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The Pharmacological Effects of Conjugates of Pharmacologically Active Amines to Complex or Simple Carriers: a New Class of Drug

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SUMMARY

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Conjugation of selected amines to rabbit serum albumin or copolymer carriers (via amide bonds by sp^3 C—N bonds generated by reduction of glutaraldehyde-amine Schiff bases) forms compounds that differ chemically from the free hormones. However, when histamine is conjugated to carrier, by either the carbodiimide or the glutaraldehyde sequence, the pharmacological properties of the conjugate resemble those of free histamine in that both stimulate accumulation of adenosine cyclic 3',5'-monophosphate (cAMP) in human leukocytes. Additionally, the effects of the histamine conjugates are blocked by antihistamines but are unaffected by adrenergic blocking agents. Amide conjugates of norepinephrine are inactive, but when this amine is conjugated via glutaraldehyde the resulting conjugate, like norepinephrine, stimulates accumulation of cAMP in leukocytes and is antagonized by the *beta* adrenergic blocker propranolol. Conjugates of congeners of norepinephrine that possess no *beta* adrenergic activity (e.g., normetanephrine) are inactive on leukocytes. Conjugates of some amines, such as tryptamine and dopamine, do not mimic the action of the corresponding free amines. In some cases, when the nitrogen moiety of the conjugate is converted from a primary to a secondary amine, the amine is able to interact with native leukocyte receptors (i.e., stimulation of adenylate cyclase occurs and is appropriately blocked by competitive antagonists of the free amine). In other cases, the actions of the conjugates could not have been predicted by knowing the pharmacological effects of the parent amine.

INTRODUCTION

Histamine and *beta* adrenergic catecholamines stimulate the synthesis of adenosine cyclic 3',5'-monophosphate in leukocytes, presumably via interaction with specific receptors. Although these leukocyte amine receptors can be defined pharmacologically with the use of amine congeners and specific antagonists, it has been

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difficult to physically detect and characterize them. When the amines are conjugated to a large carrier molecule, such as rabbit serum albumin, and the conjugates are chemically linked to Agarose beads, the beads bind leukocytes from human peripheral blood or mouse spleen (1–4). The binding phenomenon involves "receptors" similar to those defined pharmacologically, in that (a) cells do not bind to bead-carrier unless the hormones are conjugated to the carrier; (b) free amines or amine antagonists appropriately and specifically prevent binding to their corresponding bead-carrier-hormone; and (c) in preliminary experiments, cell subpopulations that failed to bind to hormone-carrier-beads also failed to accumulate cAMP when they were exposed to free hormone.

Because of these observations, we expected that amine conjugates linked to beads would stimulate cAMP[3] accumulation in the leukocytes that were bound to them. Such was not the case (3). One explanation may be that the process of linking the conjugates to beads subtly alters the pharmacological activity of the conjugate. Accordingly, we have examined the pharmacological properties of soluble conjugates of biogenic amines linked either to RSA or to a synthetic random peptide copolymer. The results indicate that conjugation of amines to these macromolecules preserves some of the pharmacological properties of the amines and critically modifies others. Some of the properties of the conjugates are predictable on chemical grounds; others are not. In some cases, drugs with entirely new pharmacological properties have been formed. A new and potentially useful class of drugs has been identified: amines bound to carrier molecules that can be selected on the basis of physical properties quite different from those of the amines themselves.

MATERIALS AND METHODS

Preparation of human leukocytes. Venous blood of normal volunteers was sedimented with 3% dextran 250 (Pharmacia) in phosphate-buffered NaCl, and the leukocytes were isolated as previously described (2). Heparin (5 units/ml of blood) was used as an anticoagulant. After the leukocytes had been isolated and washed once in cold 0.32 M sucrose, they were suspended in a convenient volume (5×10^7 cells/0.5 ml) of either phosphate-buffered NaCl solution or Eagle's minimal essential medium without glutamine. The differential count of cells prepared in this fashion was similar to that of stained smears from the peripheral venous blood of the same subject. Contamination by platelets was of the order of 1 platelet/15–20 leukocytes.

