Inhibitory effect of fluoride on insulin receptor autophosphorylation and tyrosine kinase activity

Francesc VIÑALS, Xavier TESTAR, Manuel PALACÍN and Antonio ZORZANO*

Departament de Bioquímica i Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, 08028 Barcelona, Spain

Fluoride is a nucleophilic reagent which has been reported to inhibit a variety of different enzymes such as esterases, asymmetrical hydrolases and phosphatases. In this report, we demonstrate that fluoride inhibits tyrosine kinase activity of insulin receptors partially purified from rat skeletal muscle and human placenta. Fluoride inhibited in a similar dose-dependent manner both β -subunit autophosphorylation and tyrosine kinase activity for exogenous substrates. This inhibitory effect of fluoride was not due to the formation of complexes with aluminium and took place in the absence of modifications of insulin-binding properties of the insulin receptor. Fluoride did not compete with the binding site for ATP or Mn^{2+} . Fluoride also inhibited the autophosphorylation and tyrosine kinase activity of receptors for insulin-like growth factor I from human placenta. Addition of fluoride to the pre-phosphorylated insulin receptor produced a slow (time range of minutes) inhibition of receptor kinase activity. Furthermore, fluoride inhibited tyrosine kinase activity in the absence of changes in the phosphorylation of pre-phosphorylated insulin receptors, and the sensitivity to fluoride

was similar to the sensitivity of the unphosphorylated insulin receptor. The effect of fluoride on tyrosine kinase activity was markedly decreased when insulin receptors were preincubated with the copolymer of glutamate/tyrosine. Prior exposure of receptors to free tyrosine or phosphotyrosine also prevented the inhibitory effect of fluoride. However, the protective effect of tyrosine or phosphotyrosine was maximal at low concentrations, suggesting the interaction of these compounds with the receptor itself rather than with fluoride. These data suggest: (i) that fluoride interacts directly and slowly with the insulin receptor, which causes inhibition of its phosphotransferase activity; (ii) that the binding site of fluoride is not structurally modified by receptor phosphorylation; and (iii) based on the fact that fluoride inhibits phosphotransferase activity in the absence of alterations in the binding of ATP, Mn^{2+} or insulin, we speculate that fluoride binding might affect the transfer of phosphate from ATP to the tyrosine residues of the β -subunit of the insulin receptor and to the tyrosine residues of exogenous substrates.

INTRODUCTION

The insulin receptor is a disulphide-linked heterotetrameric membrane glycoprotein consisting of two α (M_r 135 000) and two transmembrane β (M_r 95 000) subunits (Massagué et al., 1981; Massagué and Czech, 1982; Ullrich et al., 1985; Ebina et al., 1985). The α -subunits are entirely extracellular and participate in insulin binding, whereas the β -subunits contain extracellular, transmembrane and intracellular domains. The intracellular domain of the β -subunit displays tyrosine kinase activity. The N-terminal α -subunit contains the insulin-binding domain (Yip et al., 1988; Waugh et al., 1989; DeMeyts et al., 1990). Several studies suggest that the α -subunits exhibit an inhibitory influence over the β -subunit kinase domains of the insulin receptor (Ellis et al., 1987; Shoelson et al., 1988). Insulin binding results in a conformational change in the receptor molecule, which leads to a change in the α - α -subunit interaction within the receptor complex (Waugh and Pilch, 1989) and which is also transmitted to the β -subunit down to the C-terminal domain (Baron et al., 1990).

The kinase domain of the β -subunit of the insulin receptor can be separated into ATP-binding, tyrosine-autophosphorylation and substrate-binding domains. Binding of insulin activates a tyrosine-specific phosphotransferase activity, which enhances the rate of receptor autophosphorylation (Kasuga et al., 1982a,b; Petruzzelli et al., 1984). This autophosphorylation results in a conformational change which has been detected immunochemically (Herrera and Rosen, 1986; Perlman et al., 1989), and

also stimulates the ability of the receptor to phosphorylate various exogenous substrates (Rosen et al., 1983; Yu and Czech, 1984; White et al., 1985). The major sites of autophosphorylation are at tyrosine residues from regions 1160 (1158, 1162 and 1163) and 1330 (Tornqvist et al., 1987, 1988; Tavaré and Denton, 1988; Tavaré et al., 1988; White et al., 1988). The phosphorylation of tyrosines from the 1160 region correlates best with activation of the substrate kinase (White et al., 1988; Tavaré and Denton, 1988; Flores-Riveros et al., 1989). Site-directed mutagenesis analysis indicates that phosphorylation of tyrosines 1162 and 1163, but not 1158, plays a critical role in the activation of the receptor kinase (Zhang et al., 1991).

The tyrosine kinase activity of the insulin receptor appears to be essential for certain cellular responses to insulin. Thus anti-insulin-receptor antibodies, which inhibit the kinase activity of the insulin receptors, also block the ability of cells to respond to insulin (Morgan et al., 1986; Morgan and Roth, 1987). In addition, the microinjection of insulin receptors in *Xenopus* oocytes causes an increase in the phosphorylation of ribosomal S6 subunit, which is further increased by prior receptor activation, due to insulin-receptor autophosphorylation (Maller et al., 1986). Studies with receptors mutated at the ATP-binding site (Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987) or at tyrosine residues 1162 and 1163 (Ellis et al., 1986; Debant et al., 1988) have also led to the conclusion that the tyrosine phosphotransferase function of the insulin receptor is an absolute requirement for the hormone to activate the receptor signalling function in cells.

