhibition of the sarcoplasmic-reticulum $(Ca^{2+} + Mg^{2+})$-ATPase by AlF_4^- warns us against explaining all effects of these compounds in intact cells by an activation of G-proteins.

We thank Mrs. I. Willems, Mrs. M. Crabbe and Ms. L. Bauwens for expert technical assistance, and Mrs. M. Vander Aerschot and Mrs. G. Duerinckx for typing the manuscript. This work was supported by the FGWO (Belgium).

REFERENCES

1. Sternweis, P. C. & Gilman, A. G. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 4888–4891
2. Bokoch, G. M., Katada, J., Northup, J. K., Hewlett, E. L. & Gilman, A. G. (1983) J. Biol. Chem. **258**, 2072–2075
3. Katada, T., Bokoch, G. M., Northup, J. K., Ui, M. & Gilman, A. G. (1984) J. Biol. Chem. **259**, 3568–3577
4. Katada, T., Northup, J. K., Bokoch, G. M., Ui, M. & Gilman, A. G. (1984) J. Biol. Chem. **259**, 3578–3585
5. Brandt, D. R. & Ross, E. M. (1986) Biochemistry **25**, 7036–7041
6. Northup, J. K., Smigel, M. D., Sternweis, P. C. & Gilman, A. G. (1983) J. Biol. Chem. **258**, 11369–11376
7. Kanaho, Y., Moss, J. & Vaughan, M. (1985) J. Biol. Chem. **260**, 11493–11497
8. Bigay, J., Deterre, P., Pfister, C. & Chabre, M. (1987) EMBO J. **6**, 2907–2913
9. Litosh, I. (1987) Biochem. J. **244**, 35–40
10. Strnad, C. F., Parente, J. C. & Wong, K. (1986) FEBS Lett. **206**, 20–24
11. Guillon, G., Mouillac, B. & Balestre, M. (1986) FEBS Lett. **204**, 183–188

12. Seifert, R., Rosenthal, W. & Schultz, G. (1986) FEBS Lett. **205**, 161–165
13. Hepler, J. R. & Harden, T. K. (1986) Biochem. J. **239**, 141–146
14. Blackmore, P. F., Bocckino, S. B., Waynick, L. E. & Exton, J. H. (1985) J. Biol. Chem. **260**, 14477–14483
15. Blackmore, P. F. & Exton, J. H. (1986) J. Biol. Chem. **261**, 11056–11063
16. Hughes, B. P. & Barrit, G. J. (1987) Biochem. J. **245**, 41–47
17. Post, E. W. & Kotz, J. C. (1975) Int. Rev. Sci. Inorg. Chem. Ser II **1** (chap. 7), 220–248
18. Corbridge, D. E. C. (1974) The Structural Chemistry of Phosphorus, Elsevier, Amsterdam and New York
19. Pedersen, P. L. & Carafoli, E. (1987) Trends Biochem. Sci. **12**, 146–150.
20. De Smedt, H., Borghgraef, R., Ceuterick, F. & Heremans, K. (1979). Biochim. Biophys. Acta **556**, 479–489
21. Verbist, J. Wuytack, F., De Schutter, G., Raeymaekers, L. & Casteels, R. (1984) Cell Calcium **5**, 253–263
22. Jones, L. R. & Cala, S. E. (1981) J. Biol. Chem. **256**, 11809–11818·
23. Wuytack, F. & Casteels, R. (1980) Biochim. Biophys. Acta **595**, 257–263
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
25. Schuurmans Stekhoven, F. & Bonting, S. L. (1981) Physiol. Rev. **61**, 1–76
26. Verma, A. K. & Penniston, J. T. (1984) Biochemistry **23**, 5010–5015
27. Gopalakrishna, A. & Anderson, W. B. (1982) Biochem. Biophys. Res. Commun. **104**, 830–836
28. Sillen, L. G. & Martell, A. E. (1964) Stability Constants of Metal-ion complexes, Special Publication no. 17, pp. 264–265), The Chemical Society, London
29. Taylor, C. W., Merrit, J. E. Putney, J. W. & Rubin, R. P. (1986) Biochem. Biophys. Res. Commun. **136**, 362–368
30. Robinson, J. D., Davis, R. L. & Steinberg, M. (1986) J. Bioenerg. Biomembr. **18**, 521–531
31. Ray, T. K. & Forte, J. G. (1976) Biochim. Biophys. Acta **443**, 451–467

Received 13 October 1987/19 January 1988; accepted 11 April 1988