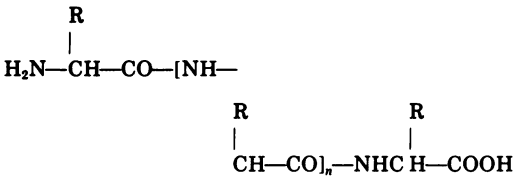
Leukocyte cAMP concentrations. Leukocytes in phosphate-buffered NaCl or in minimal essential medium were exposed to drugs or control solutions for 15 min at 37° in room air with constant gentle shaking. All incubations were done with 1 mM caffeine in each tube (control and experimental). At the end of the incubation, cells were separated from the medum by centrifugation at $800 \times g$ for 10 min at 0°. The cell pellet was resuspended in 1.2 ml of 5% trichloracetic acid. cAMP was extracted and measured by the competition binding assay of Gilman (5), slightly modified as previously described (6).

Synthesis and characterization of a random copolymer of alanine-tyrosine. A linear copolymer of DL-alanine (to prevent the possibility of α-helix formation, i.e, structural rigidity) with L-tyrosine was prepared by random copolymerization of *N*-carboxy-α-amino acid anhydrides according to Sela and Fuchs (7). The principal steps of the synthesis were to mix 14.5 g of *N*-carboxy-DL-alanine anhydride (125 mmoles) in 250 ml of anhydrous dioxane with 5.15 g of *N*-carboxy-*S*-tyrosine anhydride (25 mmoles) in 150 ml of dioxane and to initiate the polymerization by adding 0.6 g (5.1 mmoles) of triethylamine. The mixture was stirred vigorously at room temperature for 24 hr. Ether (800 ml) was added to complete the precipitation of the polymer that had started in the dioxane reaction mixture. The polymer was then filtered and washed with 1 liter of ether. The yield of polymer in different batches varied from 13 to 15 g.

[3] The abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; RSA, rabbit serum albumin; ECDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl.

Amino acid analysis after acid hydrolysis revealed that the polymer had a residue molar ratio of DL-alanine to L-tyrosine of 3.7:1, and an average molecular weight of 1540 (800–2000 range), as established by ultracentrifugation [method of Yphantis ($\bar{v}$ = 0.73) (8)].

The properties desired for the synthesized polymer were low molecular weight, a random structure, and only one reactive group on each end of the molecule. Such a polymer could serve as a contrast to RSA in order to detect contributions of the carrier to the ultimate effects of the conjugate. Initiation of polymerization with triethylamine results in a copolymer with both amino- and carboxyl-terminal moieties (7). The general structure of the polymer is



where R represents the side chain of either tyrosine or alanine. The carboxyl terminus can be used to bind the polymer to an amine via an amide linkage. The tyrosine may be used in a standard iodination process (9) to label the carrier or carrier-hormone complex for cell binding studies. Based on the molecular weight, the distance between the two termini is about 20 amino acid residues, which proved to be a long enough and flexible enough arm to accommodate the sites of the receptor-hormone interaction (2).

Synthesis of histamine-RSA, L- and D-norepinephrine-RSA, dopamine-RSA, tryptamine-RSA, and ethanolamine-RSA via carbodiimide coupling. Histamine-RSA was prepared by dissolving 700 mg of histamine dihydrochloride (Fluka), 100 mg of RSA, and 2 g of ECDI (Ott Chemical Company, Muskegon, Mich.) in 10 ml of water. The histamine conjugate is formed via nucleophilic attack by the amino function on the carbodiimide-activated carboxyl groups of the RSA (10–12). The solution stood for 2 hr at room temperature and was then dialyzed against 66 volumes of water, which was changed four times over 4 days and kept at 4°C. The histamine-RSA product was characterized by means of a [^{3}H]histamine tracer (New England Nuclear), which was followed throughout the procedure. The incorporation of histamine was calculated on the base of ^{3}H label. The various batches contained between 40 and 60 moles of histamine per mole of RSA. Batches of RSA conjugates to D- and L-norepinephrine, dopamine, tryptamine, and ethanolamine were synthesized and characterized in the same manner. Again, according to tracer incorporation, about 40–60 moles of amine were linked to 1 mole of carrier in each preparation. The histamine and tryptamine linkages to RSA clearly occur through a side chain amide bond, because the amino functions are the only nucleophilic moieties present in these molecules (10–12). The many possible functions, as well as the chemical instability, of the catecholamines make the nature of their binding to RSA less certain. In all cases, however, acid hydrolysis resulted in the reisolation of the starting amines, which is consistent with the formation of an amide bond.

