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SELECTIVE AGONISTS FOR SUBSTANCE P AND NEUROKININ RECEPTORS

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ABSTRACT

A series of neurokinin analogues and fragments have been prepared in an attempt to identify selective agonists for NK-P, NK-A and NK-B receptors. The compounds have been tested on the dog carotid artery (NK-P receptor system), the rabbit pulmonary artery (NK-A) and the rat portal vein (NK-B). C-terminal substituted analogues of the three neurokinins have provided indication that NK-P receptor selectivity is improved by the oxydation of methionine to Met(O_2), while selectivity for NK-A is favoured by replacing Met with Nle. Selectivity for NK-P receptors is further improved by the replacement of Gly^9 with Sar. Selectivity and affinity for NK-B receptors is markedly increased when Val^7 is replaced with MePhe in both the fragment NKB (4-10) and NKB. The results of the present study indicate that a) [Sar^9,Met(O_2)^{11}]SP is a potent and selective agonist for the NK-P receptors of the dog carotid artery; b) [MePhe^7]NKB is a very potent and selective stimulant of receptors for neurokinin B and c) [Nle^{10}]NKA (4-10) is a promising compound, showing some selectivity for NK-A receptor; further modifications are however needed to improve its affinity.

INTRODUCTION

Substance P and related neurokinins are a group of neuropeptides widely distributed in the central and peripheral nervous system. Once released by nervous and possibly some chemical stimuli (1), neurokinins act on specific receptors to exert various biological actions. Even before the discovery of neurokinin A and neurokinin B, growing evidence, indicating the presence of multiple receptors for these peptides, was emerging from pharmacological studies performed with substance P homologues (2), fragments (3) and analogues (4). With the discovery of substance K (5) and neuromedin K (6), later renamed neurokinin A (NKA) and neurokinin B (NKB) (7), further evidence in support of the existence of three different receptors for these peptides were brought using as a criterion the order of potency of agonists in both pharmacological (8, 9) and biochemical (10, 11) experiments. It has however been rapidly realized that the naturally occurring neurokinins, as well as their fragments and even some homologues

tachykinins are not selective enough for discriminating between one and the other receptor type. Even using selective pharmacological preparations that appear to contain almost exclusively a single receptor type (8), the natural peptides show little selectivity.

In the present study, an attempt has been made to identify chemical modification that will be suitable for improving the selectivity of neurokinin agonists for one or another receptor type. Furthermore, we have tried to identify the shortest peptide sequences that maintain full activity and selectivity, in order to eliminate some of the effects that are attributed to the N-terminal part of the natural molecules, particularly that of substance P. Results obtained with new compounds prepared in our laboratory are compared with those of selective agonists for neurokinin receptors reported by other workers (12, 13, 14).

MATERIALS AND METHODS

Peptides. [Sar^9]SP was purchased from Peninsula Laboratories; all other peptides were prepared in our laboratory using solid phase methodology as described previously (17). In brief, methylbenzhydrylamine resin was used to obtain peptides with a C-terminal amide group, while C-terminal free acid peptides were obtained with chloromethylated resin. N-Boc protection was used for the amino acid derivatives with the following side chain protecting groups: tosyl for Arg and His, cyclohexyl for Asp, 2-chloro-benzyloxycarbonyl for Lys and benzyl for Ser and Thr. Met(O) and Met(O_2) (Sigma) were treated with di-t-butyl dicarbonate in order to afford Boc-Met(O) and Boc-Met(O_2) used to obtain respectively, peptides containing C-terminal methionine sulfoxide and methionine sulfone. SP-OMe was prepared according to Watson et al. (12). Couplings were performed using the symmetric anhydride method, except for Boc-Arg(Tos) and Boc-Gln which were activated using the dicyclohexylcarbodiimide/1-hydroxybenzotriazol method. Coupling were monitored using the qualitative Kaiser (15) test. After removal of the last N-Boc protecting group, the resin was dried overnight in vacuo. The peptides were cleaved from the resin with liquid HF (10 ml/g resin) in the presence of 10% anisole and dimethyl sulfide (0.25 ml/g resin). The crude product was purified by gel filtration on Sephadex LH20 (50% CH_3CN in 0.2 M CH_3 COOH). The products were further purified by preparative HPLC, with an acetonitrile gradient in 0.05% TFA. Purety of the final products was controlled by TLC in several solvent systems and by analytical HPLC. Amino acid analyses gave the expected composition, although N-methyl amino acids could not be detected. Some of the compounds in which methionine has been oxydated or replaced with Nle and Ala have been described before (4, 16, 17).

