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Isolation and identification of pharmacologically active amino acids in skin and their structure-activity relationship on the guinea-pig ileum

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Summary

1. Aqueous extracts of stratum corneum were found to cause histamine-like contractions of the guinea-pig ileum which were not antagonized by mepyramine, atropine or bromolysergic acid diethylamide.

2. The compounds responsible for this contraction were isolated by chromatography and shown to be common amino acids, particularly L-serine and L-alanine which occur in abundance in the stratum corneum.

3. Amino acid analogues were assayed on the guinea-pig ileum against alanine as standard. With the exception of γ-amino butyric acid and L-alanine benzyl ester all analogues which had appreciable activity gave dose-response curves parallel to L-alanine.

4. The response to L-alanine benzyl ester was abolished by mepyramine and this analogue appears to be a partial agonist on the histamine receptor.

5. The effects of substitution on the equipotent molar ratios of amino acid analogues indicate that all four chemical groups attached to the α-carbon of L-alanine interact with the receptor.

6. Our results suggest that the guinea-pig ileum contains an L-α-amino acid receptor.

Introduction

In an attempt to examine the outer layers of the skin for the presence of histamine it was found that the stratum corneum contained a smooth muscle stimulating substance which was not histamine. This finding was the subject of a preliminary communication (Lewis, Rosenthal & Trahan, 1959). More recently we have taken up this problem again and isolated and identified the active principles as common amino acids. In addition, the activities of a number of amino acid analogues on the isolated guinea-pig ileum have been determined in an attempt to characterize the receptor on which they act. This part of the work was communicated to the Pharmacological Society (Lewis, McMartin & Yates, 1970).

Methods

Identification of substances in stratum corneum which contract the guinea-pig ileum

Human stratum corneum was cut from the plantar and palmar regions of volunteers and cadavers. This tissue (133 g) was extracted 3 times with 3 volumes of Analar

acetone, to give an acetone powder (51 g). A portion of this powder (30 g) was extracted six times with 200 ml of 80% ethanol and the combined ethanol washings were evaporated, redissolved in 50 ml of ion exchange water and extracted four times with 50 ml of chloroform. The aqueous phase was evaporated to give 5 g of solid. One gramme of this residue in 6 ml of water was applied to a 1·8 × 40 cm column of the sulphonic acid resin AG50 × 4 which was eluted with a linear gradient of 0·2 N to 4 N hydrochloric acid of total volume 1 litre at a flow rate of 2·5 ml/min. Fractions (20 ml) were collected and evaporated to dryness in a vacuum desiccator. Aliquots of each fraction were tested for activity on the guinea-pig ileum and those which caused a contraction in the presence of mepyramine (0·1 μg/ml) were quantitatively analysed for amino acids using the Beckman amino acid analyser.

Comparison of activities of different amino acids and amino acid analogues on the guinea-pig ileum

Materials

With the exception of L-alanine ethyl ester which was synthesized by Dr. Wade in these laboratories, and L-allothreonine which was supplied by Dr. D. F. Elliott, all amino acids and amino acid analogues were obtained commercially as indicated in the tables or text by (a) for Calbiochem, (b) for Mann Research Laboratories, (c) for Sigma, (d) for Schwarz Bioresearch Inc., (e) for K. & K. Laboratories Inc., (f) for Ralph N. Emmanuel Ltd., (g) for Hopkin and Williams Ltd., and (h) for B.D.H. Chemicals Ltd.

Bioassay

The compounds were assayed against L-alanine or glycine on the isolated guinea-pig ileum suspended in 5 ml of Tyrode solution at 34° C. A tension of 0·3–0·5 g was applied to the tissue. Isotonic contractions of the tissue were recorded using a frontal writing pen (magnification × 6). Since log dose-response curves were parallel, equipotent molar ratios could be obtained by assaying two concentrations of each analogue with appreciable activity against two concentrations of L-alanine or glycine in the order of a 2 × 2 Latin square and measuring their equiactive molar doses on a log dose-response curve.

