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Chemical and Biological Aspects of Peptide Catecholamine Conjugates

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ABSTRACT

Polymeric derivatives of D-isoproterenol have been prepared by diazotization to water-soluble, random copolypeptides of hydroxypropylglutamine and p-aminophenylalanine, molecular weight 1500, 3000, and 9600. The polymeric isoproterenol derivatives were purified by gel chromatography which reduced contamination by the parent catecholamine to undetectable levels (i. e., less than 0.01 wt %) and by 6-aminoisoproterenol (a possible decomposition product) to less than 0.4%. The derivatives were found to elicit positive chronotropic responses in isolated perfused guinea pig hearts, with mean effective doses (ED_{50}) which were between 1.3 and 2.0 orders of magnitude (for the 1500 and 9600 molecular weight derivatives, respectively) less than the ED_{50} for D-isoproterenol. Inotropic response decay times in isolated cat papillary muscles following washouts suggest that the polymer-bound drug does not diffuse into muscle tissues. In vivo biological studies (in conscious dogs) indicate that the polymeric drugs have significantly prolonged durations of action of between 5.5 ± 0.4 min for the derivative of highest (9600) molecular weight and 27.4 ± 4.9 min for the derivative of lowest (1500) molecular weight compared with 4.5 ± 0.14 min for a comparable dose of free isoproterenol. Possible explanations for the inverse relationship

between the molecular weights of the derivatives and their in vivo durations of action are suggested. Our findings demonstrate that under controlled conditions isoproterenol can retain biological activity while covalently bound to a soluble polymeric support. The in vivo biological data suggest that the tethering of drugs to polymers may provide a means of maximizing the therapeutic utility of drugs with short half-lives.

INTRODUCTION

Both synthetic and naturally occurring polymers have been used as matrices for controlling and/or modifying the activity of biological molecules in a wide variety of applications. One of the earliest studies of this type can be traced to the 1920's, when the reactions of haptenic groups were elucidated [1]. More recently, the technique of immobilized enzymes was developed by Katchalski and others [2], while the area of affinity chromatography can be traced to early experiments by Lerman [3] which were later developed by Cuatrecasas and Anfinsen [4] and others [5]. The covalent immobilization of hormones has also been used extensively to study the mechanism of action of these molecules [6-8].

The use of polymers as carriers for drugs, though proposed [9] almost as early as the other uses mentioned above, has only recently been elaborated on. A number of excellent reviews are now available on this subject [10-12]. Our interest in this area was stimulated by the possibility of "tailor making" polymers as carriers for drugs which might increase the therapeutic usefulness of these molecules. This increase in utility could arise simply from increasing the plasma retention time of the drug, through retarding its excretion or metabolism or even through specifically localizing the drug or directing it to the target tissue. Polypeptides are particularly attractive as model carriers for this application since they can readily be prepared with a wide variety of properties, including their molecular weight, charge, hydrophobic/hydrophilic balance, etc.

Our early studies were concerned with polypeptide derivatives of insulin, one of which—polyglutamyl-insulin (MW = 100,000)—was shown to have an increased duration of action in vivo [13]. Similarly, a polyglutamyl derivative of an analog of the luteinizing hormone-releasing factor was prepared which had a dramatically increased in vivo duration of action [14]. In both these cases, the poly(glutamic acid) carrier may simply retard the metabolism of the peptide hormones, since polyelectrolytes are known to be inhibitors of proteolytic enzymes.

More recently our attention has been focused on the study of polypeptide derivatives of catecholamines. These adrenergic hormones are particularly attractive to study since much is understood about their biochemistry and pharmacology and, in addition, their receptors are known to be localized at the surface of the cell membranes. Their polymeric derivatives therefore need not pass through the membrane to exert their effect. Earlier work in this area by Kaplan and coworkers [15, 16] had shown that epinephrine and isoproterenol could be immobilized by covalent binding via an azo linkage to porous glass beads which had been derivatized with an aryl amine. While the glass-immobilized catecholamines have shown interesting biological properties which are in many ways quite different from those of the parent drug [17], it has proven impossible to eliminate completely the possibility of a minute leakage of covalently or noncovalently bound catecholamine into a microvolume at the bead-tissue interface as the cause of the observed biological effects [18]. We therefore undertook the synthesis of a series of water-soluble copolypeptides containing hydroxypropylglutamine and p-aminophenylalanine to which isoproterenol was covalently coupled via diazotization [19]. The polymeric isoproterenol derivatives were exhaustively purified in order to eliminate the possibility that noncovalently attached catecholamine molecules contribute to their biological activity.

