Glucose-mediated Insulin Release: 3′,5′ cAMP Phosphodiesterase

Vyvyan Bowen, B.Sc. (Hons.), and Norman R. Lazarus, M.B., B.Ch., B.Sc., Ph.D.
Dartford, England

SUMMARY

Cyclic nucleotide phosphodiesterase from isolated mouse islets of Langerhans has been investigated and characterized. The enzyme has a pH optimum of 8.5 and is magnesium dependent, 10 mM. magnesium causing a fivefold increase in V max. Calcium is without effect on the enzyme. It is inhibited by theophylline and parachloromercuribenzoate. Glucose has no effect on the activity of the enzyme. Tolbutamide at concentrations equivalent to those found under clinical conditions is a noncompetitive inhibitor of the enzyme. DIABETES *22*:738-43, October, 1973.

Cyclic AMP has been implicated in the regulation of insulin release from mammalian pancreas.[1] Further investigation showed that potentiation of glucose-mediated insulin release by theophylline and glucagon was accompanied by a rise in intracellular cyclic AMP concentration.[2,3] Glucagon stimulates the adenylate cyclase enzyme.[4] Glucose does not.[4,5] It was of interest, therefore, to characterize the phosphodiesterase from mouse islets and to determine the effects of glucose on this enzyme both directly and indirectly.

MATERIALS AND METHODS

Reagents. Adenosine 3′,5′ cyclic phosphate (free acid) and Worthington crude collagenase were obtained from Cambrian Chemicals Limited, Beddington Farm Road, Croydon, CRO 4XB, England. D-glucose-6-phosphate disodium salt, and bovine serum albumin powder, fraction V, were obtained from the Sigma Chemical Company, St. Louis, Mo. Adenosine 5′ monophosphate was obtained from Boehringer Mannheim (London) Limited, Bilton House, Uxbridge Road, Ealing, London, W.5, and [3-H]adenosine 3′,5′ cyclic phosphate, ammonium salt (14 to 20 Ci per mmol) from the Radiochemical Centre, Amersham, Buckinghamshire, England. Aldrich Chemical Company Incorporated, Milwaukee, Wis. supplied 4-chloromercuribenzoic acid (sodium salt). Theophylline and tolbutamide were obtained from Burroughs Wellcome and Company, Temple Hill, Dartford, Kent, England, and N.E. 220 Liquid Scintillator from Nuclear Enterprises Limited, Sighthill, Edinburgh 11, Scotland. Gelman I.T.L.C. type silica gel impregnated glass fiber sheets were obtained from Gelman Hawksley, Lancing, Sussex, England. All other reagents were Analytical Reagent Grade purchased from BDH Chemicals Limited, Poole, Dorset, England.

Isolation procedure. All isolations of mouse islets of Langerhans were carried out in a bicarbonate buffered salt solution.[6] Before use the solution was gassed for ten minutes with oxygen and carbon dioxide (95:5). Islets of Langerhans were isolated as previously described.[7]

Preparation of soluble enzyme. All procedures were carried out at 4° C. Islets were homogenized in a small volume of 40 mM. Tris-HCl buffer, pH 7.6, and the resulting homogenate centrifuged at 30,000 g for fifteen minutes in a Sorvall RC2-B refrigerated centrifuge. After dialysis of the supernatant (20 mM. sodium chloride overnight), the nondiffusible material was centrifuged (10,000 g) and lyophilized (final protein concentration 4 mg. per milliliter). The solution was then used as described below.

Preparation of particulate enzyme. The islet homogenate obtained from 1,000 islets was centrifuged at 10,000 g for twenty minutes at 4° C. The supernatant was then centrifuged at 110,000 g for twenty minutes at 4° C. in an M.S.E. Superspeed 50 ultracentrifuge and the precipitate resuspended in 100 μl. of distilled water containing 0.4 per cent Triton X-100. This solution was then used in the enzyme assays.

Protein estimations were made by the method of Lowry et al.[8]

From the Diabetes Research Unit, The Wellcome Foundation, Temple Hill, Dartford, Kent, England.

