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EVALUATION OF A NEW BETA-ADRENERGIC BLOCKING AGENT, CARTEOLOL, BASED ON METABOLIC RESPONSES IN RATS—II.

BLOCKADE BY CARTEOLOL OF THE EPINEPHRINE- AND ISOPROTERENOL-INDUCED INCREASES OF TISSUE AND BLOOD CYCLIC AMP *IN VIVO*

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Abstract—Intraperitoneal injection of epinephrine caused a slight but significant increase in cyclic AMP in the liver and skeletal muscle in 5–10 min. Likewise, there was a significant increase in cyclic AMP in the muscle and adipose tissue 5 min after the injection of isoproterenol. However, no change was detected in the cardiac muscle. Pretreatment of rats with theophylline was so effective in enhancing responses of tissue cyclic AMP as to make it possible to detect a 2- to 3-fold increase of cyclic AMP even in the heart. The maximal increases in tissue cyclic AMP induced by 500 μg/kg of isoproterenol or epinephrine were blocked by carteolol, a new beta-adrenergic blocking agent, at doses of 10–100 μg/kg, regardless of whether or not the rats had been treated with theophylline. In contrast, neither the increase of liver cyclic AMP by glucagon nor the increase of adrenal cyclic AMP by adrenocorticotropic hormone (ACTH) was affected by this agent. Blood cyclic AMP rose sharply after isoproterenol, the maximal response attaining 20- to 30-fold of the preinjection level. Based on this tremendous increase of blood cyclic AMP, the dose of isoproterenol and carteolol required for the half-saturation of the beta-adrenergic receptor was calculated as 20–30 and 0.5 μg/kg of body wt respectively. These values are in the same order as the respective values obtained from the response of carbohydrate and lipid metabolites in blood.

The preceding paper [1] showed that the increases in blood concentrations of various carbohydrate and lipid intermediary metabolites induced by epinephrine and isoproterenol were blocked by a small dose of 5-(3-tert-butylamino-2-hydroxy)propoxy-3,4-dihydrocarbostyril hydrochloride (carteolol), a new beta-adrenergic blocking agent. It is now well established [2] that stimulation of beta-adrenergic receptors results in an activation of adenylate cyclase which leads to an accumulation of cyclic AMP in the cell. The cyclic AMP formed then plays an essential role in the observed beta-adrenergic responses such as metabolic and cardiovascular alterations. Thus, the changes in the tissue level of cyclic AMP are likely to reflect the agonist–receptor interaction more directly than the changes in carbohydrate and lipid metabolite levels, endocrine or exocrine secretions, muscle contraction and relaxation, etc. The present paper deals with the increases of tissue and blood cyclic AMP induced by beta-stimulants *in vivo* as well as their blockage by carteolol.

MATERIALS AND METHODS

Male Wistar rats fasted overnight were used. For the purpose of determining tissue levels of cyclic AMP, liver, skeletal muscle, heart, adrenals and adipose tissues were rapidly excised from pentobarbital-anesthetized rats and frozen in a clamp precooled in liquid N_2. All the tissues, except the heart, had been exposed immediately before sacrifice in order to freeze the tissue within 10 sec. In the case of skeletal muscle, the skin was cut around the ankle and pulled back so as to expose most of the leg muscle, which was then pressed between frozen clamps. Frozen tissue samples were powdered with liquid N_2 in a porcelain mortar. The powder was extracted with perchloric acid, diluted with 4 mM EDTA, neutralized with KOH and centrifuged to give protein-free supernatant as described by Yajima and Ui [3]. The tissue content of cyclic AMP was determined by the protein binding technique [3] with the use of the cyclic AMP binding protein extracted from rat liver.