EXPERIMENTAL

The copolypeptides of hydroxypropylglutamine and p-aminophenylalanine were prepared by the random copolymerization of γ-benzylglutamic acid and p-nitrophenylalanine N-carboxyanhydrides (NCAs) by the general procedure described previously [19]. The NCAs were copolymerized in a molar ratio of 4:1 (glutamic acid:p-nitrophenylalanine) at 10% (w/v) concentration in dry dioxane with the use of dibenzyl glutamate as initiator; anhydride to initiator (A/I) ratios were 5, 10, and 50. Conversion of the γ-benzyl glutamate and p-nitrophenylalanine residues to the hydroxypropylglutamine and p-aminophenylalanine residues was carried out as described previously [19]. The polymers were fractionated on Biogel P-2 (A/I = 5, 10) [19] or dialyzed exhaustively against water using a Spectrapor 3 membrane (Spectrum Medical Industries, Inc., molecular weight cutoff approximately 3500) (A/I = 50) [19]. The estimated molecular weights of the polymers are given in Table 1.

The polymeric isoproterenol derivatives were prepared and purified by the general procedure described previously. The in vitro biological activities of the polypeptide-isoproterenol conjugates in the isolated perfused guinea pig heart and the isolated cat papillary muscle were determined as previously described [19]. The in vivo

TABLE 1. Estimated Molecular Weights of Random Copolypeptides of Hydroxypropylglutamine with p-Aminophenylalanine

Polymer	A/I	Molecular weight	Method of determination
I	5	1500	Ebullioscopic[a]
II	10	3000	Gel permeation chromatography[b]
III	50	9600	Ultracentrifugation[c]

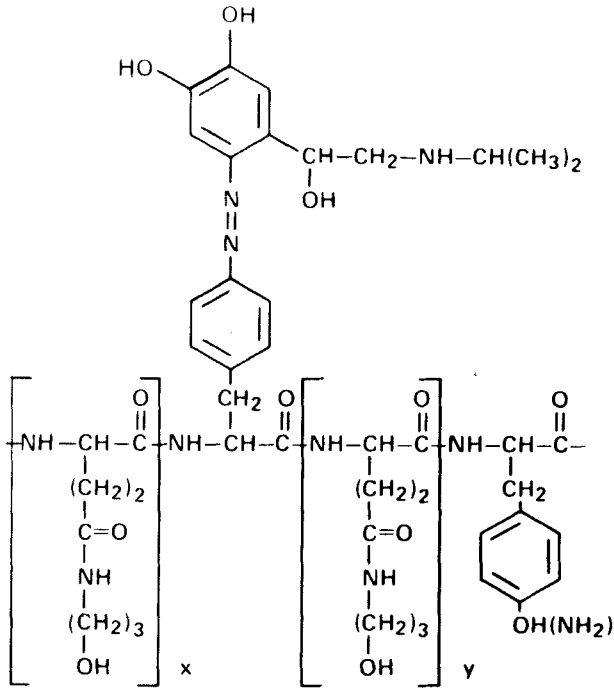
[a]Measurement performed by Huffman Laboratories, Inc.
[b]From a comparison with samples I and III on Biogel P-10.
[c]At 30°C in water by the method of Yphantis [24] with the use of a Beckman Spinco Model E ultracentrifuge.

biological activity of the derivatives was studied by monitoring cardiac functions following a bolus injection of solutions of the drugs in normal saline into the saphenous vein of chronically instrumented, conscious mongrel dogs (weight approx. 20 kg) [20].