Accepted for publication March 26, 1973.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/22/10/738/335952/22-10-738.pdf by guest on 18 September 2024

Assay of phosphodiesterase. The enzyme activity is assayed by measuring the formation of [3-H]5' AMP from [3-H]3',5' cAMP.[9] The method was found to be of sufficient sensitivity. All phosphodiesterase incubations were performed at 37° C. in the presence of 5 mM. 5' AMP for no longer than fifteen minutes.[10] The substances used ranged from 1 to 10 μCi per mmol. In all experiments the sampling procedure was the same; 20 μl. aliquots were withdrawn from the reaction vessel using a micropipette, spotted directly onto Whatman 3 MM. chromatography paper or Gelman I.T.L.C. chromatography medium and dried immediately with a hot air blower, the entire procedure taking less than fifteen seconds. The chromatographic separation was achieved by upwards elution in a propan-2-ol: 0.1 M. boric acid: .880 ammonia (7:2:1),[11] or 55:29:16 propan-2-ol: ethyl acetate: .880 ammonia,[12] which separates 5' AMP, 3' AMP and cyclic AMP. Spots were visualized under ultraviolet light, cut out and placed into scintillation vials. Then 1 ml. of water was added to each vial and the paper disc was shook to destruction. After 10 ml. of N.E. 220 dioxan-based liquid scintillator was added, the vial was shook further. All samples were counted in a Nuclear Enterprises 8312 scintillation spectrometer, corrections for quenching being made by the channels ratio method. All radioactivity added to the paper could be recovered by this method.

RESULTS

No difference in Km or magnesium dependence could be detected between freshly prepared enzyme and enzyme derived from islets stored at —20° C. for one week. Thus, either was used in the experiment described.

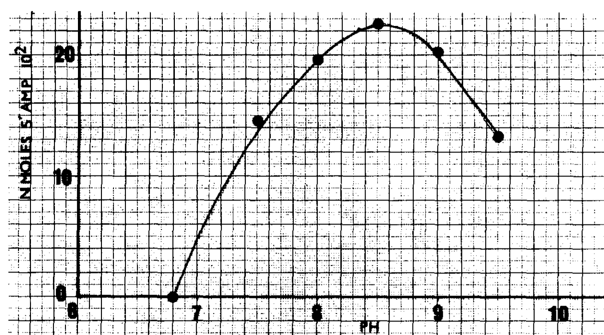

FIG. 1. Effect of pH on the activity of the enzyme. Aliquots of enzyme were incubated in Tris-HCl buffer pH 6.8 to 9.5 at 37° C. for ten minutes. Other procedures were as described under Materials and Methods.

Effect of pH. Figure 1 shows the effect of pH on mouse islet cyclic nucleotide phosphodiesterase. Aliquots of enzyme were incubated in Tris-HCl buffers containing 1 mM. cAMP and 10 mM. magnesium. The pH ranged from 6.8 to 9.5. The activity of the enzyme was sharply pH dependent, with a peak around 8.3. No 3-H-3' AMP was found in the reaction vessels after chromatographic separation of the products, identifying the sole product of reaction as 3-H-5' AMP.

Effect of Mg^{2+}. As can be seen from figure 2 the enzyme is active when magnesium is omitted from the incubation medium. Increasing the magnesium concentration up to 10 mM. leads to an increase in activity, reaching a maximum five times greater than the basal level.

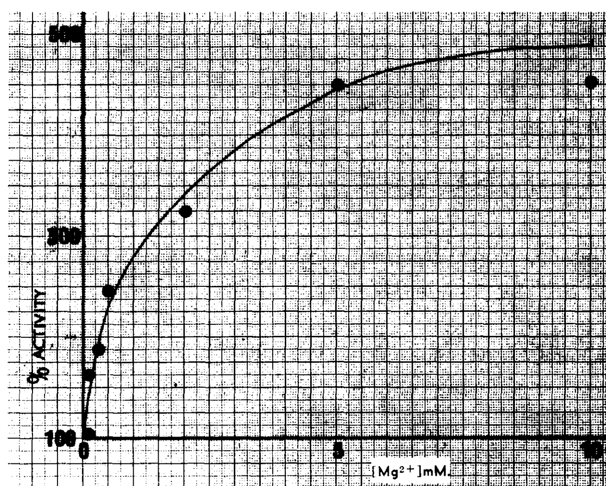

FIG. 2. Effect of magnesium concentration on the activity of the enzyme. A 10 μl. quantity of enzyme preparation was incubated for ten minutes at 37° C. in a total volume of 50 μl. of 1 mM. cAMP containing various concentrations of $MgSO_4$. Other procedures were as described under Materials and Methods.