In the experiments in which determination of blood cyclic AMP was undertaken, 2 ml blood was withdrawn from anesthetized rats through a syringe (containing 100 units heparin) inserted into the inferior vena cava, and quickly mixed with 0.2 ml of an isotonic solution of 11 mM theophylline. The presence of theophylline (or EDTA at the final concentration above 10 mM) is essential because otherwise cyclic AMP undergoes breakdown during the following procedure of assay. Plasma was separated by centrifugation in the cold and then assayed for cyclic AMP by a "cyclic AMP assay kit" obtained from Radiochemical Centre Amersham. The sources of other reagents are the same as described in the preceding paper [1].

RESULTS

Increases of tissue cyclic AMP by epinephrine and isoproterenol and their blockade by carteolol. Earlier reports have shown that a rapid increase in tissue

cyclic AMP level induced by epinephrine is followed by a sharp decline *in vivo* [4–6] in perfused liver [7–10] and heart [11], and in isolated [12, 13] and cultured [14, 15] cells *in vitro*. Therefore, the time course was first studied for changes in cyclic AMP level in rat skeletal muscle after the intraperitoneal injection of epinephrine, in an attempt to determine the time when the maximal response is observed *in vivo*. The results are shown in Fig. 1, which also records the similar time courses in the adipose tissue and liver in the rat treated with adrenocorticotropic hormone (ACTH) and glucagon respectively. In accord with previous publications [3, 5], the effect *in vivo* of beta-adrenergic agonists in causing an increase in cyclic AMP was small in magnitude in the skeletal muscle; the maximal dose of epinephrine produced less that a 2-fold increase after 5 min and lower doses were without effect (Fig. 1A). Our unpublished data showed that the increase in muscle cyclic AMP at 2 min after epinephrine was variable, though slightly larger in magnitude than the increase at 5 min in most cases. Likewise, cyclic AMP in the adipose tissue responded to 5–10 units ACTH with its peak level at 5 min (Fig. 1B). In the case of the enormous increment of liver cyclic AMP by glucagon, the maximal response at the highest dose used was delayed as the dose of glucagon was increased until it plateaued from 5 to 10 min. This time course for glucagon action is essentially the same as that reported by other investigators [5, 6, 16–18]. Based on these results in Fig. 1, the tissue levels of cyclic AMP were measured at 5 min after injection of beta-stimulants or hormones in the following experiments except where otherwise indicated.

Figure 2 shows the dose-dependent response of cyclic AMP to injected epinephrine and isoproterenol in various tissues. It is seen that the cyclic AMP level was increased in the skeletal muscle, but not in the heart muscle, by the injection of isoproterenol or epinephrine. Epinephrine exhibited a smaller effect

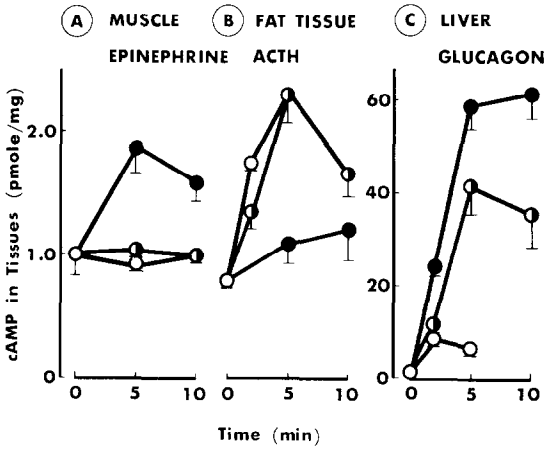

Fig. 1. Time course of the increase in tissue cyclic AMP in response to epinephrine and other hormones. Epinephrine, ACTH or glucagon was injected intraperitoneally into fasted rats at zero time at the following doses: epinephrine: 50 μg (○), 200 μg (◑) and 500 μg (●)/kg of body wt; ACTH: 2 units (○), 10 units (◑) and 50 units (●)/kg; glucagon: 50 μg (○), 200 μg (◑) and 500 μg (●)/kg. Each point represents the mean of four to six observations with S.E.M. as a vertical line.