In the incubation with cells, the effects of free drug were compared with equivalent amine concentrations of conjugated drug in separate aliquots of cells. Controls for a conjugate of the hormones were (a) a comparable concentration of free amine in one set of tubes, (b) a comparable concentration of carrier in the second set of tubes, and (c) a combination of carrier plus free amine (unconjugated) at the concentration of each comparable to the conjugate.

Synthesis of low histamine-carrier conjugates. Histamine (60 mg) was mixed with 50 mg of RSA in 2 ml of H_2O. ECDI (3 mg) was then added to the solution and allowed to stand for 2.5 hr. Histamine-RSA (low-substituted) was separated from the mixture by preparative column chromatography and with the use of Sephadex G-15, which had been equilibrated with 0.01 M sodium phosphate at pH 7 (2, 3). The resultant conjugate contained 4–5 moles of histamine per mole of RSA.

Polymer-amine conjugates. The random copolymer described above (100 mg) was mixed in 2 ml of water, and 1 N NaOH was

added by droplets until the polymer was completely dissolved. Then the pH was adjusted to 5.0 by adding 1 N HCl. About 10–20% of the polymer reprecipitated as the pH was lowered; the precipitate was removed by centrifugation at 10,000 × *g* for 20 min. Histamine (50 mg) and ECDI (25 mg) were added to the clear solution of the polymer. The coupling reaction was allowed to proceed for 2 hr at room temperature. The histamine-polymer conjugate was then isolated by column chromatography over Sephadex G-25, which had been equilibrated with 0.02 N HCl. Characterization of the conjugate by acid hydrolysis revealed that 0.5–0.7 mole of histamine was coupled to 1 mole of polymer.

Polymer conjugates to tryptamine and D- or L-norepinephrine were synthesized by similar techniques and yielded comparable products.

Synthesis of drug-RSA or drug-polymer with glutaraldehyde. RSA (100 mg) or polymer (150 mg) was dissolved as described above. Amines (70 mg) and 50 μg of 50% aqueous glutaraldehyde (Fluka) were added to either solution. When D- or L-norepinephrine was used, the solution turned yellow after 1.5 hr and a precipitate formed. The mixture was then acidified to pH 3 with 1 N HCl. A large excess of $NaBH_4$ (200 mg) was slowly added. The mixture turned colorless and was filtered over cotton wool. The final conjugate was then isolated by column chromatography (Sephadex G-25 for hormone-RSA conjugates; Sephadex G-15 for hormone-polymer-glutaraldehyde) (2, 3). Both columns were equilibrated with 0.2 N HCl. The protein-hormone conjugates were characterized by radioactive tracer; 40–50 moles of norepinephrine were coupled to 1 mole of RSA. Norepinephrine-polymer-glutaraldehyde was characterized by following trace amounts of [^{14}C]norepinephrine throughout the procedure. Either isomer of norepinephrine (0.3–0.6 mole) was bound to 1 mole of polymer. The same procedure was used to conjugate similar amounts of dopamine and normetanephrine to polymer.

Controls in synthesis of amine-polymer-glutaraldehyde preparations. It was possible that during synthesis a high molecular weight polymer of norepinephrine-glutaraldehyde had formed, which would be included on the Sephadex column used for the isolation of the norepinephrine-polymer conjugate. In order to determine whether an amine-glutaraldehyde polymer had been formed, two control procedures were performed. In the first, alanine-tyrosine copolymer was iodinated (^{125}I) by standard procedures (9). The polymer (100 mg) was allowed to react with 1 mCi of ^{125}I (New England Nuclear) diluted 1:10,000 with nonradioactive NaI (150 μg), and the iodinated polymer was purified on Sephadex G-25. The ^{125}I-polymer was coupled to [^{14}C]norepinephrine-glutaraldehyde; the doubly labeled peptide-hormone conjugate was purified on Sephadex G-25, as described above. [^{14}C]Norepinephrine bound to the ^{125}I-labeled polymer could be readily separated from the excess norepinephrine-glutaraldehyde (both formed in the reaction mixture) and [^{14}C]norepinephrine (Fig. 1). The second control was to incubate [^{3}H]norepinephrine with glutaral-