Effect of substrate concentration. Figure 3 shows the effect of increasing substrate concentration, ranging from 1 to 10 x 10^{-5} M. cAMP, on the velocity of reaction. All incubations were performed in 100 mM. Tris-HCl buffer, pH 8.0, containing 10 mM. magnesium sulfate and 5 mM. adenosine 5' monophosphate. Sampling procedures were as described under Materials and Methods. An s/v vs. s[13] linear plot gives an apparent Km approximately 10^{-5} for cAMP. Reducing the magnesium concentration has no effect on apparent Km but does affect V max.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/22/10/738/335952/22-10-738.pdf by guest on 18 September 2024

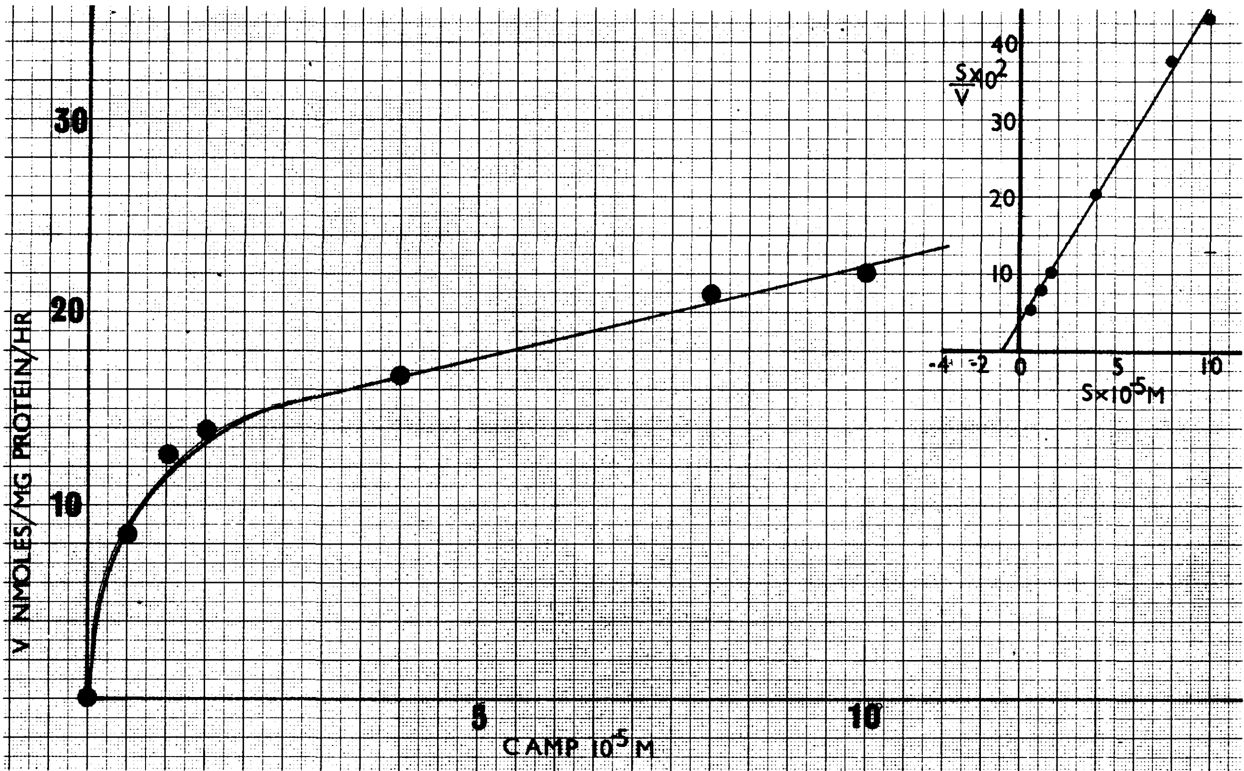

FIG. 3. Effect of substrate concentration. Aliquots of enzyme (about 0.4 mg.) were incubated in a total volume of 200 μl. of 100 mM. Tris-HCl buffer pH 8.0 at 37°C. containing various concentrations of cAMP. Other procedures were as described under Materials and Methods.