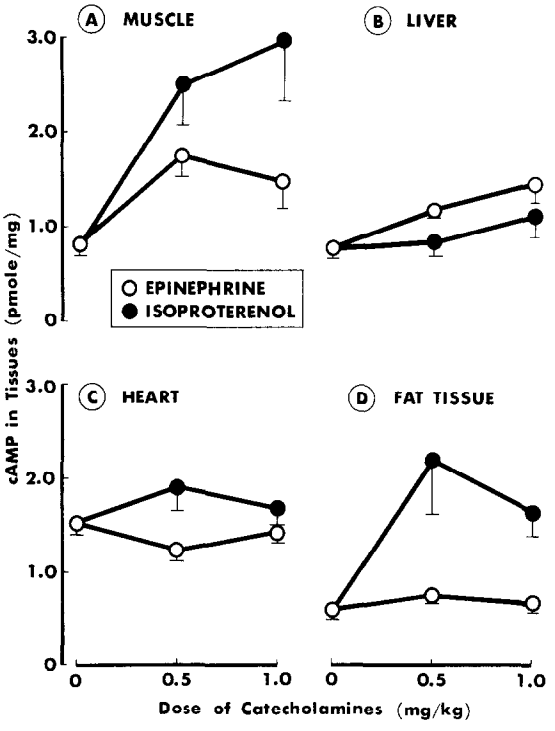

Fig. 2. Dose-dependent increases in tissue cyclic AMP after epinephrine or isoproterenol. Tissues were excised 5 min after the intraperitoneal injection of isoproterenol or epinephrine at dose of 500 μg/kg of body wt. The number of observations is four.

than isoproterenol in the muscle and was essentially without effect in adipose tissue; however, it raised the hepatic level slightly but significantly. Since the increase of the dose of epinephrine or isoproterenol from 500 μg to 1.0 mg/kg of body wt caused no significant potentiation, or tended to result in even a slight reduction, of the response of tissue cyclic AMP (Fig. 2, panels A and D), the doses of epinephrine and isoproterenol were kept at 0.5 mg/kg in the following experiments, in which the inhibition by the beta-adrenergic blocking agent was studied.

The cyclic AMP level elevated by isoproterenol in the muscle and adipose tissue was significantly reduced by the treatment of rats with carteolol 15 min prior to the catecholamine and mostly blocked when the interval between the injections of the agonist and the antagonist was prolonged to 30 min (Fig. 3). The action of epinephrine was also blocked by this beta-adrenolytic agent. These inhibitions were next studied as a function of the dose of blocker and are illustrated in Fig. 4. Carteolol, injected 30 min before the catecholamines, was very effective in reducing the isoproterenol- and epinephrine-induced elevation of tissue cyclic AMP; there was a complete blockade in all the tissues tested at 10–100 μg/kg of the antagonist.

Specificity of carteolol-induced blockade of catecholamine actions on tissue cyclic AMP. In order to rule out the possibility that carteolol diminishes tissue cyclic AMP without specific interaction with beta-adrenergic receptors, the effects of ACTH and glucagon, the agents known to raise the cyclic AMP content of their target organs without the mediation of