Biological assays. The peptides were tested as agonists and some of them also as antagonists on three pharmacological preparations described by Regoli et al. (8) as selective for one or the other neurokinin. The dog carotid artery with intact endothelium as described by D'Orléans-Juste et al. (18) is very sensitive to substance P and provides a good selective preparation for studying the substance P receptor, NK-P; the rabbit

pulmonary artery without endothelium, prepared according to D'Orléans-Juste et al. (19) is a preparation that responds by contraction to neurokinin A and contains the NK-A receptor type; the third preparation, sensitive to neurokinin B is the rat portal vein, which is considered selective for the NK-B receptor (20) type. The tissues were suspended in 10 ml organ baths, filled with oxygenated Krebs' solution at 37°C and the changes of tension were recorded isometrically on a Grass system. The pharmacological parameters of pD_2 (apparent affinity of agonists) was used according to Ariens (21) to characterize and compare the various compounds. Relative affinities of agonists were calculated and displaced in % of those of each neurokinin in the respective selective preparation.

All peptides were prepared in concentrated solutions (1-2 mg/ml) in distilled water and kept at -20°C until use. Noradrenaline solutions, used to contract the dog carotid artery, were added with 5% of ascorbic acid in order to prevent the oxydation. NKB was dissolved in 10% sulfolane.

RESULTS

Neurokinin analogues modified at the C-terminal position

In a first series of experiments, several substitutions were made in the C-terminal positions of SP, NKA and NKB. In particular, the thioether function of Methionine was oxydated to sulfoxide or sulfone; the residue was also replaced with either the isosteric Nle or with Ala. The compounds were tested on three selective pharmacological preparations, the dog carotid artery, the rabbit pulmonary artery and the rat portal vein, to evaluate their activities respectively on NK-P, NK-A and NK-B receptors. The compounds activities are expressed in terms of apparent affinity (pD_2) and in percent of the affinity of the most potent neurokinin in each preparation (relative activity), according to Regoli et al. (8, 9). The results, summarized in Table 1, indicate that the oxydation of the thioether function of Methionine in substance P favours the interaction with the NK-P receptor of the dog carotid artery. In fact, the sulfoxide derivative of SP (compound no 2 of Table 1) maintains full activity in the D.C.A. and R.P.V., but is practically inactive on the R.P.A. The sulfone derivative is even more active than SP on the D.C.A., while loosing affinity on the R.P.A. and particularly on the R.P.V. Replacement of Met^{11} of SP with the isosteric Nle bringt about variable decreases of affinity which are however more evident in the R.P.A. and R.P.V.: $[Nle^{11}]SP$ maintains almost 50% of the SP-activity on the D.C.A. (17). As reported before for the D.C.A. and other tissues (3, 4) $[Ala^{11}]SP$ shows little activity in all preparations.

As for NKA, the most favourable change appears to be the replacement of Met^{10} by Nle. $[Nle^{10}]NKA$ maintains a good affinity on the R.P.A. and R.P.V., but is almost 20 times less potent than NKA on the D.C.A. Therefore, $[Nle^{10}]NKA$ shows some selectivity for the NK-A receptor system. On the contrary, the oxydation of Met^{10} of NKA to sulfoxide or sulfone, bringt about marked decreases of affinities in the three tissues.