RESULTS AND DISCUSSION

The proposed structure of the polymeric catecholamine derivatives is as shown in I. The polymer is a random copolymer of hydroxypropylglutamine and p-aminophenylalanine. The molar ratio of hydroxypropylglutamine to p-aminophenylalanine was approximately 4:1. Incorporation of drug (measured using radiolabeled isoproterenol) was typically 2.5-10 wt %. Since the diazotized p-aminophenylalanine residues in the random copolymer are not fully substituted, we assume that the remainder were hydrolyzed to tyrosyl residues during work-up of the derivatives. Typical elution profiles for the purification of two of the derivatives (molecular weights 1500 and 9600) on Biogel P-2 are illustrated in Fig. 1.

The products of the diazotization reaction mixture were separated by this method and subsequently identified by thin-layer chromatography. The first column purification of the lower molecular weight derivative (Fig. 1-1) resulted in four peaks assigned to the polymeric derivative, an unassigned minor peak, 6-aminoisoproterenol, and free isoproterenol. It is interesting to note that isoproterenol and its 6-amino derivative were separated by this method. Figure 1-2 represents the



I

third subsequent purification of the polymeric isoproterenol derivative peak. The most significant point of this purification is the reduction of contamination by the parent D-isoproterenol to undetectable levels (i. e., less than 0.01%). A small but recurrent contamination by 6-aminoisoproterenol was present at each column purification, presumably due to photolysis of the azo linkage of the catecholamine to the polymer during storage and handling. This contamination by the 6-amino derivative represented less than 0.4% of the polymeric catecholamine peak in each case.

The purification of the higher molecular weight (9600) polymeric catecholamine derivative was essentially identical to that of the 1500 molecular weight derivative described above. The soluble parent isoproterenol peak, 6-aminoisoproterenol, and polymeric isoproterenol peaks were in the identical fractions to those observed for the lower molecular weight derivative (Fig. 1-3). Subsequent gel chromatography of this higher molecular weight derivative (Fig. 1-4) revealed no detectable D-isoproterenol, indicating less than 0.01% contamination

