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Alpha$_2$-Adrenergic Inhibition of Insulin Secretion via Interference with Cyclic AMP Generation in Rat Pancreatic Islets

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SUMMARY

Glucose-induced insulin secretion and cyclic AMP accumulation in isolated rat pancreatic islets as well as GTP-activated adenylate cyclase of the membrane-rich preparation from the islets were strongly inhibited by some *alpha*-adrenergic agonists. The relative potencies of the agonists, estimated according to their dose-dependent actions, were in such an order that clonidine > epinephrine (≃ norepinephrine) ≫ phenylephrine ≃ methoxamine, regardless of which of the three parameters (i.e., insulin release, cyclic AMP accumulation, and adenylate cyclase activity) was used for estimation. There was a highly significant correlation between the amounts of cyclic AMP accumulation and the rate of insulin release that were changed in response to these agonists. The order of the potencies of *alpha*-adrenergic antagonists to reverse epinephrine inhibition of these parameters was invariably yohimbine ≃ dihydroergotamine ≃ phenylephrine ≫ prazosin. In conclusion, the rat islet cell membrane is equipped with *alpha*$_2$-adrenoceptors which are linked to adenylate cyclase to cause diminution of the cellular content of cyclic AMP; insulin secretion may be inhibited consequently.

INTRODUCTION

Stimulation of *alpha*-adrenoceptors results in diminution of the cyclic AMP content or attenuation of its increase due to stimulating agents in various cells (see ref. 1 for review). The decrease in the cellular cyclic AMP content was found to be associated with suppression of cyclic AMP-mediated cellular functions and has been accounted for by *alpha*-adrenergic inhibition of membrane adenylate cyclase in the majority of these cells (1). Experimental evidence has been presented for involvement of *alpha*$_2$-adrenoceptors in the inhibition of adenylate cyclase of human platelets (2), hamster adipocytes (3), and mouse neuroblastoma × rat glioma hybrid cells (4), leading to a hypothesis (4, 5) that *alpha*$_2$-receptors may mediate inhibition of adenylate cyclase in a variety of cell types.

The adenylate cyclase-cyclic AMP system plays an important role in the control of pancreatic insulin secretion (6). Epinephrine at concentrations of 0.5–150 ng/ml caused a strong and concentration-dependent inhibition of cyclic AMP accumulation in islet cells in parallel with suppression of insulin release (7). Adenylate cyclase of the islet cell membrane was also inhibited by a similar concentration range of epinephrine (8). These actions of the catecholamine were efficiently antagonized by phentolamine (7, 8), making it plausible to assume, in light of the above-mentioned hypothesis, that *alpha*$_2$-adrenoceptors may exist on the membrane of rat islet cells. Indeed, it has been briefly reported that yohimbine was more potent than prazosin in reversing epinephrine-induced inhibition of pancreatic insulin secretion (9). The purpose of the present paper is to show an involvement of *alpha*$_2$-receptors in the inhibition of the adenylate cyclase-cyclic AMP system that may be responsible for the control of insulin release from isolated rat islets.

MATERIALS AND METHODS

Materials. Aprotinin and rat insulin (Novo Laboratories) were gifts from Hoechst Japan Ltd. (Tokyo) and Kodama Ltd. (Tokyo), respectively. Reagents for microradioimmunoassay of cyclic AMP, i.e., ^{125}I-labeled cyclic AMP tyrosine methyl ester, anti-cyclic AMP antiserum, and cyclic AMP, were obtained from a Yamasa cyclic AMP assay kit which was a gift from Yamasa Shoyu Company (Chiba). Clonidine, methoxamine, E643,[1] and propranolol were generously provided by Japan C. H. Boehringer & Sons (Osaka), Nippon Shinyaku Company (Osaka), Eisai Company (Tokyo), and Otsuka Pharmaceutical Company (Tokushima), respectively. Prazosin was synthesized by Dr. O. Yonemitsu, of the Faculty of Pharmaceutical Sciences, Hokkaido University.

Commercial sources of other chemicals were as follows: collagenase (Type 4), Worthington Biochemical Company (Freehold, N. J.); IBMX, Aldrich Chemical Com-

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[1] The abbreviations used are: E643, 2-[4-(*n*-butyryl)homopiperazine-1-yl]-4-amino-6,7-dimethoxyquinazoline; IBMX, 3-isobutyl-1-methylxanthine; KIU, kallikrein inhibitory units.