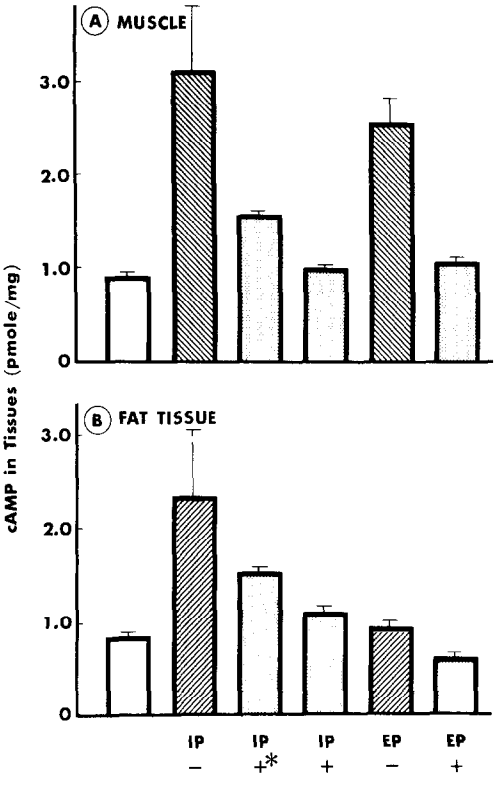

Fig. 3. Inhibition by carteolol of isoproterenol- and epinephrine-induced increases in tissue cyclic AMP. Tissue levels of cyclic AMP were measured in the control rats receiving no drug (open column), 5 min after intraperitoneal injection of isoproterenol (IP) or epinephrine (EP) (500 μg/kg, hatched column) and in the rat injected subcutaneously with carteolol in addition to isoproterenol or epinephrine (dotted column). Carteolol was injected 30 min before the beta-stimulants at a dose of 1 mg/kg (the asterisk indicates 15 min before). The number of observations is four. Key: −, without carteolol; +, with carteolol.

beta-receptors, were studied in rats pretreated with carteolol (Fig. 5). In accord with previous results obtained *in vivo* [19, 20] and *in vitro* [21], intraperitoneal injection of ACTH caused a tremendous increase in cyclic AMP in adrenal cortex. This increase was not reduced by the pretreatment of rats with carteolol. Likewise, the glucagon-induced elevation of liver cyclic AMP level was not blocked by carteolol, though there was a trend for progressive fall as the dose of carteolol was raised. It is likely that the carteolol-induced reduction of tissue cyclic AMP level took place only when the level was raised by beta-adrenergic stimulants.

Effect of carteolol on isoproterenol-induced elevation of tissue cyclic AMP in rats pretreated with theophylline. Inotropic action of beta-stimulants observed in the perfused heart of animals *in vitro* is known to be preceded by a sharp increase of cyclic AMP in the heart tissue, affording evidence that cyclic AMP mediates the cardiovascular response to the beta-receptor stimulation [22]. Nevertheless, neither epinephrine nor isoproterenol caused a detectable rise of cyclic AMP in the heart *in vivo* (Fig. 2). Probably, the time required (about 30 sec) to isolate and freeze-clamp the beating heart would have resulted in such a scatter of analytical data as to obscure small increases in tissue metabolites. In order to overcome this difficulty by potentiating the response of tissue cyclic AMP, theophylline was injected intraperitoncally into rats in combination with isoproterenol in the experiments shown in Figs. 6 and 7. It is seen in Fig. 6 that the prior injection of theophylline (100 mg/kg as aminophylline), despite its failure to affect cyclic AMP level by itself, was very effective in enhancing the isoproterenol-induced increase in cyclic AMP levels in the liver, muscle, heart and adipose tissue (compare with Fig. 2). These enhanced increases in cyclic AMP in tissues of rats pretreated with theophylline were inhibited by carteolol (Fig. 7) at roughly the same dose as in the rats receiving no theophylline (Fig. 2), i.e. there was a total inhibition at a dose of 10–100 μg/kg.

Elevation of blood level of cyclic AMP by isoproterenol and its inhibition by carteolol. Intraperitoneal injection of isoproterenol (500 μg/kg) gave rise to a striking increase in plasma concentration of cyclic AMP. The maximal response was observed 10–20 min after injection (inset of Fig. 8A). In Fig. 8A, the plasma cyclic AMP level 15 min after the injection of isoproterenol was plotted against the dose of the catecholamine. For comparison, blood levels of lactate (panel B), glycerol (panel C) and free fatty acids (panel D) observed under the same condition was also plotted. These dose–response relationships revealed the ED_{50} for isoproterenol as 20 μg/kg of body wt for cyclic AMP and 10–15 μg/kg for other metabolites. The maximal increase of plasma cyclic AMP induced by 500 μg/kg of isoproterenol was inhibited by carteolol, propranolol and pindolol in a dose-