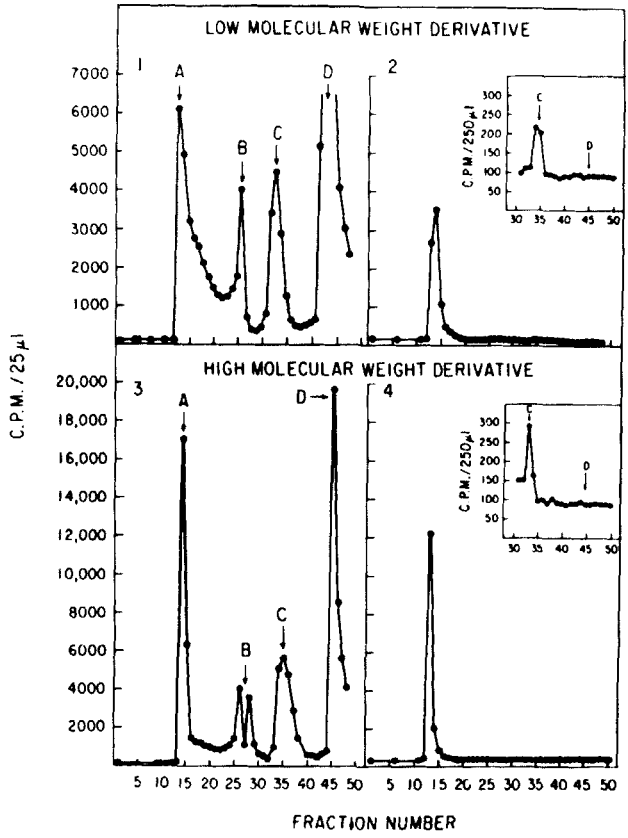


FIG. 1. Gel permeation chromatography of polymeric isoproterenol derivatives. Sections 1 and 3 represent the elution profiles for the separation of the products from the coupling reactions of the 1500 and 9600 MW derivatives, respectively. Peak assignment by thin layer chromatography indicates: (A) the polymeric isoproterenol derivatives; (B) an unassigned lower molecular weight polymeric peak; (C) 6-amino-D-isoproterenol; (D) D-isoproterenol. Sections 2 and 4 represent the third subsequent column purifications of the respective polymer peaks, with inserts illustrating peaks (C) and (D) at 100-fold increase in sensitivity. Gel chromatography was performed on a Bio-Gel P-2 (Bio-Rad) column (2.7 × 85 cm) at 4°C using 1mM ammonium bicarbonate as eluent. Fractions (12.5 ml) were monitored by radioactivity using ^{3}H-isoproterenol; isoproterenol-containing peaks were lyophilized in the dark [19].

by this amine. As in the case of the lower molecular weight derivative, 6-aminoisoproterenol was detectable at each purification step, although the degree of contamination was always less than 0.05% of the polymer-bound isoproterenol.

Thin-layer chromatography of the polymeric isoproterenol derivative peaks from the columns revealed no detectable free isoproterenol or its 6-amino derivative when the plates were scanned using a radiochromatograph (limits of sensitivity: ~ 0.4%).

The polymeric isoproterenol derivatives proved to be stable to storage at -15°C. Figures 1-2 and 1-4 represent chromatography after approximately 3 months at this temperature. Since no parent isoproterenol was detectable (i. e., less than 0.01%) in three subsequent purifications by gel chromatography and since 6-aminocatecholamine contamination was always less than 0.4%, these values represent the maximum levels of contamination of the polymeric isoproterenol derivatives by free drug molecules [19].

In Vitro Biological Characterization of Polymeric Catecholamine Derivatives

The two polymeric isoproterenol derivatives were tested for biological activity both immediately following their final gel chromatography, and also after lyophilization [19]. The results were identical in each case. The polymeric catecholamine derivatives all produced positive chronotropic responses in a dose-related manner when injected into isolated perfused guinea pig hearts. The log dose-response relationships for the 1500 and 9600 MW polymeric derivatives are compared with D-isoproterenol, 6-aminoisoproterenol, and D-epinephrine in Fig. 2. The responses are plotted as a function of the total number of moles of isoproterenol added, determined using radiolabeled catecholamine.

The mean effective dose (ED_{50}) for D-isoproterenol is 1.4×10^{-11} mole. The dose-response for the lower molecular-weight (1500) polymeric isoproterenol derivative is shifted to the right of that for isoproterenol by 1.3 orders of magnitude, with an ED_{50} of 2.8×10^{-10} mole. The dose-relationship for the higher molecular-weight (9600) derivative is shifted slightly further to the right with an ED_{50} of 1.4×10^{-9} mole. Thus, the polymeric isoproterenol derivatives, while less active than the parent isoproterenol, are considerably more active than both 6-aminoisoproterenol and D-epinephrine. The dose-response relationship for 6-aminoisoproterenol (Fig. 2) indicates an ED_{50} of 2.2×10^{-8} mole, which is very similar to that reported previously for this derivative [21]. The calculated ED_{50} for D-epinephrine is 2.0×10^{-9} mole.

