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### Research Article

Long-term clinical studies have associated tolbutamide therapy with an increased incidence of cardiovascular deaths. The effects of this and other sulfonylurea drugs on contractility and rate of isolated rabbit atria, automaticity of isolated dog Purkinje fibers, and adenyl cyclase activity in particulate preparations of rabbit and human hearts were studied. At concentrations that are attained clinically, tolbutamide (10 mg/100 ml) increased contractility of driven rabbit atria to  $124 \pm 5\%$  of control, acetohexamide (3.9 mg/100 ml) to  $140 \pm 5\%$ , chlorpropamide (8.3 mg/100 ml) to  $139 \pm 6\%$ , and tolazamide (3.1 mg/100 ml) to  $119 \pm 6\%$ . These effects were accentuated in the presence of  $2.5 \times 10^{-4}$  M theophylline and were not blocked by  $1 \times 10^{-5}$  M propranolol. Adenyl cyclase was activated by each of these drugs at concentrations below those which increase contractility. The drugs also increased the rate and slope of phase 4 depolarization in spontaneously beating Purkinje fibers, but did not alter the spontaneous rate of isolated rabbit atria. Since inotropic and chronotropic stimulation can be deleterious in some clinical settings, these findings may be of significance in interpretation of cardiovascular mortality data.

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# The Effect of Sulfonylurea Drugs on Rabbit Myocardial Contractility, Canine Purkinje Fiber Automaticity, and Adenyl Cyclase Activity from Rabbit and Human Hearts

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**ABSTRACT** Long-term clinical studies have associated tolbutamide therapy with an increased incidence of cardiovascular deaths. The effects of this and other sulfonylurea drugs on contractility and rate of isolated rabbit atria, automaticity of isolated dog Purkinje fibers, and adenyl cyclase activity in particulate preparations of rabbit and human hearts were studied. At concentrations that are attained clinically, tolbutamide (10 mg/100 ml) increased contractility of driven rabbit atria to  $124 \pm 5\%$  of control, acetohexamide (3.9 mg/100 ml) to  $140 \pm 5\%$ , chlorpropamide (8.3 mg/100 ml) to  $139 \pm 6\%$ , and tolazamide (3.1 mg/100 ml) to  $119 \pm 6\%$ . These effects were accentuated in the presence of  $2.5 \times 10^{-4}$  M theophylline and were not blocked by  $1 \times 10^{-5}$  M propranolol. Adenyl cyclase was activated by each of these drugs at concentrations below those which increase contractility. The drugs also increased the rate and slope of phase 4 depolarization in spontaneously beating Purkinje fibers, but did not alter the spontaneous rate of isolated rabbit atria. Since inotropic and chronotropic stimulation can be deleterious in some clinical settings, these findings may be of significance in interpretation of cardiovascular mortality data.

## INTRODUCTION

Oral hypoglycemic agents have been used extensively for nearly two decades, yet no reported studies of the effects of any of these drugs on fundamental cardiovascular properties such as contractility or automaticity could be found. Spurred by the recent University Group

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Diabetes Program study (1) suggesting an association of tolbutamide therapy with an increased incidence of cardiovascular deaths, we investigated the effects of this and other sulfonylurea compounds on contractility of isolated rabbit atria, automaticity of isolated canine Purkinje fibers, and adenyl cyclase activity in particulate preparations of rabbit and human hearts. Several interesting effects were found which may be of importance to an understanding of the therapeutic actions of these agents. A portion of these data has been previously reported in preliminary form (2, 3).

## METHODS

**Rabbit atrial contractility.** New Zealand white rabbits were stunned by a blow to the back of the neck. The hearts were rapidly removed and the atria dissected in oxygenated solution at 37°C. Atria were halved and one-half was used for each experiment. The tissue was suspended between two stainless steel hooks, one of which was connected by a silk thread to a Grass model FT 03C force displacement transducer (Grass Instrument Co., Quincy, Mass.). Stimuli were delivered bipolarly through one of the hooks and a platinum electrode at a constant rate of 120/min with a Grass model S44 stimulator and SIU5 stimulus isolation unit. Contractions were recorded with a Grass model 79 polygraph.

