Aluminofluoride Complexes: Phosphate Analogues and a Hidden Hazard for Living Organisms

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Abstract: There is a burgeoning body of evidence that aluminum can be implicated as an etiological factor of several neuropathological events. The molecular mechanisms underlying aluminum toxicity are still poorly understood. Reflecting on many studies, we suggest a new view on the toxicity of aluminum in a link with fluoride. Soluble aluminofluoride complexes - fluoroaluminate (AlF_x) are formed in water solutions containing fluoride and traces of aluminum. These complexes are able to simulate phosphate groups in many biochemical reactions. AlF_x are used in many laboratory investigations of guanine nucleotide binding proteins (G proteins). They affect various enzyme activities and cell signaling cascades. The hidden danger of a long-term synergistic action of aluminum and fluoride is not fully recognized at this point. We suggest that aluminum and fluoride can exacerbate the pathological and clinical problems, namely by interfering with a great number of G-protein-dependent cellular mechanisms, and by worsening excitotoxicity, microglial priming, and brain inflammation. Our suggestion opens the door to a better understanding of mechanisms of aluminum harmful effects on human health.

Keywords: Aluminum, aluminofluoride complexes, Alzheimer disease, fluoride, fluoroaluminate, G proteins, human exposure, neurotoxicity.

INTRODUCTION

Aluminum was not considered to be a toxic element until the 1970´s, although toxicity was reported periodically. In 1957, Campbell and coworkers, after reviewing 503 referenced studies, concluded that aluminum presents a hazard in only a few special circumstances [1]. It seems that such opinion is still widely accepted by biomedicine, the pharmaceutical industry, and by food producers. Much has already been written about human diseases attributed to the body burden of aluminum [2-6]. Wide ranges of toxic effects of aluminum to many cellular processes both in man and animals have been demonstrated [2]. Aluminum is now a well-established toxin, which has been implicated as an etiological factor of several neuropathological disorders [2- 7].

There are clearly a number of ways in which humans are exposed to aluminum which have been discussed by other authors in this special issue. For the general population, the major sources of aluminum are food and beverage packaging, aluminum-containing medications (e.g., antacids and buffered aspirins), vaccines, parenteral nutrition, drinking water, and water used for hemodialysis. Such items as deodorants, vaginal douches, baby skin creams, sun creams, and baby diaper wipes not only have high aluminum content, but are applied to areas where there is a far greater tendency for aluminum absorption [8-10]. Aluminum used as an adjuvant in vaccines creates a great risk of high accumulation and toxicity due to its 100% bioavailability [11].

Both active and passive defense mechanisms of the human organism designed to prevent aluminum accumulation are oversaturated in the face of abundant aluminum exposure from the environment, food chain, and man-made products. There are a number of reasons why the molecular mechanisms underlying aluminum toxicity are still poorly understood. Measurements of total aluminum in samples of plasma or serum are rarely representative of the bioavailable aluminum. Moreover, the concentration of aluminum in body fluids or tissues would be expected to be very small and therefore, difficult to confirm by experimental methods. The possible health risks of aluminum *in vivo* escaped attention because many effects might appear several years after exposure. Due to multifaceted interactions of aluminum with molecules in biological system, we can hardly find one reaction, one enzyme, one substrate or one gene, which explains the observed deterioration of health by aluminum. We now need to identify those conditions under which biologically reactive aluminum has produced a biological response in the affected system.

Intensive laboratory research of mechanisms of signal transduction demonstrates many pathways, which could change our understanding and interpretation of aluminum action on the cellular level. Since the 1980´s aluminum ions (Al^{3+}) plus fluoride have been widely used in laboratory investigations as tools for stimulation of various guanine nucleotide binding proteins (G proteins) [12]. Bigay *et al.* [13] suggested that fluoride in millimolar concentrations forms in the presence of micromolar Al^{3+} aluminofluoride

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complexes (AlF_x). These fluorometallic complexes need not to be obtained pre-made through any pharmaceutical or biotech product catalogue. They are formed in water solutions containing fluoride and traces of aluminum in the form of the soluble ionic complexes. The term fluoroaluminate is also used for these compounds. Chemical studies show that Al^{3+} binds fluoride stronger than 60 other metal ions [14].

The idea that AlF_x acts as a high affinity analogue of the terminal phosphate of GTP was suggested some 20 years ago by Bigay *et al*. and Chabre [13, 15]. G proteins take part in an enormous variety of biological signaling systems, helping control almost all important life processes [16]. Moreover, AlF_x also influences the activity of a variety of phosphatases, phosphorylases and kinases. The effects of aluminum in the presence of fluoride have been studied on various cells and tissues [12, 16-18].