The β-adrenergic antagonist, D,L-propranolol (5×10^{-7} M),

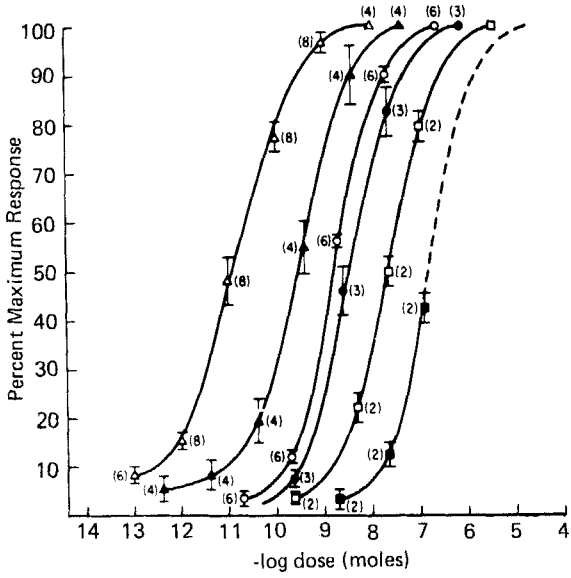


FIG. 2. Positive chronotropic effects of: (△) D-isoproterenol; (▲) 1500 molecular weight polymeric D-isoproterenol derivative; (○) 9600 MW polymeric D-isoproterenol derivative; (●) D-epinephrine; (□) 6-amino-D-isoproterenol (■) 9600 MW polymeric D-isoproterenol derivative in the presence of continuously perfused D, L-propranolol (5×10^{-7} M) in Krebs solution. The doses of the polymeric isoproterenol derivatives are plotted as the total moles of isoproterenol added. The error bars represent the standard error of the mean of each point; the number of experiments for each point is indicated in parentheses. Responses are plotted as the percentage of the maximum change in heart rate of isolated perfused guinea pig hearts [19].

inhibits the chronotropic action of the polymeric isoproterenol (9600 MW) derivative in an apparently competitive manner (Fig. 2). We have calculated a pA_2 value of 8.3 for propranolol from a calculated K_b value of 5.0×10^{-9} M. (pA_2 is the negative logarithm of the concentration of an antagonist required to give a dose ratio of two, and K_b is the apparent receptor-antagonist dissociation constant.) The calculated pA_2 value is similar to those reported by others for propranolol antagonism of isoproterenol on β-adrenergic receptor systems [22].

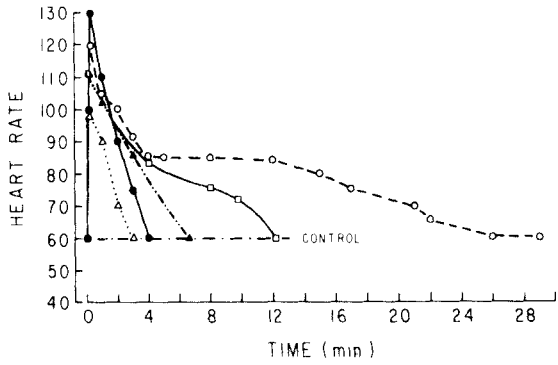


FIG. 3. Positive inotropic and chronotropic response duration in chronically instrumented conscious dogs [20] following injection of isoproterenol and polymeric isoproterenol derivatives. Heart rate is plotted as a function of time for: (△) D-isoproterenol (0.1 μg/kg); (●) D-isoproterenol (1 μg/kg); (▲) polymeric isoproterenol derivative (MW 9600) (0.1 μg/kg equivalent); (□) polymeric isoproterenol derivative (MW 3000) (0.1 μg/kg equivalent); (○) polymeric isoproterenol derivative (MW 1500) (0.1 μg/kg equivalent). Equivalent doses of polymeric derivatives were calculated on the basis of their in vitro biological activities in the perfused guinea pig heart [19].

The perfusates from the guinea pig hearts were collected within seconds of drug testing and immediately subjected to thin-layer chromatography. No 6-aminoisoproterenol or isoproterenol was detectable by this technique. The perfusate was also concentrated more than 1000-fold by lyophilization and resubjected to thin-layer chromatography, after which no detectable contaminants were found either visually or using radioscanning.