Based on the pivotal role of insulin-receptor kinase activity on insulin action, the catalytic properties of the insulin-receptor kinase require thorough characterization. In studies initially designed to investigate the interaction between regulatory G-proteins and insulin receptors, we substantiated a potent inhibitory effect of fluoride on insulin-receptor kinase activity. On the basis of this finding and the fact that the use of fluoride, a potent nucleophilic reagent (Edwards and Pearson, 1962), has yielded useful information on the kinetics of a variety of enzymes (Layne and Najjar, 1975; Bunick and Kashket, 1982; Nilsson and Brändén, 1983), we have characterized the inhibitory effect of fluoride on insulin-receptor autophosphorylation and receptor kinase for exogenous substrates.

MATERIALS AND METHODS

Materials

Pig monocomponent insulin was a gift from Mr. T. L. Jeatran, Eli Lilly and Co. Human recombinant insulin-like growth factor I (IGF-I) was obtained from Boehringer. [^{125}I -Tyr A14]moniodoinsulin was obtained from New England Nuclear. [γ - ^{32}P]ATP was prepared from [^{32}P]P $_i$ (New England Nuclear) by using a Gamma-prep kit from Promega Biotech. Wheat-germ agglutinin (WGA) bound to agarose was obtained from Vector, and NaF from Merck. BSA (fraction V, fatty-acid-free), copolymer of Glu/Tyr (4:1) and other chemicals were from Sigma.

Receptor preparations

Male Wistar rats weighing 250–300 g and fed *ad libitum* were used for all studies. Hindlimb muscle was obtained from sodium pentobarbital-anaesthetized rats and frozen in liquid nitrogen as previously described (Camps et al., 1990). After powdering in a cooled mortar, mixed skeletal muscle was homogenized with a Polytron homogenizer (setting 7, 30 s, 4 °C) in a buffer (3.5 ml/g) containing 25 mM Hepes, 4 mM EDTA, 4 mM EGTA, 1 trypsin-inhibitor unit of aprotinin/ml, 0.2 mM phenylmethanesulphonyl fluoride, 25 mM benzamidine, 1 mM bacitracin, 2 μM leupeptin, 2 μM pepstatin, 0.1 mM sodium orthovanadate and 10 mM sodium pyrophosphate, pH 7.6. After homogenization, Triton X-100 was added to give a final concentration of 1% and the homogenate was stirred for 1 h at 4 °C and centrifuged at 150 000 g for 90 min at 4 °C. The supernatant was recycled three times through a column of WGA bound to agarose (3 ml of supernatant/ml of resin bed), at 4 °C. The resin was washed with buffer (100 ml) containing 25 mM Hepes and 0.1% Triton X-100, pH 7.4. Receptors were eluted from the WGA column with the buffer containing 25 mM Hepes, 0.1% Triton X-100 and 0.3 M *N*-acetyl-D-glucosamine.

Human placental microsomes were prepared by the procedure of Shia and Pilch (1983). Full-term human placentas were placed in ice-cold PBS. After removal of chorion and amnion, the placenta was cut into pieces and homogenized with a Polytron homogenizer in homogenization buffer (30 mM Hepes, 0.25 M sucrose, 25 mM benzamidine, 10 μM leupeptin, 1 trypsin inhibitor unit of aprotinin/ml, 1 mM phenylmethanesulphonyl fluoride, pH 7.6), at a 1:4 ratio of tissue weight to buffer volume. Placental microsomes were prepared by differential centrifugation (Harrison and Itin, 1980; Pilch, 1985). Placental microsomes (10–20 mg/ml) were solubilized with 1% Triton X-100 at 4 °C for 60 min in the presence of 1 mM phenylmethanesulphonyl fluoride. The supernatant after ultracentrifugation at 100 000 g for 50 min (4 °C) was subjected to WGA chromatography.

^{125}I -ligand binding

Insulin binding was measured as described by James et al. (1986). WGA eluate (20 μl) was incubated in 30 mM Hepes buffer containing 0.1% BSA, 100 units/ml bacitracin (pH 7.6, 1 h, 22 °C, 200 μl) and 20 000 c.p.m. of [^{125}I -Tyr A14]moniodoinsulin (~ 60 pM) and increasing concentrations of unlabelled insulin. Receptors were precipitated with 0.5 ml of bovine γ -globulin (1 mg/ml) and 0.5 ml of polyethylene glycol (25%, w/v) (Hollenberg and Cuatrecasas, 1976). The precipitated ^{125}I -insulin-receptor complex was pelleted for 10 min in a micro-centrifuge. The supernatant was aspirated and the pellets were counted for radioactivity in an LKB γ -counter. Non-specific binding was estimated as ^{125}I -insulin bound in the presence of 1 μM insulin (5–10% of total binding). Binding data were expressed per μg of protein, the latter being measured by the method of Bradford (1976).