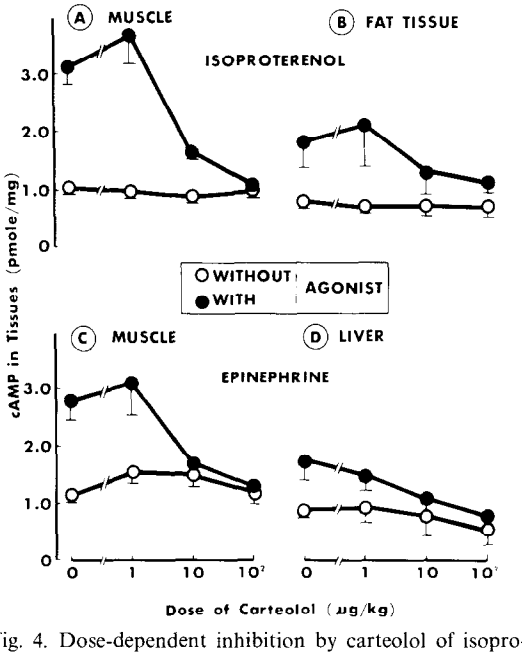

Fig. 4. Dose-dependent inhibition by carteolol of isoproterenol- and epinephrine-induced increases in tissue cyclic AMP levels. Carteolol was injected subcutaneously 30 min before intraperitoneal injection of isoproterenol or epinephrine (500 μg/kg); 5 min later, the rats were sacrificed for assay of tissue cyclic AMP. The number of observations is four.

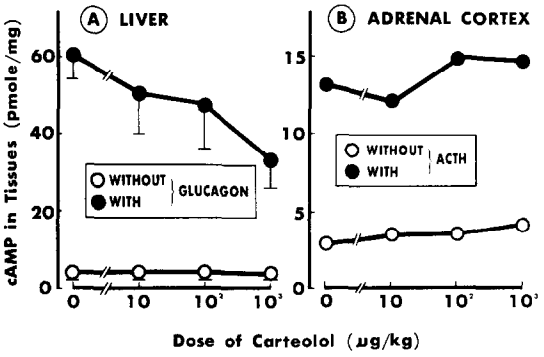

Fig. 5. Failure of carteolol to inhibit the increases of cyclic AMP induced by glucagon (500 μg/kg, intraperitoneal) in the liver and by ACTH (10 units/kg, intraperitoneal) in the adrenal cortex. The number of observations is four for panel A. In panel B, adrenal cortices from three to four rats were combined to be assayed for cyclic AMP.

dependent manner (Fig. 9). Based on these plots, ID_{50} was estimated as 10 μg/kg for carteolol, 300–400 μg/kg for propranolol and 10–20 μg/kg for pindolol. Thus, carteolol was much more potent than propranolol and slightly more potent than (or essentially as potent as) pindolol, as the inhibitor of isoproterenol-induced increase of plasma cyclic AMP.

DISCUSSION

The results presented in this paper have shown that the increase in tissue cyclic AMP elicited by isoproterenol or epinephrine *in vivo* was efficiently prevented by the prior treatment of rats with carteolol, a new beta-adrenergic blocking agent. In contrast, carteolol was without effect on the increases of tissue cyclic AMP caused by the hormones other than beta-stimulants, such as glucagon and ACTH. Thus, it is very likely that carteolol selectively reverses the beta-receptor-mediated activation of adenylate cyclase.