Replacement of Met^{10} by Ala (compound 10 of Table 1) reduces markedly all activities.

TABLE 1

ACTIVITIES OF NEUROKININS MODIFIED AT THE C-TERMINAL END ON SELECTIVE PHARMACOLOGICAL PREPARATIONS.

	PEPTIDE	PREPARATION					
		D.C.A		R.P.A		R.P.V	
		pD_2	R.A	pD_2	R.A	pD_2	R.A
1	Substance P	10.00	100	6.1	4.0	5.8	1.4
2	[Met(O)^{11}]-SP	10.06	114		0.01	5.6	0.8
3	[Met(O_2)^{11}]-SP	10.1	126	5.7	0.4	inact.	
4	[Nle^{11}]-SP	9.7	46	5.6	0.3	inact.	
5	[Ala^{11}]-SP	7.6	0.4	inact.		inact.	
6	Neurokinin A	9.4	25	8.2	100	6.4	6.0
7	[Met(O)^{10}]-NKA	7.6	0.4	6.8	3.4	5.7	1.1
8	[Met(O_2)^{10}]-NKA	8.5	2.8	7.5	17	6.6	8.5
9	[Nle^{10}]-NKA	8.0	1.1	8.0	62	6.5	6.5
10	[Ala^{10}]-NKA	6.0	0.01	6.1	0.8		1.0
11	Neurokinin B	8.9	8.0	7.4	17	7.7	100
12	[Met(O)^{10}]-NKB	8.1	1.2	6.7	3.2	5.7	1.2
13	[Met(O_2)^{10}]-NKB	8.4	2.8	6.2	1.0	6.6	9.3
14	[Nle^{10}]-NKB	8.9	8.3	6.4	1.6	6.3	4.0
15	[Ala^{10}]-NKB			5.4	0.2	inact.	

pD_2: apparent affinity, calculated from the middle point (50 % of the maximum effect) of at least 6 concentration response curves. pD_2 is expressed by the -log of the molar concentration of compound.

R.A: relative affinity indicated in the percent of the affinity of the the most active neurokinin in each preparation.

In the NKB series, all substitutions of Met^{10} are accompanied by some decrease of affinity for the NK-B receptors: in fact, all analogues shown in Table 1 are at least 10 times less potent than NKB in the R.P.V. (see compounds 12, 13, 14 and 15 of Table 1). They maintain some activity in the other two preparations and therefore they do not show any selectivity for NK-B receptors.

Selective NK-P receptor agonists

A combination of several modifications were made in the sequence of SP to

obtain potent and selective agonists for the NK-P receptor. The results are summarized in Table 2, where the activities of SP are first compared with those of some fragments and of compounds that have been shown to be selective stimulants of the receptors for substance P (8, 19, 23).

TABLE 2

ACTIVITIES OF SUBSTANCE P FRAGMENTS AND ANALOGUES ON SELECTIVE PHARMACOLOGICAL PREPARATIONS.

	PEPTIDE	PREPARATION					
		D.C.A		R.P.A		R.P.V	
		pD_2	R.A	pD_2	R.A	pD_2	R.A
1	Substance P	10.00	100	6.1	100	5.8	100
2	SP(4-11)	9.67	47	6.2	126	5.9	112
3	[pGlu^6]-SP(6-11)	9.56	36	6.0	78	6.1	174
4	SP-OMe	9.60	40	inact.		inact.	
5	[pGlu^6,Pro^9]-SP(6-11)	9.70	50	5.2	12	5.6	58
6	[Pro^9]-SP	10.16	145	5.5	24	inact.	
7	[Sar^9]-SP	10.38	240		0.1	5.8	98
8	[Sar^9,Met(O_2)^{11}]-SP	10.45	282	inact.		inact.	
9	[Pro^9,Met(O_2)^{11}]-SP	10.21	162	inact.		inact.	
10	[β-Ala^4,Sar^9,Met(O_2)^{11}]-SP(4-11)	10.35	224		0.1		0.1
11	[pGlu^6,Sar^9,Met(O_2)^{11}]-SP(6-11)	9.92	83	4.60	3.0	5.46	39

R.A.: relative affinity, expressed in percent of that of substance P in the three preparations.

pD_2: as in Table 1.

