Br. J. Pharmac. (1978), **64**, 99–108.

A NEW BIOASSAY FOR GLUCAGON

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1 The relaxant action of glucagon has been studied in strips of rabbit renal arteries partially contracted by a low concentration (1 ng/ml) of noradrenaline.

2 The preparation was relaxed in a dose-dependent manner by concentrations of glucagon varying between 25 ng/ml and 420 ng/ml.

3 The relaxant effect of glucagon (0.1 μg/ml $\simeq ED_{60}$) on this preparation was not affected by propranolol (5.0 μg/ml), cimetidine (10 μg/ml), diphenhydramine (10 μg/ml), indomethacin (5.0 μg/ml), phentolamine (1.2 μg/ml), atropine (10 μg/ml) and 8-Leu-AT_{II} (1.0 μg/ml) but was slightly potentiated by Des-Arg^9 Leu-OMe^8-Bk (25 μg/ml) and indomethacin (50 μg/ml).

4 The dose-response curve to glucagon remained parallel in the presence of papaverine (2.5 μg/ml) but was shifted to the left by a factor of 2.5 to 2.8. Theophylline (250 μg/ml) also potentiated the vascular relaxation induced by glucagon.

5 Insulin (10 μg/ml) did not influence the relaxant effect of glucagon.

6 The removal of the N-terminal amino acid (His) of glucagon reduced by 89% the biological activity of this fragment on the vascular preparation. The removal of the C-terminal amino acids Met-27, Asn-28 and Thr-29 of glucagon resulted in a fragment which was inactive either as an agonist or as an antagonist when tested at concentrations as high as 925 ng/ml.

7 It is concluded that the relaxation of partially contracted strips of rabbit renal arteries by glucagon constitutes a simple, sensitive, relatively specific and reliable bioassay which may be useful for the determination of glucagon in biological materials and for structure-activity relationship studies with this hormone.

Introduction

Several bioassays for glucagon have been developed, based on the ability of the hormone to provoke hyperglycemia *in vivo* or to stimulate glucose production, phosphorylase activity or adenylate cyclase activity *in vitro* (for a review, see Luyckx & Lefebvre, 1970). Due to their lack of specificity and/or sensitivity, these bioassays were rapidly replaced by radioimmunoassays (Unger, Eisentraut, McCall & Madison, 1961). The various advantages and disadvantages of these assays have been discussed previously (Foa, 1964; Luyckx & Lefebvre, 1970; Sokal, 1972).

Although very useful for determining picrogram amounts of circulating glucagon in the blood (Jaspan & Rubenstein, 1977), radioimmunoassays are of little value for structure-activity relationship studies, because the structural requirements for immunogenicity are unlikely to be similar to those involved in biological activity (Heding, Frandsen & Jacobsen, 1976). Consequently, researchers working on the design of glucagon analogues possessing a prolonged duration of action or a specific inhibitory action against glucagon have to rely upon bioassays to obtain relevant structure-activity relationship data.

Until now, the activation of the adenylate cyclase system by glucagon in isolated plasma membrane of rat livers (Rodbell, Birbaumer, Pohl & Sundby, 1971a; Rodbell, Krans, Pohl & Birbaumer, 1971b; Bromer, Boucher & Patterson, 1973; Lin, Wright, Hruby & Rodbell, 1975) has been used extensively as a bioassay for structure-activity studies. This assay involves the measurement of the amount of cyclic 3′, 5′-adenosine monophosphate (cyclic AMP) produced when labelled adenosine triphosphate (ATP) is incubated with plasma membranes containing adenylate cyclase in the absence or presence of drugs such as glucagon, its fragments or derivatives. The measurement of the specific binding of labelled glucagon to plasma membranes has also been used as a parallel assay in an attempt to determine whether the struc-

tural requirements for hormonal recognition and action were similar (Rodbell *et al.*, 1971b; Lin *et al.*, 1975).

Besides its well known glycogenolytic and lipolytic effects, glucacon also exerts a few cardiovascular actions: it stimulates the heart (Lucchesi, 1968; Glick, Darmley, Wechsler & Sonnenblick, 1968) and it reduces the vascular resistance in several organs (Ross, 1970), including the kidneys (Olsen, 1977). To our knowledge, no attempt has yet been made to develop a bioassay for glucagon, based on its ability to relax vascular smooth muscle.

In this study, we investigated the effect of glucagon on several smooth muscle preparations. The relaxant effect of glucagon on partially contracted strips of rabbit renal artery is proposed as a new sensitive, specific and reliable bioassay which may be useful for studying structure-activity relationships of glucagon and for various analytical purposes involving this peptide.