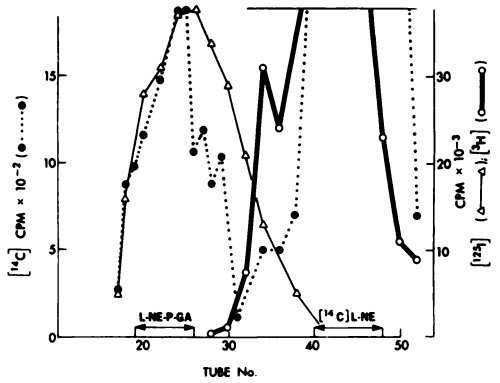


FIG. 1. *Sephadex G-25 chromatography of ^{125}I-copolymer (△) without conjugation, [^{3}H]norepinephrine reacted with glutaraldehyde, followed by $NaBH_4$ (○), and [^{14}C]norepinephrine conjugated to copolymer via glutaraldehyde. The polymer was labeled with ^{125}I (●).*

The [^{14}C]norepinephrine linked to copolymer chromatographed with labeled polymer, and the excess norepinephrine, with [^{3}H]norepinephrine. There is no evidence of high molecular weight [^{3}H]norepinephrine after reaction with glutaraldehyde. L-NE-P-GA, area of collections made for the L-norepinephrine-polymer-glutaraldehyde conjugate; [^{14}C]L-NE, area of chromatography of free [^{14}C]- norepinephrine, coinciding with [^{3}H]norepinephrine.

dehyde (followed by $NaBH_4$), but without polymer. When this mixture was chromatographed, the [^{3}H]norepinephrine chromatographed in the area of the free ^{14}C-labeled norepinephrine (Fig. 1). None was seen in the higher molecular weight fractions, where norepinephrine-polymer-glutaraldehyde was eluted from the column. We thus concluded that no high molecular weight polymerization occurred between the catecholamine and glutaraldehyde and that norepinephrine-polymer-glutaraldehyde fractions were not contaminated with such a product. Analogous experiments with dopamine produced similar results.

The properties of the conjugate did not change when stored at −20° for up to 2 months. Additionally, acid hydrolysis did not lead to the formation of any free amine, which confirms that the initial amino linkage was reduced to the stable sp^3 bond. We have previously shown that conjugates that were insolubilized and incubated with human peripheral blood leukocytes lost only negligible amounts of label (1).

When 3 times the highest concentrations of soluble conjugates used experimentally were incubated with 3 times the number of leukocytes used experimentally for twice the time periods ordinarily used, less than 1% of the low molecular weight amine fractions could be detected in the chromatographed supernatants. These low concentrations were not sufficient to stimulate leukocyte cAMP accumulation. These data, and others presented below, allow us to state that, in our experiments, the pharmacological effects were produced by the whole protein- or peptide-hormone conjugates and not by free amine impurities.

RESULTS

Amide-linked conjugates of histamine with RSA led to the accumulation of cAMP in human leukocytes (Fig. 2 and Table 1) despite loss of the free amino group. Because free biogenic amines stimulate adenylate cyclase activity in a variety of tissues, including leukocytes, we suspect that this is the most likely mechanism of action of the conjugates (13–15).

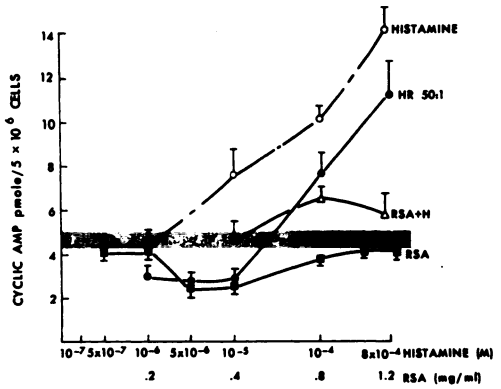


FIG. 2. *Effects of histamine (○), histamine conjugated to rabbit serum albumin (HR) at 50 moles of histamine per mole of RSA (●), RSA plus histamine (H) at concentrations of histamine and RSA equivalent to the histamine-RSA conjugate (△), and RSA alone (■) on leukocyte cAMP content*

The effects of the histamine-RSA conjugate are significantly different from histamine plus RSA ($p < 0.005$). The area represented by the shaded bar indicates the concentration of cAMP in the absence of pharmacological agents.

RSA per se consistently reduced the intracellular cAMP concentration (Fig. 2). The potency of the amide-conjugated histamine, which was calculated per mole of bound histamine, appeared to be less than the potency of free histamine. The reasons for such a discrepancy in potency are not clear, but it was not due to the actions of RSA alone, because RSA alone reduced the maximal effects of histamine (Fig. 2). Perhaps this action contributes to, but does not fully explain, the dose-response curve produced by the histamine-RSA conjugate. The pharmacological effect of histamine-RSA appeared to depend on the intact conjugate (Fig. 2). However, low-substituted histamine-RSA produced no noticeable stimulation (Fig. 3). This might have been due to the cAMP-lowering effects of the RSA in the preparation (Figs. 2 and 3).

