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Notice of Entitlement

We, AKTIEBOLAGET ASTRA, of S-151 85 Sodertalje, Sweden, being the Applicant and Nominated Person in respect of Application No. 17858/92, state the following:

The actual inventors of the invention of Application No. 17858/92 are Tamas Bartfai, Tomas Hokfelt, Ulo Langel, Bo Ahren, Stefan Lindskog, Silvana Consolo, Tiit Land and Zsuzsanna Wiesenfeld-Hallin.

The basic application listed on the PCT Request form, namely, Swedish Application No. 9101472-0, was filed in our name as assignee of the actual inventors.

The said basic application listed in the declaration under Article 8 of the PCT is the first application made in a Convention country in respect of the invention.

We have entitlement from the actual inventors by assignment.

Our address for service is care of E. F. WELLINGTON & CO., Patent and Trade Mark Attorneys, 312 St. Kilda Road, Melbourne, Victoria, 3004.

DATED this 21st day of October 1993

For and on behalf of
AKTIEBOLAGET ASTRA
By:

. . . . . .
BRUCE S. WELLINGTON
Patent Attorney for
Applicant/Nominated Person

To: The Commissioner of Patents,
Commonwealth of Australia

C/KA/8486/3

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GALANIN ANTAGONIST

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(57) Claim

1. A peptide selected from the group consisting of

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-
-Gly-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-
-Gly-Pro-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-
-Gly-Pro-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-
-Gly-Pro-Lys-X
|
Pro-Leu-Phe-Gly-Gly-Tyr-H

wherein X represents -NH_2 or -OH (amide or free acid), and functional analogues thereof (i.e. shorter or longer peptides, with or without

.../2

substitution of one or several amino acid residues for other amino acid residues) which exhibit substantially the same galanin antagonistic effect as said peptide group, and functional derivatives thereof (i.e. peptides wherein one or several amino acid residues are substituted for other chemical groups) which exhibit substantially the same galanin antagonistic effect as said peptide group.

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(54) Title: GALANIN ANTAGONIST

(57) Abstract

A galanin antagonist which is a galanin receptor ligand is described. Further, new peptides Galanin(1-12)-Pro-Substance P(5-11), Galanin(1-12)-Pro-Bradykinin(2-9), Galanin(1-12)-Pro-Pro-Pro-(Leu^5-Enkephalin(5-1)), Galanin(1-12)-Pro-Lys(ε-NH-)Pro-(Leu^5-Enkephalin(5-1)) and functional analogues and functional derivates thereof which exhibit substantially the same galanin antagonistic effect as said peptides are disclosed. Use of the galanin antagonist, a pharmaceutical preparation comprising it and a method of treating a disorder in a mammal which depends on the physiological function of galanin at the galanin receptor are also included.

GALANIN ANTAGONIST

The present invention relates to a galanin antagonist which is a galanin receptor ligand. Further, it relates to the peptides Galanin(1-12)-Pro-Substance P(5-11), Galanin(1-12)-Pro-Bradykinin(2-9), Galanin(1-12)-Pro-Pro-Pro-(Leu^5-Enkephalin(5-1)), Galanin(1-12)-Pro-Lys(ε-NH-Pro-(Leu^5-Enkephalin(5-1)) and functional analogues and functional derivatives thereof which exhibit substantially the same galanin antagonistic effect as said peptides. Use of the galanin antagonist, a pharmaceutical preparation comprising it and a method of treating a disorder in a mammal which depends on the physiological function of galanin at the galanin receptor are also included.

Background

Neuro-transmitters and hormones can induce their cellular effects by binding to and activating membrane bound receptors. The neuro-peptide galanin was isolated in 1983 from porcine upper intestine and was found to contain 29 amino acid residues (Tatemoto, K., et al, FEBS Lett., 164 (1983) 124-128). The sequences of galanin from two other mammals, rat and cow, have been described (Vrontakis M. E., et al, J. Biol. Chem. 262(1987) 16755-16758; Kaplan L. M. et al, Proc. Natl. Acad. Sci. U.S.A. 85(1988) 1065-1069 and Rökaeus Å. and Carlquist M., FEBS Lett. 234 (1988) 400-406). A comparison of the peptide sequence of galanin from the mammals rat, porcine and bovine reveals that the N-terminal amino acids 1-15 are identical. Thus, it is most likely that said conserved region will be found in galanin from other mammals, including man.

Galanin has a wide pattern of distribution, often correlating with multiple neuro-formula actions exerted in a variety of different systems.