Cyclic AMP in the skeletal muscle exhibited the largest response to isoproterenol or epinephrine among the tissues tested, but the magnitude of the increase was only 2- to 4-fold (Fig. 2) and much smaller than those provoked by glucagon [5, 6, 16–18, 23] or ACTH [19, 20] in their target organs (Fig. 5). Cyclic AMP in the liver, adipose tissue and heart responded to beta-stimulants to an even lesser extent. Recent publications [3–6, 24] have shown similar small responses of tissue cyclic AMP to beta-stimulants *in vivo*. This is not surprising in view of the previous studies *in vitro* which showed that the addition of epinephrine or isoproterenol to perfused organs [7–10], tissue slices [25–33], free cells [21, 34] and organ culture [14] gave rise to an increase in tissue cyclic AMP of a small (2- to 3-fold) magnitude and of a short duration. (On the other hand, epinephrine caused more than 10-fold increases in cyclic AMP in the pineal body *in vivo* [35, 36], and in the uterus [37], epidermis [38] and tumor cell line [15] *in vitro*.) Such a small response of tissue cyclic AMP to the addition *in vitro* of beta-stimulants forms a sharp contrast with the actions *in vitro* of glucagon [7, 39, 40], ACTH [21, 41–43] and para-

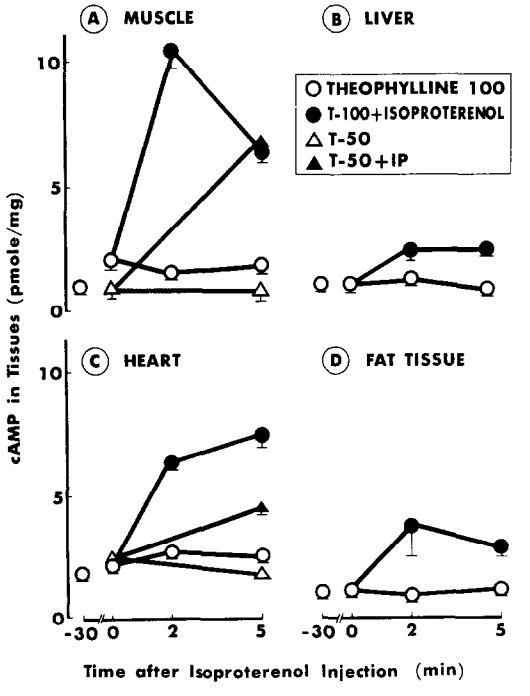

Fig. 6. Effect of theophylline with or without isoproterenol on the tissue level of cyclic AMP. Aminophylline was used as the theophylline-containing drug. Aminophylline, 100 mg/kg of body wt (open circles) or 50 mg/kg (open triangles), was injected intraperitoneally at the time indicated on abscissa as "−30." Isoproterenol, 500 μg/kg, was injected intraperitoneally at zero time (solid symbols). The number of observations is four.

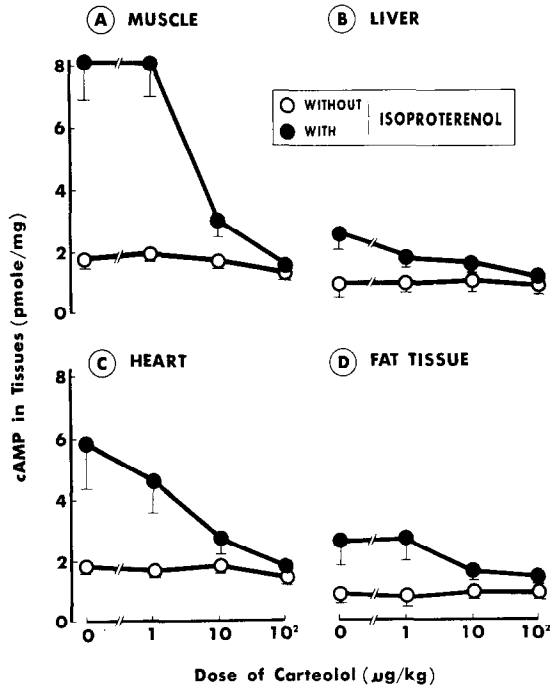

Fig. 7. Dose-dependent inhibition by carteolol of the isoproterenol-induced increase in tissue cyclic AMP in rats pretreated with theophylline. Theophylline, 100 mg/kg of body wt as aminophylline, was injected intraperitoneally 30 min before isoproterenol. Other experimental conditions are the same as in Fig. 4. The number of observations is four.