Atria were suspended in 24 ml of physiological solution of the following composition: NaCl 130 mM, KCl 4.6 mM, MgCl<sub>2</sub> 1 mM, CaCl<sub>2</sub> 1.8 mM, Tris (hydroxymethyl) amino-methane 5 mM, dextrose 11 mM adjusted to pH 7.40 with HCl. The solution was continuously oxygenated and maintained at a constant temperature of 37°C. The tissues were preloaded with 1-3 g tension and permitted to stabilize for at least 45 min before addition of drugs. Simultaneous control preparations remained stable over the entire course of each experiment. The degree of preloading had no effect on the subsequent results. All drugs were added directly to the organ bath to bring the total volume to 25 ml. In experiments with the addition of a second drug, contractions were stabilized before each subsequent addition.

**Intracellular recordings.** Mongrel dogs of either sex were anesthetized with sodium pentobarbital 30 mg/kg i.v. Hearts were rapidly excised through a midsternal incision. False tendons were removed from the right or left ventricle and mounted in a plexiglass chamber and superfused with Tris-buffered physiological solution (see above) saturated with 100% O<sub>2</sub> and maintained at 37°C. The preparation was driven by bipolar silver electrodes with the same stimulation arrangement as above. Transmembrane potentials were recorded with microelectrodes filled with 3 M KCl and tip resistances of 10–50 megohms. Potential differences were amplified with a Transidyne General MPA-2 microelectrode preamplifier (Transidyne General Corp., Ann Arbor, Mich.), displayed on a Tektronix 564B oscilloscope (Tektronix, Inc., Beaverton, Oreg.), and photographed with a polaroid camera.

**Adenyl cyclase assay.** After removal of atria from the freshly stunned rabbits, approximately 250 mg of left ventricular muscle was homogenized in 0.25 M sucrose and the particulate fraction prepared as previously described (4). Adenyl cyclase was assayed by the method of Krishna, Weiss, and Brodie (5). Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (6). The particulate fraction containing approximately 0.09–0.11 mg protein in a total volume of 0.06 ml was incubated at 37°C for 4 min with the following: ATP, 1.6 mM; ATP- $\alpha$ -<sup>32</sup>P, 2.5–3.5  $\times 10^6$  cpm; theophylline, 8 mM; MgCl<sub>2</sub>, 2 mM; Tris-Cl, 21 mM (pH 7.7); bovine or human serum albumin, 0.8 mg/ml; and drug or norepinephrine at concentrations stated in the text. The cyclic AMP-<sup>32</sup>P produced was determined as previously described (4).

**Preparation of human tissue.** Left ventricular papillary muscles were obtained at the time of mitral valve replacement in three patients whose ages ranged from 42 to 54 yr. The major hemodynamic abnormality was mitral stenosis in two patients and combined stenosis and regurgitation in the other. The valvular malformation resulted from rheumatic heart disease in all three patients but no associated aortic valve lesions were present. Two of the three patients were on maintenance digoxin therapy at the time of operation. The human heart particles were prepared and adenyl cyclase activity assayed in an identical manner to that described above for the rabbit ventricular muscle preparation.

**Tissue level of cyclic AMP.** In this experiment, one-half of the atrium was used as control and the other half was treated with 5  $\times 10^{-4}$  M tolbutamide. After development of the inotropic effect (approximately 5 min) in the treated half, each was rapidly immersed in 5% trichloroacetic acid containing 0.002 M EDTA. Cyclic AMP content was assayed by the method of Gilman based on the competition in protein binding of cyclic AMP to a cyclic AMP-dependent protein kinase from bovine skeletal muscle (7).