Hitherto no galanin antagonists, which are galanin receptor ligands, have been reported. A galanin antagonist would be a useful tool in determining the physiological significance of galanin and to develope pharmaceutical preparations for the regulation of the physiological function of galanin at the galanin receptor.

Description of the invention

One aspect of the invention is directed to a galanin antagonist which is a galanin receptor ligand. Thus, the antagonistic effect of the galanin antagonist according to the invention is exercised at galanin receptors. In an embodiment of this aspect of the invention the antagonist is selected from the group consisting of the peptides

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Gln-Gln-Phe-Phe-Gly-Leu-Met-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Lys-X
|
Pro-Leu-Phe-Gly-Gly-Tyr-H

wherein X represents $-NH_2$ or -OH (amide or free acid), and functional analogues thereof and functional derivatives thereof.

In this specification and claims, it is intended that "functional analogues" of said peptide group of the invention

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be understood to mean shorter of longer such peptides, with or without substitution of one or several amino acid residues for other amino acid residues, as long as such analogues exhibit substantially the same pharmacological function as said peptide group of the invention, i.e. are galanin antagonists at the galanin receptor.

Further, it is intended that "functional derivatives" of said peptide group according to the invention be understood to mean such peptides wherein one or several amino acid residues are substituted for other chemical groups, i.e. organic as well as inorganic molecules or elements resulting in a "peptidomimetic", which exhibit substantially the same pharmacological function as said peptide group of the invention, i.e. are galanin antagonists at the galanin receptor.

It is believed that it is the structural conformation at the galanin receptor of a peptide according to the invention which is responsible for its pharmacological function as a galanin antagonist and for its affinity to the galanin receptor (thus being a galanin receptor ligand).

Thus, it is believed that the functional derivatives and the functional analogues comprised by the invention should have a similar structural conformation at the galanin receptor as said peptide group of the invention. The surrounding of the galanin receptor could be mimicked by e.g. blood or physiological saline solution.

Another aspect of the invention is directed to the peptides

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-X,

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H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Lys-X
|
Pro-Leu-Phe-Gly-Gly-Tyr-H

wherein X represents $-NH_2$ or -OH (amide or free acid), and functional analogues and functional derivatives thereof which exhibit substantially the same galanin antagonistic effect as said peptides.

As mentioned above, the peptides may also be named Galanin-(1-12)-Pro-Substance P(5-11), Galanin(1-12)-Pro-
-Bradykinin(2-9), Galanin(1-12)-Pro-Pro-Pro-(Leu^5-Enkephalin(5-1)) and Galanin(1-12-Pro-Lys(ε-NH-)Pro-(Leu^5-Enkephalin(5-1)), respectively.

In the experimental part of this specification the above listed peptides, in amide form (i.e. X = $-NH_2$), have been named M15, M35, M36,A and M34,A, respectively.

Functional analogs may be peptides such as

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
(D-Arg)-Pro-Lys-Pro-Gln-Gln-(D-Trp)-Phe-(D-Trp)-Leu-Leu-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Gln-Phe-Phe-Gly-Leu-Met-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Ala-Leu-Ala-Leu-Ala-Leu-Ala-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Ala-Leu-Ala-Leu-Ala-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Ala-
-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Tyr-Gly-Gly-Phe-Leu-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X

wherein X represents -NH2 or -OH (amide or free acid).

A further aspect of the invention is directed to the use of a galanin antagonist which is a galanin receptor ligand for the preparation of a pharmaceutical preparation. It can be mentioned that the above listed peptides are soluble in water, which facilitates the preparation of a pharmaceutical preparation for injection.

Yet another aspect of the invention is directed to a galanin antagonist which is a galanin receptor ligand for use in a pharmaceutical preparation.

Yet a further aspect of the invention is directed to a pharmaceutical preparation comprising a galanin antagonist which is a galanin receptor ligand as an active ingredient, together with pharmaceutically acceptable additive(s). Depending on the specific type of

pharmaceutical preparation to be prepared, such additive(s) should be chosen to make up the desired preparation. Suitable additive(s) for the specific type of preparation to be prepared, such as solutions for injection, tablets or plasters, can be found in the U.S. Pharmacopoeia.

Thus the present invention also provides compositions containing an effective amount of compounds of the present invention, including the nontoxic addition salts, amides and esters thereof, which may, alone, serve to provide the above-recited therapeutic benefits. Such compositions can also be provided together with physiologically tolerable liquid, gel or solid diluents, adjuvants and excipients.

These compounds and compositions can be administered to mammals for veterinary use, such as with domestic animals, and clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy will range from about 0.01 to 1000 mcg/kg, more usually 0.1 to 1000 mcg/kg of the host body weight. Alternatively, dosages within these ranges can be administered by constant infusion over an extended period of time until the desired therapeutic benefits have been obtained.

Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified or incorporated in drug delivery systems like liposomes or microspheres. The active ingredient is often mixed with diluents or excipients which are physiologically tolerable and compatible with the active ingredient. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or

the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH-buffering agents, and the like.

The compositions are conventionally administered parenterally, by injection, for example, either subcutaneously, intramuscularly, intraperitoneally or intravenously. Additional formulations which are suitable for other modes of administration include suppositories, vagitories, intranasal aerosols, buccal formulations like adhesive tablets, gels or patches and in some cases, oral formulations. For suppositories and vagitories, traditional binders and excipients may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10% preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained-release formulations, or powders, and contain 10%-95% of active ingredient, preferably 25%-70%.

The peptide compounds may be formulated into the compositions as neutral or salt forms. Pharmaceutically acceptable nontoxic salts include the acid addition salts (formed with the free amino groups) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as ispropylamine, trimethylamine, 2-ethylamino

ethanol, histidine, procaine, and the like.

Still another aspect of the invention is directed to a method of treating a disorder in a mammal which depends on the physiological function of galanin at the galanin receptor, which comprises administering to said mammal a pharmacologically effective amount of a galanin antagonist which is a galanin receptor ligand. Since the physiological function of galanin at the galanin receptor is specific for each individual, the pharmacologically effective amount of a galanin antagonist which is to be administered to treat a disorder in a mammal has to be decided by a physician or veterinary on an individual bases.

Galanin antagonists would be useful in the regulation of at least the following:
insulin release,
growth hormone release,
acetylcholine release,
dopamine release,
Substance P release,
Somatostatin release,
Noradrenaline release.

At present, the presumed fields of medical indication are endochrinology, food intake, neurology and psychiatry: senile dementia of Alzheimer's type, schizophrenia, analgesia, intestinal diseases.

Preparation of a galanin antagonist according to the invention

Conventional methods of preparing peptides can be used to prepare the galanin antagonists of the invention, suitably modified if the antagonist is a peptidomimetic. In addition to liquid phase synthesis, conventional

procedures for synthesizing the novel compounds of the present invention include any solid phase peptide synthesis method. In such a method the synthesis of the novel compounds can be carried out by sequentially incorporating the desired amino acid residues one at a time into the growing peptide chain according to the general principles of solid phase methods.

Abbreviations used in the following:

NMP	= N-Methylpyrrolidone
HOBt	= N-hydroxybenzotriazole
MBHA	= p-Methylbenzhydrylamine
PAM	= p-Acetoxymethyl
Boc	= tert-Butyloxycarbonyl
DMSO	= Dimethylsulfoxide
DIEA	= diisopropylethylamine
Tos	= tosyl
OcHex	= cyclohexyl ester
4-MeBzl	= 4-methylbenzyl
OBzl	= bensyl ester
Bom	= t-Benzyloxymethyl
Clz	= 2-Chlorobenzyloxycarbonyl
Bzl	= O-benzyl
For	= formyl
Brz	= 2-bromobenzyloxycarbonyl
TFA	= trifluoroacetic acid
DMS	= dimethyl sulfid
DCM	= dichloromethan
DMF	= N,N-dimethylformamide

The peptides "M15", "M35" and "M36,A" of this invention were assembled in a step-wise manner on a solid support using an Applied Biosystems Model 431A Peptide Synthesizer using the standard NMP/HOBt Solvent-Activation strategy on a 0.1 mmole scale (small scale).

The peptide "M34,A" was synthesized in the same manner, except for the protective group which was ε-Fmoc and Boc for Lys. Accordingly one part of the peptide was synthesized using Boc-chemistry, and the other part was synthesized using Fmoc chemistry.

The functional derivatives of the invention were prepared in analogous manner.

tert-Boc-amino acids were coupled to tert-Boc-amino acid-PAM (Nova Chemical Company Ltd., UK) resin for free acid (X = -OH) or MBHA(Bachem Feinchemikalien AG, Switzerland) resin for amide (X = $-NH_2$) as hydroxybenzotriazole(HOBt) esters.

As individual cycle for each amino acid included deprotection of the tert-Boc-group with 50% trifluoroacetic acid in CH_2Cl_2(DCM) for 19 min, and acylation with 10-fold excess (compared to the amount of amino groups on a resin) of the protected amino acids in a mixture of 15% DMSO in N-methyl pyrrolidone (NMP) for 35 min. Between each operation several extensive washings were performed with CH_2Cl_2, DIEA and NMP. After each coupling acetylation (capping) was carried out using 10% acetic anhydride and 5% DIEA in NMP for 5 min.

