ADENYLYL CYCLASE ACTIVATION BY HALIDE ANIONS OTHER THAN FLUORIDE

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SUMMARY

Adenylyl cyclase of rat liver and fat cells is activated by chloride, bromide, and iodide in addition to fluoride, previously believed to be uniquely effective among the halide anions. Liver homogenates are activated approximately 6 fold by fluoride while chloride and bromide increase cyclase by 3 fold and iodide about 2 fold. Optimal concentrations of chloride, bromide and iodide are about 100 times higher than those required for activation by fluoride. The cyclase of fat cell ghosts is activated some 9 fold by fluoride, but the other halide anions produced effects very similar in magnitude to those seen with liver, although for fat the optimally effective concentrations were lower. These observations appear to relate adenylate cyclase to a number of other anion activated enzymes, some of which have already been studied in pure form by a number of physico-chemical techniques, and which may serve as models for understanding the action of fluoride and other anions on adenylyl cyclase.

Activation of adenylyl cyclase by fluoride was first reported in 1958 by Rall and Sutherland (1). This effect has been thought to be specific for fluoride and not to occur with the other halides or other anions (2, 3). The mechanism of action of fluoride has remained obscure, in part at least because of the difficulties of studying the membrane-bound, insoluble enzyme and of obtaining sufficient amounts of purified solubilized material for study. The biochemical literature, however, contains many examples of anion activation of other enzymes; inhibition by anions is even more common. In no case is either anion activation or inhibition limited to a single anion. Moreover, fluoride is a known activator of several other enzymes. For these reasons it seemed to us unlikely that fluoride activation of adenylyl cyclase could be specific for this ion, and we therefore undertook to re-examine this issue.

MATERIALS AND METHODS

The tissues used for preparation of adenylate cyclase of liver and fat cells were obtained from a strain of outbred Wistar rats raised in our laboratory. Homogenates of livers were made from animals of either sex of ages 4-5 months (mean weight 480 grams, males; 260 grams, females). In each individual

assay the livers from three animals of the same sex were pooled in the preparation of the homogenate. Animals were rapidly rendered unconcious with carbon dioxide and the respective tissues excised. Pooled livers were homogenized in a Virtis Homogenizer (Model 45) for 3-5 seconds with 20 volumes of an ice cold solution containing 2 mM glycylglycine, 1 mM $MgSO_4$, 10 mM NaCl, 10 mM KCl, pH 7.4 (4). Liver adenylyl cyclase was assayed by the method of White and Zenser (5) with modification of the incubation medium (legend Fig. 1). Fat cell ghosts were prepared from epididymal fat pads of animals 6-7 months old (mean weight 550 grams) by a minor modification of the method of Harwood and Rodbell (6) in which 1 mM dithiothreitol replaces mercaptoethanol and assayed for adenylyl cyclase by the method of Salomon et al. (7). Protein determinations were performed according to the method of Lowry et al. (8) using bovine serum albumin as the standard. NaF, NaCl, NaBr, and NaI were analytical grade reagents from Fisher Scientific and Merck. The sodium salts were assayed for contaminating fluoride with the Amadec-F reagent (9). No salt contained more than 0.003 % fluoride. This corresponded to 0.018 mM in the maximally effective concentration of activating anion, an amount too small to account for any of the observations in terms of contamination by fluoride.

[^{14}C] cyclic AMP was added to the incubation medium to monitor possible phosphodiesterase destruction of the [^{32}P] cyclic AMP product while [^{3}H] cyclic AMP served to monitor column losses. No destruction of the reaction product was seen, i.e., recoveries of [^{3}H] and [^{14}C] cyclic AMP were essentially identical. The product produced by activation of liver adenylate cyclase with all anions tested was [^{32}P] cyclic AMP as shown by its complete destruction with 3', 5'-cyclic adenosine monophosphate phosphodiesterase (Sigma). [^{3}H] cyclic AMP was destroyed in parallel by such treatment. The presence of the anions did not increase the blanks.

RESULTS

The results of sodium halide activation of liver adenylyl cyclase are shown in Fig. 1. Although NaF produced the greatest activation with a 6 fold increase over the basal level (no added anion), NaCl gave a 3.3 fold increase in activity. Activation by NaBr was nearly as great, and NaI almost doubled the basal activity. The Cl^- curve was quite broad as compared with the relatively narrower range for Br^- and I^-. Fluoride, although active at about one-hundredth the concentration of the other halides, is comparable to the others in being effective over a wide range with decreasing activity at higher concentrations (12). Adenylyl cyclase from fat cell ghosts showed halide activation similar to that seen with the liver enzyme (Fig. 2). Compared with liver adenylyl cyclase the magnitude of the activations over basal (3 fold) were similar for Cl^-, Br^- and I^-. With the fat cell enzyme, however, fluoride resulted in greater (9 fold) activation over basal.