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pany (Milwaukee, Wisc.); fetal bovine serum, Flow Laboratories; minimal essential medium with Earle's salts, Dainippon Seiyaku Tissue Culture Center (Osaka); ^{125}I-labeled insulin, Dainabot Radioisotope Laboratories (Tokyo); epinephrine, E. Merck (Darmstadt, Germany); phentolamine, Ciba-Geigy-Japan (Tokyo); phenoxybenzamine and dibenamine, Nakarai Chemical Company (Kyoto); bovine serum albumin (Fraction V), dextran (clinical grade, average molecular weight 80,700), norepinephrine, phenylephrine, yohimbine, dihydroergotamine, GTP, creatine phosphate, and creatine phosphokinase, Sigma Chemical Company (St. Louis, Mo.). ATP was also a Sigma product (Catalog No. A2383) prepared by phosphorylation of adenosine. Other reagents used were of analytical grade.

Preparation, culture, and incubation of pancreatic islets. Pancreatic islets were isolated by the collagenase technique from male Wistar rats weighing 200–250 g as described elsewhere (7). Where indicated, 10–50 islets were maintained in a sterile plastic Petri dish (51 mm in diameter) containing 4 ml of the culture medium which consisted of minimal essential medium (with Earle's salts) supplemented with 10% heat-inactivated fetal calf serum, antibiotics (100 IU of sodium penicillin G and 100 μg of streptomycin sulfate per milliliter), 7 mM glucose, aprotinin (200 KIU/ml), and 1 mM ascorbic acid. The dish was placed for 2 hr in a CO_2 incubator gassed with air containing 5% CO_2 at 37°.

Islets, freshly isolated or immediately after 2-hr maintenance in culture as described above, were thoroughly washed and then incubated for 30 min in 200 μl of Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 5.5 mM glucose, 0.5% bovine serum albumin, aprotinin (200 KIU/ml), and 1 mM ascorbic acid. After this preincubation period, islets were further incubated in a plastic tube containing 200 μl of fresh medium of the same composition further supplemented with 0.5 mM IBMX, glucose, and adrenergic agents to study their insulin secretory and cyclic AMP responses. Both incubations were carried out with shaking at 120 strokes/min at 37° under a gas mixture of 95% O_2 and 5% CO_2.

Insulin and cyclic AMP determinations. After incubation of islets, an aliquot (20 μl) of the incubation medium was assayed for insulin content by the radioimmunochemical method described earlier (10), using rat insulin as the standard. Cyclic AMP was measured as described earlier (7) by the method of Honma *et al.* (11).

Preparation of the membrane-rich fraction from islets and assay of its adenylate cyclase activity. The crude membrane preparation was prepared from islets cultured for 2 hr as described previously (8); the protein content of this preparation was about 0.25 mg/ml. The adenylate cyclase activity of this preparation was measured by following the synthesis of cyclic AMP from nonradioactive ATP in the presence of an ATP-regenerating system as described elsewhere (8).

RESULTS

Enhanced insulin secretory and cyclic AMP responses to glucose of islets cultured for 2 hr. Figure 1 shows insulin release and cyclic AMP accumulation in islets which were incubated for 30 min with various concentrations of glucose. More insulin was released from, and more cyclic AMP accumulated in, islets that had been maintained in culture for 2 hr than noncultured islets, especially in the presence of glucose at concentrations higher than 10 mM. Thus, the glucose-induced increments in insulin release and cyclic AMP were much larger after culture than before culture. Since it was these increments that were susceptible to the inhibitory action of *alpha*-adrenergic agonists, the magnitude of the adrenergic effects was larger in cultured islets than in noncultured islets. For this reason, islets cultured for 2 hr were used instead of fresh islets in the subsequent experiments.

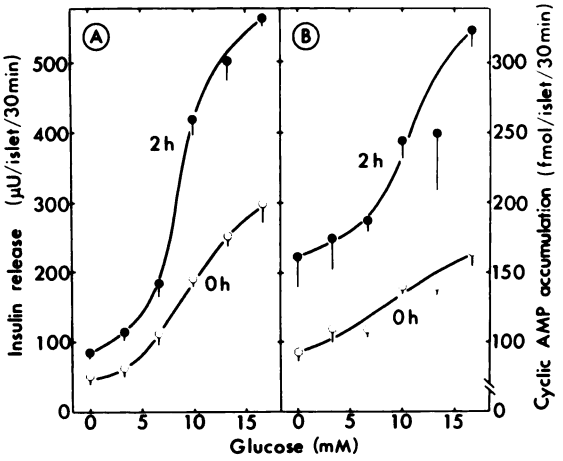

FIG. 1. *Insulin release (A) and cyclic AMP accumulation (B) in response to glucose during incubation of pancreatic islets*

Pancreatic islets maintained in culture for 2 hr (●) or 0 hr [i.e., the fresh islets (○)] were incubated for 30 min in the medium supplemented with glucose at concentrations indicated. The data are means ± standard error of the mean from four different batches of islets.