Effect of Ca^{++}. Since calcium is obligatory for insulin release,[14] the effect of calcium on islet cyclic nucleotide phosphodiesterase was determined. Varying the magnesium to calcium ratio from 10.0 to 1.0 has no effect on the activity of the enzyme.

Effect of theophylline. The enzyme was incubated at 37° C. in 100 M. Tris-HCl buffer pH 8.0 containing 10 mM. $MgSO_4$ and 5 mM. 5′ AMP with various concentrations of theophylline. The total volume of the reaction mixture was 40 μl. Other details are described under Materials and Methods. Figure 4 shows that the enzyme was 50 per cent inhibited by 10 mM. theophylline.

Dependence on thiol groups. As can be seen from figure 5 the activity of the enzyme is over 90 per cent inhibited after five minutes by the addition of 0.5 x 10^{-4} M. parachloromercuribenzoate.

Effect of glucose on phosphodiesterase activity. Addition of glucose or glucose-6-phosphate to the incubation medium during the assay of the enzyme had no

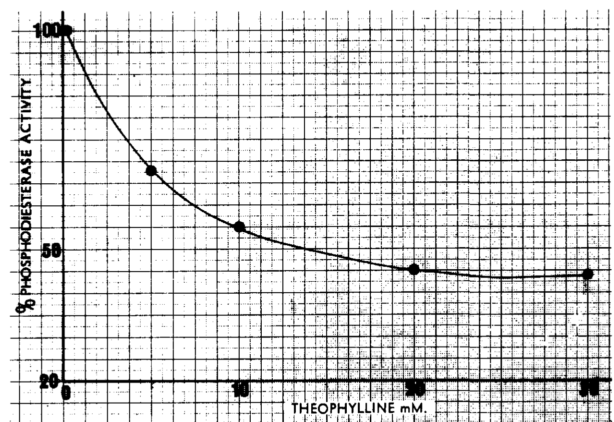

FIG. 4. Effect of theophylline. Aliquots of enzyme were incubated for ten minutes at 37° C. in 100 mM. Tris-HCl buffer pH 8.0 containing 1 mM. cAMP, with various concentrations of theophylline. Other procedures were as described under Materials and Methods.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/22/10/738/335952/22-10-738.pdf by guest on 18 September 2024

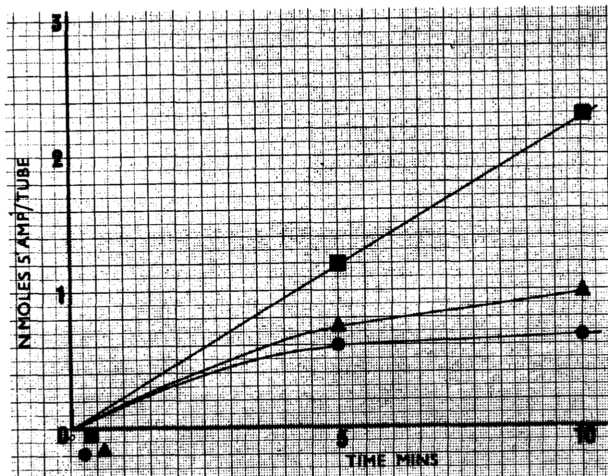

FIG. 5. Dependence on thiol groups. Aliquots of enzyme were incubated at 37° C. in 100 mM. Tris-HCl buffer pH 8.0 containing various concentrations of parachloromercuribenzoate. (■ 0 mM.; ▲ 0.05 x 10^{-4} M.; ● 0.5 x 10^{-4} M.).