**Chemicals.** The sodium salt of tolbutamide, as supplied by the manufacturer (Orinase Diagnostic, Upjohn Co., Kalamazoo, Mich.) was twice recrystallized in ethanol to remove any traces of Ca<sup>++</sup> or other contaminants. The recrystallized product was found to be calcium-free by atomic absorption spectrophotometry. All other sulfonylurea drugs were supplied in crystalline form by the manufacturers.<sup>1</sup>

<sup>1</sup> Acetohexamide was generously supplied by Dr. J. M. McGuire, The Lilly Research Laboratories, Indianapolis, Ind.; chlorpropamide was supplied by Pfizer, Inc., Brooklyn, N. Y. Tolazamide was generously supplied by Dr. Paul W. O'Connell, The Upjohn Co., Kalamazoo, Mich., and phenformin was obtained from K & K Laboratories, Inc., Plainview, N. Y.

Propranolol was obtained from Ayerst Laboratories, New York and norepinephrine from Sterling-Winthrop Research Institute, Rensselaer, N. Y. All chemicals were of reagent grade, and triple glass distilled water was used for the preparation of solutions.

## RESULTS

**Contractility of rabbit atrium.** Upon addition of sulfonylurea drugs to the bathing media, an increase in contractility is seen after 1 min and the effect stabilizes within 10 min. At higher concentrations of the drugs, progressive increases in contractile force are sometimes seen for up to 30 min. In some experiments, spontaneous beating occurred and such experiments were discarded. Table I summarizes the effects of these drugs on contractility before and after 1  $\times 10^{-5}$  M propranolol. In these preparations, this concentration of propranolol did not significantly alter base line contractility since the mean postpropranolol amplitude was 98.7% of control. The sulfonylurea drugs tested exhibit a significant positive inotropic effect at these concentrations which is not abolished by beta blockade. Phenformin, a biguanide, produces no significant change in contractile force. The concentration of drugs listed in Table I are all within range of clinically attained serum levels (8).

The concentration response relationship of tolbutamide in the presence and absence of 2.5  $\times 10^{-4}$  M theophylline is shown in Fig. 1. This concentration of theophylline is known to inhibit phosphodiesterase (9), and in this preparation, produces a significant inotropic effect (126% of control). The maximum response to tolbutamide is not determined in these experiments since such results are limited by the solubility of the drug at higher concentrations. The contractile response to tolbutamide is enhanced in the presence of theophylline; however, the wide range of data when the two drugs

TABLE I  
Effects of Oral Hypoglycemic Agents on Contractility of Isolated Rabbit Atria before and after Beta Blockade

Drug	Concentration M	% Control response*	% Control response* after 1 $\times 10^{-5}$ M propranolol
			%
Tolbutamide	5 $\times 10^{-4}$	124 $\pm$ 5	123 $\pm$ 14
Acetohexamide	1 $\times 10^{-4}$	140 $\pm$ 5	142 $\pm$ 14
Chlorpropamide	3 $\times 10^{-4}$	139 $\pm$ 6	148 $\pm$ 12
Tolazamide	1 $\times 10^{-4}$	119 $\pm$ 6	129 $\pm$ 10
Phenformin	1 $\times 10^{-3}$	85 $\pm$ 8	
Norepinephrine	5 $\times 10^{-5}$	214 $\pm$ 21	100 $\pm$ 21

\* The per cent of control contractions after stabilization after drug addition expressed as mean  $\pm$  SEM for 5–18 experiments with each drug.

are combined makes true potentiation or synergism difficult to establish.

**Effect on automaticity.** The recordings in Fig. 2 are representative of the effects of tolbutamide on automaticity of isolated canine Purkinje fibers. In quiescent fibers  $1 \times 10^{-8}$  M tolbutamide induces spontaneous activity within 15 min which is reversible upon washing out the drug. The spontaneous rate in a series of 12 Purkinje fibers increased from  $4.2 \pm 1.9/\text{min}$  (mean  $\pm$  SEM) to  $36.4 \pm 6.6/\text{min}$  after  $1 \times 10^{-4}$  M tolbutamide and decreased to  $7.2 \pm 1.7/\text{min}$  15 min after return to control solution. In addition, peculiar bursts of rapid spontaneous activity with partial depolarization lasting 3–10 sec were occasionally observed. None of these effects were abolished by  $1 \times 10^{-4}$  M propranolol. No consistent changes were observed in action potential configuration or upstroke velocity of phase 0 in the driven fibers before and after tolbutamide. Each of the other sulfonyleurea drugs produced similar effects. De-