Knowledge of mechanism of action of AlF_x on the molecular and cellular level will draw us nearer to an understanding of the detrimental effects of aluminum and fluoride combinations in the environment. Strunecka and Patocka [17] proposed that fluoride could complex with any preexisting Al^{3+} within body fluids to produce the AlF_x and this could lead to a combination of chronic activation of G protein-regulated systems (i.e., G protein-coupled receptors (GPCRs) and suppression of other critical enzymes. Such interference with G protein signaling by the synergistic action of Al^{3+} and fluoride in the environment (i.e., water, and food chains), might impair many physiological functions of human body. In this regard it is interesting to note that the origins of many human diseases are linked to malfunctioning of G protein-regulated signaling [19-20]. Accordingly, GPCRs have been the targets of many therapeutic drugs for at least a decade [21].

2. BASIC CHEMISTRY OF ALF_X

2.1. The Discovery of AlF_x

Fluoride anions are well known as the activators of the purified guanine nucleotide-binding regulatory component of adenylyl cyclase (AC) [22]. The breakthrough for explanation of the fluoride effects on AC led to the observation that Al^{3+} was required for activation of the regulatory component of AC by fluoride [23]. This observation had at first been ignored because aluminum is a normal component of glass from which it might be picked up by a solution containing fluoride. The requirement for aluminum was highly specific: of 28 other metals tested, only beryllium promoted activation of the guanine nucleotide-binding regulatory component of AC by fluoride [13, 15]. The use of a controlled concentration of Al^{3+} plus fluoride in a reconstituted system enabled the experiments to be more sensitive and stable.

Bigay *et al.* and Chabre were the first to elucidate the mechanism of action of Al^{3+} plus fluoride on G proteins [13,15]. In their experiments with transducin, they discovered that the predominant species of AlF_x is a tetrafluoroaluminate anion AlF_4^-, which shows structural similarity to the phosphate group PO_4^{3-}. Their experimental studies and calculations provided evidence that AlF_4^- acts as a high affinity analogue of the γ-phosphate of nucleotides, which mimics the role of the γ-phosphate only if the β-phosphate is present and remains unsubstituted [15]. This effect is more readily seen with G proteins because GDP is always tightly bound in the site after the hydrolysis of GTP. $AlCl_3$ together with NaF became the widely used tools for the study of involvement of G proteins in various cells and tissues [12]. A γ-phosphate model was proposed and quickly expanded to other phosphoryl-transfer enzymes [24-25].

2.2. AlF_x : The Phosphoryl Transfer Transition State Analogue

Transfer of phosphate groups is the basic mechanism in the regulation of the activity of numerous enzymes, energy metabolism, cell signaling, movement, and regulation of cell growth. Phosphate is an important component of phospholipids in the cell membranes. Phosphate is a ligand of considerable physiological importance that also forms complexes with aluminum. The anion PO_4^{3-} does not exist in aqueous solution at pH values under 12, and at physiological pH values, HPO_4^{2-} is the dominant species [26]. Aluminum can form salts with inorganic phosphate such as aluminum orthophosphate and sodium aluminum phosphate (the latter a common food additive) [27]. The orthophosphate anion with the tetrahedron structure is the basic anionic unit of all phosphates [13].

Analogies between a phosphate group and aluminofluoride complex consist of atomic and molecular similarities. The fluorine atom has the same size and the same valence orbitals as oxygen. Of course, fluorine is more electronegative than oxygen and with a similar capacity for forming hydrogen bonds. Aluminum is close to phosphorus in the periodic table, and their valence electrons are in the same shell. An Al–F bond is the same length as a P–O bond in phosphate, i.e. 1.5 to 1.6 Å [15]. Like phosphorus, aluminum has possible coordination numbers of 1–6, due to the possible hybridization of its outer shell 3p electrons with the 3d orbitals. These complexes can bind to proteins by hydrogen bonds to the fluorine atoms just as oxygen atoms of a phosphate ion.

However, an important functional difference between the phosphate group and the structurally analogous AlF_x complexes exist [13,15]. With phosphate, oxygen is covalently bound to the phosphorus and does not exchange with oxygen from solvent. In AlF_x, ionic bonds are formed between the electropositive aluminum and the highly electronegative fluorine. While the reaction of a bound phosphate compound with orthophosphate is endergonic and slow, the corresponding reaction with AlF_x is rapid and spontaneous [15]. AlF_x bind ionically to the terminal oxygen of GDP β-phosphate. Enzyme-bound GDP or ADP could therefore form complexes with AlF_x that imitate ATP or GTP in its effect on protein conformation. This effect often causes a structural change that locks the site and prevents the dissociation of the trisphosphate.