As previously reported (6), L-norepinephrine was a more potent stimulator of intracellular leukocyte cAMP accumulation than histamine. However, when L-norepinephrine was conjugated to RSA via ECDI by the procedures described above,

TABLE 1

Effects of blocking agents on cAMP accumulation by amines and conjugated amines

The effects of free histamine and tryptamine and those conjugated via ECDI were tested in the presence and absence of the indicated pharmacological antagonists. Each value represents the mean and standard deviation of five separate experiments, in which duplicate tubes of 5×10^6 cells were assayed and averaged. Incubation times were 15 min, and all incubations were performed in the presence of 1 mM caffeine. Duplicates did not differ from each other by more than 10%. The molar concentrations of conjugates refer to the concentration of bound amine rather than the concentration of the conjugate. Therefore the effects of conjugates can be directly compared with those of free amines to relate their potencies.

Amine or conjugated amine	Concentration	Antagonist	Concentration	Cellular cAMP
	mM		*mM*	*% control*
H[a]	0.1	None		246 ± 62
None		D	0.4	97 ± 26
H	0.1	D	0.4	97 ± 7[b]
H	0.1	D	0.1	132 ± 60[b]
H	0.1	D	0.01	274 ± 61
None		B	0.4	97 ± 5
H	0.8	B	0.4	83 ± 21[b]
H	0.8	B	0.1	109 ± 17[b]
H	0.8	B	0.01	98 ± 21[b]
		P	0.4	96 ± 10
H	0.8	P	0.4	224 ± 44
HR	0.8	None		209 ± 61
HR	0.8	D	1	101 ± 28[b]
HR	0.8	D	0.4	127 ± 50[b]
HR	0.8	D	0.01	174 ± 19
HR	0.8	B	0.4	107 ± 25[b]
HR	0.8	B	0.1	110 ± 29[b]
HR	0.8	B	0.01	183 ± 13
HR	0.8	P	0.4	217 ± 24
T	0.8	None		81 ± 26
TR	0.8	None		332 ± 56
TR	0.8	D	0.4	231 ± 61
TR	0.8	B	0.4	348 ± 125
TR	0.8	P	0.4	302 ± 70
None		M	0.4	99 ± 6
TR	0.8	M	0.4	289 ± 64

[a] The abbreviations used in the table are: H, histamine; D, diphenhydramine; B, burimamide; P, propranolol; HR, histamine-RSA (50:1); TR, tryptamine-RSA (50:1); M, methysergide.

the pharmacological effect was absent (Fig. 3). When conjugates of dopamine were made via ECDI to RSA, the free amine was active but the conjugate was not (Fig. 3).

The conjugation process, surprisingly, could actually create some pharmacological effects not typical of the unconjugated amine. For example, tryptamine had no effect of its own on leukocyte cAMP, but, when conjugated to RSA via ECDI, it produced cAMP accumulation equivalent to the effects of 0.8 mM free or conjugated histamine (Fig. 3; Tables 1 and 2). These effects were not inhibited by methysergide, which is a competitive blocker of serotonin (Table 1).

When histamine-RSA was incubated with cells, we noted, both by direct observation of the incubation tubes and by light microscopy, that the leukocytes aggregated. We tested the possibility that cell aggregation was responsible for activation of membrane adenylate cyclase. Each pharmacologically active RSA conjugate did aggregate cells (Table 2). However, aggregation per se did not seem to be the critical determinant of a pharmacological effect, since ethanolamine-RSA produced impressive cell aggregation but did not raise cAMP levels in leukocytes. Similarly, polylysine, a highly charged substance that produced cell aggregation, did not generate accumulation of intracellular cAMP (Table 2).