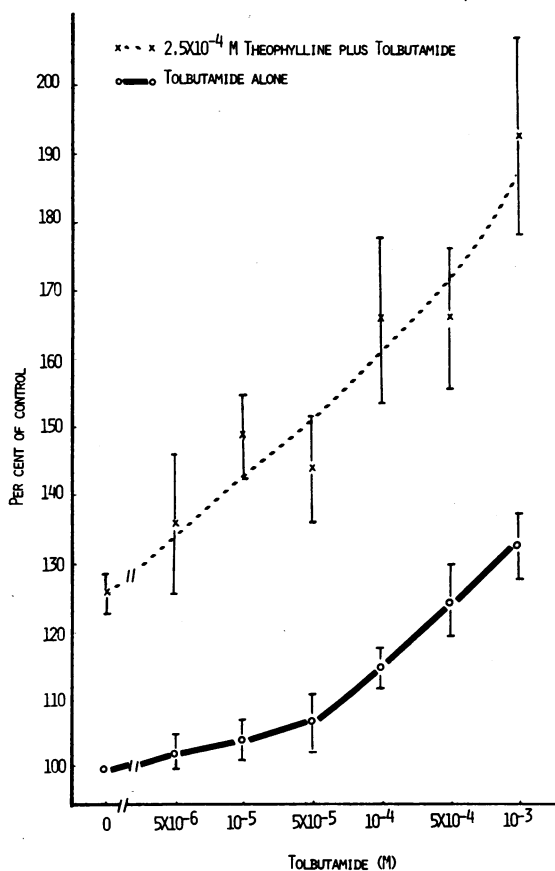


FIGURE 1 The effect of increasing concentrations of tolbutamide on contractility of isolated rabbit atria in the presence and absence of theophylline. The increase in contractile force is expressed as the per cent of the stable control value for each experiment. Each point represents the mean  $\pm$  SEM for at least eight experiments with different atria.

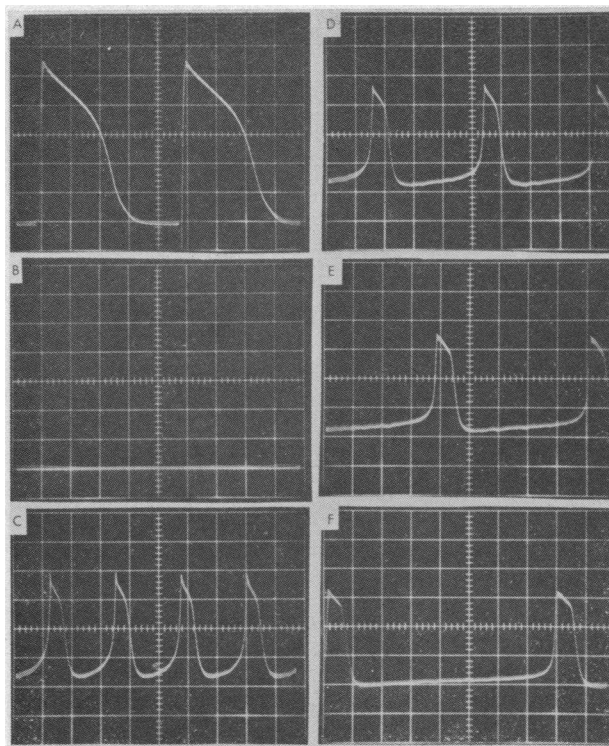


FIGURE 2 Typical effect of tolbutamide on intracellular electrical activity of canine Purkinje fiber. Panel A demonstrates normal control action potential configuration while stimulated 2/sec. Panel B demonstrates stable resting membrane potential without spontaneous activity over a 5 min interval. Panel C shows spontaneous diastolic depolarization after 15 min exposure to  $1 \times 10^{-8}$  M tolbutamide. Panels D-F demonstrate progressive slowing of the spontaneous activity at 5, 10, and 20 min after return to control solution. Each vertical division is 20 mv in each panel. In panel A time scale is 100 msec/division, in panel B, 5 sec/division, and in panels C-F, 200 msec/division.

spite these changes in automaticity of isolated Purkinje fibers, no change in the rate of spontaneously beating rabbit atria have been seen. In a series of 15 such atria, tolbutamide  $5 \times 10^{-4}$  M did not significantly alter the spontaneous rate.