With the excention of Boc-Lys(Fmoc) in the case of the peptide 34,A, all amino acids used were tert-Boc-protected at the N-terminal (obtained from Nova Chemical Company Ltd., UK), and their reactive side chains were protected with Tos in

Arg, OcHex in Asp, 4-MeBzl in Cys, OBzl in Glu, Bom in His, ClZ in Lys, Bzl in Ser and Thr, For in Trp, and Brz in Tyr.

All the solvents and other reagents for automatic peptide synthesis were from Applied Biosystems.

The reagents used in deprotection and cleavage steps were of analytical grade and used without further purification:

1,2-ethaneditiol, acetic anhydride, trifluoromethyl sulfonic acid (TFMSA), dimethyl sulfide (DMS) and p-cresol from Fluka, diethyl ether and acetonitrile from Merck and HF from AGA Gas.

The fully assembled peptide-resins were dried under high vacuum overnight. Deprotection from formyl-group on Trp and benzyl-groups was performed using "low TFMSA" method. For that 100 mg of peptide-resin was treated with 2 ml of the mixture of TFMSA(10%), TFA(50%), DMS(30%), p-cresol(8%) and dimercaptoethan(2%) for 2 h at 0°C while shaking, the procedure was followed by washing with EtOH, DCM, DMF, DIEA/DCM, DMF and DCM.

After drying under vacuum the peptide was cleaved from resin and deprotected by mixing of 10 ml liquid HF (containing 20% of 1:1 mixture of p-cresol and p-thiocresol) with 1 g of peptide-resin at 0°C for 60 min. The resin was washed 2 times with 10 ml of Et_2O, peptide extracted with 20% acetonitrile/water and filtered. Lyophilization of the aqueous filtrate yielded the crude peptide.

Preparative purifications were carried out on the crude product by HPLC on reversed phase C18 column. 10 mg of crude peptide was dissolved in 1 ml of 20% acetonitrile/water and eluted with a gradient (45 min) of 20-60 % mixture of 0.1% TFA/acetonitrile in 0.1 &

TFA/water at a flow rate of 2.0 ml/min. The fractions were collected according to the absorption detected at 238 nm.

Purity of the individual peptides was checked by analytical HPLC and determined to be 99 %. Molecular weights of the peptides were determined using Plasma Desorption Mass Spectrometer (PDMS) Model Bioion 20, Applied Biosystems, the calculated molecular weight values were obtained in each case for the purified peptide.

Table.

Purity of the individual peptides was checked by analytical HPLC and determined to be 99 %. Molecular weights of the peptides were determined using Plasma Desorption Mass Spectrometer (FDMS) Model Bioion 20, Applied Biosystems, the expected values were obtained in each case.

Galanin(1-12)-Pro-SP(5-11) amide (M15): (MW = 2199) IC_{50} = 0,1 nM

1 20

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met amide

1 5 10 12 5 7 11

Galanin(1-12)-Pro-Spantide amide (C 7): (MW = 2827) IC_{50} = 0,2 nM

1 13 22

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-(D-Arg)-Pro-Lys-Pro--Gln-Gln-(D-Trp)-Phe-(D-Trp)-

23 24 spantide

-Leu-LeuNH$_2$

Galanin(1-12)-Pro-Pro-Pro-SP(5-11) amide (M37): (MW = 2392) IC_{50} = 40 nM

1 5 12 22

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met amide

5 11

Galanin(1-13)-Ala-Leu-Ala-Leu-Ala-Leu-Ala amide (M 38): (MW = 1970)
IC_{50} = 0,2 nM

1 5 13 20

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Ala-Leu-Ala-Leu-Ala-Leu-Ala amide

Galanin(1-12)--Pro-Pro-Pro-Ala-Leu-Ala-Leu-Ala amide (M 40):(MW = 1980)
IC_{50} = 13 nM

1 5 13 20

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Ala-Leu-Ala-Leu-Alaamide

Galanin(1-13)-Bradykinin(3-9) amide (M20): (MW = 2135) IC_{50} = 1 nM

1 13 20

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg amide

3 9

Galanin(1-13)-Bradykinin(2-9) amide (M 35): (MW = 2232) IC_{50} = 0,2 nM

1 13 21

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg amide

2 9

Galanin(1-13)-NPY(25-36) amide (M 32): (MW = 2961) IC_{50} = 0,05 nM

1 13 25

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr amide

25 36

Galanin(1-12)-Ala-NPY(25-36) amide (M 88): (MW = 2935) IC_{50} = 1 nM

1 13 25

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Ala-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr amide