Receptor autophosphorylation

Receptor preparations (10–40 μl of WGA eluate) were pre-incubated for 30 min at 22 °C with increasing insulin or IGF-I concentrations in a buffer containing 30 mM Hepes, 4 mM MnCl_2 , 10 mM MgCl_2 , pH 7.6, as described by Camps et al. (1990). Phosphorylation was initiated by addition of 50 μM [γ - ^{32}P]ATP (5–10 μCi) in a final volume of 100 μl . Autophosphorylation reactions were terminated after incubation for 4 min at 22 °C by the addition of an equal volume of Laemmli (1970) sample buffer containing 0.1 M dithiothreitol. Samples were then subjected to SDS/PAGE.

Electrophoresis and autoradiography

We carried out denaturing SDS/PAGE using a discontinuous buffer system (Laemmli, 1970). All samples were prepared by adding an equal volume of Laemmli sample buffer containing 0.1 M dithiothreitol. The final concentrations of sample buffer constituents were 50 mM Tris, 50 mM dithiothreitol, 10% glycerol, 1% SDS, pH 6.8. M_r standards were myosin (M_r 200 000), β -galactosidase (M_r 116 000), phosphorylase *b* (M_r 97 000), BSA (M_r 66 000) and ovalbumin (M_r 45 000). All samples were run on a 7.5% resolving gel. Autoradiography of Coomassie-Blue-stained dried gels was performed at -70 °C with Kodak X-Omat AR film using Cronex Lightning Plus enhancing screens. Quantification of autoradiograms was performed by scanning densitometry within the linear response range.

Exogenous kinase activity

Phosphorylation of an exogenous substrate was carried out with receptor preparations which were preincubated for 1 h in 30 mM Hepes buffer, pH 7.6, containing 50 mM magnesium acetate, 4 mM MnCl_2 and various concentrations of insulin or IGF-I. The receptor kinase activity was initially activated by addition of 50 μM [γ - ^{32}P]ATP (5–10 μCi) for 10 min. The reaction was initiated by addition of the exogenous substrate (copolymer of Glu/Tyr, 4:1; 0.25 mg/ml). The reaction was stopped after 30 min by applying samples to filter-paper squares (2 cm $\times$ 2 cm; Whatman 3 MM), which were immediately washed in 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. Papers were washed at least five times over 2–3 h, dried and counted for radioactivity by Čerenkov counting. Non-specific binding of [γ - ^{32}P]ATP to the paper accounted for 5–10% of the measured radioactivity associated with the copolymer in the presence of insulin.

Table 1 Inhibitory effect of fluoride on tyrosine kinase activity of insulin receptors partially purified from rat skeletal muscle

Insulin receptors were partially purified from rat skeletal-muscle homogenates after solubilization in Triton X-100 and ultracentrifugation using lectin affinity chromatography. The WGA eluate was preincubated for 1 h at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 50 mM magnesium acetate and 4 mM MnCl_2 and in the absence (Basal) or in the presence of 100 nM insulin, and with the following additions: AlF_4^- (10 mM NaF + 10 μM AlCl_3), 10 μM AlCl_3 , 10 mM NaF, 0.5 mM deferoxamine (Dfx) or 10 mM KF. Samples were further incubated with 50 μM [$\gamma\text{-}^{32}\text{P}$]ATP (5–10 μCi) for 10 min and then the reaction was initiated by addition of the substrate (Glu/Tyr copolymer). See the Materials and methods section for further details. Data are means $\pm$ S.E.M. of triplicate estimations, and the results shown are representative of three different experiments.

Additions	Kinase activity (pmol of ^{32}P /30 min per μg of protein)	
	Basal	+ Insulin
None	19 $\pm$ 3	47 $\pm$ 5
AlF_4^-		3 $\pm$ 1
AlCl_3		41 $\pm$ 4
NaF		3 $\pm$ 1
Dfx		46 $\pm$ 4
AlF_4^- + Dfx		3 $\pm$ 2
NaF + Dfx		0.6 $\pm$ 1
KF		5 $\pm$ 1

Figure 1 Fluoride completely inhibits insulin-stimulated insulin-receptor kinase activity

Insulin receptors partially purified from rat skeletal muscle were incubated for 30 min at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 4 mM MnCl_2 and 50 mM magnesium acetate in the absence or presence of different concentrations of insulin (0–100 nM) and in the absence ($\square$) or presence ($\blacksquare$) of 10 mM NaF. Samples were further incubated with 50 μM [$\gamma\text{-}^{32}\text{P}$]ATP (5–10 μCi) for 10 min and then the reaction was initiated by addition of substrate (Glu/Tyr copolymer). Data are means $\pm$ S.E.M. of triplicate estimations, and the results shown are representative of four different experiments.