Results

Smooth muscle stimulating substances in the skin of human and other species

Unidentified activity. Aqueous extracts of the outer layers of the skin of human forearm or the dorsal skin of rats, rabbits or guinea-pigs removed by stripping with Cellotape contained a principle which caused a contraction of the isolated guinea-pig ileum. Further, aqueous extracts of the stratum corneum removed from the plantar and palmar regions contained the activity whereas extracts of the skin after the stratum corneum had been removed contained no such activity. Figure 1 illustrates the activity of 100 μg and 200 μg of dried powder obtained from an aqueous extract of human stratum corneum on the guinea-pig ileum compared with histamine standards of 3 ng and 5 ng. The contractions were rapid and similar to those produced by additions of histamine but they were not reduced by mepyr-

amine 0·1 μg/ml. The experiment of Fig. 2 illustrates another difference between the active principle of the extract and histamine. When either the unknown principle or histamine is added to the guinea-pig ileum the tissue gradually becomes more sensitive to the agonist. In the experiment of Fig. 2 the tissue had already been sensitized to histamine but not to the extract. It can be clearly seen that whereas the sensitivity of the tissue subsequently remains constant to histamine, its sensitivity to the extract increases, indicating a different site of action for the two principles.

The extract of corneum was not found to be active on other isolated smooth muscle preparations such as the rat uterus, rat colon and the rat stomach strip. In addition its action on the guinea-pig ileum was not antagonized by atropine 0·1 μg/ml or a combination of bromolysergic acid diethylamide (BOL) 0·1 μg/ml and morphine 0·1 μg/ml.

The active principle was not soluble in organic solvents such as acetone, chloroform and ether, but was readily soluble in 80% aqueous alcohol. A standard

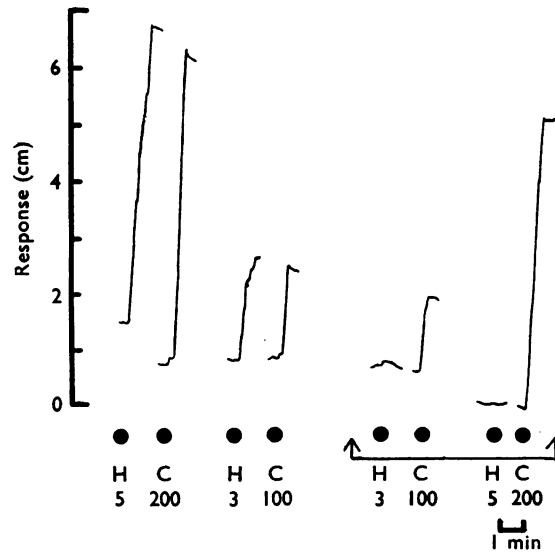


FIG. 1. Responses of the isolated guinea-pig ileum suspended in 5 ml Tyrode solution to histamine (H) 5 and 3 ng and to extract of human stratum corneum (C) 200 and 100 μg. Between the arrows the organ bath contained mepyramine 0·1 μg/ml.

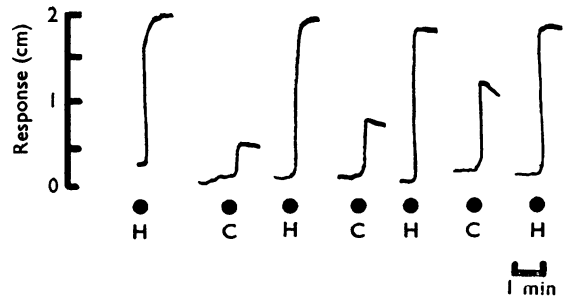


FIG. 2. Responses of the isolated guinea-pig ileum suspended in 5 ml Tyrode solution to histamine (H) 10 ng and extract of human stratum corneum (C) 100 μg.

preparation was therefore made, removing lipid by extracting with acetone and finally taking up the resulting dry powder into 80% alcohol. It was found that usually 100–200 μg of the dried alcoholic extract caused a contraction of the guinea-pig ileum.

In order to collect enough material to continue further isolation procedures stratum corneum was collected mainly from the plantar and palmar surfaces of cadavers in Cook County Hospital, Chicago.

Identification of the active principles. Fractionation of the crude extract of stratum corneum on short sulphonic acid resin columns showed that the smooth muscle-stimulating activity could be readily separated into two main components. The first was eluted with 0·1 N HCl and the contractions caused by compounds in this fraction were not antagonized by mepyramine while the component which was eluted later (2–4 N HCl) caused contractions which were antagonized by mepyramine. These later fractions therefore probably contained histamine. It seems unlikely that the histamine is present in the stratum corneum itself since it is not present when the corneum is removed by stripping with Cellotape or cutting carefully to avoid contamination with the underlying layers of epidermis or dermis.