Methods

In this study, we used various tissues derived from albino Wistar rats (200 to 300 g), albino rabbits (1.2 to 1.5 kg), guinea-pigs (500 to 600 g) and chickens (200 to 250 g). The animals were killed by stunning and bleeding through the carotid arteries. The following tissues were selected to explore the myotropic action of glucagon: rat duodenum (Horton, 1959); rat ascending colon (Regoli & Vane, 1964); rat stomach strip (Vane, 1957); rat uterus (Amin, Crawford & Gaddum, 1954); the rat jejunum and aorta strip; rabbit aorta strip (Furchgott and Bhadrakom, 1953); anterior mesenteric vein, inferior vena cava and renal artery of the rabbit; guinea-pig tracheal chain (Castillo & De Beer, 1947); guinea-pig ileum; and the chick rectum (Mann & West, 1950). The isolated organs were suspended in 40 ml organ baths containing a warm (37°C) Krebs solution of the following composition (in g/l): NaCl 6.9, KCl 0.35, $CaCl_2$ 0.28, KH_2PO_4 0.16, $MgSO_4$ 0.15, dextrose 2.0 and $NaHCO_3$ 2.1; and disodium edetate (Na_2EDTA) 0.02. This solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2.

Assessment of the myotropic effect of glucagon on various smooth muscle preparations

The contractions or relaxations of intestinal tissues were recorded with isotonic transducers (Harvard no. 356) on a 4 channel polygraph (Harvard). The tissues were stretched with 1 to 3 g loads and equilibrated for 30 to 45 min before exposure to drugs. In the case of vascular or tracheal tissues, the contractions or relaxations were recorded with isometric transducers (Harvard no. 363). The initial resting tension applied to the tissues varied between 0.5 to 0.75 g for the veins and between 1 to 2 g for the arteries or tracheal chains. The tissues were equilibrated for 60 to 90 min before the application of drugs.

The contractile effect of glucagon on intestinal tissues was tested by cumulative application of increasing concentrations of the peptide to the tissues. The stimulating effect of a cholinomimetic (carbachol) (25 to 100 ng/ml) was also measured to make sure that the tissues used for testing glucagon were suitable (in a good working range). The possible relaxant effect of glucagon was evaluated on intestinal tissues of which the basal tone was slightly raised by carbachol (25 to 100 ng/ml). Adrenaline (50 to 250 ng/ml) or glucagon (1 ng to 1 μg/ml) were then applied cumulatively to the contracted tissues. The myotropic effect of glucagon on vascular tissues was evaluated similarly except that noradrenaline (1 to 10 ng/ml) and nitroglycerin (1 to 10 μg/ml) were used respectively as control stimulant and relaxant. Carbachol (50 to 100 ng/ml) and adrenaline (50 to 250 ng/ml) were used for the same purpose on the guinea-pig tracheal chain.

Test for the specificity of glucagon receptors in the rabbit isolated renal artery

In a first series of experiments, we evaluated the contractile or relaxant effects of several vasoactive drugs (Table 2) on the rabbit isolated renal arteries. Increasing concentrations of each drug were applied cumulatively to unstimulated (for vasoconstrictors) or noradrenaline-pretreated (for vasodilator drugs) tissues until the maximum effect was reached.

In a second series of experiments, the vasodilator effect of glucagon (0.1 μg/ml) on the rabbit isolated renal arteries was measured in the absence (control relaxation) and presence of one of the following inhibitors: propranolol (5 μg/ml), cimetidine (10 μg/ml), diphenhydramine (10 μg/ml), indomethacin (5 or 50 μg/ml), phentolamine (1.2 μg/ml), atropine (10 μg/ml), 8-Leu-AT_{II} (1 μg/ml) and Des-Arg^9 Leu-OMe-BK (25 μg/ml). The antagonists were left in contact with the tissues for 20 min before adding glucagon.

Dose-response curves to glucagon, Des-His^1 glucagon and Des-Met^{27} Asn^{28} Thr^{29} glucagon on the rabbit isolated renal artery

Rabbit isolated renal arteries contracted by noradrenaline (1 ng/ml) were challenged twice with increasing concentrations of glucagon. The data derived from these experiments were used to construct dose-response curves. In some experiments, dose-response curves were obtained both with glucagon and one of its fragments on the same tissues, in the presence

of papaverine (2.5 μg/ml). Responses to glucagon were also measured in the absence and presence of theophylline (250 μg/ml). The phosphodiesterase inhibitors were applied to the tissues 20 min before repeating the injections of glucagon.