RESULTS

Effect of fluoride on insulin-receptor autophosphorylation and tyrosine kinase activity for exogenous substrates

In a preliminary set of experiments designed to assess the role of regulatory G proteins on insulin action, we investigated the effect of AlF_4^- (10 mM NaF + 10 μM AlCl_3) on insulin-receptor kinase activity by using partially purified insulin receptors from rat skeletal muscle (Table 1). These receptors respond to insulin by causing a near 3-fold stimulation of tyrosine kinase activity for exogenous substrates (Table 1). The presence of AlF_4^- caused an almost total blockade of the insulin-stimulated tyrosine kinase

Figure 2 Fluoride inhibits insulin-stimulated insulin-receptor autophosphorylation

Insulin receptors partially purified from rat skeletal muscle were incubated for 30 min at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 4 mM MnCl_2 and 10 mM MgCl_2 in the absence or presence of 100 nM insulin (INS) or 10 mM NaF. Autophosphorylation was initiated by addition of 50 μM [$\gamma\text{-}^{32}\text{P}$]ATP (5–10 μCi). The reaction was terminated after incubation for 4 min at 22 °C by addition of an equal volume of Laemmli sample buffer containing 0.1 M dithiothreitol. Then the samples were subjected to electrophoresis in 7.5% polyacrylamide gels as described in the Materials and methods section. The autoradiogram of a representative gel is shown. The position of the 97 kDa marker is shown to the left.

activity of the insulin receptor, as assessed by the phosphorylation of the copolymer Glu/Tyr (4:1). This effect was also obtained with 10 mM NaF, whereas 10 μM AlCl_3 did not cause any effect on kinase activity (Table 1). Furthermore, the inhibitory effect of 10 mM NaF on insulin-receptor kinase activity was fully shown in the presence of 0.5 mM deferoxamine (a chelator of aluminium) (Table 1). Giving further support to the inhibitory role of NaF on insulin-receptor kinase activity, we found that the inhibition produced by AlF_4^- was not blocked in the presence of a large excess of deferoxamine, enough to chelate all Al^{3+} ions present in the medium (Table 1). KF (10 mM) also blocked tyrosine kinase activity of the insulin receptor, substantiating a role for the fluoride anion as the inhibitory agent of the insulin-receptor kinase (Table 1).

Fluoride inhibited the tyrosine kinase activity of partially purified receptor preparations both in the absence and in the presence of different concentrations of insulin (Figure 1). In fact, fluoride (10 mM) caused the complete inhibition of insulin-stimulated tyrosine kinase activity (Figure 1). The inhibitory action of fluoride on tyrosine kinase activity of insulin receptors was also detected by using histone 2b, another exogenous substrate of the insulin-receptor kinase (results not shown).

In the absence of NaF, insulin caused a 2.5-fold increase in the insulin-receptor autophosphorylation (Figure 2). On the basis of digestion assays by different proteases, we have estimated that the maximally phosphorylated insulin receptor has 40–50 % of the phosphate on the C-terminal domain and the rest in the 1160 region (results not shown), which is in keeping with previous observations obtained with insulin receptors from placenta or skeletal muscle (Goren et al., 1987; Camps et al., 1990). The presence of 10 mM NaF led to inhibition of insulin-stimulated β -subunit autophosphorylation (Figure 2), and 0.5 mM

Figure 3 Dose-dependency of the inhibitory effect of fluoride on insulin-stimulated autophosphorylation and tyrosine kinase activity of the insulin receptor

(a) Insulin receptors from rat skeletal muscle were incubated for 30 min at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 4 mM MnCl_2 , 10 mM MgCl_2 , 100 nM insulin and in the presence of various concentrations of NaF. Autophosphorylation was initiated by addition of 50 μM [γ - ^{32}P]ATP (5–10 μCi). The reaction was terminated after incubation for 4 min at 22 °C by addition of an equal volume of Laemmli sample buffer containing 0.1 M dithiothreitol. Then the samples were subjected to electrophoresis in 7.5% polyacrylamide gels as described in the Materials and methods section. The autoradiogram of a representative gel is shown. The position of the 97 kDa marker is shown to the left. Quantification of autoradiograms by scanning densitometry is also shown in (b) (■). (b) Insulin receptor preparations from rat skeletal muscle were incubated for 1 h at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 50 mM magnesium acetate, 4 mM MnCl_2 , 100 nM insulin and various concentrations of NaF. Samples were further incubated with 50 μM [γ - ^{32}P]ATP for 10 min. The reaction was initiated by addition of the substrate (Glu/Tyr copolymer). See the Materials and methods section for further details. Data are means of five independent experiments (□).

deferoxamine did not prevent the effect of NaF on insulin-receptor autophosphorylation (results not shown).

The effect of fluoride in inhibiting autophosphorylation of the insulin receptor or the tyrosine kinase activity for exogenous substrates was concentration-dependent (Figure 3). In fact, both autophosphorylation and tyrosine kinase activities showed similar sensitivity to fluoride. Thus half-maximal inhibition was attained at approx. 6 mM and maximal inhibition at 8–10 mM NaF (Figure 3b). These data fitted well to the Hill equation, showing a Hill coefficient of 5.5 ± 0.5 (results not shown).