2.3. The Structure and Species of AlF_x

The average stoichiometry of AlF_x depends on the fluoride concentration and the pH of the solution [14, 28-29]. For most of the physiological and biochemical studies involving the putative AlF_x, the fluoride source is usually NaF (or KF) and the Al^{3+} source is $AlCl_3$. Moreover, Al^{3+} is a frequent

contaminant of commercial chemicals and it can also be picked up from the glass surface, depending on the substance stored in the glass container [26]. Phenomenological observations seemed to verify that pH determines the complexation state of AlF_x [15, 25]. The theoretical calculation of the Al^{3+}-fluoride predominance is demonstrated in Fig. **1**. However, the exact structure and the proportions of species such as AlF_3 and AlF_4^- that are able to simulate PO_4^{3-} group in biochemical reactions are still disputed [24, 30]. Our calculation is in agreement with Martin [14]. Free Al^{3+} concentration in aqueous solution changes dramatically with pH. The maximum concentration of $Al(OH)_3$ is reached at about neutral pH. At pH ≥ 7.0 the predominat species is aluminate ion $Al(OH)_4$. At neutral pH, the main species of fluoroaluminate are mixtures of AlF_3 and AlF_4^-. The more acidic the solution, the more free Al^{3+} is available, the less the OH^- group competes with Al^{3+} binding to fluoride. This is the condition in most cellular and biochemical studies. AlF_3 is the most thermodynamically stable compound of fluoride and Al^{3+}.

Fig. (1). Dependency of the complexation state of AlF_x on pH and fluoride concentration for Al^{3+} 10 μM.

The assumption that AlF_x acts through its tetrahedral phosphate-like complex was supported by the analogy with beryllium because all beryllium complexes are strictly tetrahedral and cannot take on the pentavalent conformation adopted by phosphate in transition states [15]. However, both ATPase and GTPase pathway must go through a pentacoordinated transition state for the γ-phosphate. The later studies of the crystal structures of nucleotide binding proteins provided evidence that AlF_x may also act as the phosphoryl transfer transition state analogue with a pentavalent phosphorus [24]. The X-ray crystallography of heterotrimeric G proteins bound to GDP and AlF_x [31-32] provided evidence that AlF_4^- could be the active site species. Al^{3+} is bound to four fluoride ligands in a square-planar coordination with two oxygen ligands at the apical position of the resulting octahedron (Fig. **2**). One oxygen ligand is the γ-phosphate oxygen, the leaving group in the transfer reaction, whereas the other is the oxygen from water believed to represent the attacking nucleophile of the hydrolysis reaction. The crystal structures of nucleotide binding proteins complexed with AlF_x appear to indicate that AlF_4^- acts as a transition state analogue, although it is the AlF_3 that mimics the phosphoryl group being transferred [25, 33]. The reason for the differences is not clear at present, however, research evidence suggests that the preference for AlF_4^- versus AlF_3 may be pH dependent. Indeed, the complexation state of AlF_x could simply balance the overall charge at the active site to the AlF_x binding complex. As shown by Schlichting and Reinstein [25], the pK_a of the last ionisable oxygen atom of the β-phosphate of a nucleoside diphosphate is 6.8 in the absence and ~5 in the presence of Mg^{2+}, respectively. Thus, the authors suggest that for charge conservation AlF_4^- would bind at pH values below the pK_a, whereas neutral AlF_3 would bind at pH values above the pK_a.

Fig. (2). Conformational changes of the γ-phosphate in a phosphoryl-transfer reaction transition state and various species of AlF_x (AlF_4^{1-} and AlF_3). Dotted lines indicate that the degree of bond making and bond breaking determines whether the transition is more dissociative, with a metaphosphate-like intermediate, or associative, with a pentavalent intermediate. Charges have been omitted for clarity. N = guanosine or adenosine

2.4. Fluoride plus Al^{3+}: The Tools in the Discovery of the Role of G Proteins

Liver membranes, multi-receptor fat cell system, and the light-activated rhodopsin system provided the first insight that AC is both inhibited and stimulated by two independent processes involving GTP and fluoride [34-36]. Thus appeared the nomenclature now popularly known as G_t, G_s, and G_i classes [20]. In a detailed study of the light-activated rhodopsin system it was suggested that hydrolysis of GTP is a very rapid process, whereas the rate limiting step is the release of inorganic phosphate from its binding sites on transducin, the G protein responsible for activation of phosphodiesterase in rod outer segments. Beginning with trans-

ducin it emerged that G proteins are constructed of three types of subunits; an α-subunit uniquely capable of binding and degrading GTP and a tightly knit complex of β- and γ-subunits. This discovery, eventually established for all G proteins coupled to receptors [16] opened up a new chapter in signal transduction, which, in recent years, has helped to explain the pleiotropic actions of hormones [20].