Drugs

Des-His[1] glucagon and Des-Met[27] Asn[28] Thr[29] glucagon and glucagon were kindly provided by Dr R. S. Dolman, Eli Lilly, Toronto, Ontario. The following drugs were used: papaverine hydrochloride, noradrenaline, adrenaline bitartrate, 5-hydroxytryptamine creatine sulphate, dopamine hydrochloride, adenosine triphosphate Na salt (ATP), adenosine 5'-diphosphate Na salt (ADP), adenosine 5'monophosphoric acid Na salt (AMP), adenosine, adenosine 3',5'-cyclic monophosphoric acid Na salt (cyclic AMP), guanosine 3',5'-cyclic monophosphoric acid Na salt (cyclic GMP) (Sigma), isoprenaline hydrochloride (Withrop), histamine dihydrochloride (Fisher), theophylline (Anachem), acetylcholine chloride (Roche), prostaglandin E_2 (PGE_2) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Upjohn), vasopressin (Parke-Davis), propranolol hydrochloride (Ayerst), phentolamine hydrochloride (Ciba), diphenhydramine (Parke-Davis), cimetidine (Smith, Kline and French), indomethacin (Merck), atropine sulphate (Sigma); 1-Asp, 5-Ile-angiotensin II (AT_{II}), 1-Asp, 5-Ile-8-Leu-AT_{II} (8-Leu-AT_{II}), bradykinin (Bk) and Des-Arg[9] Leu-OMe[8]-Bk were synthesized in our laboratory (Park, Choi, Rioux & Regoli, 1974; Park, St-Pierre, Barabé & Régoli, 1977) using the solid-phase method of Merrifield (1963). Substance P was synthesized in our laboratory by (St-Pierre, Fournier, Gaudreau & Regoli (unpublished method).

Indomethacin was dissolved in Trizma base (Sigma) (24.2 g/l). PGE_2 and $PGF_{2\alpha}$ were dissolved in absolute ethanol. Glucagon and its fragments were dissolved in the diluting solution for injection provided by Eli Lilly Co. It contains glycerin, 1.6 v/v with phenol, 0.2% w/v, as a preservative; the pH of this solution is approximately 4.0. Daily dilutions of all drugs were made with 0.9% w/v NaCl solution (saline). Concentrations of all drugs are expressed in g/ml of the base except for the nucleotides (g/ml of the salt). Catecholamines were dissolved in saline HCl (0.01 N). The statistical significances were evaluated by Student's *t* test for paired or independent samples and *P* values of 0.05 or less were considered to be significant.

Results

The effect or absence of effect of glucagon on several smooth muscle preparations

The results shown in Table 1 indicate that glucagon (1 ng to 1 μg/ml) was inactive both as a stimulant or a relaxant on several smooth muscle preparations obtained from rats, rabbits, guinea-pigs or chickens. However, glucagon was found to relax at least two vascular tissues: the rat isolated aorta and the rabbit isolated renal artery. For further studies, we chose the rabbit isolated renal artery because, in most experiments, the responses to glucagon were more consistent and more stable than in strips of rat aorta.

Table 1 Sensitivity of various tissues to glucagon†

Preparation	*Dose range (per ml)*	*Myotropic effect*
Rat duodenum (3)	1 ng–1 μg	No effect
Rat jejunum (3)	1 ng–1 μg	No effect
Rat stomach strip (3)	1 ng–1 μg	No effect
Rat ascending colon (3)	1 ng–1 μg	No effect
Rat uterus (3)	1 ng–1 μg	No effect
Rat aorta strip (7)	50 ng–1 μg	Relaxation
Rabbit aorta strip (10)	1 ng–1 μg	No effect
Rabbit renal artery (12)	25 ng–1 μg	Relaxation
Rabbit inferior vena cava (13)	1 ng–1 μg	No effect
Rabbit anterior mesenteric vein (3)	1 ng–1 μg	No effect
Guinea-pig ileum (4)	1 ng–1 μg	No effect
Guinea-pig tracheal chain (2)	1 ng–1 μg	No effect
Chick rectum (2)	1 ng–1 μg	No effect

† The relaxant effect of glucagon was assessed on tissues contracted either with carbachol (non vascular tissues) or with noradrenaline (vascular tissues). The number of individual determinations is given in parentheses. Glucagon did not contract any of these preparations.

Moreover, strips of rabbit renal arteries were usually more sensitive to glucagon than strips of rat aorta. The solvent used for glucagon was carefully tested in various concentrations and was found inactive on the rabbit isolated renal arteries and the rat aorta.