On the basis of the potent inhibitory effects of fluoride on insulin-receptor autophosphorylation and exogenous kinase activities, we analysed whether insulin binding was modified by the anion. To this end, ^{125}I -insulin equilibrium-binding analysis was performed in receptor preparations incubated in the absence or presence of 10 mM NaF (a concentration that maximally inhibits

Figure 4 Fluoride does not inhibit insulin receptor binding

Insulin receptor preparations were incubated for 1 h at 22 °C in a buffer containing 25 mM Hepes, 0.1 mg/ml BSA, 100 units/ml bacitracin, ^{125}I -insulin, various concentrations of unlabelled insulin and without (□) or with (■) 10 mM NaF, in a total volume of 200 μl . See the Materials and methods section for further details. The data shown (Scatchard plot) are from a representative experiment ($n = 3$). Each data point is the mean of triplicate determinations.

Figure 5 Double-reciprocal plot of fluoride-induced inhibition of tyrosine receptor kinase with respect to ATP

Insulin receptor preparations from rat skeletal muscle were incubated for 1 h at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 50 mM magnesium acetate, 4 mM MnCl_2 and 100 nM insulin. Samples were further incubated with 10, 50 or 100 μM [γ - ^{32}P]ATP for 10 min, followed by a further 10 min incubation in the absence (□) or presence (■) of 6 mM NaF. The reaction was initiated by the addition of the substrate (Glu/Tyr copolymer). See the Materials and methods section for further details. A Lineweaver-Burk plot of a representative experiment is presented.

insulin-receptor kinase activity). The data show that fluoride did not modify insulin-binding parameters (Figure 4). Thus K_d values for high-affinity binding were 0.68 and 0.59 nM in control and NaF-treated groups respectively; high-affinity binding-site values (B_{max}) were 1.57 and 1.61 fmol/μg of protein in control and NaF-treated groups respectively.

The inhibition by fluoride with respect to ATP concentration was studied. The results obtained are shown in Figure 5 as a double-reciprocal plot and indicate that the inhibition of tyrosine kinase of the insulin receptor is non-competitive with regard to ATP. Furthermore, fluoride did not cause any measurable hydrolysis of ATP under the incubation conditions, as assessed by nucleotide adsorption to Norit A (results not shown). Therefore, inhibition by fluoride of receptor tyrosine kinase activity is not a consequence of degradation of ATP. The extent of the inhibition induced by fluoride was also independent of the concentration of Mn^{2+} present in the medium (results not shown), again suggesting that fluoride does not compete with the binding site of the cation.

We next preincubated insulin-receptor preparations with 10 mM NaF for 30 min at 22 °C. Samples of insulin receptors

Table 2 Bio-Gel chromatography eliminates the effect of fluoride on insulin-receptor kinase activity

Insulin receptors from rat skeletal muscle were incubated for 30 min at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 4 mM $MnCl_2$ and 50 mM magnesium acetate in the absence of insulin, in the presence of 100 nM insulin or in the presence of 100 nM insulin and 10 mM NaF. Then tyrosine kinase activity for exogenous substrates was assayed as detailed in the legend to Figure 1 and in the Materials and methods section (A). Receptors previously incubated in the absence or presence of NaF in 30 mM Hepes, pH 7.4, for 30 min at 22 °C were desalted by Bio-Gel chromatography (0.5 cm $\times$ 5 cm column). After desalting, basal and insulin-stimulated kinase activity for exogenous substrates was assayed in control receptors (B) and in receptors previously incubated with NaF (C). Data are expressed as a percentage of basal and are means $\pm$ S.E.M. of triplicate estimations. The results shown are representative of three different experiments.

	Kinase activity (% of basal)	
	Insulin	Insulin + fluoride
(A) Untreated insulin receptors	195 $\pm$ 21	72 $\pm$ 7
(B) Insulin receptors + desalting	183 $\pm$ 8	
(C) Insulin receptors preincubated with 10 mM NaF + desalting	213 $\pm$ 32

Figure 6 Fluoride inhibits autophosphorylation of insulin and IGF-I receptors from human placenta

Receptor preparations from human placenta were incubated for 30 min at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 4 mM $MnCl_2$ and 10 mM $MgCl_2$ in the absence or presence of 100 nM IGF-I, 100 nM insulin (INS) or 10 mM NaF. Autophosphorylation was initiated by addition of 50 μ M [γ - ^{32}P]ATP (5–10 μ Ci). The reaction was terminated after incubation for 4 min at 22 °C by addition of an equal volume of Laemmli sample buffer containing 0.1 M dithiothreitol. Then the samples were subjected to electrophoresis in 7.5% polyacrylamide gels as described in the Materials and methods section. The autoradiogram of a representative gel is shown. The position of the 97 kDa marker is shown to the left.

previously treated with NaF were desalted by passage through a Bio-Gel column. Insulin receptors not treated with NaF were also run through the column as a control. Before chromatography in Bio-Gel, incubation in the presence of fluoride caused a marked inhibition of tyrosine receptor kinase activity (Table 2). However, after desalting, kinase activity of receptors preincubated with fluoride was responsive to insulin and indistinguishable from the activity shown by desalted control receptors

(Table 2). Thus, under conditions in which insulin receptors are incubated in the presence of fluoride in 30 mM Hepes buffer, pH 7.4, the effect of fluoride on receptor kinase is eliminated by desalting.