Samples were fractionated using a longer column which was eluted with a linear molarity gradient of HCl. Fig. 3 shows the smooth muscle-stimulating activity of the fractions obtained in this experiment when tested in the presence and absence of mepyramine against standards of histamine and L-serine. The early peak of biological activity could be split into two separate peaks (aliquots 18–21 and

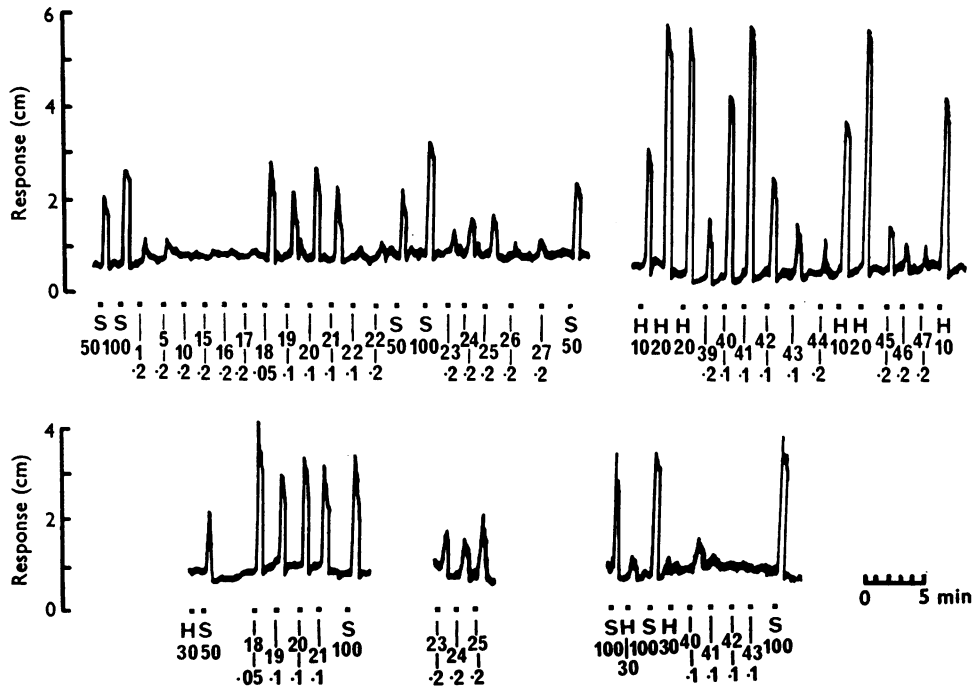


FIG. 3. Biological activity of fractions obtained after ion exchange chromatography. Responses of the isolated guinea-pig ileum suspended in 5 ml Tyrode solution to serine (S) 50 or 100 μg, histamine (H) 10, 20 or 30 ng and to aliquots of samples which are indicated by the sample number and volume (ml) added to the bath. Lower tracing shows responses in the presence of mepyramine 0·1 μg/ml.

23–25) which were both resistant to mepyramine whilst the second peak of activity (aliquots 40–43) was antagonized by mepyramine. The early peaks were found to contain large amounts of common amino acids. When pure amino acids were tested on the guinea-pig ileum it was found that some of them, notably alanine and serine, had slight but definite smooth muscle-stimulating activity, which was not affected by concentrations of mepyramine that abolished the responses of the tissue to histamine.

When samples of fractions representing each peak of biological activity were examined analytically it was found that they contained only amino acids. Table 1 shows the amino acid content and approximate biological activity relative to L-serine of the main fractions which were active in the presence of mepyramine.

It is concluded from these results and from the relative activities of the other amino acids given in the subsequent tables, that the smooth muscle-stimulating activity of extracts of the stratum corneum is due mainly to the presence of the amino acids L-serine and L-alanine, and to smaller amounts of glycine and proline.