**Activation of adenylyl cyclase.** Fig. 3 demonstrates that tolbutamide increased the accumulation of cyclic AMP in particulate preparations from both rabbit and human ventricular muscle. Significant effects ( $P < 0.05$ ) occurred at  $1 \times 10^{-8}$  M in the rabbit and  $1 \times 10^{-7}$  M in the human. Adenylyl cyclase from the human hearts appears to be more sensitive to tolbutamide than that from the rabbit, half maximal activity being approximately  $3 \times 10^{-8}$  M in the human and  $7 \times 10^{-7}$  M in the rabbit. The effect of other sulfonyleureas and phenformin on adenylyl cyclase as compared to tolbutamide are shown in Table II. The concentrations of these drugs were chosen arbitrarily, based on the concentration-response relation-

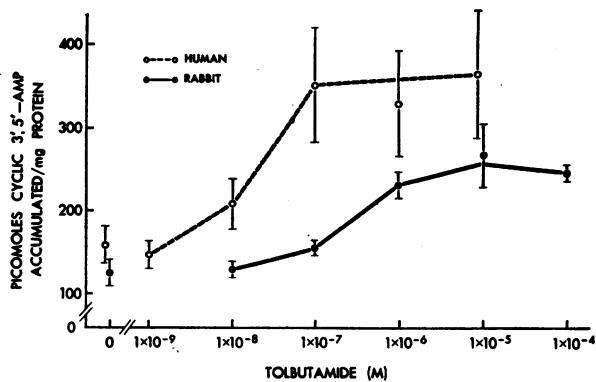


FIGURE 3 The effect of graded doses of tolbutamide on adenylyl cyclase activity in rabbit and human hearts. Each value represents the means  $\pm$ SE of 6-13 samples from four rabbit hearts and the mean  $\pm$ SE of 6-10 samples from three human hearts. The human heart particles were prepared from papillary muscles obtained during mitral valve replacements.

ship for tolbutamide. All of the sulfonylureas produced significant increases in adenylyl cyclase activity comparable to that produced by tolbutamide. Phenformin at  $1 \times 10^{-8}$  M and  $1 \times 10^{-4}$  M did not significantly increase cyclic AMP accumulation in human or rabbit heart. The apparent increase produced by phenformin at  $1 \times 10^{-6}$  M in the rabbit heart was not significant due to widely scattered data in this particular experiment and was not studied further since this dose was ineffective in the human.

Fig. 4 demonstrates that propranolol at  $1 \times 10^{-5}$  M failed to abolish the accumulation of cyclic AMP produced by tolbutamide  $1 \times 10^{-6}$  M. In contrast, the same concentration of propranolol abolished the activation of adenylyl cyclase by norepinephrine,  $5 \times 10^{-5}$  M. Furthermore, maximal concentrations of tolbutamide and norepinephrine in combination did not produce an additive

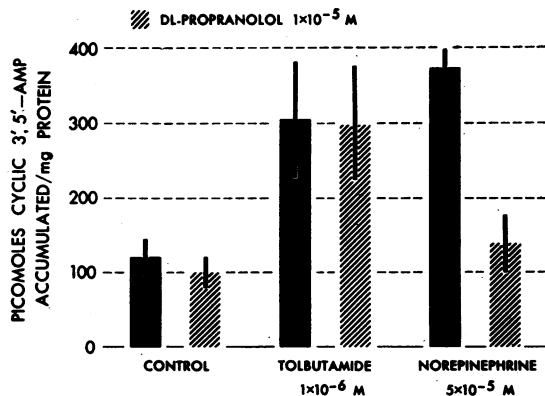


FIGURE 4 The effect of DL-propranolol on the tolbutamide mediated increase in adenylyl cyclase activity. Each value represents the mean  $\pm$ SE of three samples.