Inhibition of insulin release and cyclic AMP accumulation by alpha-adrenergic agonists. Alpha-adrenergic agonists added to the incubation medium caused a dose-dependent inhibition of glucose-induced insulin release and cyclic AMP accumulation in islets cultured for 2 hr (Fig. 2). Epinephrine and norepinephrine were roughly equipotent full agonists. Clonidine, an *alpha*$_2$-agonist, was more potent whereas phenylephrine and methoxamine, *alpha*$_1$-agonists, were less potent than epinephrine or norepinephrine, although the intrinsic activities of these *alpha*$_1$- or *alpha*$_2$-selective agonists were less than those of the nonselective physiological neurotransmitters. This order of potency and efficacy was the same whether the insulin data or cyclic AMP data were used as the indices.

In Fig. 3 are plotted the insulin data in Fig. 2A as a function of the corresponding cyclic AMP data in Fig. 2B. The linear plot (the line is not illustrated in Fig. 3) intersects the abscissa at the 25–30% point, which reflects the basal cellular content of cyclic AMP that is not susceptible to adrenergic inhibition. The degrees of the inhibition of insulin release from islets as caused by various agonists at their various concentrations were strictly correlated with the degrees of decline in their own cyclic AMP contents. The correlation coefficient, r, was 0.95 and highly significant ($p < 0.001$).

Reversal by alpha-adrenergic antagonists of epineph-

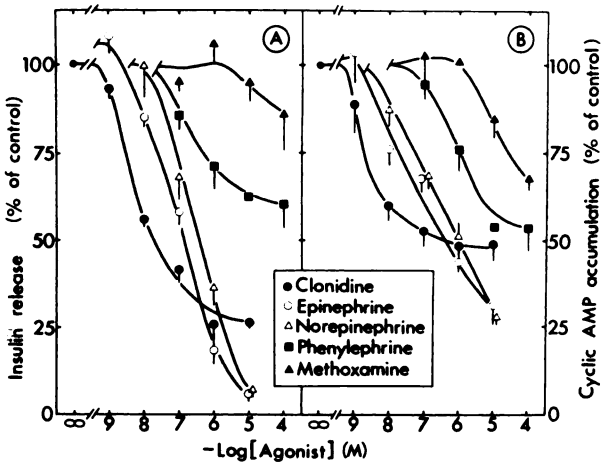

FIG. 2. *Inhibition of insulin release and cyclic AMP accumulation as a function of concentrations of alpha-adrenergic agonists*

Islets maintained in culture for 2 hr were incubated for 30 min in the medium supplemented with 16.5 mM glucose and *alpha*-adrenergic agonists at concentrations indicated on the abscissa. Insulin release (A) and cyclic AMP (B) are shown as percentes of control values obtained in the absence of any agonist. The data are means ± standard error of the mean from three to six different batches of islets.

rine-induced inhibition of insulin secretion and cyclic AMP accumulation. As is shown by *open symbols* in Fig. 4, *alpha*-adrenolytic agents were essentially without effect on insulin release and cyclic AMP accumulation unless they were added together with adrenergic agonists. However, when added to islets in the presence of epinephrine, yohimbine, an *alpha*$_2$-antagonist, and phentolamine, a nonselective *alpha*-antagonist, caused a dose-dependent reversal of epinephrine-induced inhibition of insulin release and cyclic AMP accumulation (Fig. 4). In contrast, prazosin, an *alpha*$_1$-antagonist, was ineffective in epinephrine inhibition even at the highest concentration used.