Specificity of glucagon receptors in the rabbit isolated renal artery

As shown in Table 2, the rabbit isolated renal artery was sensitive to the myotropic action of several endogenous drugs. The preparation was stimulated by various catecholamines (noradrenaline (NA), adrenaline, dopamine), acetylcholine, various amines (histamine, 5-hydroxytryptamine), prostaglandin E_2 and $F_{2\alpha}$, and various peptides such as angiotensin II, bradykinin, vasopressin and substance P. However, a few compounds exerted a relaxant effect on this preparation, including isoprenaline, histamine, ATP, ADP, AMP, adenosine, cyclic AMP, and glucagon. The relaxant effect of these compounds was observed on tissues in which the basal tone was raised by a vasoconstrictor such as NA. A relaxant action of histamine was observed in tissues contracted by NA and only when an H_1-receptor blocking agent (diphenhydramine) was present. When used at concentrations higher than 250 ng/ml, isoprenaline provoked the contraction of the preparation. Cyclic GMP was inactive both as a stimulant and as a relaxant.

The specificity of glucagon receptors in the rabbit isolated renal artery was studied by comparison of the relaxant effect of an ED_{60} concentration of glucagon before and after exposure of the tissues to various antagonists. The results are summarized in Table 3. The relaxant effect of glucagon in the rabbit isolated renal artery was not modified by propranolol or cimetidine even at concentrations which blocked completely the relaxant actions of isoprenaline and histamine respectively. Diphenhydramine, phentolamine, atropine or 8-Leu-AT_{II} did not influence the vascular effect of glucagon even when used at concentrations which were shown in preliminary studies to block

Table 2 Myotropic effects of various compounds on strips of rabbit renal artery

Compounds	*Type of effect*	*Dose range (per ml)*	*n*
Noradrenaline	Contraction	0.2 ng–3.1 μg	9
Adrenaline	Contraction	0.2 ng–10 μg	10
Isoprenaline†	Relaxation	5 ng–0.25 μg	6
5-hydroxytryptamine	Contraction	1 ng–17 μg	6
Histamine	Contraction	6 ng–28 μg	7
Histamine††	Relaxation	100 ng–1 μg	7
Dopamine	Contraction	6 ng–85 μg	7
Acetylcholine	Contraction	63 ng–130 μg	4
Prostaglandin E_2	Contraction	1 ng–2.5 μg	9
Prostaglandin $F_{2\alpha}$	Contraction	1 ng–2.5 μg	9
Angiotensin II	Contraction	0.2 ng–0.13 μg	10
Bradykinin	Contraction	130 ng–13 μg	6
Vasopressin	Contraction	0.3 mu–33.1 mu	4
Substance P	Contraction	630 ng–13 μg	4
Glucagon†††	Relaxation	25 ng–0.5 μg	22
Adenosine triphosphate	Relaxation	25 ng–3.8 μg	8
Adenosine diphosphate	Relaxation	100 ng–1.3 μg	8
Adenosine monophosphate	Relaxation	50 ng–1.3 μg	8
Adenosine	Relaxation	25 ng–1.3 μg	8
Cyclic 3′,5′-adenosine monophosphate	Relaxation	250 ng–135 μg	7
Cyclic 3′,5′-guanosine monophosphate	No effect	25 ng–640 μg	8

n = number of experiments.
† The relaxant effect of isoprenaline was assessed on tissues contracted by noradrenaline (1 ng/ml).
†† The relaxant action of histamine was obtained on strips contracted by a submaximal concentration of noradrenaline (1 ng/ml), in the presence of diphenhydramine (2.5 μg/ml).
††† The relaxant actions of glucagon and the various nucleotides were assessed on strips contracted by a submaximal concentration of noradrenaline (1 ng/ml).

completely the vasoconstrictor actions of high concentrations of their respective agonists, namely histamine, NA, acetylcholine and angiotensin II. A possible interference by intramural prostaglandins was investigated by the use of indomethacin, a powerful inhibitor of prostaglandin synthesis (Vane, 1971). The relaxant effect of glucagon in the rabbit isolated renal artery was not modified by indomethacin (5 μg/ml) but was significantly potentiated by indomethacin (50 μg/ml). Des-Arg^9 Leu-OMe^8-Bk (25 μg/ml), a recently developed antagonist of bradykinin (Regoli, Barabé & Park, 1977), antagonized the stimulant effect of bradykinin on the rabbit isolated renal artery (G. Gagnon, D. Regoli and F. Rioux, unpublished results) and also potentiated the vascular effect of glucagon on this preparation.

The potentiating effects of phosphodiesterase inhibitors on the vasodilator effect of glucagon in the rabbit isolated renal artery

The relaxant effects of glucagon on strips of rabbit renal arteries stimulated by NA are illustrated in Figure 1. In Figure 1a, a stable plateau of contraction was observed when the tissue was exposed to NA. In Figure 1b and c, the preparation was challenged twice with increasing concentrations of glucagon. The potentiation of the relaxant action of glucagon by papaverine (2.5 μg/ml) a well known inhibitor of vascular phosphodiesterase (Triner, Nahas, Vulliernoz, Overweg, Verosky, Habif & Ngai, 1971) is shown in Figure 1d. Theophylline also potentiated the vascular relaxant effect of glucagon (tracing not shown).