In Vivo Studies of Polymeric Isoproterenol Derivatives

The biological activity of the polymeric isoproterenol derivatives was compared with that of D-isoproterenol by monitoring cardiac responses to the drugs in conscious dogs. The results are shown in Fig. 3 and are summarized in Table 2. The positive chronotropic and inotropic responses to D-isoproterenol are characterized by an immediate rise to peak heart rate within 30 sec of injection followed immediately, without a plateau phase, by a rapid return to control

TABLE 2. Positive Inotropic and Chronotropic Response Duration in Conscious Dogs Following Injection of Isoproterenol and Polymeric Isoproterenol Derivatives

	Dose (μg/kg)	Average time to return to control levels (min)
D-Isoproterenol	1.0	4.5 ± 0.14 (n = 5)
Polymeric isoproterenol (9600 MW)	0.1[b]	5.5 ± 0.4 (n = 5)
Polymeric isoproterenol (3000 MW)	0.1[b]	12.8 ± 1.6 (n = 5)
Polymeric isoproterenol (1500 MW)	0.1[b]	27.4 ± 4.9 (n = 7)

[a]Measured in chronically instrumented mongrel dogs (approx. 20 kg) [20].

[b]Equivalent dose calculated on basis of in vitro biological activity (perfused guinea pig heart) [19].

levels within 3-4 min. Increasing the dose of isoproterenol from 0.1 μg/kg (a moderate dose) to 1 μg/kg (a high dose) results in only a slight prolongation of the chronotropic and inotropic action. Average duration for the response to 1 μg/kg D-isoproterenol was 4.5 ± 0.14 min (N = 5) (see Table 2).

In contrast to the short time course of action of the parent isoproterenol, a bolus injection of the lowest molecular weight (1500) polymeric isoproterenol derivative results in positive chronotropic and inotropic responses which are relatively well sustained, decreasing only slowly to control levels usually within 30 min (see Fig. 3). The average duration of the response to the 1500 MW derivative was 27.4 ± 4.9 min (n = 7) (Table 2).

The duration of the inotropic and chronotropic responses to the polymeric isoproterenol derivatives was found to be inversely related to their molecular weight over the molecular weight range tested. The durations of inotropic and chronotropic responses to a 3000 MW polymeric isoproterenol derivative were markedly different from those obtained with the other derivatives, averaging 12.8 ± 1.6 min (n = 5)—a value significantly different from that obtained with the 1500 MW derivative [$p < 0.01$ (Student's t test)]. By increasing the molecular weight of the polymer carrier to 9600, response durations were decreased to an average of only 5.5 ± 0.4

min (n = 5)—a value significantly different from those obtained with the 1500 and 3000 MW derivatives ($p < 0.01$).

Diffusion Studies with Polymeric Isoproterenol Derivatives

Because the response onset in cardiac muscle involves catecholamine-induced propagation, the drug washout times were studied, free isoproterenol being compared to the high molecular weight (9600) polymeric isoproterenol derivative. The results were dramatic. Following a single washout of the polymeric derivative, after 10 min incubation, the force of contraction returned to control levels, usually within 2 min. In contrast, a dose of free isoproterenol which produces an essentially identical response took on average over 20 min to return to control levels following a single washout. As a control soluble isoproterenol was added together with unsubstituted (MW 9600) polymer. The resulting washout time was essentially identical to that for free isoproterenol. These results are summarized in Table 3.

Subjecting the high molecular weight polymeric derivative to simulated muscle bath conditions produced no detectable isoproterenol and less than 0.4% 6-aminoisoproterenol [19]. From these results we conclude that insufficient free drug was produced during the course of the assay to produce the observed biological activity.

Because of the shift in the dose-responses to the right of the parent D-isoproterenol it was critical to be able to rule out the presence of traces of this compound. A contamination of greater than 5% and 1% of D-isoproterenol would be required to explain the respective dose-responses for the 1500 and 9600 MW derivatives. Similarly, 300% and 500% contaminations, respectively, of 6-aminoisoproterenol would be required for the observed biological responses. Gel chromatography (Fig. 1) and thin-layer chromatography have indicated that the highest possible contamination by D-isoproterenol is less than 0.01%, which is at least 1.5-2 orders of magnitude less than the minimum amount required to explain our results [19]. The chromatography immediately prior and subsequent to biological testing indicates that no detectable free D-isoproterenol was present in our preparations. We believe that our controls more than adequately rule out the possibility of contamination by free isoproterenol or 6-aminoisoproterenol explaining our results.

