Activation and Inhibition of Fat Cell Adenylate Cyclase by Fluoride

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Incubation (30°) of fat cell particulate fractions with fluoride before assay in the effective absence of fluoride results in activation of adenylate cyclase. Whereas the effect of fluoride (1.3 to 7 mM) when added to the assay was maximal in <2 min, 10 or 15 min of incubation before assay was usually required to produce maximal activation with any given concentration of fluoride. Under both conditions 3 to 5 mM fluoride produced maximal activation. After incubation with fluoride for 5 to 20 min cyclase activity was constant for at least 15 min of assay without fluoride: maximal activity was greater than that produced by fluoride added to the assay system and the concentration required to produce significant activation was lower. Fluoride activation in the assay or during prior incubation could be prevented by pyrophosphate. When added during the early minutes of assays with fluoride, 1.5 mM pyrophosphate, which had little effect on the activity of enzyme previously incubated with fluoride, rapidly reduced activity to essentially basal levels; when added after 10 min its effect was equally rapid but much smaller. It appears that activation is initially reversible by removal of fluoride as it is by addition of pyrophosphate, but becomes largely irreversible by these means with continued exposure to fluoride.

Fluoride in the assay system inhibited cyclase activated by isoproterenol or choleragen or by incubation with fluoride prior to assay; inhibition, dependent on fluoride concentration, was maximal at 5.3 mM. Since maximal activity produced by incubation with fluoride before assay exceeded that of nonincubated preparations assayed with fluoride, and was reduced to the latter level when assayed in the presence of fluoride, we infer that inhibition is reversible at a time when fluoride activation is relatively irreversible. Pyrophosphate (1.5 mM), which prevented fluoride activation, did not reduce fluoride inhibition of isoproterenol-, fluoride-, or choleragen-activated cyclase. When 3 mM MnCl₂ was present in the assay, inhibition by fluoride was not observed. In descriptive terms, MnCl₂ appeared to cause rapid reversal of fluoride inhibition. Thus, fluoride inhibits, in an apparently similar manner, fat cell adenylate cyclase whether it is activated by isoproterenol, fluoride, or choleragen. Although fluoride activation and inhibition can apparently be dissociated or modified differentially, until the mechanism(s) of action of fluoride is elucidated it cannot be concluded that these are totally independent processes.

Since Rall and Sutherland (1) first observed that fluoride increased cyclic-AMP formation by particulate fractions from liver, fluoride activation of adenylate cyclases from most mammalian tissues has been demonstrated. Apparently irreversible (or very slowly reversible) fluoride activation of cyclase preparations from parotid (2), adrenal (3, 4), brain (5), liver (6, 7), skeletal muscle (8), heart (9), fat (10), and other tissues (5) has been reported. Perkins and Moore (5) found that adenylate cyclase from rat cerebral cortex incubated with fluoride before assay was activated at a rate that was dependent on temperature and fluoride concentration. As they pointed out, the activation produced by incubation of the enzyme with fluoride before assay was apparently slow relative to the essentially immediate effects that are usually observed when fluoride is present in the assay system. Our studies are consistent with the view that fluoride activation of the fat cell adenylate cyclase is a relatively rapid process whether it occurs in the assay system or during prior activation but the extent of reversibility of activation declines with increasing time of exposure to fluoride.

Harwood and Rodbell (11) first demonstrated inhibition of hormone-activated cyclase by fluoride in concentrations that under other conditions caused maximal stimulation. They suggested that fluoride "uncouples the process of hormonal activation subsequent to hormone receptor interaction." In our studies fluoride, in addition to inhibiting isoproterenol stimulation of the enzyme, inhibited the markedly elevated cyclase activity from fat cells incubated with choleragen and the cyclase activated by incubation with fluoride before assay. The characteristics of the inhibitory effects of fluoride in all of these situations were similar. Thus fluoride inhibition of adenylate cyclase is not specifically related to hormonal activation. It

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1 The abbreviation used is: cyclic AMP, adenosine 3'-5'-monophosphate.
Activation and Inhibition of Adenylate Cyclase by Fluoride