Structures of G_t and G_i have been determined in their GTP-, GDP- and AlF_x-liganded states [20] . Gα-subunit is composed of two distinct domains, a Ras-like GTPase domain and a predominantly helical domain that is unique to the Gα-subunit. The bound guanine nucleotide is held at the interface of these domains. Three switch regions within the GTPase domain (switches I, II, and III) change conformation in response to the guanine nucleotide-liganded state of the Gα-subunit. All three of these switch regions form significant contacts with Gβγ and effectors. In the GTP-bound state, the switch regions are held in place by contacts to the terminal γ-phosphate of the nucleotide. These regions appear to be less ordered in crystals of the GDP-bound G proteins [20]. The determination of the three-dimensional structures of heterotrimeric G proteins bound to GDP and AlF_x [31-32] confirmed that AlF_x is located in the γ-phosphate-binding site of these proteins. The studies of the crystal structures of nucleotide binding proteins complexed with fluoride and Al^{3+} indicate that factors other than pH, such as the location of positively charged amino acid of the active site of the phosphoryl-transferring enzyme may cause deviation from the strict pH dependence of AlF_3 versus AlF_4^- in biological systems [24].

3. ALF_X IN LABORATORY STUDIES

3.1. AlF_x as the Phosphate Analogue

The low cost and availability of fluoroaluminates has probably contributed to their widespread use as a tool in laboratory studies [17]. Fluoride in the presence of trace amounts of aluminum can affect various cells and tissues of animals as well as humans with powerful pharmacological efficacy (i.e., blood elements [37-41], lymphocytes and other cells of the immune system [42-44]). Both aluminum and fluoride are considered nephrotoxic substances. The effects of AlF_x on the kidney have been studied *in vitro* using glomerular mesangial cells, proximal tubular cells, and the collecting tubule cells of rat kidney [45]. Fluoride and Al^{3+} in kidney tubular cells were found to affect ion transporting processes, stimulate AC, inhibit amiloride-sensitive Na^+/H^+ exchange regulated by cAMP-dependent protein kinase, enhance epidermal growth factor-stimulated prostaglandin production, and mimic vasopressin and bradykinin induced Ca^{2+} mobilization [46-47]. Acid phosphatases were suggested as the potential cellular targets of fluoride action in the renal tissue [48]. NaF and AlF_x have been shown to act as bone cell mitogens [49-51]. The mitogenic action of AlF_x shows several different characteristics than that of fluoride. Exposure of osteoclasts to AlF_x resulted in a marked concentration-dependent inhibition of bone resorption [52-53].

AlF_x affect several important cellular processes such as ion transport, calcium influx and mobilization, neurotransmission, growth and differentiation of cells, and protein phosphorylation [12, 17]. It has been reported that AlF_x affects the assembly of cytoskeletal proteins and impairs the polymerization-depolymerization cycle of tubulin [15, 37]. Shape changes and disorganization of the spectrin network were observed after addition of 1 mM NaF and 10 μM $AlCl_3$ in human red blood cells [39]. Rapid and dynamic changes of the cytoskeletal network are of vital importance for many cells.

It is important to emphasize that Al^{3+} affects a wide range of enzymes activities either by inhibition or activation. In whole-cell systems, the intracellular effects of extracellular Al^{3+} challenge are often biphasic, being stimulatory at low doses and inhibitory at high doses. The hormetic dose-response curve occurs as a response to a disruption of homeostasis [26]. The explanation is that at low doses biological systems display an overcompensation response, which results in the apparent low-dose stimulation related toxicity. At higher doses with greater toxicity, the system often displays a more limited capacity for a compensatory response, usually insufficient to return to control level. Here we show that micromolar concentrations of aluminum ($AlCl_3$) in the presence of fluoride can both inhibit (Table **1**) and stimulate (Table **2**) various enzymes in various cells. It is likely that these effects are due to the action of AlF_x as analogue of phosphate groups.