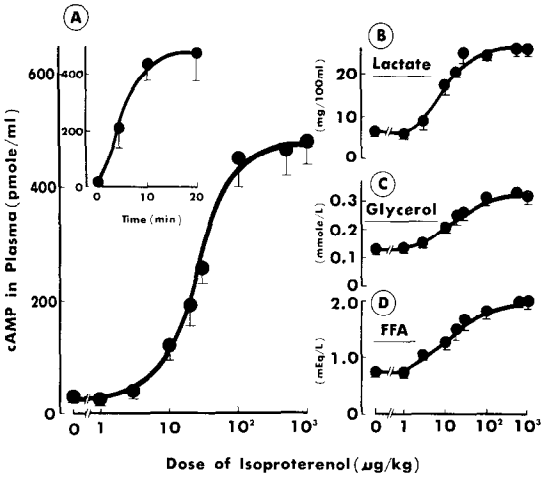

Fig. 8. Effect of intraperitoneal injection of isoproterenol on plasma level of cyclic AMP of fasted rats. Inset of panel A shows the time course of plasma cyclic AMP after isoproterenol injection (500 μg/kg of body wt). Panels A, B, C and D represent the dose–response relationships between isoproterenol and plasma levels of cyclic AMP, lactate, glycerol and free fatty acids (FFA) 15 min after the injection of the agonist respectively. The number of observations is three to four. Plasma levels of lactate, glycerol and FFA were determined as described in the preceding paper [1].

thyroid hormone [44–46] on cyclic AMP levels in their target organs, which responded with more than a 10-fold increase. Our failure to detect the elevation of hepatic cyclic AMP level in isoproterenol-treated rats (Fig. 2) is apparently in keeping with Butcher and Potter [47], who reported that sensitivity of adenylate cyclase to isoproterenol is mostly lost at 20 days of postnatal age in rats. However, hepatic cyclic AMP increased in response to isoproterenol if the rat had been treated with theophylline (Fig. 6). This strongly suggests that sensitivity of adenylate cyclase to isoproterenol, at least in part, is still preserved in the adult rat liver.

Prior treatment of rats with theophylline, a strong inhibitor of phosphodiesterase, was effective in enhancing the response of tissue cyclic AMP to isoproterenol to such a degree as to make it possible to detect its increase even in cardiac muscle. This increase of cyclic AMP in the heart was inhibited by carteolol, in keeping with the recent finding [48, 49] that carteolol is a potent blocking agent of the inotropic action of isoproterenol in the dog. The exaggerated increase in tissue cyclic AMP induced by isoproterenol in the theophylline-treated rats was prevented by roughly the same dose of carteolol as employed in inhibiting the smaller increase in the rats receiving no theophylline; ID_{50} for carteolol action on the increase of cyclic AMP induced by 0.5 mg/kg of isoproterenol was 10–100 μg/kg for either case. This fact lends support to the concept that carteolol lowers the tissue cyclic AMP level by selectively acting on the formation of cyclic AMP rather than on its breakdown via phosphodiesterase.

The plasma level of cyclic AMP changes in response to alterations of nutritional and hormonal states [19, 20, 50–56]. Hence, plasma cyclic AMP is very likely to have originated from tissues. Moreover, the accumulation of cyclic AMP elicited by beta-stimulants *in vitro* in the perfused organ [9, 57, 58] or isolated cells [59, 60] resulted in its release into the perfusing or bathing medium. Since the basal level of cyclic AMP in plasma is less than one-twentieth of the basal level in tissues, the per cent increase of its plasma concentration induced by beta-stimulation could be larger than that in tissue concentrations, if a considerable portion of the intracellular cyclic AMP generated by activated adenylate cyclase leaves the cell as observed *in vitro*.* In support of this expectation, there was a 20- to 30-fold increase in plasma cyclic AMP in response to the maximal dose of isoproterenol (Fig. 8) in contrast to a smaller than 4-fold increase in tissue cyclic AMP even after treatment with theophylline. Accordingly, the analysis of plasma cyclic AMP is more advantageous than that of tissue cyclic AMP for assessing the potency of beta-adrenergic agonists or antagonists quantitatively. Thus, the dose of isoproterenol required for half-maximal activation of adenylate cyclase (ED_{50}), as well as the dose of carteolol required to reduce the isoproterenol-induced activation to one-half the maximal response (ID_{50}), was obtained with considerable precision with plasma cyclic AMP but not with tissue cyclic AMP. The calculation based on these ED_{50} and ID_{50} values afforded 0.5 μg/kg as the dosage of carteolol that reflects the dissociation constant of the beta-receptor–carteolol complex, which is inert in activation of