The results of Table 2 indicate that C-terminal fragments of SP such as SP (4-11) and SP (6-11) are less selective than the natural undecapeptide, because they are less potent than SP on the D.C.A., but maintain fairly high activities on the R.P.A. and particularly on the R.P.V. SP methyl ester (23) is selective for the NK-P receptors, but has only 40% of the SP-activity on the D.C.A. The hexapeptide [pGlu^6,Pro^9]SP (6-11), which has been recommended as a selective agonist for SP receptors by Laufer et al. (24), is still fairly active on the R.P.A. and R.P.V.: furthermore, the compound has 50% of the SP-activity on the D.C.A. [Pro^9]SP, a compound recently described by Ploux et al. (25), is more potent than SP on the NK-P receptor system and shows some selectivity

because it is inactive on the R.P.V. The compound maintains however some activity on the NK-A system of the R.P.A. Another compound, [Sar^9]SP, described several years ago by Sandberg et al. (13), is almost 2.5 times more potent than SP on the D.C.A. and is practically inactive on the R.P.A.; [Sar^9]SP maintains however full activity on the R.P.V. and is therefore not selective enough. [Sar^9]SP shows a pharmacological spectrum rather different from [$Met(O_2)^{11}$]SP, the compound (Table 1) which is completely inactive on the R.P.V.

Based on these findings, the two modifications in positions 9 and 11 of SP were combined, in order to eliminate the activities in the R.P.A. with Sar^9 and in the R.P.V. with $Met(O_2)^{11}$. [Sar^9,$Met(O_2)^{11}$]SP is a potent activator of NK-P receptors of the D.C.A. (3.5 times more potent than SP) and is inactive on the other two preparations. [Sar^9,$Met(O_2)^{11}$]SP is therefore to be considered a potent and selective agonist of the NK-P receptor. Replacement of Gly^9 by Sar appears to be more favourable than by Pro, since [Pro^9,$Met(O_2)^{11}$]SP, although selective for NK-P receptors, is only half as active as [Sar^9,$Met(O_2)^{11}$]SP on the D.C.A..

Despite its selectivity, [Sar^9,$Met(O_2)^{11}$]SP is an undecapeptide that contains the N-terminal Arg and Lys which are responsible for the release of histamine (26) and some other effects of SP (28). We therefore decided to prepare and test shorter sequences, containing the same modifications. As shown in Table 2, the octapeptide (compound 10) is potent on the D.C.A. and quite selective, while the shorter hexapeptide (compound 11) regains some activity on the R.P.A. and especially on the R.P.V. Further studies are needed to improve affinity and selectivity of hexapeptide sequences for the NK-P receptor.

Selective NK-B receptor agonists

Further experiments were performed in an attempt to identify chemical changes that will favour occupation and activation of NK-B receptors. The results of this study are summarized in Table 3, where the activities of neurokinin B in the three pharmacological preparations are compared with those of analogues of two heptapeptides (NKB (4-10) and DiMeC7). As shown before by Regoli et al. (8), the shortening of the NKB sequence by the elimination of three residues at the N-terminal (as in NKB (4-10)), give a compound that is 10 fold less active than NKB on the R.P.V., but is almost as active as NKB on the D.C.A. and more potent on the R.P.A. NKB (4-10) is however the minimum active sequence of NKB and it was therefore selected for further design of NK-B selective agonists (see below). Such a design was based on the observation that DiMeC7, the compound reported by Sandberg et al. (13), several years ago, although weak on the R.P.V., shows some selectivity on the NK-B receptor (9, 23) system.