25 36

Galanin(1-12)-Pro-Pro-Pro-Leuenkephalin(1-5) amide (M 36): (MW = 2078)
IC_{50} = 40 nM

1 12 20

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Tyr-Gly-Gly-Phe-Leu amide

1 5

Galanin(1-12)-Pro-Pro-Pro-Leuenkephalin(5-1) amide (M 41): (MW = 2078)
IC_{50} = 20 nM

1 12 20
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Leu-Phe-Gly-Gly-Tyr amide
5 1

Galanin(1-13)-Lys amide (Pro-Leuenkephalin(5-1)) (M34,A) (MW = 2223)
IC_{50} = 10 nM

1 13 14
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Lys amide
N-teminus I
Pro-Leu-Phe-Gly-Gly-Tyr
5 1

IC_{50} has been determined in displacement experiments in rat hypothalamus

PHARMACOLOGICAL EXPERIMENTS

1) Galanin inhibits glucose-induced insulin release, measured according to Ahrén, B., et al (Biochemical and Biophysical Research Communications, Vol. 140. No. 3, 1986, 1059-1063). By using the same procedure as the cited Ahrén B., et al, a peptide according to the invention, M15, is now shown to block the galanin-inhibited insulin release in equimolar concentration.

Animals and preparation of cells.

Adult obese hyperglycemic mice (ob/ob) of both sexes were taken from a local non-inbred colony and starved overnight. The animals were killed by decapitation and the islets were isolated by collagenase isolation technique. A cell suspension was prepared essentially as described by Lehrnmark, Å., in Diabetologia 10, 1974, 431-438. Briefly, the islets were dissociated into single cells and small clusters by shaking in a Ca^{2+}-Mg^{2+}-deficient medium supplemented with EGTA (Ethylene glycol-bis(beta-aminoethyl ether)N,N,N',N',-tetra-acetic acid, SIGMA). Thereafter, the cells were incubated at 37°C, pH 7.4, overnight in 12 ml RPMI 1640 medium (Tissue culture medium from SIGMA, which contains L-glutamine and does not contain sodium bicarbonate) supplemented with 10% NU-serum TM (Collaborative Research Inc., Lexington, Ma, U.S.A.), 100 IU/ml penicillin, 60 µg/ml gentamycin and 100 µg/ml streptomycin. To avoid attachment of the cells to the culture flasks during incubation, the suspension was shaken gently.

Media

The basal medium used was a HEPES buffer (N-[2-Hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid], SIGMA), pH 7.4, physiologically balanced in cations with

Cl^- as the sole anion. In all cases the basal medium was supplemented with 1 mg/ml albumin.

Measurements of insulin release.

The kinetics of insulin release were studied by perfusing pancreatic beta-cells (approx. 1×10^6) with 20 mM or 5 mM glucose mixed with Bio-Gel P-4 polyacrylamide beads (200-400 mesh, Bio-Rad Lab., Richmond, Ca, U.S.A.) in a 0.5 ml column. The flow rate was 0.5 ml/min and 1-3 min fractions were collected and analyzed for insulin radioimmunologically, using crystalline rat insulin as a reference.

Results.

After 60 min incubation in the presence of glucose (8.3 mM), galanin (10^{-7} M) completely abolished insulin release from

34±2 µU/ml to 3±1 µU/ml ($P < 0.001$; N=32). The peptide of the invention,

M 15, alone (10^{-6} M - 10^{-9}M) dose-dependently counteracted the galanin-induced inhibition of insulin release.

Insulin release in the presence of glucose, galanin and M15 at 10^{-6} M, 10^{-7} or 10^{-8} was 28±2 µU/ml (N=16), 17±4 µU/ml (N=16), and 10±3 µU/ml (N=16), respectively. M15 at 10^{-9} M was without effect.

As a result of the above experiments it can be concluded that the peptide of the invention, M15, counteracts the galanin-induced inhibition of insulin release in islets.

2) Galanin inhibits acetylcholine release, measured according to Fisone G., et al (Proc. Natl. Acad. Sci. vol 84, 1987, 7339-7343). By using substantially the same procedure as the cited Fisone G., et al, a peptide according to the invention, M15, is now shown to counteract the inhibitory effects of galanin on the scopolamine-induced release of acetylcholine in vivo.