Our results suggest that the amide conjugates of amines with RSA act via native specific receptors for the corresponding free amines. The maximal effects of free amine plus the maximal effects of the corresponding RSA-conjugated amine were not additive (data not shown). The effects of histamine-RSA were competitively blocked by specific inhibitors of free histamine (Table 1). Diphenhydramine and burimamide blocked the effects of both free and conjugated histamine. Conversely, propranolol, a *beta* adrenergic antagonist that has no effect on free histamine activity, did not alter the activity of the histamine conjugate but did appropriately antagonize conjugates of norepinephrine via glutaraldehyde and free norepinephrine. The activity of the tryptamine-RSA conjugate was not affected by any of the blocking agents used, including methysergide, a competitive blocker of 5-hydroxytryptamine effects. However, the finding that tryptamine-RSA had pharmacological ef-

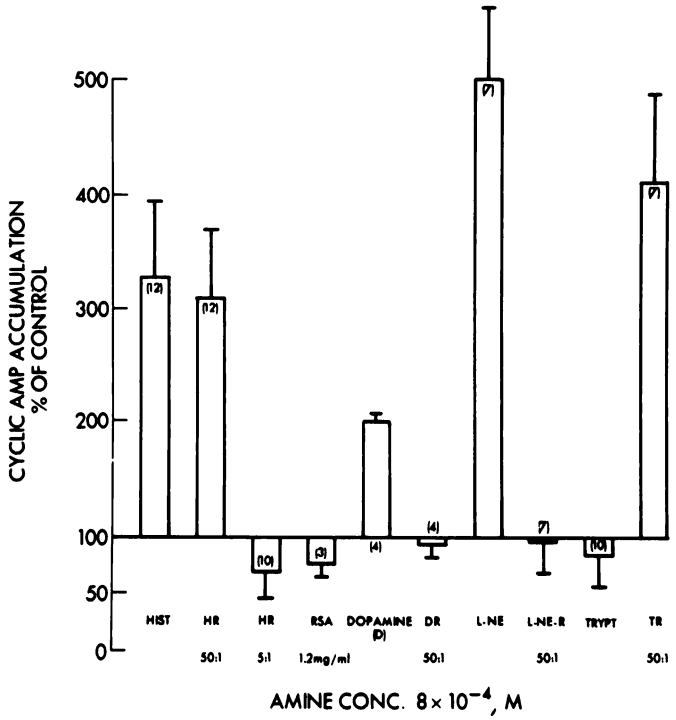


FIG. 3. *Effects of free amines and those conjugated via ECDI to RSA on cAMP accumulation in leukocytes*
The molar ratio of the conjugate to carrier is given under the abscissa. Numbers in parentheses denote the number of determinations. HIST, histamine; HR, histamine-RSA; DR, dopamine-RSA; L-NE, L-norepinephrine; L-NE-R, L-norepinephrine-RSA; TRYPT, tryptamine; TR, tryptamine-RSA.

fects meant that the conjugates to the complex RSA molecules were pharmacologically as well as chemically distinctly different from the free amines.

To determine whether the carrier contributed to the pharmacological effect, we tested the activity of amines linked to alanine-tyrosine copolymer (Figs. 4 and 5). We found that when histamine was conjugated to polymer via ECDI, its effects were similar to both free histamine and histamine-RSA (Table 2). When histamine, D- or L-norepinephrine, dopamine, and normetanephrine were linked via glutaraldehyde to polymer and their effects were tested, we found the following.

1. The effects of histamine-copolymer-glutaraldehyde were equipotent to similar concentrations either of histamine linked to polymer or RSA via ECDI or of free histamine (data not shown). The blocking effects of burimamide and diphenhydramine were as effective and specific against histamine-copolymer-glutaraldehyde as they were against histamine-RSA, histamine-RSA-glutaraldehyde, and histamine-copolymer.

2. The effects of free D-norepinephrine were considerably less than the effects of L-norepinephrine (Fig. 5) and might have been accounted for either by the contamination of the D with the L isomer or by the direct effects of the D isomer. When either D- or L-norepinephrine was linked via ECDI to the polymer, no pharmacological effects were seen (corresponding to the conjugates of norepinephrine with RSA). However, when D- or L-norepinephrine was linked to the polymer via glutaraldehyde, their potencies were somewhat less than those of the corresponding free amines; their effects were blocked by propranolol and not by antagonists of other amines (Fig. 5).