The drug washout and response decay experiments (Table 3) represent both a unique control experiment and also an example of an area in which polymeric drug derivatives may find great utility. Our results show that the higher molecular weight (9600) polymeric isoproterenol derivative can be removed from the muscle baths with

TABLE 3. Polypeptide-Isoproterenol Conjugate (MW 9600) vs. D-Isoproterenol Response Decay Times Following Drug Washout in Isolated Cat Papillary Muscles[a]

Drug added				
Type	Dose (mole isoproterenol)	Incubation time (min)	% Response (Δ force/control force)[b]	Response decay time following single washout (contractions to reach control force)[b]
Polypeptide-isoproterenol (MW 9600)	1.1×10^{-7}	10	58.9 ± 11.6 (n = 6)	19.5 ± 2.6 (n = 6)
D-isoproterenol	$1.5 - 6.0 \times 10^{-8}$	10	65.0 ± 8.9 (n = 8)	239.7 ± 39.2 (n = 8)
D-Isoproterenol and unsubstituted polymer (MW 9600)	6×10^{-8}	10	40.5 ± 5.5 (n = 2)	335 ± 25 (n = 2)
Polypeptide-isoproterenol (MW 9600)	2.7×10^{-7}	40	77.5 ± 12.5 (n = 2)	91 ± 5 (n = 2)
D-Isoproterenol	6.0×10^{-8}	40	97.0 ± 21 (n = 2)	330 ± 12 (n = 2)

[a]Isolated right ventricular cat papillary muscles were prepared as described [16, 17]. They were subjected to the indicated compounds for 10 or 40 min. The papillary muscle bath was drained, refilled with fresh oxygenated Krebs solution (prewarmed to 30°C), drained and refilled with fresh Krebs solution. The force of contraction was monitoried until the control inotropic state was regained. The dose of soluble isoproterenol was chosen to match the inotropic response obtained with the polymer-immobilized isoproterenol. The papillary muscles were maintained at 30°C and stimulated to contract at 12 contractions/min [19].

[b]Mean ± SEM; n = number of experiments.

a single washing, resulting in an immediate response decay to control levels. This is in sharp contrast to the results obtained with soluble isoproterenol (Table 3) where, following one bath change, there is a very slow response decay to control levels. We feel that these results can be explained in terms of the different diffusion rates of the two types of drug. These observations are a further indication that we are not dealing with a "dissociated" species of isoproterenol. If the observed responses were due to an isoproterenol contaminant, the washout differentials would be identical to that obtained with a mixture of soluble isoproterenol and unsubstituted polymer (Table 3).

The positive inotropic and chronotropic responses obtained in dogs with the polymeric catecholamine derivatives suggest that the in vivo activity is due to the covalently coupled species of isoproterenol and not released drug. Significant differences in response duration depended only upon the molecular weight of the polymer since the same total amount of isoproterenol was given in each case. In addition, the profiles of the responses due to the polymeric isoproterenol derivatives were very different from those obtainable with soluble isoproterenol alone, further implicating the covalently coupled species as the biological stimulus.

The explanation for the inverse relationship between the molecular weights of the polymeric isoproterenol derivatives and their in vivo durations of action is at present not clear. It is possible that the duration of action is related to both tissue penetration (diffusion) which is molecular weight-dependent, and also to rates of metabolism. Since it is known that the rate of cellular uptake of macromolecules by endocytosis is inversely proportional to molecular weight [23], it is possible that the higher molecular weight polymeric isoproterenol derivatives are more rapidly taken up by endocytosis than the lower molecular weight derivatives. It is possible that the different rates of tissue penetration and metabolism via endocytosis are both responsible for the different in vivo durations of action of the polymeric drugs. Other factors may also be involved. We are currently studying the distribution and rates of metabolism of both the polymers and the polymeric isoproterenol derivatives.

CONCLUSION

Our studies have demonstrated that isoproterenol can retain activity while covalently bound to a polymeric support. The interesting in vivo biological activity of these polymeric drugs encourages us to continue our studies which are currently directed towards a clearer understanding of the role of the polymer support in determining the biological activity of the polymer-drug conjugates.

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