Effect of fluoride on IGF-I receptor kinase activity

To examine whether fluoride leads to inhibition of other tyrosine kinases, we obtained preparations of human placental microsomes enriched in IGF-I and insulin receptors as reported (Rubin et al., 1983). In keeping with previous observations (Rubin et al., 1983), IGF-I caused, in a dose-dependent manner, the phosphorylation of the M_r -95000 band (results not shown). With 100 nM IGF-I, 10 mM NaF completely inhibited the autophosphorylation of IGF-I receptors (Figure 6). Under these conditions, fluoride also inhibited insulin-stimulated phosphorylation of the M_r -95000 band (Figure 6), consistent with inhibition of insulin-receptor autophosphorylation. IGF-I stimulated the tyrosine kinase activity for exogenous substrates, and concentrations of IGF-I as low as 1 nM led to a 75% increase of kinase activity whereas approximately a 4-fold and an 8-fold increase over basal activity, was attained with 10 nM and 100 nM IGF-I respectively (results not shown). The dose-dependent activation of tyrosine kinase was in agreement with previous reports (Rubin et al., 1983; Sasaki et al., 1985). At all IGF-I concentrations tested, fluoride (10 mM) led to the complete inhibition of IGF-I-stimulated tyrosine kinase activity (results not shown).

Effect of fluoride on the kinase activity of the pre-phosphorylated receptor

In the next series of experiments, we assessed whether the effect of fluoride inhibiting the receptor kinase activity was due to a specific blockade of the autophosphorylation process. To this end, insulin receptors were maximally phosphorylated by incubation with 100 nM insulin for 1 h, followed by incubation for 10 min with 50 μ M [γ - ^{32}P]ATP. Phosphorylated receptors were incubated for different time periods with or without 10 mM NaF, and the phosphorylation level and the tyrosine kinase activity were determined. Under control conditions, the phosphorylation level of insulin receptors was maintained for at least 30 min after maximal phosphorylation had been attained (results not shown). The phosphorylation level of the β -subunit of the insulin receptor was not modified by the presence of NaF even 30 min after its addition (results not shown). Under these conditions, in which phosphorylation of the β -subunit of the insulin receptor was maximal, fluoride inhibited tyrosine kinase activity for exogenous substrates, in a time-dependent manner (Figure 7). Thus the half-maximal inhibitory effect of 10 mM NaF was attained 0.5 min after its addition, and the maximal inhibitory effect was obtained after approx. 2 min (Figure 7). Similarly to the unphosphorylated receptor (see Figure 3), 10 mM NaF caused 85% inhibition of insulin-stimulated tyrosine receptor kinase (Figure 7), and 6 mM NaF caused approximately half-maximal inhibition (34% of insulin-stimulated tyrosine receptor kinase) (results not shown). Thus the unphosphorylated and the phosphorylated insulin receptor show similar sensitivity to fluoride.

Protective effect of receptor kinase substrates, free tyrosine and phosphotyrosine against fluoride action

The time-dependent effect of NaF on kinase activity suggested the direct interaction of fluoride with the insulin receptor as the

Figure 7 Fluoride inhibits the kinase activity of the pre-phosphorylated receptor

Insulin receptors were preincubated for 1 h at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 50 mM magnesium acetate, 4 mM MnCl_2 and 100 nM insulin. Then receptors were maximally phosphorylated by incubation in the presence of 50 μM [γ - ^{32}P]ATP (5–10 μCi) for 10 min. Phosphorylated receptors were incubated for the times indicated in the presence of 10 mM NaF, and then the phosphorylation reaction was initiated by addition of substrate (Glu/Tyr copolymer) during 30 min. See the Materials and methods section for further details. Data are means $\pm$ S.E.M. of triplicate estimations, and the results shown are representative of three different experiments.

Figure 8 The inhibitory effect of fluoride on receptor kinase activity is prevented by exogenous substrates

Insulin receptors partially purified from rat skeletal muscle were maximally phosphorylated by incubation for 1 h at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 50 mM magnesium acetate, 4 mM MnCl_2 and 100 nM insulin, followed by 10 min incubation with 50 μM [γ - ^{32}P]ATP (5–10 μCi). At that time, some receptors were incubated in the presence of 10 mM NaF for 10 min and then with the copolymer Glu/Tyr for 30 min (●). Some other receptors were incubated with the copolymer Glu/Tyr, and at 10 min after its addition, 10 mM NaF was added (see the arrow) (■) or not (□), and kinase activity was allowed to proceed for 20 min further. Data are means $\pm$ S.E.M. of 3–4 independent experiments.

initial event leading to inhibition of the receptor kinase activity. In order to map the effect of fluoride, we next addressed whether the effect of fluoride was prevented by substrates of the kinase. To this end, insulin receptors were phosphorylated in the presence of insulin for 10 min and the copolymer Glu/Tyr (4:1) was then added to allow phosphorylation to be catalysed by the receptor. At 10 min after copolymer addition, 10 mM fluoride was added or not (Figure 8), and the reaction was allowed to proceed for a further 20 min. In parallel, a control group was run in which pre-phosphorylated insulin receptors were incubated with fluoride for 10 min and then with Glu/Tyr copolymer, and under these conditions, as expected, NaF totally inhibited kinase activity (Figure 8). When NaF was added 10 min after initiation of