ED_{50} values for glucagon were obtained on strips of rabbit renal arteries stimulated by increasing doses of NA in the absence and presence of papaverine or theophylline. The results are summarized in Table 4. The concentrations of glucagon needed to produce 50% of the maximal relaxation (ED_{50}) of rabbit isolated renal arteries stimulated by NA (5 ng/ml) or NA (30 ng/ml) were respectively 3.4 and 7.4 times greater than with NA (1 ng/ml) thus indicating that glucagon was much more active as a relaxant on tissues slightly contracted (0.8 to 1 g) than on those strongly contracted (1.5 to 2 g) by NA. Both papaverine (2.5 μg/ml) and theophylline (250 μg/ml) were found to reduce the ED_{50} values for glucagon. Papaverine was found to be more than 100 times as active as theophylline in potentiating the vascular effect of glucagon in the rabbit isolated renal artery.

Table 3 The effect of various inhibitors on the vasodilator effect of glucagon in the rabbit isolated renal artery†

Antagonist	*Agonist*	*Dose (ng/ml)*	*Relaxation (% of maximum) Before*	*After*	
Propranolol†† (5 μg/ml)	Isoprenaline	250	54.6 ± 4.4	0	(7)
	Glucagon	100	53.4 ± 5.6	57.4 ± 6.2	(7)
Cimetidine††† (10 μg/ml)	Histamine	350	78.4 ± 6.5	0	(7)
	Glucagon	100	58.2 ± 5.1	54.3 ± 6.1	(12)
Diphenhydramine (10 μg/ml)	Glucagon	100	64.2 ± 8.3	57.8 ± 7.1	(6)
Indomethacin (5 μg/ml)	Glucagon	100	62.1 ± 5.5	70.0 ± 5.7	(10)
(50 μg/ml)	Glucagon	100	59.6 ± 5.8	78.5 ± 6.9*	(10)
Phentolamine†† (1.2 μg/ml)	Glucagon	100	62.7 ± 5.2	57.5 ± 5.3	(8)
Atropine (10 μg/ml)	Glucagon	100	60.4 ± 5.3	61.7 ± 5.6	(9)
8-Leu-AT$_{II}$ (1 μg/ml)	Glucagon	100	45.0 ± 2.5	54.4 ± 8.0	(5)
Des-Arg^9 Leu-OMe^8-Bk (25 μg/ml)	Glucagon	100	52.2 ± 4.4	73.9 ± 6.9*	(7)

† Except when otherwise stated, the vasodilator effect of glucagon was assessed on tissues contracted by a submaximal concentration of noradrenaline (1 ng/ml). In parentheses, the number of experiments.
†† The effects of propranolol and phentolamine were evaluated on tissues contracted by a submaximal concentration of histamine (15 ng/ml).
††† The vasodilator effects of histamine or glucagon were evaluated in the presence of diphenhydramine (2.5 μg/ml) (control relaxation) and compared to those obtained in the presence of diphenhydramine (2.5 μg/ml) plus cimetidine (10 μg/ml).
*$P < 0.05$.

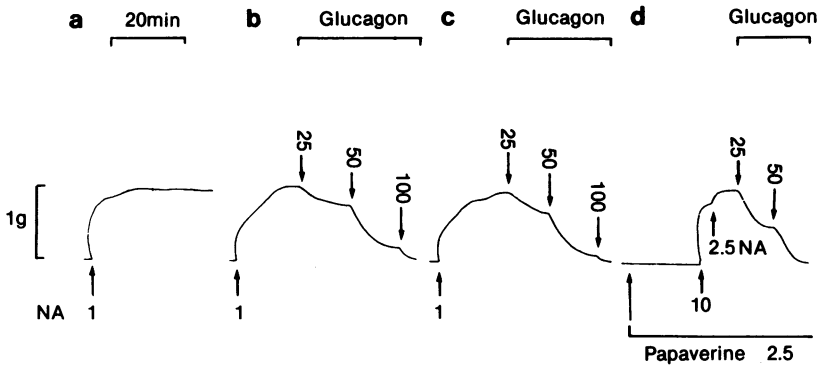

Figure 1 Typical tracing showing the relaxant effect of various concentrations of glucagon (b and c) on a strip of rabbit renal artery exposed to noradrenaline (NA, 1 ng/ml) or (d) NA (10 plus 2.5 ng/ml) in the presence of papaverine (2.5 μg/ml). All doses shown on figure are ng/ml, except for papaverine (μg).