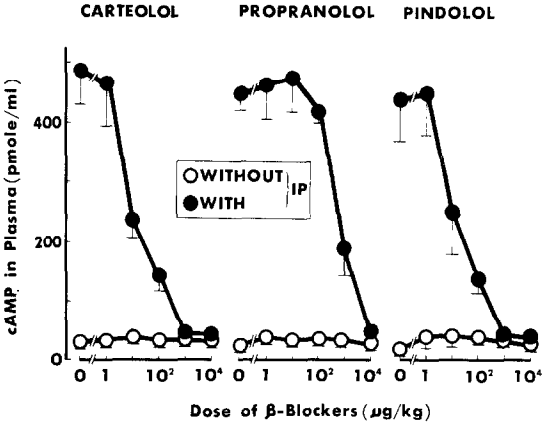

Fig. 9. Inhibition of isoproterenol-induced increases in plasma cyclic AMP by carteolol, propranolol and pindolol. The beta-blockers were injected subcutaneously 30 min prior to 500 μg/kg of isoproterenol. Plasma cyclic AMP was determined 5 min after isoproterenol. The number of observations is four.

* In view of our results (Fig. 2) that the cateholamine-induced increase in cyclic AMP was more marked in skeletal muscles than in other types of tissue, most of the cyclic AMP accumulated in blood in response to beta-stimulants appears to have originated from skeletal muscles. A simple calculation shows that the release of only 10 per cent of baseline cyclic AMP in skeletal muscle (occupying 45 per cent of body weight) into the extracellular fluids (occupying 25 per cent of body weight) can result in a 4- to 8-fold increase in the blood level of cyclic AMP if renal excretion is neglected. Thus, it is not surprising that tremendous increases in blood cyclic AMP occurred without detectable changes in tissue nucleotide in the rat treated with a smaller dose of beta-stimulants.

adenylate cyclase.* This value ("corrected ED_{50}") is of the same order as the value similarly calculated on the basis of the isoproterenol- or epinephrine-induced alteration of the blood level of carbohydrate and lipid metabolites [1]. It is concluded, therefore, that the blood level of cyclic AMP can serve as a good index *in vivo* for the interaction of agonists or antagonists with the beta-adrenergic receptor, and that carteolol is a very potent blocking agent of the receptor.

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* Based on an assumption that the change in blood cyclic AMP induced by an agonist or an antagonist is proportional to the amount (concentration) of the agonist–receptor complex formed and that the antagonist–receptor complex is totally inactive in producing cyclic AMP, the dissociation constant of the antagonist–receptor complex (K_i) can be calculated as

$$K_i = \frac{\text{50\% inhibitory concentration}}{1 + \dfrac{\text{concentration of agonist employed}}{K_a}}$$

where K_a is the concentration of the agonist needed to induce the half-maximal increase, and "50% inhibitory concentration" refers to the concentration of antagonist that induces 50 per cent inhibition of the effect of the agonist at the concentration employed. "Corrected ID_{50}," which corresponds to K_i, is then calculated by using "ID_{50} for carteolol," "the dose of isoproterenol employed for assessing the ID_{50}," and "ED_{50} for isoproterenol" instead of "50% inhibitory concentration," "the concentration of agonist employed," and "K_a," respectively, in the above equation (see Ref. 1).

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