Phosphoryl transfer reactions are involved in processes of energy transduction. ATP generation in mitochondria requires the association of an F_1 subunit with an F_0 transmembrane subunit transporting protons [57]. The binding of ADP and inorganic phosphate in a catalytic site of F_1 triggers conformational changes, which lock both of them into the site and induce the formation of pyrophosphate bonds by eliminating a water molecule. So, an AlF_x analogue of pyrophosphate, $R–O–PO_2–O–AlF_3$ can arise, which may be bound at the site for the γ-phosphate. The inhibition of mitochondria ATPase activity in the presence of AlF_4^- was reported [57]. This inhibition was not reversed by elution of fluoride from solution or by the addition of strong aluminum chelators. No significant release of the complex occurred over a period of days. AlF_x inhibits many ATPases, phosphatases, and phosphorylases (Table **1**). The interference of AlF_x with the energy transformation processes may thus affect the energy metabolism of the entire organism [17]. Lunardi *et al*. [57] reported that the inhibition of mitochondrial F-ATPase by fluoride requires the presence of Al^{3+}. While, prior incubation with the Al^{3+} chelator desferrioxamine markedly slowed the mitochondrial F-ATPase inactivation by fluoride, adding 1 μM $AlCl_3$ accelerated this reaction. Missianen *et al*. [61] studied the fluoride effect on the Ca^{2+}-Mg^{2+}-ATPase of the endoplasmic reticulum and provided evidence that the time course of inhibition and the concentrations of fluoride and Al^{3+} required for this inhibition differed for enzymes from different tissues. The mechanism of fluoride inhibition of a P-type cation-transport ATPases has been suggested by the action of AlF_x [13, 57, 61].

The experimental observations of the effects of AlF_x on intact cells indicate that these complexes, in many cases form in the system after the addition of fluoride and Al^{3+} into the extracellular solution [84]. It also appears that AlF_x exert their effects at very low concentrations [85].

Table 1. Inhibitory Effects of AlFx on Enzymatic Activities in Various Cells . $AlCl_3$ was Present in Micromolar Concentrations

Enzyme	Source	NaF	References
acid phosphatase	osteoclasts	mM	[52]
adenylyl cyclase	liver	up to 10 mM	[54]
	fibroblasts	5 mM	[55]
AChE	red blood cells	0.01–10 mM	[56]
F-ATPase	mitochondria	mM	[57]
glucose-6-phosphatase	liver	μM	[58]
glycogen synthase	hepatocytes	2–15 mM	[59]
IMP-ase	fibroblasts	mM	[60]
Ca^{2+}-Mg^{2+}-ATPase	mitochondria	1–10 mM	[61]
PKC	retina	mM	[62]
PLD	liver, brain, lymphocyte	mM	[63]

Abbreviations: acetylcholinesterase (AChE), inositol monophosphatase (IMP-ase), protein kinase C (PKC), phospholipase D (PLD).

Table 2. Stimulatory Effects of AlF_x on Enzymatic Activities

Enzyme	Source	NaF	References
adenylyl cyclase	lymphoma cell	10 mM	[23]
	smooth muscle	10 mM	[64]
	heart	1–10 mM	[65-66]
	turkey RBC	10 mM	[67]
	brain	10 mM	[68]
	kidney	10 mM	[69]
alkaline phosphatase	bone cells	10–100 μM	[70-71]
cytidylate cyclase	rat brain	mM	[72]
ERK	bone	1–10 mM	[50]
$K^+[ACh]_M$ channel	heart	>1 mM	[73]
K^+ATP channel	heart	mM	[74]
MAP kinases	lung	5–7.5 mM	[75]
glycogen phosphorylase	hepatocytes	1–50 mM	[54, 76]
PI 3-kinase	human HepG2 cells and HeLa cells	30 mM	[77]
PKC	lung	5–7.5 mM	[75]
PLA_2	platelets	5–10mM	[78]
	macrophages	5–10 mM	[79]
	endothelial cells	5–20 mM	[80]
PLC	hepatocytes	1–50 mM	[81]
	red blood cells	1 mM	[39]
	rabbit femoral artery	10 mM	[64]
PLD	platelets	5–10 mM	[38]
	rat atria	10 mM	[82]
	canine brain cortex	AlFx	[83]
tyrosine kinase	osteoblasts	1 –10 mM	[49]
		10 –100 μM	[70]

Abbreviations: extracellular signal-regulated kinase (ERK), mitogen activated kinases (MAP kinases), protein kinase C (PKC), phospholipase A_2 (PLA_2), phospholipase C (PLC), phospholipase D (PLD), red blood cells (RBC).

3.2. AlF_x and G Proteins

There are hundreds of G protein-coupled receptors (GPCRs) [86]. Physiological agonists of GPCRs include neurotransmitters and hormones, such as dopamine, epinephrine, norepinephrine, serotonin, acetylcholine, glucagon, vasopressin, melatonin, TSH, neuropeptides, opioids, excitatory amino acids, prostanoids, purines, photons and odorants [20, 86]. Laboratory investigations support the hypothesis that G proteins are potential AlF_x targets [13, 87]. The AlF_x acts as the first messenger triggering processes of neurotransmission and in potentiating the action of various hormones [12, 24]. AlF_x may therefore mimic the action of many neurotransmitters, hormones, and growth factors.