Microdialysis Experiments

Surgery and basic methodology. Experiments were carried out with female CD-COBS (Charles River, Como, Italy) rats, weighing 200-260 g. Animals were anesthetized with equitensin (1% pentobarbital, 4% chloral hydrate). Guide cannula was implanted stereotoxically into the ventral hippocampus according to the following coordinates from the atlas of Paxinos and Watson (Paxinos G. and Watson C. (1986) The Rat Brain in Stereotoxic Coordinates, 2nd edn. Academic Press, Sydney.): 5 mm posterior to the bregma, 4.0 mm lateral to the midline, and 6.8 mm below the surface of the dura mater. A stylet was then inserted to keep the guide cannula patent until the next day. At the time the stylet was removed and replaced with a microdialysis probe (CMA 10, Carnegie Medicine AB, Stockholm, Sweden) containing a membrane having an exposed area of 3 x 0.5 mm. The microdialysis probe extended 3 mm beyond the end of the guide cannula; it was perfused at a constant rate of 2 µl(min with Ringer's solution (NaCl, 147 mM; $CaCl_2$, 2.2 mM and KCl, 4.0 mM), containing 10 µM physostigmine sulfate and adjusted to pH 7.0 with NaOH. The initial 40 min perfusate was discarded. Thereafter, perfusates were collected every 20 min during a total perfusion period of 260 min. At the end of the collection period, the samples were immediately frozen on solid CO_2 and lyophilized. Acetylcholine (Ach) content was quantified by a specific radioenzymatic method described in detail by Consolo S.,

et al. (J. Neurochem. 48, (1987), 1459-1465.) and Wu C.F., et al (Neurobiol. Aging 9, (1988), 357-361.) and involving (a) the conversion of choline to phosphorylcholine in the presence of choline phosphokinase and ATP, (b) the enzymatic hydrolysis of acetylcholine to choline and acetic acid, (c) the reacetylation of the resulting choline to [^{3}H]acetylcholine with the addition of [^{3}H]acetylcoenzyme-A, 99.9 GBq/mmol, and acetyl-coenzyme A:ChAT, and (d) the separation and scintillation counting of the resulting [^{3}H]acetylcholine by extraction into tetraphenylboron-containing ketone phase by liquid-liquid ion exchange chromatography. Phosphorylation, hydrolysis and acetylation reactions were validated routinely.

The concentration of acetylcholine in each sample was calculated by linear regression based on the radioactivity of the standards (linear from 10 fmol-25 pmol acetylcholine) with the slope equal to 7800 net dpm/pmol. The coefficient of variation of the replicate perfusate samples or acetylcholine standards was approximately 3%.

In vitro recovery of acetylcholine through three dialysis tubings was determined as previously described (Wu et al., ibid). The average recovery was 17.6± 0.5%.

At the termination of the release experiments, the placements of the dialysis probes were verified histologically by use of staining for Nissl substance.

Effect of galanin and M 15 on the scopolamine-induced release of ACh, measured "in vivo" from the rat ventral hippocampus

Saline	2.0 pmoles
Scopolamine	13.1 pmoles
Scopolamine + Galanin	6.9 pmoles
Scopolamine + M15	9.4 pmoles

Galanin (1.56 nmoles) and M15 (3.12 nmoles) were injected i.c.v. 2 min before scopolamine (0.3 mg/kg, s.c.). ACh release was measured in the perfusate collected during the following 80 min, in four 20 min-fractions. The values are expressed as pmoles of ACh measured during the 20 min fraction corresponding to the peak of the scopolamine-effect and corresponding to the second 20 min-fraction. The data are the means of experiments carried out in two rats (n=2).

As a result of the above experiments it can be concluded that the peptide of the invention, M15, counteracts the galanin-induced inhibition of scopolamine-induced release of acetylcholine in hippocampus.

3) Effects of intrathecal galanin and C-fiber stimulation on the flexor reflex has been described by Wiesenfeld-Hallin Z., et al (Brain Res. 486 (1989)205-213). By using substantially the same procedure as the cited reference, a peptide according to the invention, M15, is shown to have an antagonistic effect on intrathecal galanin-induced facilitation of the flexor reflex. An other peptide of the invention, M35, has given similar results in preliminary experiments.

Electrophysiological study

In acute experiments, the magnitude of the polysynaptic hamstring flexor reflex in response to activation of high threshold afferents was examined in decerebrated, spinalized, unanaesthetized rats by recording the electromyogram (EMG) from the posterior bicepts femoris/semitendinosus muscles. The animals were briefly anaesthetized with Brietal®, and a tracheal cannula was inserted. The rats were mounted in a stereotaxic frame, decerebrated by aspiration of the forebrain and midbrain and then ventilated. The spinal cord was exposed by laminectomy at thoracic level and sectioned at Th_{8-9}. An i.t. catheter (PE 10) was implanted caudally to the transection with its tip on the left side of the lumbar spinal cord (L_{4-5}). The flexor reflex was elicited by test stimuli to the sural nerve or its innervation area with single electric shocks (0.5 ms, 10 mA) of sufficient strength to activate C-fibres (Wall and Woolf, 1984). In some experiments a CS (conditioning stimulus) (1 Hz, 20 s) of the same strength as the test stimuli was administered to the sural nerve.