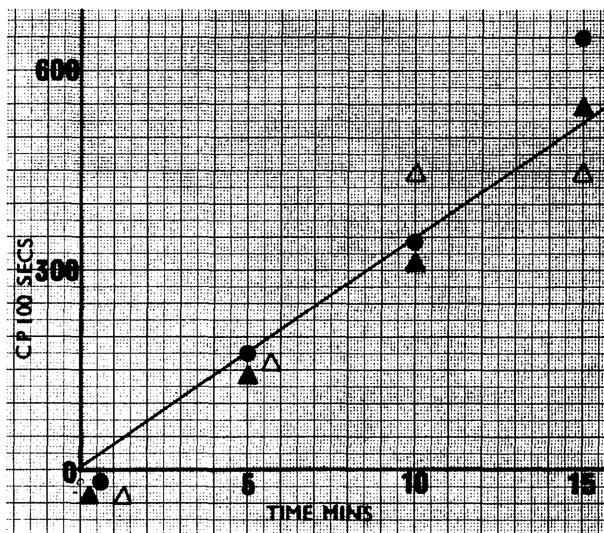

FIG. 6. Effect of incubation of islets in glucose on phosphodiesterase activity. Groups of 500 islets were incubated in 2 mM. glucose for five minutes (▲) or fifteen minutes (△) or in 17 mM. glucose for five minutes (●). Islets were harvested and the phosphodiesterase prepared. The enzyme was then assayed by incubation in 100 mM. Tris-HCl buffer pH 8.0 containing 1 mM. cAMP at 37° C.

effect on its activity. Groups of 500 islets were incubated in the presence of 2 mM. and 17 mM. glucose for five minutes. The islets were harvested and the enzyme prepared and assayed as described under Materials and Methods. The results (figure 6) show that incubation of islets in 2 mM. or 17 mM. glucose has no effect on phosphodiesterase activity. When a heated (60° C.) enzyme preparation (no dialysis step) from islets exposed to 17 mM. glucose was added to enzyme obtained from nonincubated islets at an equivalent concentration (10 μl. of 100 μg. per milliliter protein), there was no effect on enzyme activity.

Effect of tolbutamide. As can be seen from figure 7, tolbutamide at a concentration equivalent to that found under clinical conditions inhibits the phosphodiesterase. From the v/s vs. V plot presented it can be seen that the inhibition is of a noncompetitive nature.

Particulate enzyme. A comparison between the 'particulate' and the soluble phosphodiesterase did not show any difference between the two enzymes. Both have an apparent Km for cAMP of approximately 1.0 x 10^{-5}M.

DISCUSSION

The cAMP phosphodiesterase from isolated mouse islets of Langerhans appears to be very similar to those described in other tissues. It survives storage at —20° C. for several weeks with no apparent loss of activity. Two types of enzyme exist in islet tissue; the particulate and the soluble. As far as Km for cAMP and inhibition by theophylline are concerned, the two enzymes behave in a similar manner. Other investigators[15] have found the existence of a soluble and particulate phosphodiesterase with apparently identical properties in

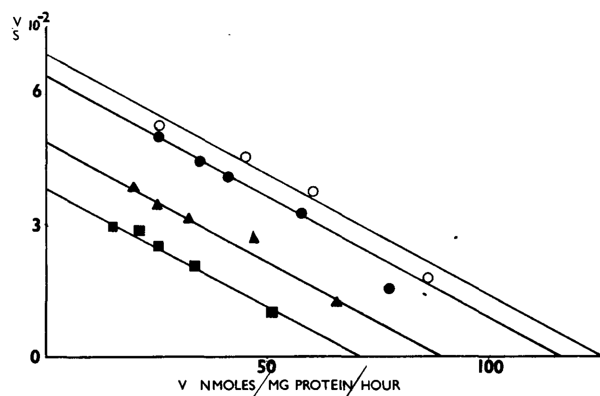

FIG. 7. Effect of tolbutamide. Aliquots of enzyme were incubated with a range of substrate concentrations (0.05 to 0.5 x 10^{-4} M. cAMP) containing various concentrations of tolbutamide (O 0 mM.; ● 0.5 mM.; ▲ 5 mM.; ■ 10 mM.).