The observation that AlF_x can activate heterotrimeric G proteins has been useful for the study of G protein involvement in numerous biological systems, for the elucidation of three-dimensional structures of G proteins and several GTPases and for understanding the mechanism of GTP hydrolysis [12]. Nucleotide exchange and GTP hydrolysis are fundamental to the regulation of all types of G proteins that have been examined to date.

As shown in Table **3**, numerous laboratory results demonstrate that micromolar $AlCl_3$ in the presence of fluoride can affect the activity of AC and phospholipase C (PLC), the major GPCRs effector enzymes. Consequently, the levels of the second messenger molecules, inositol phosphates, and cytosolic free calcium ions, in various cells and tissues can also be altered by the combination of $AlCl_3$ and fluoride. Given the ubiquity of biochemical pathways that depend on GPCRs and calcium signaling, the spectrum of physiological processes that could be adversely impacted by AlF_x is extremely vast.

The principle of amplification of the initial signal during its conversion into a functional response has been a widely accepted tenet in cell physiology. Hence, the effects of AlF_x could also be amplified during signal transduction (Fig. **3**).

The amplification of AlF_x signal could detrimentally affect many essential biochemical processes that are dependent on GPCRs signaling. Indeed, the discoveries of receptor diversity, numerous G proteins, and PLC families [20], broadens enormously the possibilities of AlF_x interactions with signal transduction pathways. The diversity of molecules involved in these processes is manifested at all levels of molecular signaling. Endocrine glands, such as the parathyroid gland, the thyroid, the pituitary gland, and the pineal gland, are extremely sensitive to AlF_x [12]. Regarding the crucial role of these glands in regulation of growth, development, and metabolism of many tissues, AlF_x might influence the proper function of the entire human body. Enormous possibilities for multiple molecular interactions of aluminum and fluoride exist in the brain [12, 20] and clearly warrant further investigation. Regarding the role of phosphates in cell metabolism and life processes, we can predict hundreds of reactions that might be influenced.

Considering that many G protein-dependent reactions are fundamental for cellular and physiological processes, the common denominator of which is the transfer of a phosphoryl group, we can conclude that fluoroaluminates represent a hidden hazard for human health.

Fig. (3). AlF_x acts as the messenger of false information. Its message is greatly amplified during the conversion into the functional response of a cell. The second messenger molecule could be cAMP, $Ins(1,4,5)P_3$, and DAG. Moreover, AlF_x can participate as the analogue in the phosphoryl-transfer reactions involved in the signaling cascade.

4. ALF_X: HIDDEN DANGER FOR HUMAN HEALTH

4.1. Physiological Implication of Aluminum Burden

The synergistic action of Al^{3+} plus fluoride has important pathological implication. The effects of fluoride or Al^{3+} alone substantially differ from the effects of AlF_x. For example, Al^{3+} in micromolar concentrations avidly binds with fluoride to form AlF_x [13, 57, 84]. This means that the effects of AlF_x could result in pathophysiological consequences at lower concentrations than either Al^{3+}or fluoride acting alone. Indeed, the addition of aluminium chloride to 15 mM reduced the concentration of NaF required for 50% inhibition of the Mg^{2+}-dependent ATPase activity of the human erythrocyte ghosts [84]. The toxicological potential of Al^{3+} is thus markedly increased in the presence of fluoride and vice versa. For example, it has also been also observed that Al^{3+}-induced neural degeneration in rats is greatly enhanced when the animals were fed low doses of fluoride. The presence of fluoride caused more Al^{3+} to cross the the blood-brain barrier and be deposited in the brain of rat [85].

It is hard to predict what would happen in the human body after a chronic exposure to an increased content of Al^{3+} and fluoride in body fluids and in various tissues. Chronic exposure of humans to AlF_x can begin in the foetus (i.e., if a mother drinks fluoridated water). The severity and the development of AlF_x intoxication symptoms will depend on a person´s age, genetic background, nutrition status, kidney function, and many other factors.

There are many examples of aluminum-induced neurotoxicity [6]. Most of the ill effects caused by the synergistic action of aluminum and fluoride were first recognized among workers in aluminum factories, where fluoride and aluminum are present in high concentrations [88-90].

Elevated aluminum levels have been implicated as the cause of dialysis encephalopathy in renal failure patients undergoing long-term hemodialysis [91]. Since many hemodialysis units rely on systems to purify fluoridated tap water without removal of the fluoride, it is likely that many patients are also being exposed inadvertently to increased concentrations of fluoride [92]. Of note, given that patients with renal failure are not able to remove AlF_x from the blood, they are at increased risk of AlF_x toxicity.