The effect of papaverine on the dose-response curves to glucagon or its fragments as measured in the rabbit isolated renal artery

Increasing concentrations of glucagon could be tested 2 or 3 times on the same strips of rabbit renal artery without any evidence of desensitization (Figure 2). The intervals of time between each set of glucagon injections was fixed at 30 min. The recovery of the contraction induced by NA was found to be a reliable index that the relaxant effect of glucagon was dissipated. The onset of action of glucagon (ED_{50}) on strips of rabbit renal artery contracted by NA (1 ng/ml) was usually rapid (10–15 s); the relaxation reached a plateau in 10 to 20 min.

The dose-response curve obtained with glucagon in the isolated rabbit renal artery pretreated or not with papaverine (2.5 μg/ml) is illustrated in Figure 3. In the absence of papaverine, the minimal effective dose of glucagon approximated 25 ng/ml while in the presence of papaverine, concentrations of glucagon as low as 2.5 to 5.0 ng/ml could be measured. The dose-response curve to glucagon was shifted to the left by a factor of 2.5 to 2.8 in the presence of papaverine but remained parallel. The linear portion of the dose-response curve obtained with glucagon alone extended from 45 to 175 ng/ml; in the presence of papaverine, it ranged from 20 to 75 ng/ml.

In a limited number of experiments, we evaluated the relaxant effect of glucagon, Des-His^1-glucagon and Des-Met^{27} Asn^{28} Thr^{29}-glucagon in strips of rabbit renal artery pretreated with NA (15 ng/ml) and papaverine (2.5 μg/ml). The dose-response curve obtained with Des-His^1-glucagon (2 experiments) was

Table 4 ED_{50} values for glucagon tested on rabbit isolated renal arteries contracted by various concentrations of noradrenaline (NA) in the absence or presence of papaverine (2.5 μg/ml) or theophylline (250 μg/ml)†

Compounds	*Concentration of NA (ng/ml)*	*ED_{50} for glucagon (ng/ml)*	
NA	1	83.3 ± 3.8	(22)
NA	5	282.1 ± 39.1***	(7)
NA	30	615.0 ± 177.2**	(6)
NA plus papaverine††	15	29.1 ± 2.0***	(9)
NA plus theophylline††	50	62.8 ± 5.9*	(7)

† The results are expressed as means ± s.e. means. In parentheses, the number of individual determinations. The increases in tension induced by the various doses of NA were: 0.8 to 1 g for NA (1 ng); 1.5 to 1.7 g for NA (5 ng) and 2.1 to 2.3 g for NA (30 ng).

†† The contractions induced by NA (15 or 50 ng/ml) in the presence of papaverine or theophylline were equivalent to those produced by NA (1 ng/ml) alone.

When compared to ED_{50} for glucagon in the presence of NA (1 ng/ml); *$P < 0.01$; **$P < 0.005$; ***$P < 0.001$.

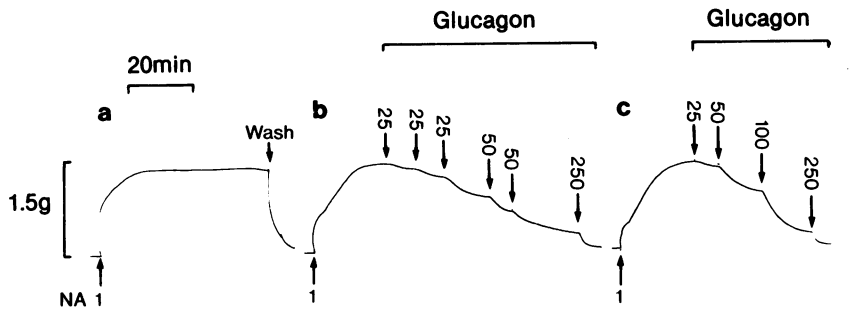

Figure 2 Typical tracing illustrating the contraction of a strip of rabbit renal artery exposed to noradrenaline (NA, 1 ng/ml) (a) and the relaxations induced by cumulative applications of glucagon. The effects of the first (b) and second (c) set of glucagon injections were obtained on the same tissue. All doses shown on figure are ng/ml.

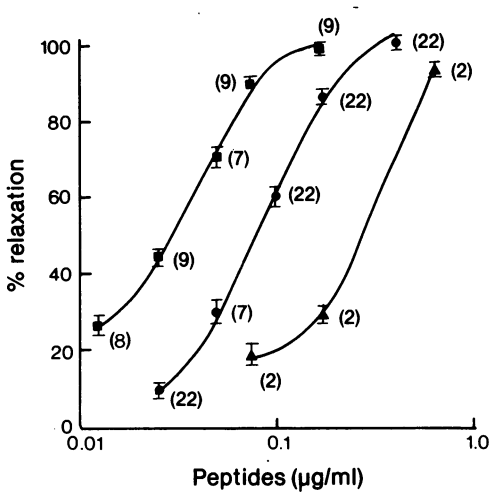

Figure 3 Dose-response curves obtained with glucagon and Des-His[1] glucagon on strips of rabbit renal arteries partially contracted by noradrenaline (1 ng/ml) in the absence or presence of papaverine (2.5 μg/ml). The number of individual determinations are indicated in parentheses. (●) Glucagon; (■) glucagon in the presence of papaverine; (▲) Des-His[1] glucagon in the presence of papaverine.