The activity of amino acid analogues on the guinea-pig ileum

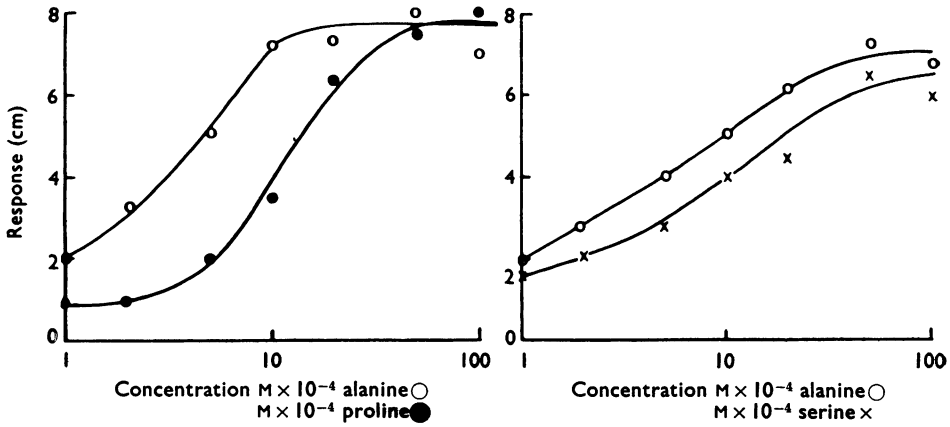
L-Alanine was the most potent amino acid of those tested for ability to contract the isolated guinea-pig ileum. The maximum contraction which could be obtained with this compound varied from one sample of tissue to another and sometimes tissues which were sensitive to histamine were almost totally insensitive to L-alanine. The response to amino acids showed the phenomenon of specific sensitization as shown for the crude extract in Figure 2. Once the tissue had been sensitized, responses to amino acids were usually reproducible for several hours although unpredictable changes in sensitivity occasionally occurred. The rate of contraction and relaxation was rapid and very similar to the response produced by histamine. However the amino acids differ from histamine in that their action is resistant to anti-histamines and their log dose-response curves have a different shape. Figure 4 illustrates three experiments which show that the log dose-response curves of L-alanine, L-serine and L-proline are similar to each other but quite different from that of histamine.

Two amino acid analogues, γ-amino butyric acid (GABA) and L-alanine benzyl ester, gave anomalous results and appear to be acting differently from all the other amino acids examined. GABA which is known to act on smooth muscle (Curtis & Watkins, 1965) was not antagonized by anti-histamines but gave a much steeper dose-response curve than that for L-alanine as shown in Fig. 5 and a larger maximal contraction. β-Alanine which has a structure intermediate between L-alanine and GABA was inactive on the guinea-pig ileum so did not act on either receptor.

The other analogue, L-alanine benzyl ester was antagonized by mepyramine 0·1 μg/ml. Its maximum response was much smaller than that to histamine but in relatively high concentrations it reduced the sensitivity to histamine as shown in Fig. 6 but not to L-alanine. This finding indicates that L-alanine benzyl ester is a partial agonist, in the sense defined by Stephenson (1956). This analogue was the only one among the derivatives or amino acids tested that was antagonized by mepyramine which gave similar pA_2 values with the two agonists, varying between 9·70 and 9·91. According to Arunlakshana & Schild (1959) this indicates that the agonists act on the same receptor.

TABLE 1. *Activity on guinea-pig ileum and amino acid composition of fractions of the eluate from a sulphonic acid resin column to which an aqueous extract of acetone powder of human stratum corneum had been applied.*

Fraction Number	Activity equivalent to serine (mg/ml)	Amino acid content (mg/ml)							
		Serine	Threo-nine	Aspartic acid	Glutamic acid	Glycine	Alanine	Proline	Valine
18	3·5	2·4	0·4	0·2					
20	1·5				0·1	3·3	0·8		
21	1·0					0·1	0·4		
24	0·3							0·2	0·4



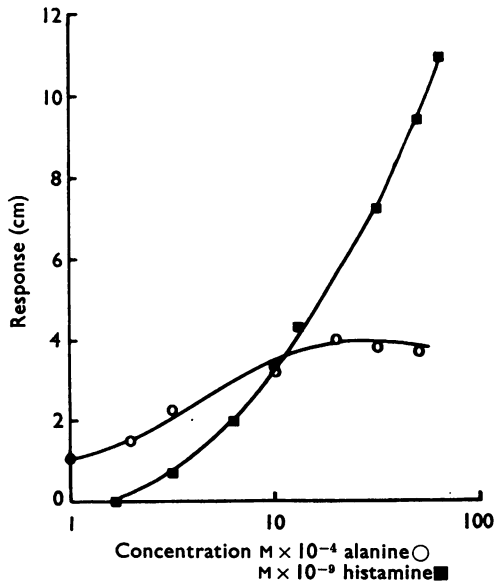


FIG. 4. Log dose-response curves plotted from three different experiments on isolated guinea-pig ileum. Responses to L-alanine and L-proline, L-alanine and L-serine, L-alanine and histamine are shown.

The more active amino acids, L-alanine and L-serine, were tested on a number of pharmacological preparations in addition to the guinea-pig ileum. They had negligible activity on the rat uterus, colon, duodenum and fundic strip, and on blood flow through the cat hind limb.