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/22/10/738/335952/22-10-738.pdf by guest on 18 September 2024

other tissues, and it was thought justifiable to consider them as one enzyme in the experiments described. As with other phosphodiesterases, the enzyme is strongly inhibited by p-chloromercuribenzoate, showing that the enzyme is dependent on sulfhydryl groups for catalytic activity. The thiol groups involved may or may not be at the active site of the molecule. The pH optimum of the phosphodiesterase is approximately 8.5, and the enzyme is dependent on magnesium for the exhibition of full activity, while calcium is without effect. The apparent Km for cAMP is approximately 10^{-5} M. Enzymes with more than one Km have been reported[15] when two different ranges of substrate differing by three orders of magnitude (10^{-3} M. and 10^{-6} M.) have been used in the determination. In the experiments described, however, only one range of substrate concentrations was utilized (10^{-5} M.). The enzyme is 50 per cent inhibited by 10 mM. theophylline. The methyl xanthines are poor inhibitors of cyclic nucleotide phosphodiesterases, and there are several synthetic compounds which are far more potent.[16]

The intracellular level of cAMP may be controlled both by its rate of formation and rate of hydrolysis. Phosphodiesterase inhibitors potentiate glucose-mediated insulin release. This shows that the intracellular concentration of cAMP does play a role in the release of insulin from the beta cell.[1] Montague and Cook[17] have suggested, however, that glucose does not cause insulin release by increasing the beta cell cAMP concentration. Atkins and Matty[4] and Davis and Lazarus[5] have further shown that glucose has no effect on islet adenyl cyclase activity. Also, glucose has no direct effect[18] nor indirect effect on the activity of the cyclic nucleotide phosphodiesterase.

Since heat-stable modifiers of phosphodiesterase activity are known to exist,[19,20] the addition of heated supernatant obtained from islets exposed to 17 mM. glucose to enzyme from nonincubated islets was tested. There was no effect. Although this is not conclusive, it does suggest that glucose does not give rise to any stable modifier of phosphodiesterase activity. Thus it is concluded that glucose-mediated insulin release is not caused via the cyclic adenylate system.

Tolbutamide is shown to be a noncompetitive inhibitor of the cyclic nucleotide phosphodiesterase, in agreement with the observations of Rosen et al.[21] and Chaudhuri and Winer.[22] Goldfine et al.[23] reported competitive inhibition of the phosphodiesterase enzyme by tolbutamide, which is surprising in view of the dissimilarity between cyclic AMP and tolbutamide. Tolbutamide in a perfused system causes mainly first phase insulin release,[24] and it is possible that this release is mediated both by modulation of the cyclic nucleotide phosphodiesterase and by stimulation of the cyclase system.

REFERENCES

[1] Malaisse, W. J., Malaisse-Lagae, F., and Mayhew, D.: A possible role for the adenyl cyclase system in insulin secretion. J. Clin. Invest. *46*:1724-34, 1967.

[2] Turtle, J. R., Littleton, G. K., and Kipnis, D. M.: Stimulation of insulin secretion by theophylline. Nature *213*:727-28, 1967.

[3] Turtle, J. R., and Kipnis, D. M.: An adrenergic receptor mechanism for the control of cyclic 3'5' adenosine monophosphate synthesis in tissues. Biochem. Biophys. Res. Commun. *28*:7979-8002, 1967.

[4] Davis, B., and Lazarus, N. R.: Glucose mediated insulin release from mouse islets of Langerhans: adenylate cyclase. Biochem. J. *128*:96-7P, 1972.

[5] Atkins, T., and Matty, A. J.: Adenyl cyclase and phosphodiesterase activity in the isolated islets of Langerhans of obese mice and their lean littermates. The effect of glucose, adrenaline and drugs on adenyl cyclase activity. J. Endocrinol. *51*:67-78, 1971.

[6] Gey, G. O., and Gey, M. K.: The maintenance of human normal cells and tumor cells in continuous culture. I. Preliminary report: cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation. Am. J. Cancer *27*:45-76, 1936.