The effects of other *alpha*-adrenolytic agents on epinephrine inhibition of insulin release and cyclic AMP generation are illustrated in Fig. 5. Dihydroergotamine

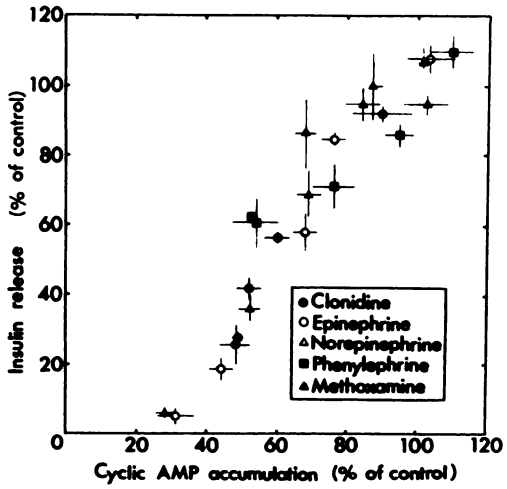

FIG. 3. *Correlation of insulin release and cyclic AMP accumulation in islets in the presence of various concentrations of alpha-adrenergic agonists*

Insulin release from a batch of islets is plotted against the cyclic AMP content based on the data in Fig. 2.

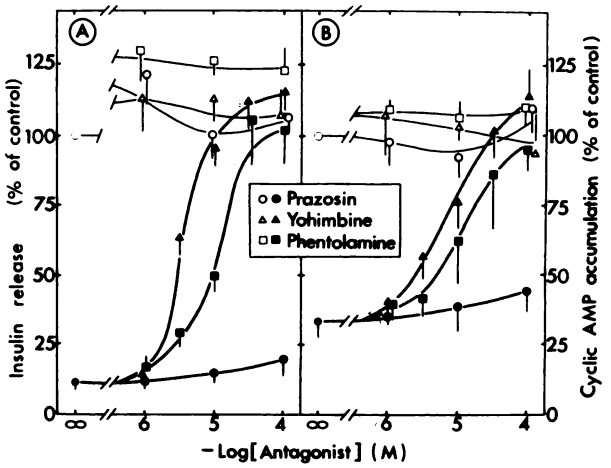

FIG. 4. *Alpha*$_2$*-adrenoceptor-mediated reversal of epinephrine-induced inhibition of insulin release and cyclic AMP accumulation*

Islets maintained in culture for 2 hr were further incubated in the medium supplemented with 16.5 mM glucose in the presence (●) or absence (○) of 10 μM epinephrine. *Alpha*-adrenergic antagonists were also added at concentrations indicated. Insulin release (A) and cyclic AMP (B) are shown as percentes of the control value obtained when neither epinephrine nor *alpha*-antagonists were included in the medium. The data are means ± standard error of the mean from three or four different batches of islets.

was as potent as, but phenoxybenzamine and tolazoline were less potent than, yohimbine in reversing the epinephrine inhibition. E643 and dibenamine were similar to prazosin in that they were very weak antagonists of the epinephrine action. The order of potency or efficacy among the agonists or antagonists that exerted strong influences on insulin release was essentially the same as the order determined on the basis of their effects on cyclic AMP accumulation. However, there was some

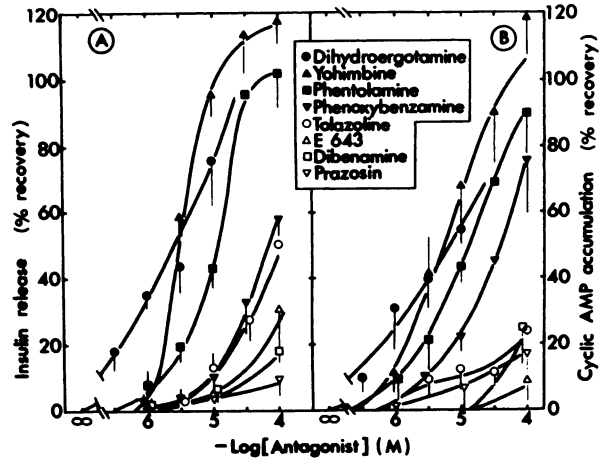

FIG. 5. *Reversal of epinephrine-induced inhibition of insulin release and cyclic AMP accumulation by various alpha-adrenergic antagonists*

Islets maintained in culture for 2 hr were further incubated in the medium supplemented with 16.5 mM glucose and 10 μM epinephrine. *Alpha*-adrenergic antagonists were added at concentrations as indicated. The increments in insulin release (A) or cyclic AMP (B) caused by an *alpha*-antagonist in the presence of epinephrine are shown as percentes of the difference between the value obtained with epinephrine alone and the value obtained with the antagonist alone. The data are means ± standard error of the mean from three to six different batches of islets.

disparity between the insulin data and cyclic AMP data as to the action of weak antagonists such as tolazoline and E643 (Fig. 5). As a whole, the data in Figs. 2, 4, and 5 indicate that *alpha*$_2$-adrenoceptors are responsible for catecholamine-induced inhibition of insulin secretion and decrease of the intracellular accumulation of cyclic AMP.