METHODS

Fat cells were prepared as previously described from epididymal fat pads of Osborne-Mendel rats (125 to 200 g) that were permitted free access to food and water until decapitated (19). For most experiments cells were washed once in Krebs-Ringer phosphate medium containing 30 mg/ml of bovine serum albumin, and then three times in 0.25 mM sucrose with 10 mM Tris/HCl, pH 7.4. Cells were suspended in 5 ml of the same solution and homogenized in a 4 ml Dounce homogenizer (seven strokes with a B pestle). The homogenate was centrifuged at 100,000 × g for 40 min. The fat cells were removed with a spatula, the supernatant decanted, and the sides of the tube wiped dry. The particulate fraction was suspended in 0.25 mM sucrose, 10 mM Tris, pH 7.4, using a Dounce homogenizer. In some experiments it was diluted with the same solution and adenylate cyclase activity was assayed immediately. In other experiments, it was adjusted to contain 0.20 mM sucrose, 8 mM Tris, pH 7.4, and 5 mM MgSO4. Some portions were diluted, usually 33-fold, with 0.25 mM sucrose, 10 mM Tris buffer, pH 7.4, and immediately assayed for adenylate cyclase activity. Other portions were incubated at 30°C with or without fluoride before dilution and assay. Adenylate cyclase activity (counting concentrations of fluoride introduced into the assay with the incubated fractions were always less than 0.17 mM, a concentration that had no demonstrable effect on adenylate cyclase activity (see "Results").

In some experiments, freshly prepared cells were incubated at 37°C in Krebs-Ringer phosphate medium containing 30 mg/ml of bovine serum albumin for several hours in the presence or absence of cholera toxin or forskolin before preparation of particulate fractions and assay of adenylate cyclase activity.

For assay of adenylate cyclase activity, particulate fractions (<125 μg of protein) in either 15 or 55 μl of 0.55 mM sucrose with 10 mM Tris, pH 7.4, were incubated at 30°C for 10 min (unless otherwise stated) in a final volume of 75 μl containing 3.3 mM ATP (approximately 2 × 10^6 cpm of [3H]ATP), 6.6 mM MgCl2, 23.3 mM Tris/HCl, pH 8.0, 0.5 mM cyclic AMP, 0.3 mM dithiothreitol, 200 μg of bovine serum albumin, 16.7 mM sodium phosphoenolpyruvate, and 16 μg of pyruvate kinase (including 5.5 nmol of (NH4)2SO4). (In many of the time course studies, rather than individual incubations of 75 μl, much larger volumes, e.g. 0.9 ml, were incubated; 75-μl samples were removed at the indicated times to end the assay.) The reaction was terminated by addition of 0.5 ml of a 10% sodium dodecyl sulfate solution containing 7.0 mM ATP, 2.5 mM cyclic AMP, and 10 mM Tris buffer, pH 7.4. The reaction mixture was transferred to AG 50W-X8 (100 to 200 mesh) columns (0.5 × 3.0 cm), and cyclic AMP was eluted in 3.0 ml as described by Krishna et al. (13). After two precipitations with ZnSO4, and Ba(OH)2, the supernatants were transferred to columns (0.8 × 2.5 cm) of AG 1-X2 (100 to 200 mesh). The columns were washed with 10 ml of water and 10 ml of 0.005 n HCl; cyclic AMP was eluted with 6 ml of 0.03 n HCl. One portion of each eluate was used for radioassay of cyclic [3H]AMP and another for measurement of absorbance at 259 nm, to assess and correct for recovery of cyclic AMP. Enzyme activity was constant for 15 to 20 min in the standard assay system over the range of protein concentrations used except that in the presence of fluoride, there was sometimes a delay of <2 min before the constant cyclic AMP accumulation was established (see for example Figs. 8, 10, and 12). This delay, which was not demonstrably related to fluoride concentration, was not always observed. Whether or not it was, when adenylate cyclase activity is reported as nanomoles of cyclic AMP/10 min, this refers to the amount of cyclic AMP accumulated between 6 and 10 min of assay. Data reported are means of duplicate assays; all observations have been repeated at least twice with different particulate preparations. Protein was determined by the method of Lowry et al. (14), using bovine serum albumin as standard. Cholera (Lot 1071) was prepared by Dr. R. Finkelstein, University of Texas Southwestern Medical School and supplied by Dr. Carl Miller, National Institute of Allergy and Infectious Diseases.