As shown by the results of Table 3, DiMeC7 maintains less than 5% of the NKB activity on the D.C.A. and 6% on the R.P.V., but is inactive on the NK-A receptor of the R.P.A. These findings lead us to utilize into the heptapeptide NKB (4-10) the chemical modifications which are present in DiMeC7. We therefore prepared compounds 4, 5 and 6 of Table 3. When

tested on the three preparations, it was apparent that the replacement of Gly by Sar favours the occupation and activation of NK-P receptors, since [Sar^8]NKB (4-10) is potent in the D.C.A., but rather weak on the R.P.A. and R.P.V.

TABLE 3

BIOLOGICAL ACTIVITIES OF NEUROKININ B PEPTIDES.

		D.C.A		R.P.A		R.P.V	
		pD_2	R.A	pD_2	R.A	pD_2	R.A.
1	Neurokinin B	8.90	100	7.45	100	7.68	100
2	NKB(4-10)	8.70	63	7.95	316	6.67	10
3	DiMeC7	7.50	4.0	inact.		6.35	4.7
4	[MePhe^7,Sar^8]-NKB(4-10)	7.61	5.1	inact.		7.03	22
5	[Sar^8]-NKB(4-10)	9.26	229	6.72	19	6.44	6
6	[MePhe^7]-NKB(4-10)	7.09	1.5	5.50	1.1	7.58	79
7	[Sar^7]-NKB(4-10)	7.21	2	6.31	7.2	5.86	1.5
8	[Pro^7]-NKB(4-10)	6.20	0.2	5.65	1.6	5.85	1.5
9	[Phe^7]-NKB(4-10)	8.63	54	6.31	7	5.86	1.5
10	[MeVal^7]-NKB(4-10)	7.64	5.5	5.65	1.6	7.68	100
11	[MePhe^7]-NKB	7.15	1.8	5.24	0.6	8.30	417
12	Senktide	6.39	0.3	5.46	0.7	7.61	85

pD_2 as in table 1
R.A.: relative activity expressed in percent of that of NKB.

The combination of the two substitutions in positions 7 and 8, as in [MePhe^7,Sar^8]NKB (4-10), gives a compound slightly better then DiMeC7, since it is more active (by 0.7 log units) on the R.P.V., it is inactive on the R.P.A. and it has practically the same activity as DiMeC7 on the D.C.A. These results suggest that the most favourable changes for NK-B receptor selectivity might be the substitution of Val^7 with MePhe. This assumption was confirmed by the biological activities obtained with [MePhe^7]NKB (4-10), which is almost as active as NKB on the R.P.V., but maintains only 1% of NKB activities in the other two preparations. Val^7 of NKB (4-10) could also be favourably replaced with MeVal: in fact, compound 10 of Table 3 was found to be as active as NKB on the R.P.V. and was equivalent to [MePhe^7]NKB (4-10) in the other two preparations. Compound 10 is therefore as selective as compound 6. When Val^7 of NKB

(4-10) was replaced with other residues, such as Phe, Pro or Sar, the affinities of the resulting compounds (no 8, 9 and 10 of Table 3) on the NK-B system of the R.P.V. were markedly decreased. Some of these compounds (no 8 and 10) showed fairly good affinities in the D.C.A. and R.P.A. Selectivity for NK-B receptor system appears therefore to be favoured by the presence of a N-methyl residue in the center position of the sequence NKB (4-10). The validity of this statement is supported by the comparison of the biological activities of NKB (4-10) with those of its analogue [MePhe^7]NKB (4-10) and [MeVal^7]NKB (4-10), in Table 3 (see also Drapeau et al., 1987) (22).

To further improve the agonist affinity for NK-B receptors, a decapeptide analogue of NKB, containing MePhe in position 7, was prepared and tested. As shown in Table 3, this compound is more active than NKB (more than 4 folds) on the R.P.V., but is 150 and 250 times less potent than NKB respectively in the D.C.A. and R.P.A. [MePhe^7]NKB is the most active agonist for NK-B receptors that we have tested: it is soluble in water and appears to be promising for physiological and pharmacological studies.