Figure 9 The inhibitory effect of fluoride on receptor kinase activity is prevented by free tyrosine and phosphotyrosine

Insulin receptors partially purified from rat skeletal muscle were incubated for 1 h at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 50 mM magnesium acetate, 4 mM MnCl_2 , 100 nM insulin and various concentrations of free tyrosine or phosphotyrosine. This was followed by 10 min incubation with 50 μM [γ - ^{32}P]ATP (5–10 μCi). At that time, receptors were incubated in the absence (□) and presence (■) of 10 mM NaF. Kinase activity was initiated by addition of Glu/Tyr copolymer for 30 min. Data are means of duplicate estimations, and the results shown are representative of three different experiments.

exogenous-substrate phosphorylation, the anion only caused a very moderate inhibitory effect, and therefore a very substantial protective effect of Glu/Tyr copolymer was found (61% of fluoride effect under control conditions, $n = 3$ independent observations) (Figure 8).

A protective effect against the inhibitory action of fluoride was also observed after addition of free tyrosine or phosphotyrosine (Figure 9). Free tyrosine did not modify receptor kinase activity; however, concentrations of tyrosine as low as 0.16 mM were effective in protecting against the inhibitory effect of 10 mM NaF (Figure 9a) (0.33 mM tyrosine contained an equivalent amount of tyrosine molecules compared with the concentration of copolymer used for the protection studies of Figure 8). Phosphotyrosine at concentrations in the range 0.3–1.25 mM caused a marked inhibition of tyrosine kinase activity of insulin receptor (Figure 9b), and under these conditions it also protected against the inhibitory action of fluoride (Figure 9b). To determine the specificity of the protective action of tyrosine and phosphotyrosine, receptors were preincubated in the presence of 0.66 mM phosphoserine or 0.66 mM L-phenylalanine and subsequently exposed to fluoride. Under these conditions, phosphoserine and L-phenylalanine were unable to protect the receptor against inhibition induced by 10 mM NaF (Table 3). Incubation in the presence of 1 mM phosphate or 1 mM pyrophosphate already caused a marked inhibition of insulin-stimulated insulin-receptor kinase (results not shown), which precluded the study of their actions against the effect of fluoride.

Table 3 Inhibitory effect of fluoride on receptor kinase activity is not prevented by phosphoserine and phenylalanine

Insulin receptors partially purified from rat skeletal muscle were incubated for 1 h at 22 °C in 30 mM Hepes buffer, pH 7.6, containing 50 mM magnesium acetate, 4 mM $MnCl_2$, 100 nM insulin and 0.66 mM phosphoserine or L-phenylalanine. This was followed by 10 min incubation with 50 μ M [γ - ^{32}P]ATP (5–10 μ Ci). At that time, receptors were incubated in the absence and presence of 10 mM NaF. Kinase activity was initiated by the addition of Glu/Tyr copolymer during 30 min. Data are means $\pm$ S.E.M. of triplicate estimations and the results shown are representative of three different experiments.

	Kinase activity (pmol of ^{32}P /30 min per μ g of protein)	
	Basal	+ Fluoride
Control	2.28 $\pm$ 0.01	0.56 $\pm$ 0.04
0.66 mM phenylalanine	2.32 $\pm$ 0.01	0.45 $\pm$ 0.09
0.66 mM phosphoserine	2.78 $\pm$ 0.06	0.68 $\pm$ 0.05

DISCUSSION

In this study we have observed that incubation of partially purified detergent-solubilized insulin receptor and IGF-I receptor preparations in the presence of NaF caused an almost total inhibition of receptor kinase activity (receptor autophosphorylation and tyrosine kinase activity for exogenous substrates). It has been reported that fluoride and trace amounts of contaminating aluminium can form AlF_4^- complexes which, by acting as phosphate analogues, can modify a variety of processes such as reactions of phosphate transfer (Chabre, 1990). However, the inhibitory effect of fluoride on the insulin receptor kinase was not a consequence of the formation of AlF_4^- , since chelation of all possible contaminating aluminium with a large excess of deferoxamine did not abolish the effect of fluoride.

The following critical observations were made regarding the fluoride-induced inhibition of tyrosine kinase activity of insulin receptors: (a) receptors from rat muscle and human placenta were inhibited by fluoride; (b) the inhibitory effect of fluoride on insulin-receptor autophosphorylation and tyrosine kinase activity showed a similar dose-response dependence; (c) the fluoride effect was non-competitive with regard to ATP or Mn^{2+} concentrations; (d) the effect of fluoride inhibiting the kinase activity was slow; (e) fluoride inhibited the tyrosine kinase activity of unphosphorylated and phosphorylated insulin receptors to a similar extent; and (f) prior addition of copolymer Glu/Tyr, free tyrosine and phosphotyrosine, but not phosphoserine and phenylalanine, provided a marked protection against the inhibitory effect of fluoride.