TABLE II

Effect of Other Hypoglycemic Agents on Rabbit and Human Myocardial Adenylyl Cyclase

Drug	Concentration	Amount cyclic 3', 5'-AMP accumulated/mg protein per 4 min*
	M	pmoles
A. Control (rabbit)		190 $\pm$ 60
Tolbutamide	$1 \times 10^{-4}$	430 $\pm$ 60
Acetohexamide	$1 \times 10^{-7}$	270 $\pm$ 30
Acetohexamide	$1 \times 10^{-6}$	370 $\pm$ 3
Acetohexamide	$1 \times 10^{-5}$	440 $\pm$ 10
Acetohexamide	$1 \times 10^{-4}$	500 $\pm$ 40
B. Control (rabbit)		99 $\pm$ 22
Tolbutamide	$1 \times 10^{-4}$	165 $\pm$ 11
Chlorpropamide	$1 \times 10^{-4}$	165 $\pm$ 22
Tolazamide	$1 \times 10^{-4}$	209 $\pm$ 33
C. Control (rabbit)		120 $\pm$ 20
Tolbutamide	$1 \times 10^{-4}$	230 $\pm$ 30
Phenformin	$1 \times 10^{-6}$	190 $\pm$ 60
Phenformin	$1 \times 10^{-4}$	150 $\pm$ 16
D. Control (human)		130 $\pm$ 13
Tolbutamide	$1 \times 10^{-6}$	286 $\pm$ 39
Phenformin	$1 \times 10^{-6}$	156 $\pm$ 22
Phenformin	$1 \times 10^{-4}$	130 $\pm$ 39

\* Each value represents the mean  $\pm$ SE of four samples except C. Control and phenformin  $1 \times 10^{-4}$  M which represent the mean  $\pm$ SE of eight samples.

response (control  $152 \pm 48$ , tolbutamide  $1 \times 10^{-4}$  M  $344 \pm 50$ , norepinephrine  $5 \times 10^{-5}$  M  $464 \pm 40$  and the two agents in combination  $408 \pm 32$  pmoles cyclic AMP accumulated per mg protein).

We have measured the effects of tolbutamide on the concentration of cyclic AMP in intact rabbit atria. At a concentration of tolbutamide which enhances myocardial contractility ( $5 \times 10^{-4}$  M) the concentration of cyclic AMP increased approximately two- to threefold from a control value of 1.8 pmoles/mg tissue to 4.4 pmoles/mg tissue.

It is of interest that we have observed species differences in the activation of adenylyl cyclase by tolbutamide. In two separate experiments, adenylyl cyclase in particulate preparations of cat ventricle was not activated by tolbutamide  $1 \times 10^{-4}$  M.

## DISCUSSION

The data in this investigation demonstrate that the sulfonylurea drugs increase contractility of isolated rabbit atria, increase automaticity of isolated dog Purkinje fibers, and activate adenylyl cyclase in particulate preparations from rabbit or human hearts. The im-

plications of these findings are of importance in light of the findings of the University Group Diabetes Program study (1) since they suggest several possible mechanisms for the apparent increase in cardiovascular mortality.

Although inotropic agents are widely used in the treatment of congestive heart failure, there are no studies as to the effects of long-term administration of inotropic agents to subjects with normal myocardial contractility. Cardiac work and myocardial oxygen consumption are increased by a number of interventions that increase myocardial contractility. These include administration of calcium (10), catecholamines (11), digitalis (12), and paired electrical stimulation (13). It has also recently been shown that a number of inotropic agents including isoproterenol, ouabain, glucagon, and bretylium tosylate administered to dogs before experimental coronary occlusion increased the severity and extent of the ischemic injury (14). The state of myocardial contractility is thought to play an important role in the pathogenesis of another pathologic entity, acute dissecting aneurysm of the aorta (15). Therefore, the inotropic effect of the sulfonylurea drugs might increase myocardial work and oxygen consumption and increase the severity of several pathologic processes affecting the cardiovascular system of humans. Tolbutamide also increases automaticity of isolated Purkinje fibers. This is one of two basic mechanisms that have been postulated for the genesis of cardiac arrhythmias (16). These combined effects might be particularly disadvantageous in diabetics, a patient population known to have an increased incidence of coronary artery disease and generalized atherosclerosis.