While the present work was under way, Wormser et al. (14) published the senktide, an analogue of the sequence SP (5-11), containing a N-methyl residue in the center position. This compound was also prepared and tested in our laboratory and the results of the biological activities are presented in Table 3. Senktide appears to be quite selective for the NK-B receptor, but its affinity on the R.P.V. is 5 fold less than that of [MePhe^7]NKB. Senktide is more active than [MePhe^7]NKB on the NK-A receptor system of the R.P.A.

Selective NK-A receptor agonists

Table 4 summarizes some preliminary results obtained with NKA-related

TABLE 4

ACTIVITIES OF NEUROKININ A, ITS FRAGMENTS AND ANALOGUES ON SELECTIVE PHARMACOLOGICAL PREPARATIONS.

		D.C.A.		R.P.A		R.P.V.	
		pD_2	R.A.	pD_2	R.A.	pD_2	R.A.
1	Neurokinin A	9.40	100	8.20	100	6.45	100
2	NKA(4-10)	8.62	16.6	8.50	191	6.80	224
3	[Nle^{10}]-NKA	8.04	4.4	8.00	60	6.50	112
4	[Nle^{10}]-NKA(4-10)	7.00	0.4	7.90	49		<1.0

pD_2: as in Table 1
R.A: relative activity expressed in percent of that of NKA.

peptides, prepared with the purpose of improving agonists affinity and selectivity for the receptors of neurokinin A. As shown before (see Table 1 and the paper by Drapeau et al. (17)), the substitution of Met^{10} by Nle as in [Nle^{10}]NKA, is accompanied by a marked decrease of affinity (by approximately 25 folds) in the D.C.A., with minor changes in the R.P.A. and R.P.V. Because of previous observations (8, 9) that the C-terminal heptapeptide NKA (4-10) is more potent than NKA on the R.P.A. and in other preparations (for instance the human isolated bronchus (27)), the C-terminal Met was replaced with Nle to obtain the sequence [Nle^{10}]NKA (4-10). The results presented in Table 4 confirm previous findings that NKA (4-10) is more selective than NKA, since it is twice as active as NKA on the R.P.A., but is 6 folds less potent on the D.C.A. [Nle^{10}]NKA (4-10) is even more selective, since it maintains almost 50% of activity on the R.P.A., but is practically inactive on the R.P.V. and is two and a half orders of magnitude less active than NKA on the D.C.A.

CONCLUSIONS

Selective agonists for NK-P receptors have been obtained by reducing the conformational freedom of the undecapeptide SP with the replacement of Gly^{9} by Sar. Selectivity and potency have been further improved by oxydizing Met^{11} to $Met(O_2)$. Histamine releasing ability and metabolic degradation have been eliminated or reduced by using the same modifications in the octapeptide SP (4-11), without loosing much affinity. [β-Ala^{4},Sar^{9},$Met(O_2)^{11}$]SP (4-11) appears to be a potent and selective NK-P agonist: the shorter hexapeptide sequence is however not selective enough.

The chemical change essential to improve selectivity of NKB-related peptides for the NK-B receptor has been found to be the replacement of Val^{7} by a N-methyl residue, preferably MePhe. High selectivity for NK-B receptors has been shown by [$MePhe^{7}$]NKB (4-10) and especially by [$MePhe^{7}$]NKB. This compound is selective for NK-B, it is extremely active, and it is soluble in water. If adequately protected from metabolic degradation, [$MePhe^{7}$]NKB could provide an excellent ligand for NK-B receptors studies.

An attempt has been made to improve selectivity of NKA-related peptides for the NK-A receptors system. Replacement of Met^{10} by Nle confers some selectivity as does the reduction of the decapeptide chain by three residues from the N-terminal. [Nle^{10}]NKA (4-10) represents a first step towards the identification of a selective NK-A receptor agonist.

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