The flexor reflex was recorded as EMG activity via stainless steel needle electrodes inserted in the ipsilateral hamstring muscles. The number of action potentials elicited during the reflex was integrated over 2 s. The integrated reflex was recorded on a Gould chart recorder (Model 2400 S). During the experiments the heart rate and rectal temperature of the rat were monitored and maintained within normal limits. The proper location of the i.t. catheter was confirmed after each experiment by laminectomy.

Galanin (Bachem, Bubendorff, Switzerland) and Somatostatin (Ferring, Malmö, Sweden) were dissolved in 0.9% saline. All peptides were injected i.t. in a volume

of 10 ul followed by 10 ul saline to flush the catheter.

Data collection

A stable baseline reflex magnitude was established for at least 20 min before each i.t. injection or nerve CS. The effect of i.t. peptides or the nerve CS on the flexor reflex was expressed as percent change in reflex magnitude compared to baseline which was defined as 100%. To test the interaction of galanin with other peptides or the sural CS, it was administered 1 5 min prior to the injection of the other peptides when the facilitatory effect of galanin had subsided or simultaneously with the sural CS.

The antagonistic effect of intrathecal (i.t.) M-15 on the i.t. galanin-induced facilitation of the flexor reflex was measured. The peak facilitatory effect of 30 pmol galanin was 167.0±42.8% over baseline reflex magnitude. The antagonism of M-15, injected 5-10 min after galanin was calculated as the percentage reduction of the peak facilitatory effect of galanin. Data is expressed as meant S.E.M.

Dose of M-15	n	% antagonism
30 pmol	4	35.8±20.7
300 pmol	4	79.3±8.4
3 nmol	3	93.0±3.4

The experiments were repeated using the peptide of the invention, M35, and similar results were achieved.

As a result of the above experiments it can be concluded that peptides of the invention counteract the intrathecal galanininduced facilitation of the flexor reflex in a dose-dependent manner.

4) Ligand binding studies were conducted. Displacement of ^{125}I-galanin by galanin, galanin fragment (1-13), Substance P fragment (4-11), galanin fragment (1-13) + Substance P fragment (4-11) in equimolar concentrations and the peptides of the invention M15, M35 and M34,A was studied. The peptides of the invention proved to bind specifically to the galanin receptor.

Preparation of ^{125}I-monoiodo-Tyr^{26}-porcine galanin

Synthetic porcine galanin(1-29) was iodinated by chloramine-T-method to yield ^{125}I-monoiodo-Tyr^{26}-porcine galanin (specific activity 1800-2000 Ci/mmol), (as described by Land T., et al in: Methods in Neurosciences, Ed. M. Conn, 5, 1991, 225-234), and employed in ligand binding studies carried out at equilibrium.

Preparation of membrane fraction of Rin m 5F cells

The establishment and cell culture of Rin m 5F (a rat pancreatic ß-tumor cell-line) cells have been described earlier (Gazdav A.F., et. al. Proc. Natl. Acad. Sci. USA, 77 (1980) 3519-3523). Briefly, the cells were grown in RPMI-1640 (Gibco) medium containing 10 % (v/v) fetal calf serum, 2.06 mM L-gluthamine, 100 IU/ml penicillin and 100 µg/ml streptomycin in 10 % CO_2 - 90 % air 37°C. They were passaged every 5 days. The cells were detached from the surface of the bottles using 0.25% trypsin containing 1 mM EDTA and centrifuged at 2000 x g for 5 min at 4°C. The pellet was exposed for 15 min to hypoosmotic 5mM HEPES buffer (pH 7.4). The suspension was centrifuged at 20000xg for 15 min, the resulting pellet was resuspended in bacitracin-containing (1 mg/mg) 5 mM HEPES-buffered Krebs-Ringer solution (137 mM NaCl, 2.68 nM KCl, 1.8 mM $CaCl_2$, 1 g/l glucose), pH 7.4, and used immediately in equilibrium binding experiments.