[3H]ATP was purchased from New England Nuclear; ATP, cyclic AMP, and L-isoproterenol-t-biurate from Sigma, ion exchange resins from Bio-Rad; dithiothreitol from Calbiochem.

RESULTS

The activity of adenylate cyclase preparations incubated in the presence of fluoride and then assayed in the effective absence of fluoride was constant for at least 15 min with no evidence of reversal of the activation during the assay period (Fig. 1). After incubation of the cyclase for 5 min with 0.5 mM fluoride activity was increased about 100% and higher concentrations of fluoride produced further increases in adenylate cyclase activity (Fig. 2). With further incubation, adenylate cyclase activity increased more slowly at a rate not obviously dependent on fluoride concentration (Fig. 2) although in a few experiments (e.g. Fig. 4) there was little or no increase in activity after 5 min. As shown in Fig. 3, the maximal activity achieved by incubation with fluoride was greater than that produced by fluoride added to the assay system, and the concentration required to produce significant activation was lower. Fluoride present in the assay system inhibited the activity of the enzyme that had been previously incubated with 5 mM fluoride (Fig. 4). With concentrations of fluoride >5.0 mM in the assay system there was no difference between the activities of preparations previously incubated with or without fluoride. No inhibition was observed in the presence of 1.3 mM fluoride, a concentration that enhanced the activity of preparations not previously incubated with fluoride.

As shown in Fig. 5, activity in the presence of maximally effective concentration of isoproterenol (0.05 to 0.1 mM) was greater than that in the presence of 5 mM fluoride. When 5 mM fluoride was present with isoproterenol, the rate of cyclic AMP accumulation was equivalent to that observed with fluoride alone, and when fluoride was added after 3 min to assays containing isoproterenol, the new lower rate was established with no discernible delay. In the presence of 0.1 mM isoproterenol, 2.5 mM fluoride significantly decreased cyclase activity, and concentrations of fluoride greater than 4 mM depressed activity to a level equal to that produced by fluoride alone (Fig. 6).

Basal activity and that in the presence of 5.3 mM fluoride were essentially independent of ATP concentration between 1.5 and 10 mM (MgCl2 concentration = 2 times ATP). With increasing ATP concentration above 1.5 mM, however, the effect of isoproterenol on cyclase activity decreased sharply. At all concentrations of ATP, activity in the presence of 0.1 mM isoproterenol plus 5.3 mM fluoride was similar to that observed with fluoride alone. Increasing MgCl2 from 6.6 to 17.3 mM with 3.3 mM ATP slightly increased basal adenylate cyclase and slightly decreased activity in the presence of isoproterenol. Activities in the presence of fluoride and of fluoride plus isoproterenol which were equal were unaffected (data not shown).

Activated adenylate cyclase from cells that had been incubated with cholera toxin was inhibited by fluoride to a level

8 Data are presented as a miniprint supplement immediately following this paper. Figs. 1 through 12 will be found on p. 6209. For the convenience of those who prefer to obtain the supplementary material in the form of 12 pages of full size photocopies, these same data are available as JBC Document Number 767-756. Orders for supplementary material should specify the title, authors, and reference to this paper, and the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.80 per set of photocopies.
activation of the adenylate cyclase that had been activated by incubation with fluoride prior to assay was also apparently abolished by 3 mM MnCl₂ which only slightly increased the activity of the enzyme assayed without fluoride. Although MnCl₂ had relatively little effect on the choleragen-activated cyclase in the absence of fluoride, 0.5 or 1 mM MnCl₂ apparently abolished fluoride inhibition of these preparations and with 3 mM MnCl₂, fluoride stimulation of the choleragen-activated cyclase was observed (data not shown).

DISCUSSION

Incubation of fat cell particulate fractions with suitable concentrations of fluoride for as little as 2.5 min resulted in activation of adenylate cyclase which was not reversed during a subsequent 15- or 20-min assay in the effective absence of fluoride. Maximal activation was sometimes attained in 5 min but usually required 10 or 15 min of incubation with fluoride. Concentrations of fluoride that were without effect in the assay produced significant activation during prior incubation. Maximal activation achieved by incubation of adenylate cyclase with fluoride was greater than that observed when fluoride was added to the assay system as noted by Perkins and Moore (5) in studies with the enzyme from rat cerebral cortex. In all probability the quantitative differences in the degree of activation demonstrable by the two procedures are due to the inhibitory effects of fluoride present in the assay system. Fluoride activation, either during incubation or in the assay was prevented by pyrophosphate which had relatively little effect on the activity of the enzyme that had been incubated for 15 min with fluoride. When added during the early minutes of assay with fluoride, pyrophosphate caused a rapid reduction of activity to essentially basal levels, whereas when added 10 min after fluoride, its effect was equally rapid but of much smaller magnitude. These findings are consistent with the view that pyrophosphate interferes with activation of adenylate cyclase by fluoride, but does not inhibit the enzyme already activated by fluoride.