It is generally known that both parenteral nutrition as well as infant formulas contain higher amount of aluminum [93]. In addition, fluoride is present in drinking water in Canada, USA, and some other developed countries. Fluoridation of drinking water as well as the use of aluminum sulfate as a flocculating agent in water treatment plants, in addition to the wide use of fluoride and Al^{3+} in medicine, industry, and agriculture, started the era of supplementation of human body with these ions as never before in the history of the human race [94-95]. Dental fluorosis as the sign of fluoride overload is endemic in at least 25 countries across the globe. Millions of people live in endemic fluorosis areas [12].

4.2. Alzheimer´s Disease (AD)

The hypothesis that the accumulation of aluminum in the brain is the risk factor for AD has been postulated and discussed elsewhere [56, 96-100]. Some authors reported that a higher amount of aluminum was found in human AD brains than in the brains of age-matched healthy controls. This suggestion was further supported by a positive correlation between the incidence of AD and concentrations of aluminum in drinking water [101]. Indeed, 9 out of 13 published epidemiological studies have shown a statistically significant association between aluminum in drinking water and AD [98]. For example, Rondeau *et al*. examined the association between exposure to aluminum from drinking water and risk of cognitive decline, dementia, and AD among elderly subjects followed for 15 years. These researchers found that a high daily intake of aluminum in drinking water ($\geq$ 0.1 mg/day) or a higher geographical exposure to aluminum was significantly associated with increased risk of dementia and that high consumption of aluminum from drinking water may be a risk factor for AD [102-105].

Aluminum is universally added to potable water supplies for water purification purposes, to reduce organic matter, turbidity, and microorganisms [106]. In several developed countries (i.e.,U.S., Canada, Australia, New Zealand), fluoride is also added to drinking water while most of Europe abandoned this practice [107]. AlF_x complexes can easily form in fluoridated water supplies, which contain fluoride anions and trace amounts of aluminum.

In a landmark study, Varner *et al*. [85] have shown that in rats chronic dietary exposure to AlF_x complexes caused severe damage to cerebrovascular endothelia and neurons, in a region-specific manner reminiscent of AD. What was surprising in the Varner *et al*. study was that only a small amount of AlF_x was sufficient to induce these marked neurodegenerative changes, namely 0.5ppm, which is the level of AlF_x relevant to human exposure from drinking water. Notably, the level of brain and kidney aluminum in the treated animals was almost double that of the control group. In addition, neuronal density in the hippocampus was decreased in area CA3 of the left hemisphere of the AlF_x group compared to the controls. Areas of the right hippocampus were also negatively affected by the AlF_x treatment showing a significant increase in damaged and grossly abnormal cells. The AlF_x group also showed significantly more immunoreactivity for β-amyloid in the lateral posterior thalamic areas of both hemispheres relative to the controls. The integrity of the cerebrovasculature appeared to be markedly compromised by the AlF_x. Concurrent with this, the fluorescence-based Morin method for aluminum detection showed marked aluminum fluorescense along brain areas that were exclusively associated with cerebral vasculature.

Varner *et al*. [85] noted striking parallels between aluminum-induced alterations in cerebrovasculature in AlF_x-treated rats and those associated with AD and related forms of dementia, where microvascular abnormalities show a regional specificity, which is concordant with neuronal degeneration patterns. The authors also suggested that alterations in the cerebrovasculature could be a primary event in AD and related neurodegenerative diseases. Altogether the study by Varner *et al*. suggests that most of the pathologic changes characteristic of AD could result from a synergistic action of aluminum and fluoride, a hypothesis consistent with our own observations [56].

The risk for AD is known to increase with aging [6]. In addition, Ca^{2+} homeostasis in the cell is also known to deteriorate with aging [108], making the elderly more vulnerable to both excitotoxicity and AlF_x toxicity. Consistent with these observations, Ca^{2+} dyshomeostasis is recognized as one of the hallmarks of AD [109]. We demonstrated that platelets from patients with AD have disturbed Ca^{2+} homeostasis [110]. For example, the basal values of cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) in the absence of extracellular Ca^{2+} were significantly lower in the platelets of patients with early stages of AD in comparison with age-matched and young controls. After the addition of 1 mM calcium into the incubation medium the $[Ca^{2+}]_i$ markedly increased in the platelets of AD patients compared to age-matched and young control subjects [110].