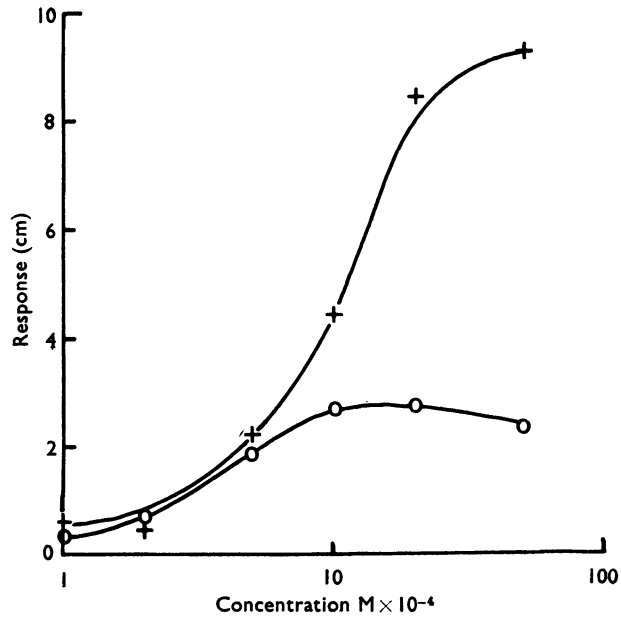


FIG. 5. Log dose-response curves on the isolated guinea-pig ileum of L-alanine (○) and γ-amino butyric acid (GABA) (+).

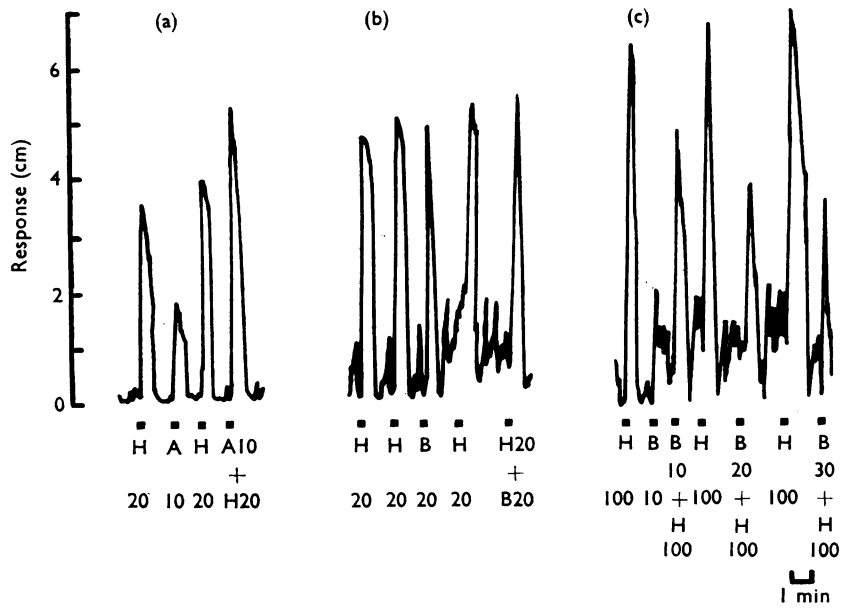


FIG. 6. Responses of the isolated guinea-pig ileum suspended in 5 ml Tyrode solution to histamine (H), L-alanine (A) and L-alanine benzyl ester (B), in ng. Responses to L-alanine and histamine were additive in (a); responses to L-alanine benzyl ester and histamine were not additive in (b) and were antagonistic in (c).

Structure-activity relationship among amino acids

Amino acids and their analogues were chosen so as to include substitutions of all four chemical groups attached to the α-carbon of alanine—the amino acid which was most active on the guinea-pig ileum. The equipotent molar dose ratio relative to L-alanine of compounds which gave dose-response curves parallel to L-alanine was calculated by measuring the distance between the slopes. Each compound was tested on at least two samples of tissue and in each case with the exception of L-alanine ethyl ester the equipotent molar dose ratio was found to vary only slightly from one tissue to another.

The α-methyl group

Replacement of this group by H- to give glycine led to an appreciable loss of activity (Table 2). When the methyl group was replaced by aliphatic groups of increasing size or by aromatic groups, activity rapidly became too small to measure but the products did not show any antagonistic properties. However this loss of activity is not simply associated with the size of the side-chain because analogues having side-chains with hydroxyl, amide, sulphur containing and basic groups frequently had greater activity than the corresponding aliphatic molecule with a side-chain of similar bulk and branching structure. Compounds with basic side-chains were active although β-amino-alanine was less active than its hydroxyl containing equivalent, serine, and β-guanidino-alanine was less active than its neutral, polar counterpart, L-asparagine. In contrast analogues with acidic side-chains had no detectable activity.