There are many possible mechanisms whereby inotropic and chronotropic effects may be produced. Since the inotropic and chronotropic effects of several hormones are postulated to be mediated by activation of adenylyl cyclase and the resultant increase in intracellular cyclic AMP (4, 17-19), this system has been examined as a potential mechanism for these actions of the sulfonylurea drugs. Furthermore, tolbutamide has recently been demonstrated to activate adenylyl cyclase in a pancreatic islet cell adenoma, a finding which may be related to its insulinogenic action on the pancreas (20).

The accumulation of cyclic AMP observed in the experiments above could occur as a result of phosphodiesterase inhibition as well as adenylyl cyclase activation. Pertinent to this, tolbutamide has been shown to inhibit phosphodiesterase in kidney homogenates (21), pancreatic beta cell preparations, and purified beef heart preparations (22). However, the lowest concentration of tolbutamide utilized in any of these studies,  $3 \times 10^{-4}$  M produced only minor inhibition of phosphodiesterase and is 30-1000 times greater than the concentrations of tolbutamide which maximally activate adenylyl cyclase

in our system. Furthermore, we have previously shown that the phosphodiesterase in the rabbit and human hearts was not inhibited by  $1 \times 10^{-4}$  M tolbutamide (3).

Sutherland, Robinson, and Butcher have defined four criteria to be fulfilled before the adenylyl cyclase-cyclic AMP system can be considered to be the mediator of the physiological effects of an agent on its target tissue (23). First, the agent should increase adenylyl cyclase in broken cell preparations. Second, the level of cyclic AMP in the intact tissue should increase in response to the agent. Third, the physiologic response of the agent should be enhanced by phosphodiesterase inhibitors. Fourth, cyclic AMP or one of its derivatives should mimic the effects of the agent. The first three of the above criteria have been fulfilled for tolbutamide. Nevertheless, our data cannot establish the definitive mechanism of these actions since definitive experiments to settle the controversial role of the adenylyl cyclase-cyclic AMP system in myocardial contractility and automaticity are lacking. In addition, it should be noted that there are quantitative discrepancies between the concentrations of tolbutamide which activate adenylyl cyclase in broken cell preparations and which produce the inotropic and chronotropic effects in intact tissues. The reason for these discrepancies is unclear. The data presented do indicate that the catecholamines and tolbutamide exert their effects on the heart by separate and distinct receptor mechanisms. The beta-adrenergic blocking agent propranolol abolishes the catecholamine effects on both contractility and adenylyl cyclase but does not alter the effects of tolbutamide.

Although the concentrations of sulfonylurea drugs used in this study either approximate or are below the concentrations of drug measured in the sera of patients receiving therapeutic doses of these drugs (8), it is possible that such measurements are erroneously high reflecting inactive metabolites or drug bound to plasma proteins. The actual active concentration of the drug in the region of the receptor for any given action is unknown. However, if one assumes that 75% of the measured serum concentration is actually inactive then the reported values for tolbutamide of 8-10 mg/100 ml shrink to 2-2.5 mg/100 ml or  $7.5-9.5 \times 10^{-5}$  M. Even at these revised estimates for active tolbutamide concentrations in patients, adenylyl cyclase from both rabbit and human would be maximally activated and a significant inotropic effect produced (110-115% of control). Certainly, any final conclusions regarding the therapeutic importance of these findings must await definitive studies in intact humans. It is interesting to speculate, however, that if these effects are confirmed in intact humans, there may be some patients in whom the inotropic effects will be beneficial. On the other hand, in patients with coronary artery disease and angina pectoris, where the

balance between myocardial oxygen supply and demand is critical, it is reasonable to expect that these drugs might be deleterious. An analysis of each clinical situation will be necessary to determine the ramifications of this additional pharmacologic action.

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