The previously reported lack of activation of heart muscle adenylyl cyclase with anions other than fluoride is possibly due to the use of low

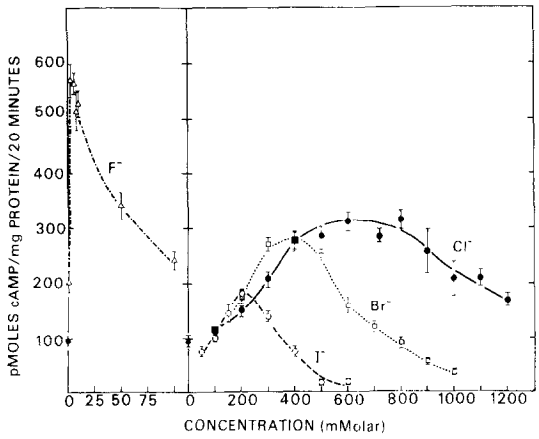

Fig. 1. Anion activation of liver adenylyle cyclase. Activation follows the order $F^- > Cl^- > Br^- > I^-$. Each point represents the mean ± S.E.M. of six experiments (no sex difference was apparent). Note the 4-fold expanded concentration scale for F^- relative to that for the other anions. Basal ■; F^- △ ; Cl^- ●; Br^- □; I^- ○. Enzyme assays were performed in duplicate (30° C; 20 minutes). Reactions were started by addition of 20 µl homogenate (110-150 µg protein) to 30 µl of assay medium. The final 50 µl mixture contained 5 mM $MgCl_2$; 20 mM Tris-HCl buffer, pH 7.6; 2 mM theophylline; 2 mM cyclic AMP; 1 mM ATP; 1 µC [α-^{32}P]ATP (10-15 C/mM) and 0.05 µC[^{3}H]cyclic AMP; 20 mM creatine phosphate; 1 mg/ml creatine phosphokinase; and the shown concentrations of salts which do not include the chloride content (40 mM) of the incubation mixture. Reactions were terminated by the addition of 100 µl of solution (pH 7.6) containing 35 mM sodium dodecyl sulfate, 45 mM ATP, and 11.5 mM cyclic AMP; followed by 50 µl containing 0.005 C[^{14}C]cyclic AMP and boiling for 5 minutes.

concentrations (8 mM) of the anions (3). In one study NaCl and LiCl (150-300 mM) were found to inhibit anti-diuretic hormone sensitive renal medullary adenylyl cyclase. Lower concentrations (25-150 mM) gave minimal stimulation, but this was not shown to be significant and was not attributed to the chloride ion (10). Such results indicate that the exact pattern of adenylyl cyclase activation by anions can be expected to vary from one tissue to another.

Since a variety of anions other than the halides are known to affect enzyme activity generally, we examined several of these anions with liver adenylyl cyclase. Sodium thiocyanate (10-1000 mM), trichloroacetate and trifluoroacetate

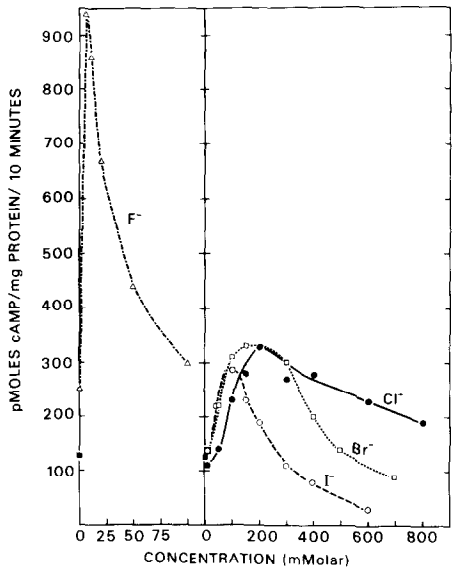

Fig. 2. Anion activation of fat cell adenylyle cyclase. The points are means of duplicate determinations from a representative experiment. Scales and symbols as in Fig. 1. Incubations were at 30° C for 10 minutes. Reactions were started by addition of 20 µl of fat cell ghosts (40-60 µg protein) to 30 µl incubation medium. The final 50 µl mixture contained 5 mM $MgCl_2$; 25 mM Tris-HCl buffer, pH 7.4; 2 mM cyclic AMP; 10 mM theophylline; 1.6 mM ATP; 1 µC[α-^{32}P]ATP (10-15 C/mM); 0.01 µC [^{3}H]cyclic AMP; 20 mM creatine phosphate; 1 mg/ml creatine phosphokinase; and the shown concentrations of salts. The assays were terminated as described in Fig. 1.