The α-hydrogen

Only a limited number of compounds were available to determine the importance of this group (Table 3). However, from a comparison of L-alanine with α-amino isobutyric acid and glycine with D-alanine it is clear that replacement of the α-H of L-amino acids even by a methyl group results in a considerable reduction of activity.

The α-amino group

The absence of detectable activity in lactic acid shows that the amino group cannot be replaced by hydroxyl (Table 4). N-Methylation of glycine did not greatly affect its activity while the quaternary ammonium derivative betaine had detectable but greatly reduced activity, and the guanidine analogues no activity. N-Acetylation of serine also reduced activity considerably.

The carboxyl acid group

A free carboxylic acid group is not a prerequisite for activity since the methyl and ethyl esters of L-alanine and the amide of glycine had some activity although considerably less than the corresponding carboxylic acids (Table 5).

Miscellaneous amino acids

The cyclic compounds proline and cycloleucine retained an appreciable activity, probably because they possess aliphatic chains in fixed positions (Table 6). β-Amino acids, however, were found to be inactive. The following peptides, obtained from Schwarz Bioresearch Inc., were tested and found to have equipotent molar dose ratios relative to L-alanine of less than 50: L-alanylglycine, glycyl-L-alanine, L-serylglycine, glycyl-L-serine, glycylglycine, triglycine, glycyl-glycyl-L-alanine and L-alanylglycylglycine.

TABLE 2. *Activity on the guinea-pig ileum of analogues of* L-*alanine derived by alteration of the* α-*methyl group*

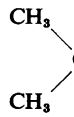
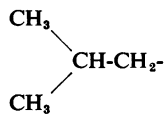
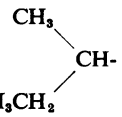
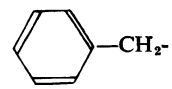
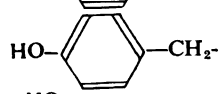
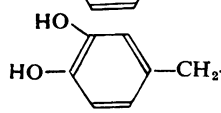
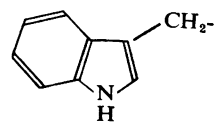
	Group replacing α-methyl of L-alanine	Name		Equipotent molar dose relative to L-alanine
(a)	Aliphatic			
	H-	Glycine	(b)	6·4, 7·4
	CH_3-	L-Alanine	(a)	1·0
	CH_3-CH_2-	L-α Amino butyric acid	(a)	4·7, 4·0
		L-Valine	(b)	7·3, 12·5
	$CH_3CH_2CH_2$-	L-Norvaline	(e)	8·3, 13·2
		L-Leucine	(b)	23, >20
		L-Isoleucine	(b)	20, >20
(b)	Aromatic			
		L-Phenylalanine	(b)	>50, >50, >50
		L-Tyrosine	(b)	>50, >50
		L-DOPA	(a)	>50, >50
		L-Tryptophan	(b)	>50, >50
(c)	Alcohol			
	$HOCH_2$-	L-Serine	(b)	1·1, 1·4, 2·0
	$HOCH_2CH_2$-	L-Homoserine	(a)	2·2, 2·4
		L-Threonine	(b)	25, >50, >50
		L-*Allo*threonine		2·0

TABLE 2 *continued*

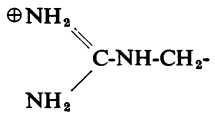
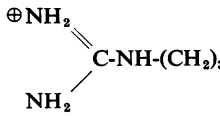
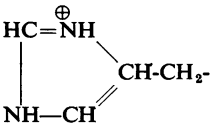
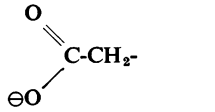
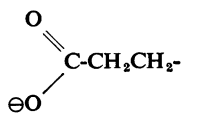
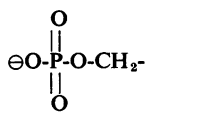
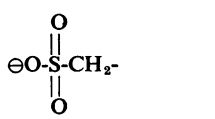
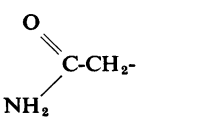
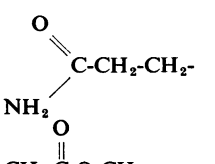
	Group replacing α-methyl of L-alanine	Name		Equipotent molar dose relative to L-alanine
(d)	Basic			
	$\oplus NH_3\text{-}CH_2\text{-}$	L-β-Aminoalanine	(a)	3·6, 3·4
		L-β-Guanidinoalanine	(a)	8·7, >50, >50
	$\oplus NH_3\text{-}(CH_2)_4\text{-}$	L-Lysine	(b)	>50, >50
		L-Arginine	(b)	>50, >50
		L-Histidine	(b)	6·9, 9·1
(e)	Acidic			
		L-Aspartate	(b)	>50, >50
		L-Glutamate	(b)	>50, >50
		L-O-Phosphoserine	(a)	>50, >50
		L-Cysteic acid	(a)	20
(f)	Other groups			
	$SH\text{-}CH_2\text{-}$	L-Cysteine	(b)	2·2, 2·4
	$CH_3\text{-}S\text{-}CH_2\text{-}CH_2\text{-}$	L-Methionine	(b)	1·6, 1·8
		L-Asparagine	(b)	2·3, 2·7
		L-Glutamine	(b)	5, 5·5
		L-O-Acetylserine	(a)	5, 5·2