Inhibition of adenylate cyclase by alpha-adrenergic agonists and its reversal by alpha-antagonists. As has been reported for adenylate cyclase from other mammalian tissues (1), GTP is strictly required for islet membrane adenylate cyclase to be susceptible to epinephrine inhibition. In the presence of 10 μM GTP, epinephrine and norepinephrine caused a dose-dependent inhibition of adenylate cyclase (Fig. 6). Clonidine was more potent, and methoxamine and phenylephrine were much less potent, than epinephrine or norepinephrine (Fig. 6). The order of the intrinsic activity, epinephrine (norepinephrine) > clonidine > phenylephrine, was the same as the order observed when the effects of these agonists were studied with intact islet cells (Fig. 2).

The effects of antagonists are shown in Fig. 7. No antagonist tested exerted a significant influence on adenylate cyclase activity when it was added alone with the exception of dihydroergotamine, which was slightly inhibitory at concentrations higher than 0.1 μM (Fig. 7C). However, when added together with epinephrine, phentolamine, yohimbine, and dihydroergotamine diminished epinephrine-induced inhibition in a dose-dependent manner. In contrast, prazosin and E643, *alpha*$_1$-adrenolytic agents, were not capable of abolishing the epinephrine inhibition. As had been expected, propranolol was without effect on the action of epinephrine.

Apparent dissociation constants for the putative agonist-receptor or antagonist-receptor complexes as measured by their action on insulin release, cyclic AMP accumulation, and adenylate cyclase. A dose-response relationship for agonist inhibition of receptor-mediated cell functions (Figs. 2 and 6) affords a rough estimation of the ID_{50} value (the concentration of the agonist required to cause the 50% inhibition), which should reflect an apparent dissociation constant of the agonist-receptor complex if an assumption is made that the observed change in the cell function would be proportional to the concentration of the complex formed. The Hill coefficients calculated from the dose-response curves in Figs. 2 and 4–7 were roughly equal to unity, excluding a possibility of a cooperative complex formation and hence making this assumption plausible. In the case of antagonists, the dissociation constant of the putative antagonist-receptor complex can be calculated according to an equation in Table 1 (12) from the concentration of the antagonist required to cause a 50% reversal of the epinephrine inhibition.

Table 1 shows apparent K_d values thus estimated for *alpha*-adrenergic agonists and antagonists based on their effects on insulin release, cyclic AMP accumulation, and adenylate cyclase activity. The K_d value for an agonist or antagonist in most cases was of essentially the same order of magnitude regardless of which of these three parameters of cell function was used for estimation. This may lend strong support to the idea that an interaction of these agonists or antagonists with their own receptors triggers a sequential change in these cell functions. Clonidine exhibited 100–1000 times stronger affinity for the receptors than did phenylephrine or methoxamine. Thus, stimulation of *alpha*$_2$-adrenoceptors on the islet cell membrane resulting from inhibition of receptor-linked adenylate cyclase lowers the cellular content of cyclic AMP which presumably in turn is responsible for the regulation of insulin secretion.

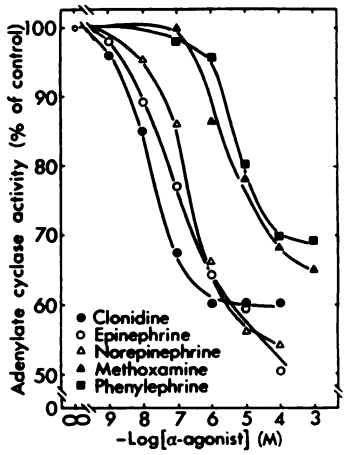

FIG. 6. *Inhibition by alpha-adrenergic agonists of adenylate cyclase activity in the membrane-rich preparation of rat islets*