It is evident from Table **3** that AlF_x can affect Ca^{2+} homeostasis in many different cells including neurons. In doing so, AlF_x could contribute to AD. Consistent with this interpretation, Xu *et al*. [87] showed that AlF_x is capable of

Table 3. The Effects of AlF_x (mM NaF + µM $AlCl_3$) on Components of Signaling Pathways

Cells/ tissue	AC	PLC	InsPs	$[Ca^{2+}]_i$
Hepatocytes	↓↑	↑	↑	↑
Platelets		↑	↑	↑
Red blood cells	↑	↑	↑	↑
Neutrophils		↑		↑
Leukocytes			↑	↑
Fibroblasts	↓	↑	↑	↑
Macrophages		↑		↑
Lung cells		↑	↑	
Kidney cells	↑			↑
Neurons	↓↑	↑	↑↓	↑
Astrocytes		↑		↑
Osteoclasts	↑	↑	↑	↑

Abbreviations: adenylylcyclase (AC), phospholipase C (PLC), inositol phosphates level (InsPs), and cytosolic Ca^{2+} level ($[Ca^{2+}]_i$). Fluoride was used as NaF in millimolar concentrations and aluminum as $AlCl_3$ (micromolar). For references see [12, 17-18].

blocking PKC-dependent processing of the amyloid β precursor protein (AβPP), a signaling pathway which increases the production of non-amyloidogenic soluble β-APP at the expense of the neurotoxic β-amyloid species. Specifically, by interfering with the action of G proteins in the trans-Golgi network, AlF_x inhibited the budding of secretory vesicles containing the AβPP and thus prevented their redistribution towards the plasma membrane where they would undergo further processing to produce the soluble neuroprotective β-APP. Altogether these observations suggest that AlF_x can promote neural degeneration, as it antagonizes the neuroprotective G protein/PKC-dependent neuroprotective AβPP pathway.

AD is a complex multifactorial disorder. Experimental evidence [85] clearly shows that AlF_x at levels relevant to human exposure can act as the initial signal in triggering AD-like pathology *in vivo*. Furthermore, by influencing Ca^{2+} homeostasis [87] and energy metabolism [57, 84] these complexes can accelerate aging and impair the functions of the nervous system. In summary, chronic, lifetime exposure to AlF_x from drinking water may represent a serious risk factor for the development of neurodegenerative diseases in countries which still utilize water fluoridation.

5. CONCLUSIONS

The discovery of AlF_x as a new class of phosphate analogues has provided numerous demonstrations of their profound effects on many cellular functions. This is not surprising when considering the role of G proteins in signal transduction and the ubiquity of phosphate in cell metabolism. The action of aluminum in the presence of fluoride is not restricted to G proteins. The phosphate analogue model can apply to free phosphates and nucleoside phosphates. Regarding the role of phosphates in cell metabolism, we can predict hundreds of reactions, which might be influenced by AlF_x. These compounds may thus represent a significant hazard to energy metabolism, immunological responses, cardiovascular function and growth and differentiation of the cells as well as general homeostasis. Moreover, the effects AlF_x may be amplified during the process of signal transduction and thus potentiate AlF_x-mediated deleterious actions, including immunoexcitotoxicity (Fig. **3**). The origins of many human diseases are known to be due to malfunctioning of signaling components. Indeed, pharmacologists estimate that up to 60 % of all medicines used today exert their effects through G protein signaling pathways [111] .

Aluminum has long been implicated as etiological factor of several neuropathological diseases [5]. One reason to suspect AlF_x as the specific culprit in these diseases is that both Al^{3+} and fluoride are known to exist in appreciable concentrations in drinking water as well as many commercial food-products, cosmetics and parenteral nutrition [10-11, 112]. Furthermore, the trend toward fluorinating pharmaceuticals increases fluoride exposure via medication [113]. In light of the published findings, scientists and physicians should not neglect the potential of AlF_x to harm human health.

6. ABBREVIATIONS

$[Ca^{2+}]i$ = Cytosolic Ca^{2+} level

AβPP = Amyloid β precursor protein

AC = Adenylyl cyclase

AChE = Acetylcholinesterase

AD = Alzheimer´s disease

ADP = Adenosine diphosphate

AlF_4^{1-} = Tetrafluoroaluminate anion

AlF_x = Aluminofluoride complexes

ATP = Adenosine triphosphate

ERK = Extracellular signal-regulated kinase

G proteins = Guanine nucleotide binding proteins

GDP = Guanosine triphosphate

GPCRs = G protein-coupled receptors

GTP = Guanosine triphosphate

InsPs = Inositol phosphates level

IMP-ase = Inositol monophosphatase

PKC = Protein kinase C

MAP kinases = Mitogen activated kinases

PLA_2 = Phospholipase A_2

PLC = Phospholipase C

PLD = Phospholipase D

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CONFLICT OF INTEREST

No conflict of interest is declared.

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