Utilization of Radiometric Analysis for Measurement of Activation of Adenyl Cyclase by Sympathomimetic Amines

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Abstract □ A simple new assay utilizing radiolabeled adenosine triphosphate for the measurement of adenyl cyclase activity was developed. The effectiveness of various sympathomimetic amines in activating adenyl cyclase was measured by means of the new assay technique.

Keyphrases □ Sympathomimetic amines—adenyl cyclase activation □ Adenyl cyclase activity—analysis □ Radioactive ATP—cyclase activity analysis

Certain actions of epinephrine appear to be due to its ability to catalyze the conversion of adenosine triphosphate to cyclic adenosine-3′,5′-monophosphate in the presence of an enzyme system known as adenyl cyclase (1). Sutherland's isolation and characterization (2–5) of this enzyme system has provided means by which an action of sympathomimetic drugs may be observed directly without the complications of an *in vivo* study. The direct observation of drug effects may allow the delineation of more precise structure-activity relationships and a more exact definition of the receptor site.

Belleau (6) has evolved a mechanism by which sympathomimetic amines may catalyze the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (AMP). Bloom and Goldman (7) have elaborated on the mechanism and presented a three-dimensional drawing of the transition state. In order to substantiate more fully the proposed mechanism, the action of amines on the isolated enzyme system must be examined. The present study attempted the development of a simple, rapid assay, which could be used for the measurement of the effect of drugs on the adenyl cyclase enzyme system.

EXPERIMENTAL

Isolation of Adenyl Cyclase—The method of Sutherland *et al.* (2) was used for extraction and isolation of adenyl cyclase. Three beef brains were used and yielded (after fractionation of DEAE cellulose[1]) 76 mg. of protein in 190 ml. of solution. The adenyl cyclase activity in the presence of 0.01 *M* sodium fluoride was 1.58 units/mg. protein.[2]

Assay for Adenyl Cyclase Activity—The incubation buffer contained, per liter, the following: 0.744 g. magnesium sulfate, 2.318 g. caffeine, 0.716 g. sodium fluoride, and 8.674 g. of tris buffer, and the pH of the solution was adjusted to 7.4. The sodium fluoride was omitted when assaying for drug effects. An adenosine triphosphate (Na salt) solution was prepared containing 30 mg./ml. of ATP uniformly labeled[3] in all three phosphorus atoms with ^{32}P. The amount of labeled ATP solution used in later experiments was doubled to compensate for the decay of the phosphorus. A mixture consisting of 1.4 ml. of incubation buffer, 0.1 ml. of labeled ATP solution, and 1.0 ml. of the enzyme solution was shaken and incubated for 15 min. at 37°. The reaction was stopped by placing the reaction tubes in a boiling water bath for 3 min. An ion-exchange resin[4] (about 2 g.) and 0.1 ml. of 4 *N* KOH were added and the mixture was allowed to equilibrate for 30 min. to remove the organic phosphorus compounds. The pyrophosphate was determined by adding 0.1 ml. of the supernatant to 15 ml. of scintillation cocktail in a 20-ml. glass scintillation vial and counting with an ambient-temperature liquid scintillation counter,[5] set for a counting error of 1%. The scintillation cocktail consisted of 1 l. of spectroquality[6] *p*-dioxane, containing 80 g. of recrystallized naphthalene and 5 g. of the scintillator PPO.[7] The counts were corrected for efficiency by the use of an external standard, decay, the fact that only two of the originally tagged phosphorus atoms were counted, and for the counts

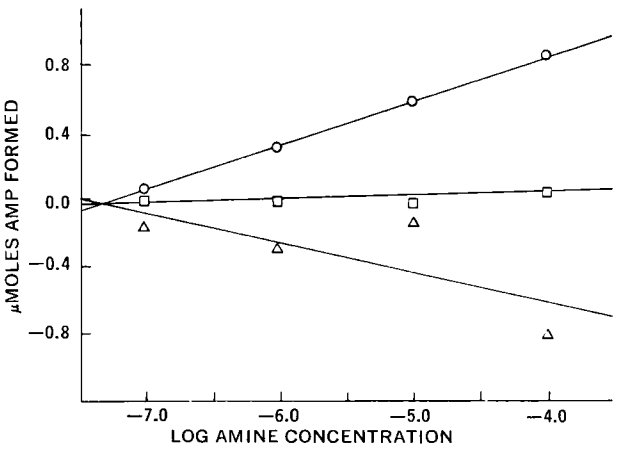

Figure 1—*Activation of adenyl cyclase by sympathomimetic amines modified in the side chain. Key:* ○, *epinephrine;* □, *adrenalone;* △, *epinine.*

[1] Mannex DEAE cellulose from Mann Biochemicals, New York, N. Y.

[2] One unit of cyclase activity is that amount which will form 1 μmole of cyclic AMP in 15 min.

[3] The labeled ATP, sodium salt in 50% ethanol, was purchased from Schwarz Bio Research, Orangeburg, N. Y., cat. no. 1462-07 (radiochemical purity greater than 97%). This material was used within 1 month from the time of manufacture. The ATP solution had a specific activity of 44.61 μc./ml. at the time of preparation.

[4] Amberlite 401 S.C.P., Rohm & Haas, Philadelphia, Pa., distributed by Mallinckrodt Chemical Works, St. Louis, Mo.