The crude membrane preparation of cultured rat islets was assayed for adenylate cyclase. The assay mixture contained the membrane preparation (1–2 μg of protein), 25 mM Tris (pH 7.6), 1 mM ATP, 10 μM GTP, 5 mM $MgCl_2$, 1 mM IBMX, aprotinin (100 KIU/ml), bovine serum albumin (1 mg/ml), and an ATP-regenerating system [phosphocreatine (5 mM) and creatine phosphokinase (50 units/ml)] in a total volume of 25 μl. The mixture was incubated for 10 min at 37°. Adrenergic agonists were included in the assay mixture at concentrations indicated. Enzyme activity, the mean of duplicate determinations, is expressed as percentage of the control value (91.4 ± 8.9 pmoles of cyclic AMP per milligram of protein per minute) obtained without any agonist.

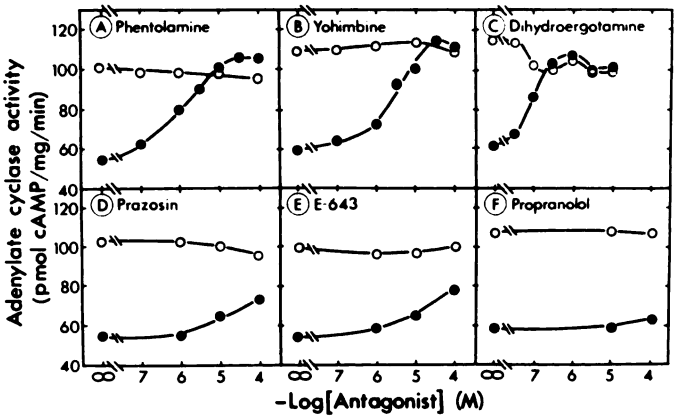

FIG. 7. *Effects of various adrenergic antagonists on epinephrine-induced inhibition of adenylate cyclase*

The crude membrane preparation of cultured rat islets was assayed for adenylate cyclase as described in Fig. 6. The assay mixture was supplemented with adrenergic antagonists at concentrations indicated in the absence (○) or presence (●) of 1 μM epinephrine. The data are means ± standard error of the mean of duplicate determinations.

DISCUSSION

In the present experiments, we used islets cultured for 2 hr instead of freshly isolated ones to study adrenergic regulatory mechanisms, since glucose-induced insulin secretion and cyclic AMP accumulation, both of which are susceptible to adrenergic inhibition, were greater after

TABLE 1

Apparent dissociation constants (K_d) of agonist (or antagonist)-receptor complex as estimated on the basis of inhibition (or its reversal) of insulin secretion, cyclic AMP accumulation, and adenylate cyclase activity

The K_d values for agonists are ID_{50} values estimated from plots in Figs. 2A and B and 6. In the case of antagonists, the values were calculated from ED_{50} values estimated from plots in Figs. 5A and B and 7 according to the equation

$$K_d = ED_{50}/(1 + [C]_{ep}/K_{d(ep)})$$

where $[C]_{ep}$ is the concentration of epinephrine in Figs. 5 (10 μM) and 7 (1 μM) and $K_{d(ep)}$ is the K_d value for epinephrine recorded in Line 2 of each column.

Drug	K_d based on		
	Insulin	Cyclic AMP	Adenylate cyclase
		μM	
Agonists			
Clonidine	0.01	0.003	0.01
Epinephrine	0.1	0.05	0.1
Norepinephrine	0.5	0.1	0.1
Phenylephrine	1	1	5
Methoxamine	10	10	5
Antagonists			
Dihydroergotamine	0.03	0.1	0.01
Yohimbine	0.03	0.08	0.1
Phentolamine	0.1	0.1	0.1

culture than before culture. This advantage of 2-hr cultured cells over fresh cells was observed only when their response to glucose was tested in the presence of IBMX, a potent inhibitor of cyclic AMP phosphodiesterase. In the absence of the methylxanthine, culture of islets resulted in a decrease, rather than an increase, in insulin release and cyclic AMP accumulation during subsequent incubation with 16.7 mM glucose (13), in accord with a previous report by Rabinovitch *et al.* (14). The decreased response to glucose may result from activation of phosphodiesterase during culture (15). Hence, the changes in the cellular content of cyclic AMP observed here in the presence of IBMX are likely to reflect generation of cyclic AMP by membrane adenylate cyclase.