TABLE 3. *Activity on the guinea-pig ileum of analogues derived from* L-*alanine by replacement of the* α-*methyl group by* R_1 *and the* α-*hydrogen by* R_2

R_1	R_2	Name		Equipotent molar dose relative to L-alanine
CH_3-	H-	L-Alanine	(a)	1·0
H-	H-	Glycine	(b)	6·4, 7·4
H-	CH_3-	D-Alanine	(a)	22, >50
CH_3-	CH_3-	α-Amino isobutyric acid	(a)	5, 5·2
H-	CH_2OH-	D-Serine	(a)	20, 12·5
CH_3-	CH_2OH-	DL-α Methyl Serine	(a)	13, 12
CH_2OH-	CH_3-			

TABLE 4. *Activity on the guinea-pig ileum of analogues derived from glycine* L-*alanine or* L-*serine by modification of the* α-*amino group*

	Group replacing the α-amino group	Name		Equipotent molar dose relative to L-alanine
(a)	Analogues of L-alanine			
	$\oplus NH_3$-	L-Alanine	(a)	1·0
	HO-	DL-Lactate	(c)	>25
(b)	Analogues of glycine			
	$\oplus NH_3$-	Glycine	(b)	6·4, 7·4
	CH_3-NH_2-	Sarcosine	(a)	6·25, 5·6
	$(CH_3)_3 \overset{\oplus}{N}$-	Betaine	(a)	30, 47
	$\oplus NH_2$ \ C-NH- / NH_2	Guanidinoacetate	(h)	>50
	$\oplus NH_2$ CH_3 \ | C—N— / NH_2	Creatine	(g)	>50
(c)	Analogue of serine			
	$\oplus NH_3$-	L-Serine	(b)	1, 1·4, 2·0
	CH_3-CO-NH-	L-N-Acetylserine	(a)	20, 20

TABLE 5. *Contracting activity on the guinea-pig ileum of analogues of amino acids with a modified α-carboxylic acid group*

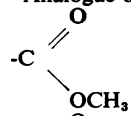
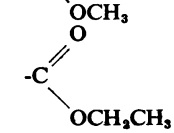
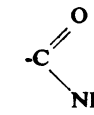
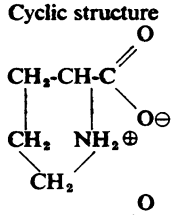
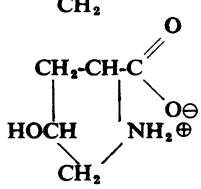
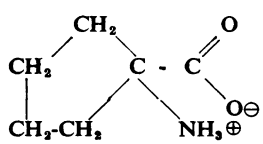
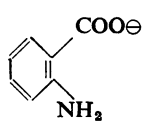
	Group replacing α-carboxylic acid group	Name		Equipotent molar activity relative to L-alanine
(a)	Analogue of alanine			
		L-Alanine methyl ester	(c)	20, 22
		L-Alanine ethyl ester		3, 10
	$-PO_3\ominus$	DL-α-Amino ethyl phosphoric acid	(a)	>50
(b)	Analogues of glycine			
	$-SO_3\ominus$	Amino methane sulphonic acid	(f)	>50, >50
	$-PO_3\ominus$	Amino methane phosphoric acid	(a)	>50
		Glycine amide	(a)	40

TABLE 6. *Activity on the guinea-pig ileum of miscellaneous compounds related to amino acids*