parallel to that obtained in several tissues with glucagon (Figure 3). ED_{50} values for Des-His[1]-glucagon were 0.28 μg in one tissue and 0.25 μg/ml in the other one. The potency of Des-His[1]-glucagon was approximately 11% compared to glucagon (100%). Des-Met[27] Asn[28] Met[29]-glucagon was found inactive both as an agonist or antagonist at doses up to 0.925 μg/ml (3 experiments). The small amount of Des-His[1]-glucagon available did not allow us to test this compound for possible inhibitory action against glucagon. Insulin (10 μg/ml) did not modify the relaxant effect of glucagon in the rabbit isolated renal artery (4 experiments).

Discussion

The results show that the rabbit isolated renal artery stimulated by a low dose of NA is relaxed in a dose-dependent manner by glucagon. The selective sensitivity of this tissue to glucagon makes it a very suitable preparation for the assay of this peptide in pancreatic tissue extracts or biological fluids. Interference with the assay by other endogenous substances may be prevented by the use of selective antagonists of compounds such as catecholamines, acetylcholine, histamine, 5-hydroxytryptamine, angiotensin and bradykinin. Other substances such as substance P and vasopressin may act as contaminants, but the high concentrations required to stimulate the rabbit isolated renal artery are unlikely to be found in pancreatic tissue extracts or in biological fluids. Their presence could be verified by use of specific assay organs such as the rabbit anterior mesenteric vein for substance P (Bérubé, Marceau, Drouin, Rioux & Regoli, 1978) or the rabbit rectum for vasopressin (Gilmore & Vane, 1970). Other contaminants such as prostaglandins could be removed by organic extraction at pH 3.5 to 4.0 while nucleotides could be separated from glucagon by gel filtration. The possible interference with the assay by endogenous compounds such as neurotensin, vasoactive intestinal peptide (VIP), gastric inhibitory peptide (GIP) secretin and pancreozymin remains to be determined.

Principal features of bioassay methods for glucagon have been reviewed by Sokal (1972). The sensitivity and specificity of the bioassay for glucagon described in this paper were comparable to those reported in the adenylate cyclase assay (Makman & Sutherland, 1964; Rodbell *et al.*, 1971a, b), the liver slice assay (Vuylsteke & De Duve, 1957) and the perfused liver

assay (Sokal, 1972). The specificity of our bioassay could be greatly improved by the use of selective antagonists, or by the introduction of simple purification procedures such as those described before. The absence of effect of insulin on the response of the rabbit isolated renal artery to glucagon may be considered a real advantage over the assays which involve the measurement of glucose output from a perfused liver. The simplicity and low cost of our bioassay might also be seen as an advantage compared to other assays (Sokal, 1972).

Obviously, no bioassay is perfect. The main criticism which could be raised against the use of vascular tissue relaxation to assess the biological activity of glucagon is the possibly unphysiological character of the biological activity being measured. In fact, most cardiovascular actions of glucagon are seen with doses usually 50 to 200 times higher than the 50 to 150 pg/ml plasma which represents the fasting range in healthy humans (Jaspan & Rubenstein, 1977). However, it is interesting to recall that the circulating amount of glucagon may be greatly increased following hypoglycemia caused by insulin (Unger, Eisentraut, McCall & Madison 1962), starvation (Aguilar-Parada, Eisentraut & Unger, 1969), exercise (Böttger, Schlein, Faloona, Knochel & Unger, 1972) severe infection (Rocha, Santeusanio, Faloona & Unger, 1973), severe trauma and burns (Lindsey, Wilmore, Moylan, Faloona & Unger, 1972) diabetes (Alford, Bloom & Nabarro, 1977) and glucagonoma (Jaspan & Rubenstein, 1977). Even under such conditions, one cannot be absolutely certain that the circulating levels of immunoreactive glucagon (IRG) are sufficiently high to affect the cardiovascular system because IRG was shown recently to contain a number of fractions of different molecular weight and of unknown biological activity (Jaspan & Rubenstein, 1977). The report by Richardson & Withrington, (1976) that low concentrations of glucagon ($\simeq$9.2 ng/ml) inhibit significantly the vasoconstriction induced by noradrenaline in the hepatic arterial vascular bed of the dog, raised the possibility that glucagon may act as a physiological antagonist of vasoconstrictors in other vascular beds. The present report which describes the inhibitory effect of glucagon on strips of rabbit renal arteries contracted by NA is consistent with the results of other workers who showed an increase in renal blood flow following glucagon infusions in dogs (Stowe & Hook, 1970; Olsen, 1977; Ueda, Nakanishi, Mizayaki & Abe, 1977).