[5] Model LS-100, Beckman Instruments, Inc., Fullerton, Calif.

[6] Matheson, Coleman & Bell, Columbus, Ohio.

[7] 2,5-Diphenyloxazole, Pilot Chemicals, Inc., Watertown, Mass.

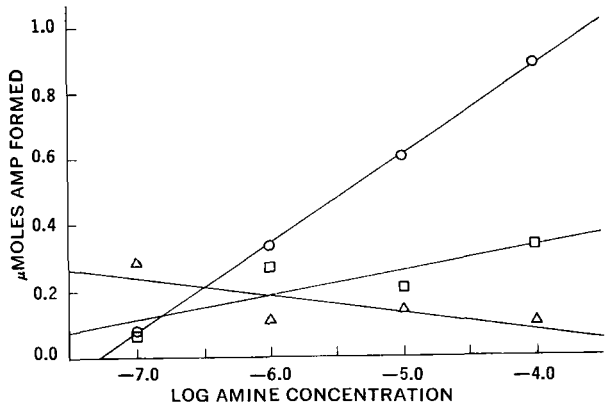

Figure 2—*Activation of adenyl cyclase by sympathomimetic amine modified in the aromatic ring. Key:* ○, *epinephrine;* □, p-*hydroxyphenylmethylamino ethanol;* △, m-*hydroxyphenylmethylamino ethanol.*

obtained in a blank assay without any enzyme present. The specific activity was used to obtain the number of micromoles of pyrophosphate formed in 15 min. (this would also be the number of micromoles of cyclic AMP formed). The precision of the analysis was checked by repeating the assay a number of times on a given enzyme solution and gave a standard deviation of 5%. The effect of the various sympathomimetic amines was determined by adding 50 μl. of an appropriate amine solution to give a final amine concentration of 10^{-7} to 10^{-3} *M*. As the enzyme preparation aged, the increase in activation by the amines began to fall off if the amine was present in a concentration above 10^{-4} *M* so only lower concentrations of amines were used. The relative effectiveness of the amines was determined by plotting the logarithm of the concentration of the drug *versus* the increase in the micromoles of cyclic AMP formed over a control which contained no activator. The log plot of epinephrine was linear with little deviation of the points from the lines, whereas other drugs exhibited more scatter. The series of amines were divided into two groups in which only one area of the molecule was varied: Fig. 1—epinephrine, adrenalone, and epinine—varied the substituent adjacent to the phenyl group; Fig. 2—epinephrine, *p*-hydroxyphenylmethylamino ethanol, and *m*-hydroxyphenylmethylamino ethanol—varied the substituents on the aromatic ring.

RESULTS AND DISCUSSION

The data from the series of amines including epinephrine, adrenalone, and epinine (Fig. 1) indicated the presence of β-hydroxy group was necessary for increased activation. When this secondary alcohol was replaced by a carbonyl group (adrenalone), no activation took place. If the secondary alcohol is replaced by a methylene group (epinine), the compound becomes an inhibitor. The previously reported (8) pD_2 values for these compounds for β-agonism predict the relative activities but do not predict the inhibitory action of epinine. A possible explanation for the importance of the β-hydroxy group, based on the model proposed by Bloom and Goldman (7), may be the reduction of the anionic charge by the formation of a hydrogen bond to one of the oxygen atoms on the phosphorus atom which the ribose hydroxyl group is about to attack. An alternate explanation may be that the β-hydroxy group is necessary in order for the complex to achieve the proper conformation for attack of the ribose hydroxyl group. Other interpretations are possible and more experimental evidence is needed before the exact role of this group is known.

Bloom and Goldman believe that the phenolic hydroxyl groups play an important role in binding the amine to the magnesium-ATP complex. The authors' data confirmed the need for both phenolic hydroxyl groups since considerable activation was lost when either one of the phenolic groups was removed as shown in Fig. 2. This effect is in agreement with pD_2 values for the amines (8) epinephrine 4.8, the *m*-hydroxyl analog 2.3, and for the *p*-hydroxyl analog 2.2.

The results of the determination of activation properties of sympathomimetic amines by measuring the effect on activation of adenyl cyclase are in general agreement with the pD_2 values obtained from guinea pig atrium preparations (8).

REFERENCES

(1) E. W. Sutherland and G. A. Robison, *Pharmacol. Rev.*, **18,** 145(1966).

(2) E. W. Sutherland, T. W. Rall, and T. Menon, *J. Biol. Chem.*, **237,** 1220(1962).

(3) T. W. Rall and E. W. Sutherland, *ibid.*, **237,** 1228(1962).

(4) F. Murad, Y. M. Chi, T. W. Rall, and E. W. Sutherland, *ibid.*, **237,** 1233(1962).

(5) L. M. Klainer, Y. M. Chi, W. L. Friedberg, T. W. Rall, and E. W. Sutherland, *ibid.*, **237,** 1239(1962).

(6) B. Belleau, *Pharmacol. Rev.*, **18,** 131(1966).

(7) B. B. Bloom and I. M. Goldman, *Adv. Drug Res.*, **3,** 121. (1966).

(8) E. J. Ariens, in "First International Pharmacological Meeting, Stockholm, 1961," vol. 7, Macmillan, New York, N. Y., 1963, p. 247.

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