The loss of reversibility of the fluoride-activated state with continuing incubation could account for the apparent "time course" of fluoride activation when the enzyme was incubated with fluoride and then assayed in its absence. It seems probable that when the enzyme is exposed to fluoride (at 30°C) in the assay system or simply in buffer at pH 7.4, the adenylate cyclase is rapidly activated to a level dependent on the concentration of fluoride. It appears that for the first few minutes the activation is rapidly and almost completely reversible following removal of fluoride or addition of pyrophosphate, but with continuing incubation at 30°C the fluoride-activated enzyme becomes progressively less susceptible to inactivation by these means. Based on studies of a different type, it has been suggested that fluoride activation of leukocyte adenylate cyclase is enzyme-dependent (17) and the loss of reversibility of the fluoride activation could result from inactivation of enzymes (or depletion of cofactors) required for this process. Other explanations, however, are equally possible.

Stimulatory effects of fluoride on fat cell adenylate cyclase are maximal with concentrations of 3 to 5 mM and decline markedly with higher concentrations in the assay system (16, 18, 19). The inhibitory effects of fluoride at high concentration have been attributed to removal of magnesium but this is not the explanation for the inhibition caused by the concentrations used in our studies. Harwood and Rodbell (11) first demonstrated that fluoride in concentrations that produced maximal
stimulation could also inhibit hormone-activated fat cell cyclase. When assays were carried out at 20° or 25°, fluoride activation of the enzyme was minimal whereas responsiveness to epinephrine, adrenocorticotropic hormone, glucagon, and secretin was preserved, and 10 mM fluoride seemingly abolished the effects of all of these agents (11). Fluoride inhibition is not, however, specifically related to hormonal activation of the fat cell cyclase. As described above, fluoride can also inhibit the choleragen-activated fat cell cyclase and the enzyme activated by incubation with fluoride prior to assay. At 30°, 5.3 mM fluoride in the assay system caused either stimulation or inhibition depending on the level of cyclase activity in the absence of fluoride. Thus, fluoride increased the activity of the enzyme from control cells and either increased or decreased to the same level the activity of the cyclase previously incubated with fluoride or that derived from cells exposed to choleragen. Similarly fluoride suppressed the activity of the isoproterenol-activated enzyme to the level produced by fluoride alone, as reported by Harwood and Rodbell (11). In descriptive terms, it appeared that regardless of the level of activity in the absence of fluoride or the manner in which the enzyme was activated (by isoproterenol, by prior incubation with fluoride, or by incubation of cells with choleragen), the activity observed in assays with 5 mM fluoride was established or determined by the fluoride.

When fluoride was present in the assay the effects of MnCl₂ were striking. Fluoride activation of the adenylate cyclase from control cells was almost doubled with 3 mM MnCl₂ and fluoride inhibition in the presence of isoproterenol was apparently abolished. When assayed with 3 mM MnCl₂, the activity of the enzyme previously incubated with fluoride (assayed with or without fluoride) and that of the unincubated enzyme assayed with fluoride were essentially the same. The choleragen-activated enzyme inhibited by fluoride without MnCl₂ was stimulated by fluoride when 3 mM MnCl₂ was added. It might appear that MnCl₂ could prevent or reverse the inhibitory effects of fluoride and permit maximal expression of fluoride activation. The small effect of MnCl₂ on the enzyme previously incubated with fluoride could mean that even with extensive dilution before assay the concentration of fluoride remaining was sufficient to cause some inhibition. It should be noted, however, that 3 mM MnCl₂ also markedly increased the basal activity of the enzyme from control cells although it had little effect when isoproterenol was present or when choleragen-stimulated preparations were assayed without fluoride. Whether it acts to relieve fluoride inhibition or to enhance fluoride activation of the fat cell cyclase, MnCl₂ appears to have different effects on the two processes. The effect of MnCl₂ added to assays in the presence of fluoride was essentially immediate, and if it reflects reversal of fluoride inhibition, then this reversal, like the onset of fluoride inhibition, is rapid.