3. Dopamine only weakly stimulated accumulation of cAMP in leukocytes; epinine produced greater stimulation (Fig. 4). The effects of each of these free amines on

TABLE 2

Effects of amines and amine conjugates on cell aggregation or cAMP levels

The effects of free amines and their conjugates with rabbit serum albumin or the copolymer were assessed on cell aggregation (assayed by light microscopy) and accumulation of cAMP in human leukocytes. Histamine-tryptamine-, and ethanolamine-RSA conjugates were made with ECDI. Norepinephrine-RSA and polymer conjugates were made with glutaraldehyde. ECDI conjugates of norepinephrine were pharmacologically inactive and did not aggregate cells. Each value represents the average and standard deviation of duplicates in five separate experiments. Each tube contained 5×10^6 cells; incubations were performed for 15 min, all in the presence of 1 mM caffeine. Controls contained cells plus caffeine.

Amine or conjugate[a]	Cell aggregation	Cyclic AMP accumulation
		% control
Histamine-RSA (50:1)	+	209 ± 61
Histamine-RSA (5:1)	−	72 ± 18
Histamine-polymer (0.5:1)	−	212 ± 17
Histamine	−	226 ± 98
D-Norepinephrine-RSA-glutaraldehyde (50:1)	−	107 ± 20
L-Norepinephrine-RSA-glutaraldehyde (50:1)	+	429 ± 45
L-Norepinephrine-polymer-glutaraldehyde (0.5:1)	−	315 ± 32
L-Norepinephrine	−	629 ± 85
Tryptamine-RSA (50:1)	+	324 ± 130
Tryptamine	−	80 ± 25
Ethanolamine-RSA	+	114 ± 13
Ethanolamine	−	95 ± 3
Polylysine	+	95 ± 9

[a] Each amine, free or conjugated, was at a concentration of 0.8 mM.

the leukocytes were blocked by propranolol (Fig. 4), but were unaffected by antihistamines and an *alpha* aderenergic blocking agent. However, the effects of dopamine-polymer conjugates made via either ECDI or glutaraldehyde were not reversed by any blocking agents (Fig. 4).

DISCUSSION

Our data indicate that selected conjugates of histamine and norepinephrine to RSA or the random copolymer have pharmacological activity that resembles the activity of the corresponding free amines in their ability to stimulate cAMP in leukocytes. The following observations provide evidence for the specificity of this pharmacological effect. (a) The effects of both the

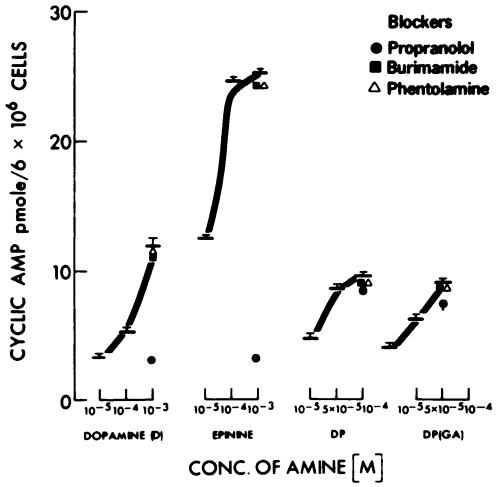


FIG. 4. *Effects of dopamine and epinine on leukocyte accumulation of cAMP in the presence and absence of 0.5 mM propranolol, burimamide, and phentolamine*

The conjugates of dopamine (D) via ECDI and glutaraldehyde (GA) to the polymer (P) were also tested with and without the same blocking agents.

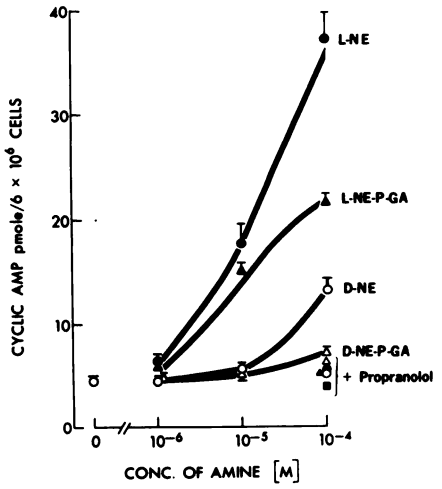


FIG. 5. *Effects of free L-norepinephrine (L-NE) and D-norepinephrine (D-NE) and the same amines conjugated to the copolymer (P) via glutaraldehyde (GA) on accumulation of cAMP in leukocytes*

All preparations were equally inhibited by propranolol at 0.5 mM.

conjugates and free amines are blocked by the appropriate competitive antagonist (i.e., antihistamines inhibit only free and conjugated histamine, and propranolol inhibits only the free and conjugated *beta* adrenergic catecholamines). (b) The stereoselectivity of the pharmacological effects of the conjugated norepinephrine mimics the stereoselectivity of free D- and L-norepinephrine. (c) There is no pharmacological effect of either conjugated or free normetanephrine [the carriers (RSA and copolymer) do not stimulate accumulation of cAMP in human leukocytes]. (d) The conjugated amines are not cleared from their carriers in quantities sufficient to account for the pharmacological properties of the conjugates.