	Structure	Name		Equipotent molar dose relative to L-alanine
(a)	Cyclic structure			
		L-Proline	(b)	2·8, 2·6
		L-Hydroxyproline	(b)	10·5, 12·5, 16·5
		Cycloleucine	(c)	4, 4·3
(b)	β-Amino acids			
	$\overset{\oplus}{N}H_3CH_2\text{-}CH_2\text{-}COO\ominus$	β-Alanine	(b)	>50, >50
	$\overset{\oplus}{N}H_3CH_2\text{-}CH_2\text{-}SO_3\ominus$	Taurine	(b)	>50, >50
		Anthranilic acid	(a)	50, >50

Discussion

The present results show that the smooth muscle-stimulating activity of the stratum corneum which is resistant to antihistamines is due to the presence of certain amino acids. The most commonly found amino acids are L-alanine and L-serine which cause a histamine-like contraction of the guinea-pig ileum at concentrations of 10–20 μg/ml.

Although amino acids are not very potent in this respect, L-serine and L-alanine are present in the human stratum corneum in such large amounts (Burke, Lee & Buethner-Janusch, 1966) that on a sensitive tissue an extract of stratum corneum could give contractions suggesting a histamine content of 1 μg/g.

A number of experimental observations indicate that amino acids act on a different receptor from other compounds which are known to cause rapid contractions of the ileum. For example the response of the tissue to amino acids was not affected by concentrations of mepyramine, atropine or BOL given together with morphine, which abolished or greatly reduced the responses of the tissue to histamine, acetylcholine or 5-hydroxytryptamine. Secondly the dose-response curve of histamine was different from that for amino acids although this is not conclusive evidence for a different receptor. On some occasions an extreme example of this difference was observed when a tissue with normal sensitivity to acetylcholine or histamine was completely insensitive to amino acids. Further, amino acids had little activity on many preparations known to be sensitive to biologically active amines. Finally the guinea-pig ileum could be specifically sensitized to amino acids and later independently sensitized to histamine and vice versa.

Since no partial agonists or antagonists of the amino acid response were found in the present experiments it is not possible to discuss the structure-activity relationship of amino acid analogues in terms of affinity and efficacy. In the ensuing discussion changes in potency resulting from structural alterations will therefore be taken to indicate that the modified part of the molecule interacts with the receptor without necessarily reacting with its 'active site'.

The observed relationship between structure and activity of amino acids suggests that the receptor is particularly sensitive to L-α-amino acids with a short side-chain. L-Alanine was the most active amino acid tested and modification of any of the four substituents of the α-carbon resulted in reduction in activity which suggests that these four substituents may all be in contact with the receptor.

Alteration of the methyl group leads to an appreciable loss of activity which indicates that it plays an important role either in binding the amino acid to the receptor (hydrophobic interaction) or in producing the optimum drug-receptor conformation by virtue of its space-filling property.

The increased activities of certain analogues with polar side-chains when compared with those containing non-polar side-chains of equal bulk suggest that there must be at least one region on the receptor with which polar non-acidic side-chains replacing the α-methyl group of L-alanine can interact. The specificity of this interaction is illustrated by the pronounced difference in the activity of the two isomers L-threonine and L-*allo*threonine which differ only in the configuration of the methyl and hydroxyl groups attached to the α-carbon. However, it is difficult to explain the high activity of methionine in this way since this amino acid is normally considered to have a non-polar side-chain.

Since there is a considerable reduction in activity when the α-hydrogen of L-α-amino acids is replaced, the α-hydrogen might well be in close proximity to the receptor during activity. Further the appreciable activity of the cyclic compounds proline and cycloleucine indicates that they are accommodated by the drug-receptor complex. The low activity of hydroxyproline suggests that the hydrogen which has been replaced by hydroxyl is also close to the receptor when proline is acting.

The present findings indicate that full activity involves interaction of the α-amino and α-carboxylic acid groups with the receptor. It would appear that it is necessary to have a small positively charged group present in the amino position of L-α-amino acid analogues. This group is therefore probably close to part of the receptor where it might be bound by ionic forces. Further, although a free carboxylic acid group is not a prerequisite of activity the greater activity of L-alanine compared to its esters suggests that charge interaction is involved to some extent and this is most likely to involve a cationic site on the receptor.

The present results therefore suggest that there is a specific receptor for L-α-amino acids in the guinea-pig ileum. The high concentrations necessary to cause contraction might be considered to preclude a physiological role for such a receptor. However, plasma concentrations of L-alanine *in vivo* (Felig, Pozefsky, Markiss & Cahill, 1970) are of the same order as those found to be active *in vitro* so that it is possible that the biological activity observed could have some physiological relevance.

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