Although both activation and inhibition of adenylate cyclase can be produced over the same general range of fluoride concentrations, these two effects differ in certain characteristics. As reported by Harwood and Rodbell (11), activation does not occur at temperatures below 25°, but fluoride inhibition is demonstrable. Similarly, in our studies fluoride inhibition of the cyclase activated by isoproterenol, by choleragen, or by prior incubation with fluoride was unaffected by 1.5 mM pyrophosphate, which prevents fluoride activation of fat cell, muscle (8), and hepatic (7, 15) adenylate cyclase. In addition, from studies with the cyclase incubated with fluoride before assay it appears that inhibition may be largely reversed by removal of fluoride at a time when activation has become relatively irreversible. Whether the opposing, and seemingly under certain conditions separable, effects are the result of fluoride acting at a single site or are independent remains to be determined. It is clear that when assayed in the presence of fluoride the activity of the fat cell adenylate cyclase (and presumably also those of other cyclases) is a resultant of both its inhibitory and stimulatory effects.

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REFERENCES
Activation and Inhibition of Adenylate Cyclase by Fluoride

Figure captions:

Figure 1. Activity of adenylate cyclase previously incubated with fluoride. One portion of the particulate fraction was diluted 1:10 and samples (100 µg protein) were assayed without fluoride. Another portion was incubated for 20 min. at 37°C on 0.5 µM fluoride, and diluted samples (10-15 µg protein) were assayed without added fluoride.

Figure 2. Activation of adenylate cyclase during incubation with fluoride. Particulate fractions were incubated with or without fluoride. In the unincubated tissues, a 1:20 dilution of the particulate fraction samples (10-15 µg protein) were assayed without added fluoride.

Figure 3. Effect of fluoride concentration on activation produced by incubation on the adenylate cyclase. One portion of the particulate fraction was diluted 1:10. The particulate fraction samples (10-15 µg protein) were assayed with the indicated concentrations of fluoride (µM). Another portion was incubated for 3 min. in the presence of the indicated concentration of fluoride, and after dilution samples (10-15 µg protein) were assayed without added fluoride.

Figure 4. Effect of fluoride on adenylate cyclase activity. Samples (15 µg protein) were assayed with or without fluoride (0.5 µM) for 2 hr. Particulate fractions from control cells (10, 20 µg protein), and from cells exposed to phorbol (10, 50 µg protein), were assayed with fluoride as indicated.

Figure 5. Effect of fluoride on adenylate cyclase activity. Samples (150 µg protein) were assayed with or without fluoride (0.5 µM) for 2 hr. Particulate fractions from control cells (10, 20 µg protein), and from cells exposed to phorbol (10, 50 µg protein), were assayed with fluoride as indicated.

Figure 6. Effect of fluoride and cAMP phosphodiesterase on adenylate cyclase activity. Samples (15 µg protein) were assayed with or without fluoride (0.5 µM) for 2 hr. Particulate fractions from control cells (10, 20 µg protein), and from cells exposed to phorbol (10, 50 µg protein), were assayed with fluoride as indicated.

Figure 7. Effect of fluoride on adenylate cyclase activity. Samples (150 µg protein) were assayed with or without fluoride (0.5 µM) for 2 hr. Particulate fractions from control cells (10, 20 µg protein), and from cells exposed to phorbol (10, 50 µg protein), were assayed with fluoride as indicated.

Figure 8. Effect of fluoride on adenylate cyclase activity. Samples (150 µg protein) were assayed with or without fluoride (0.5 µM) for 2 hr. Particulate fractions from control cells (10, 20 µg protein), and from cells exposed to phorbol (10, 50 µg protein), were assayed with fluoride as indicated.

Figure 9. Effect of fluoride on adenylate cyclase activity. Samples (150 µg protein) were assayed with or without fluoride (0.5 µM) for 2 hr. Particulate fractions from control cells (10, 20 µg protein), and from cells exposed to phorbol (10, 50 µg protein), were assayed with fluoride as indicated.
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V C Manganiello and M Vaughan


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