The pharmacological specificity described above suggests two conclusions. (a) It seems reasonable to assume that at least histamine conjugated via ECDI or glutaraldehyde and norepinephrine conjugated via glutaraldehyde can be manipulated without losing their pharmacological properties (despite the conjugation they appear to have access to their native receptor sites). (b) If, as we suspect, access of the large RSA conjugates to intracellular sites is restricted (16), these results are consistent with the assumption that receptors for amines are on the leukocyte plasma membrane surface (17).

The conjugates of histamine via ECDI to RSA or polymer are pharmacologically active, but those of norepinephrine are not. (This may be because the amine function of norepinephrine is more critical for pharmacological activity than is the case with histamine.) The histamine conjugates via ECDI may remain active because of the remaining basicity of the aromatic (imidazole) moiety; no corresponding basic functionality is found in the amide-linked catecholamines. Therefore the pharmacological activity of conjugates of catecholamines made by linking them to the carrier via glutaraldehyde was tested. Such a linking procedure results in conversion of the primary amine to a secondary amine (2). We have shown elsewhere (2) that when conjugates of norepinephrine made via glutaraldehyde instead of ECDI are linked to Sepharose beads, the former but not the latter can bind leukocytes. Their soluble counterparts have appropriate pharmacological activities.

The norepinephrine preparations conjugated through glutaraldehyde were pharmacologically active; they were almost as potent as norepinephrine (Fig. 5). The effects on leukocytes of either the free or conjugated amines were specifically and competitively antagonized by *beta* adrenergic antagonists.

However, the assumption that linking an amine via glutaraldehyde would simply convert it to another compound with effects like the amines is not warranted. Dopamine and its secondary amine congener, epinine, can stimulate leukocytes. The effects are blocked by propranolol. Although both conjugates of dopamine to the copolymer were pharmcologically active, their actions were unaffected by propranolol (Fig. 4). "Normal" basic amino function is belied by the effect of the conjugates of dopamine linked to the polymer via glutaraldehyde. Therefore the pharmacological properties of each conjugate must be tested. Their effects cannot be assumed on the basis of the experiments with histamine and norepinephrine conjugates.

The results with the dopamine conjugates show that these conjugates do not necessarily have properties identical with those of the amine and that the carrier and conjugation processes may be critical determinants of the effects of the conjugate. Thus agents that may have no pharmacological effects when free may develop them when conjugated (i.e., tryptamine and RSA are inactive; conjugates of tryptamine to RSA via ECDI are active). There is evidence that the histamine conjugates do not faithfully mimic the actions of free histamine. Burimamide is a more potent inhibitor than diphenhydramine of the effects of free histamine on leukocytes (Table 1). These data have been used to indicate that the histamine receptor on leukocytes is an H_2 rather than an H_1 receptor (17, 18). In our preliminary experiments the conjugates of histamine were about equally inhibited by both burimamide and diphenydramine, perhaps indicating that

the binding of the conjugate influences the environment of the membrane receptor so that it responds in an unusual way.

We noticed that when histamine-RSA was mixed with antihistamines, cAMP accumulation was blocked but cell aggregation was not. This result can be interpreted in at least two ways. (a) There may be both specific interactions with the hormone receptors and nonspecific binding sites for the whole conjugate. In this case, the antihistamine would not prevent binding of agonist to the cells and aggregation of cells, as suggested by Matthyssens *et al.* (19). (b) The minimal number of binding sites needed for an aggregation matrix might be less than the minimal number necessary to stimulate cAMP accumulation. For instance, if the antihistamines blocked 99% of the sites, aggregation might still be possible, without allowing adenylate cyclase activation.

The properties of binding of these conjugates require further study. So far, we can state only that some conjugates of certain hormones behave quite similarly to the parent compounds and others do not. The determinants of their behavior are not obvious. However, a new class of drugs whose effects may mimic the parents has been observed. More extensive examination of their pharmacological properties in different test systems and their distribution and metabolism *in vivo* (compared to the parent amine) seems justified.

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