Changes in the cyclic AMP content of islets were invariably associated with same-directional changes in insulin release therefrom under a variety of conditions. These insulin and cyclic AMP data were obtained after 30-min incubation of islets. The insulin data should be representative of the rate of secretion since insulin in the medium increased linearly for 30 min (7), whereas the cyclic AMP data may reflect a steady state of accumulation reached within 5–15 min and maintained during the subsequent incubation for up to 30 min in the presence of methylxanthines (7). Although a direct causal relationship is not clear, in a strict sense, between the instantaneous (cyclic AMP accumulation) and the integral (insulin release) parameters, it seems highly probable that cyclic AMP accumulation is one of the determinants of the rate of insulin secretion.

A regression line relating insulin release to cyclic AMP accumulation under the influence of *alpha*-adrenergic agonists is not exactly linear but slightly sigmoidal (Fig. 3), in support of our recent findings (13) that cyclic AMP-dependent insulin secretion obeys saturation kinetics. Rabinovitch *et al.* (16) proposed that epinephrine inhibits insulin release by a dual *alpha*-adrenergic mechanism that is cyclic AMP-dependent at its higher concentrations and cyclic AMP-independent at lower concentrations. As is discussed below, cyclic AMP appears to be an essential mediator of $alpha_2$-adrenergic receptors. Conceivably, an $alpha_1$-adrenergic mechanism independent of the adenylate cyclase-cyclic AMP system would also function in rat fresh islet cells, whereas it might be obscured in cultured islets for unknown reasons.

The percentage of inhibition of membrane adenylate cyclase by an *alpha*-agonist was somewhat smaller than the percentage of inhibition of cyclic AMP accumulation in intact cells elicited by the same agonist (compare Fig. 6 with Fig. 2). Similar refractoriness of islet membrane adenylate cyclase was shown by its failure to respond to glucose (see ref. 6 for review), which is one of the potent stimulants of cyclic AMP generation in intact islet cells (Fig. 1). Conceivably, glucose may increase the cellular level of cyclic AMP by stimulating an influx of extracellular calcium which in turn activates membrane adenylate cyclase through the mediation of calmodulin (17, 18). Since epinephrine is inhibitory to the calcium influx (7), a certain calcium-dependent mechanism would be also involved in the *alpha*-receptor-mediated regulation of adenylate cyclase in the intact islet cell; as a result, adenylate cyclase would be less susceptible to the *alpha*-adrenergic inhibition in the calcium-free membrane preparation.

In the present study, insulin release and cyclic AMP accumulation in intact islet cells as well as membrane adenylate cyclase activity were measured as parameters of the receptor-linked cell functions. Dose-response curves drawn for various *alpha*-adrenergic agonists and antagonists based on their effects on these parameters are applicable to estimation of their relative affinities for islet cell *alpha*-adrenoceptors. The orders of affinities for agonists [clonidine > epinephrine (norepinephrine) ≫ phenylephrine ≃ methoxamine] and for antagonists (phentolamine ≃ yohimbine ≃ dihydroergotamine ≫ prazosin) were in good agreement with the order of potency previously determined with inhibition of membrane adenylate cyclase of human (2) and rabbit[2] platelets and hamster (3) and human (19) adipocytes and hybrid cells (4). Thus, adrenoceptors responsible for regulation of these islet cell functions are considered to belong to the presynaptic type or the $alpha_2$ subclass of membrane *alpha*-receptors.

The dose-dependent action of various *alpha*-agonists and antagonists on islet insulin release has been reported by Nakaki *et al.* (20). Moreover, Nakaki *et al.* (20) compared the effects of yohimbine with those of prazosin[3] and the effects of clonidine with those of methoxamine on the cyclic AMP content of islets and concluded that $alpha_2$-adrenergic receptors are responsible for the inhibition of cyclic AMP generation and insulin release. Thus, the present results not only confirm the reports of Nakaki *et al.* (9, 20) but also further show that there is

[2] T. Katada, I. Matsuoka, and M. Ui, manuscript in preparation.

[3] E643, which resembles prazosin in structure, is an $alpha_1$-selective antagonist according to N. Nakahata (personal communication).

a highly significant correlation between the cyclic AMP content and insulin release under the influence of $alpha_2$-receptor function. Together with the findings that membrane adenylate cyclase is directly regulated by $alpha_2$-agonists and antagonists, we are led to a conclusion that $alpha_2$-adrenergic receptors are coupled to membrane adenylate cyclase, which in turn may play an indispensable role in regulating insulin secretion in rat pancreatic islet B-cells.

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