From the facts that fluoride (i) inhibits tyrosine kinase activity in a time-dependent manner, and (ii) inhibits autophosphorylation and tyrosine kinase activity for exogenous substrates with a similar dose-response, we conclude that fluoride inhibits the phosphotransferase catalysis by interacting directly with the insulin receptor. Furthermore, the dose-dependency of fluoride-induced inhibition of the kinase fitted well to the Hill equation ($h \sim 5$), suggesting the existence of several binding sites for fluoride on the insulin receptor which show positive cooperativity. Fluoride behaved as a slow-binding inhibitor of the insulin-receptor kinase activity. A recent report has also described a slow inhibitory effect of fluoride on Ca^{2+} -ATPase of sarcoplasmic reticulum (Murphy and Coll, 1992). Such a slow fluoride-insulin-receptor interaction implies the existence of structural

rearrangement of the protein in the process of kinase inhibition, which in turn might be explained by the sequential binding of multiple fluoride ions on the insulin receptor molecule.

Further evidence for a direct interaction of fluoride with the insulin receptor comes from protection experiments using the Glu/Tyr copolymer, free tyrosine or free phosphotyrosine. Previous incubation of insulin receptors with Glu/Tyr copolymer, tyrosine or phosphotyrosine markedly protected against the inhibitory effect of fluoride on insulin-receptor kinase activity. The protective effects of tyrosine and phosphotyrosine against fluoride-induced inactivation of the receptor kinase were observed at concentrations much lower than the concentrations of fluoride itself, which indicated that the protective action was not due to an abstraction of fluoride by tyrosine but rather to interaction of tyrosine with the insulin receptor. In addition, the effects of tyrosine and phosphotyrosine were specific, and similar concentrations of phosphoserine and phenylalanine did not provide any protective effect against the fluoride-induced tyrosine kinase inactivation. We have also observed that the Glu/Tyr copolymer caused effective protection when incubated with the insulin receptor before fluoride addition. However, the Glu/Tyr copolymer did not cause any protection when added after fluoride. This indicates that copolymer-induced protection is due to the interaction between the exogenous substrate and the insulin receptor, rather than being merely due to the introduction of unknown fluoride-reacting groups. Whether Glu/Tyr copolymer protects against fluoride inhibition because of competition for a common site or due to copolymer-induced conformational changes of the insulin receptor remains unknown.

When insulin receptors were incubated in the presence of fluoride in Hepes buffer, pH 7.4, the fluoride effect was eliminated by Bio-Gel chromatography, consistent with the view of fluoride acting as a reversible inhibitor. However, we have also observed that the effect of fluoride on receptor kinase activity is dependent on the order of addition of the synthetic substrate, Glu/Tyr copolymer. That is, prior addition of Glu/Tyr copolymer protects against fluoride-induced inhibition of the kinase, and this protection is not observed if the copolymer is added after fluoride. These latter results were obtained under incubation conditions which included the presence of 100 nM insulin, 50 mM magnesium acetate, 4 mM $MnCl_2$ and 50 μ M ATP. These findings suggest that fluoride is a reversible inhibitor and that under some selective conditions it tightly binds to the insulin receptor. In this regard, it has been reported that fluoride is a tight-binding inhibitor of the Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum (Murphy and Coll, 1992).

We have observed that when fluoride is added to the unphosphorylated insulin receptor, it blocks both receptor autophosphorylation and tyrosine kinase activity for exogenous substrates with similar sensitivity. In addition, we have shown that fluoride, when incubated with the maximally phosphorylated insulin receptor, also inhibits tyrosine kinase for exogenous substrates in the absence of changes in the phosphorylation state of the receptor. On the basis of our findings, we suggest that fluoride, after binding to a specific site of the catalytic locus of the receptor, blocks a process which is common to the phosphorylation of the tyrosine residues of the insulin receptor and the phosphorylation of exogenous substrates. Taking into account that fluoride does not compete with ATP or Mn^{2+} , the step probably blocked by fluoride is the transfer of the γ -phosphate from ATP to the tyrosine residue located either on the β -subunit of the insulin receptor or on the exogenous substrate.

As mentioned above, our results indicate that fluoride binds to the insulin receptor, probably somewhere on the cytosolic domain

of the β -subunit of the insulin receptor (kinase domain) and of the IGF-I receptor. From the fact that the unphosphorylated and phosphorylated insulin-receptor forms showed a similar sensitivity to fluoride, we postulate that fluoride acts on a site of the receptor that belongs to the catalytic locus of the kinase and which is not structurally modified by insulin-induced receptor autophosphorylation. That allows us to rule out the phosphotyrosine residues from region 1160 of the β -subunit of the insulin receptor as possible direct targets of fluoride. Furthermore, taking into account that insulin-receptor autophosphorylation causes conformational changes in the receptor (Herrera and Rosen, 1986; Perlman et al., 1989), our data allow us to suggest that the fluoride-binding site(s) must remain relatively unmodified by the conformational alterations induced by autophosphorylation. The elucidation of the specific binding site(s) of fluoride on the insulin receptor might help the understanding of some of the catalytic steps involved in receptor tyrosine kinase activity.

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