Glucagon is a well known activator of adenylate cyclase in the liver (Park & Exton, 1972) and fat cells (Birbaumer & Rodbell, 1969); this activation leads to intracellular accumulation of cyclic 3',5'-AMP, the proposed second messenger in the glucagon-induced glycogenolysis or lipolysis. A few pieces of evidence have accumulated which suggest that the cardiostimulatory property of glucagon is also due to adenylate cyclase activation (Levey & Epstein, 1969; Entman, Levey & Epstein, 1969). Although the relationships between intracellular cyclic AMP accumulation and smooth muscle relaxation have been clearly defined (Anderson, 1973), there has to date been no report supporting the idea that such mechanism could explain the relaxant effect of glucagon. In our opinion, the potentiation of the relaxant effect of glucagon in the rabbit isolated renal artery by papaverine and theophylline, two phosphodiesterase inhibitors (Triner *et al.*, 1971), constitutes pharmacological evidence that glucagon is acting through the activation of the vascular adenylate cyclase system. Biochemical evidence to support this interpretation is still lacking. The mechanism by which indomethacin or Des-Arg^9 Leu-OMe^8-Bk potentiate the relaxant effect of glucagon is unknown.

Smooth muscle preparations have been used successfully in the past to study relationships between the chemical structure and the biological activity of neurotransmitters (Ariens, 1966) and of peptides such as vasopressin and oxytocin (Walter, Schwartz & Rudinger, 1967; Rudinger, 1971) angiotensin (Regoli, Park & Rioux, 1974) and bradykinin (Schröder, 1970; Regoli *et al.*, 1977). In this paper, we have provided some evidence that structure-activity relationship studies could also be performed with glucagon using the relaxation of the rabbit isolated renal artery as a measure of the biological activity of this peptide or of its fragments. Des-His^1 glucagon was found to possess only 11% of the activity of the parent hormone in our 'vascular relaxation assay'. This value is 5 times higher than that reported for Des-His^1 glucagon in the adenylate cyclase assay but very similar to the 7 to 10% activity found in the hepatic receptor-binding assay (Hruby, Wright, Lin & Rodbell, 1976). Although our data are derived from a limited number of experiments, it is clear that the N-terminal amino acid (His) contributes significantly to the vascular relaxant activity of glucagon. The absence of effect of Des-Met^{27} Asn^{28} Thr^{29} glucagon in the 'vascular relaxation assay' at concentrations up to 925 ng/ml is consistent with the very low activity of his compound *in vivo* and in the hepatic receptor-binding assay (Bromer, 1976). These results give further support to the idea that the entire molecule of glucagon is responsible for its full biological activity (Faloona & Unger, 1974). Whether or not structure-activity data derived from studies in which the vascular relaxant effect of glucagon is used as a bioassay, may be applied to other biological systems such as liver glycogenolysis, remain to be determined.

In conclusion, we believe that the relaxation of partially contracted strips of rabbit renal artery by glucagon constitutes an inexpensive, sensitive, relatively specific and reliable bioassay which may be useful

not only for the determination of glucagon in pancreatic tissue extracts, in biological fluids or in commercial preparations of insulin, but also for structure-activity relationship studies oriented toward the design of long acting glucagon derivatives or glucagon antagonists.

This work was supported by a grant from the Banting Research Foundation. We thank Dr R.S. Dolman from Eli Lilly Co., Toronto, Ontario for his generous gift of glucagon. Des-His[1] glucagon and Des-Met[27] Asn[28] Thr[29] glucagon were obtained from Eli Lilly Co., Indianapolis, Ind. USA, through the courtesy of Dr R.S. Dolman. We also thank Dr J.E. Pike from the Upjohn Co., Kalamazoo, Mich., USA, for the gift of prostaglandin E_2 and $F_{2\alpha}$, and Smith and Kline and French Co., Montreal, Que., for the supply of cimetidine. Substance P was kindly provided by Dr Serge St-Pierre, Department of Physiology and Pharmacology, University of Sherbrooke, Sherbrooke, Que., Canada. D.R. is an associate of the Medical Research Council of Canada and F.R. is a scholar of the Canadian Heart Foundation. We also wish to thank Mrs T. Carrier for typing the manuscript. Reprint requests to F.R.

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(Received February 21, 1978.
Revised April 30, 1978.)