[7] Davis, B., and Lazarus, N. R.: Insulin release from mouse islets: effect of glucose and hormones on adenylate cyclase. Biochem. J. *129*:373-79, 1972.

[8] Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, P. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*:265-75, 1951.

[9] Cheung, N. Y.: Properties of cyclic 3'5' nucleotide phosphodiesterase from rat brain. Biochemistry *6*:1079-87, 1967.

[10] Gulyassay, P. F., and Oken, R. L.: Assay of cyclic 3'5' nucleotide phosphodiesterase in tissue homogenates. Proc. Soc. Exp. Biol. Med. *137*:361-65, 1971.

[11] Drummond, G. I., and Perrott-Yee, S.: Enzymatic hydrolysis of adenosine 3'5' phosphoric acid. J. Biol. Chem. *236*:1126-29, 1961.

[12] Woods, W. D., and Waitzman, M. B.: Isolation of cyclic 3',5'-adenosine monophosphate on a neutral silicic acid-glass microfiber matrix. J. Chromatog. *47*:536-42, 1970.

[13] Hanes, C. S.: Studies on plant amylases. I. The effect of hydrolysis by the amylase of germinated barley. Biochem. J. *26*:1406-21, 1932.

[14] Milner, R. D. G., and Hales, C. N.: The role of calcium and magnesium in insulin secretion from rabbit pancreas studied in vitro. Diabetologia *3*:47-49, 1967.

[15] Cheung, W. Y.: Role of cAMP in cell function. *In* Advances in Biochemical Pharmacology, vol. 3, Greengard, P., and Costa, E., editors. New York, Raven Press, 1970, pp. 51-65.

[16] Sheppard, H., and Wiggan, C.: Analogues of 4-(3,4-dimethoxybenzyl)-2 imidazolidinone as potent inhibitors of

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/22/10/738/335952/22-10-738.pdf by guest on 18 September 2024

rat erythrocyte adenosine 3'5' phosphate phosphodiesterase. Mol. Pharmacol. 7:111-15, 1971.

[17] Montague, W., and Cook, J. R.: The role of adenosine 3':5'-cyclic monophosphate in the regulation of insulin release by isolated rat islets of Langerhans. Biochem. J. *122*: 115-20, 1971.

[18] Ashcroft, S. J. H., Randle, P. J., and Taljedal, I-B.: Cyclic nucleotide phosphodiesterase activity in normal mouse pancreatic islets. FEBS Letters *20*:263-66, 1972.

[19] Goren, E. N., and Rosen, O. M.: The effect of nucleotides and a nondialyzable factor on the hydrolysis of cyclic AMP by a cyclic nucleotide phosphodiesterase from beef heart. Arch. Biochem. Biophys. *142*:720-23, 1971.

[20] Cheung, W. Y.: Cyclic nucleotide phosphodiesterase, evidence for and properties of a protein activator. J. Biol. Chem. *246*:2859-69, 1971.

[21] Rosen, O. M., Hirsch, A. H., and Goren, E. N.: Factors which influence cyclic AMP formation and degradation in an islet cell tumor of the Syrian hamster. Arch. Biochem. Biophys. *146*:660-63, 1971.

[22] Chaudhuri, T. K., and Winer, N.: Effect of chlorpropamide on renal phosphodiesterase. J. Lab. Clin. Med. *76*: 863, 1970.

[23] Goldfine, I. D., Perlman, S., and Roth, J.: Inhibition of cyclic 3'5' AMP phosphodiesterase in islet cells and other tissues by tolbutamide. Nature *234*:295, 1971.

[24] Grodsky, G. M., Bennett, L. L., Smith, D., and Nemechek, K.: The effect of tolbutamide and glucose on the timed release of insulin from the isolated perfused pancreas. Tolbutamide after 10 years. Proc. Brook Lodge Symposium, Butterfield, W. J. H., and Van Westerling, W., editors, 1967, pp. 11-21.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/22/10/738/335952/22-10-738.pdf by guest on 18 September 2024