(1-1000 mM) did not produce appreciable activation of liver adenylyl cyclase above the basal level. The higher concentrations of all three ions were inhibitory. Iodate has been previously reported to inhibit heart muscle adenylyl cyclase (3). We also observed progressive inhibition by iodate (0.1-100 mM).

With liver homogenates combinations of an optimal amount of fluoride (5 mM) with maximally activating concentrations of Cl^- (600 mM), Br^- (400 mM), and I^- (200 mM) resulted in activations equal to or slightly below that expected with fluoride alone. Combined optimal concentrations of Cl^- with Br^-, Cl^- with I^- and Br^- with I^- gave activation below the levels of each of the individual components.

The lack of an additive effect between fluoride and the other anions suggests that all the anions act through a common mechanism.

A number of experiments were performed to evaluate the possibility that the results with NaCl, NaBr, and NaI might be due to a non-specific solute effect or to enhancement of contaminating fluoride by high concentrations of solute. Activation by fluoride was examined in the presence of a range of concentrations of sucrose, glycerol and mannitol including the highest which could be conveniently prepared. Sucrose (100-400 mM), glycerol (200-1200 mM and mannitol (50-200 mM) alone had no effect on basal liver adenylyl cyclase. Activation by fluoride (0.001-50 mM) was unaffected by up to 800 mM sucrose, 1200 mM glycerol, and 400 mM mannitol. No activations by fluoride were seen between 0.001-0.1 mM, the entire range of possible fluoride contamination. Moreover, 600 mM chloride did not enhance the activity of fluoride over a wide range of concentrations (0.001-50 mM).

With both liver homogenates and fat cell ghosts basal levels of cyclase were proportional to the amount of tissue protein used and linear with time. Fluoride-activated samples sometime showed acceleration of the reaction toward the end of the incubations. With liver, rates in the chloride-activated samples tended to slow after 10 minutes. Explanations for these deviations from linearity are not immediately apparent, but can only diminish rather than exaggerate the observed chloride (or other anion) activations. It is conceivable that high anion concentrations slow the reactions toward the end of the incubations by inhibiting the ATP-regenerating system. Possible decrease of ATPases by high concentrations of anions cannot explain the observed cyclase activations via increased ATP concentrations, since our values are increases above basal which are linear with time and are already exhibiting maximal rates with the ATP-regenerating system used. Further studies of the factors affecting the time course are underway, as are activations by other simple anions. Experiments in progress with adenylyl imidodiphosphate may circumvent possible complexities resulting from use of the ATP-regenerating system.

DISCUSSION

Anion activation of an enzyme was first recognized nearly a century ago when chloride was noted to stimulate the activity of α-amylase. Since that time numerous other examples have been recognized (e.g., 11-15). For at least one enzyme (yeast aldolase) fluoride is most effective. Recent studies of the mechanism of anion activation of α-amylase (16) and dopamine-β-hydroxylase (17) should be especially considered in relation to the anion activation of adenylyl cyclase. Dopamine-β-hydroxylase and adenylyl cyclase prove to be activated in remarkedly similar fashion over comparable concentration ranges. Dopamine-β-hydroxylase follows the order $Cl^- > Br^- > F^- > I^-$ with activation by F^- occurring at much higher concentration than needed for adenylyl cyclase. Although fluoride activation of dopamine-β-hydroxylase is about 3 fold, it is less than the 7-9 fold increase seen with Br^- and Cl^-. Anions other than chloride also activate α-amylase, but for that enzyme fluoride is only minimally effective.

The mechanism of activation of dopamine-β-hydroxylase involves binding of the anion to a specific cationic group adjacent to the enzyme's active site; binding of chloride to α-amylase has also been demonstrated. Anion-induced conformational change could not be detected by conventional techniques with either enzyme, but a subtle conformational change is suspected for α-amylase, since chloride increases the binding of calcium by the enzyme. Activation of dopamine-β-hydroxylase by Cl^- is not accompanied by subunit association or dissociation, and α-amylase is monomeric. Moreover, thiocyanate, a potent dissociator of protein subunits, fails to activate adenylyl cyclase. The possibility does remain, however, that the anions may yet prove to act by affecting the intermolecular relationships of components of the receptor-transducer-catalytic complex (2) of adenylyl cyclase. Anion promoted association reactions for example, are known, and in at least one such case fluoride is most effective among the halides (18).

The present results appear to relate cyclase to a number of other enzymes also showing activation by anions. If these other enzymes can

be considered to be models for cyclase, their study may help in understanding the mechanism of adenylyl cyclase activation by the halide anions.

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