Displacement of ^{125}I-galanin by galanin and galanin receptor ligands

Displacement experiments were carried out in a final volume of 400 µl of bacitracin-containing (1 mg/ml) HEPES-buffered (5 mM) Krebs-Ringer solution, 0.05 % (w/v) of BSA (pH 7.4) in the presence of 0.1 - 0.2 nM ^{125}I-galanin, the membrane preparation and the increasing concentrations (10^{-12}-10^{-6}M) of unlabeled porcine galanin or of other galanin receptor ligands. Samples were incubated for 30 min at 37°C. Incubation was terminated by the addition of 10 ml of ice-cold HEPES-buffered Krebs-Ringer solution, followed by rapid filtration over Whatman GF/C filters, precoated for 5 - 6 hours in 0.3 % (v/v) of polyethyleneimine solution. Filters were washed with 10 ml of assay solution. Radioactivity retained on the filters was determined in a Packard gamma counter. Specific binding was defined as that displaceable by 1 µM galanin (or appropriate concentration of a displacer).

The IC_{50} values of the displacing ligands were calculated from the computer-generated IC_{50} values as described by Land, T., et. al. (ibid).

Fitting of the experimental data was carried out on a Macintosh SE by means of a nonlinear least squares method using the Macintosh program "KaleidGraph".

Displacement of ^{125}I-galanin from membranes of Rin m 5F by galanin receptor ligands

	IC_{50}
Galanin (1-29)	1 nM
M15	0.1 nM
Galanin fragment (1-13)	100 nM
Substance P fragment (4-11)	no displacement > 10 µm

Galanin fragment (1-13) + Substance P fragment (4-11) in equimolar concentrations	100 nM
M35	0.1 nM
M34,A	10 nM

As is evident from the above experiments, the peptides of the invention are galanin receptor ligands.

The pharmacological experiments 1), 2) and 3) thus show that the peptides of the invention are galanin antagonists, and the experiment 4) shows that they are galanin receptor ligands. Thus, the galanin antagonists of the invention are galanin receptor ligands, unlike other compounds which earlier have been shown to antagonize a specific effect of galanin in a specific tissue by virtue of interaction with a reaction step beyond the galanin receptor - which was involved in the biological action of galanin in that given cell type. Such antagonism is not specific for galanin action, and is not exerted at galanin receptor. Neither it is applicable to several tissues where galanin acts, whereas the antagonists according to the present invention are bona fide antagonists in the pharmacological meaning and exert their action at the receptor site on the outside of the cell by competing with the endogenous ligand.

Preparation of membranes from rat hypothalamus

Adult male rats (Sprague-Dawley, 180-200 g) were decapitated, the hypothalami quickly dissected and homogenized (10% mass/vol.) in 0.05 M TRIS-Cl buffer, pH 7.4. The homogenate was diluted tenfold and centrifuged at 1000 x g for 10 min. The supernatant was centrifuged at 10 000 x g for 45 min and the pellet resuspended in 5 mM Hepes buffered Krebs-Ringer solution (137 mM NaCl, 2.68 mM KCl, 1.8 mM CaCl2 1 g/l glucose), pH 7.4 to yield

a final protein concentration of 1.0 - 1.5 mg/ml.

Ligand binding studies in rat hypothalamus

Displacement experiments were carried out in a final volume of 400 µl of Hepes-buffered (5 mM) Krebs-Ringer solution, 0.05 % (w/v) of BSA (pH 7.4) supplemented with bacitracin (1 mg/ml) in the presence of 0.1 - 0.2 nM 1251-galanin, the membrane preparation from rat hypothalami and increasing concentrations (10-12-10-6 M) of unlabeled galanin or of other galanin receptor ligands. Samples were incubated for 30 min at 37°C. Incubation was terminated by the addition of 10 ml of ice-cold Hepes-buffered Krebs-Ringer solution, followed by rapid filtration over Whatman GF/C filters, precoated for 5-6 hours in 0.3% (v/v) of polyethylencimine solution. The filters were washed with 10 ml of assay solution. Radioactivity retained on the filters was determined in a Packard gamma counter. Specific binding was defined as that displaceable by 1 µM galanin. Rat and porcine galanin resulted in indistriguishable displacement curves with the membranes from rat hypothalami.

The 1C50 values of the displacing ligands were calculated by fitting of the experimental data on a Macintosh SE by means of a nonlincar least squares method using the program "KaleidaGraph".

The claims defining the invention are as follows:

1. A peptide selected from the group consisting of

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-
-Gly-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-
-Gly-Pro-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-
-Gly-Pro-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-
-Gly-Pro-Lys-X
|
Pro-Leu-Phe-Gly-Gly-Tyr-H

wherein X represents -NH_2 or -OH (amide or free acid), and functional analogues thereof (i.e. shorter or longer peptides, with or without substitution of one or several amino acid residues for other amino acid residues) which exhibit substantially the same galanin antagonistic effect as said peptide group, and functional derivatives thereof (i.e. peptides wherein one or several amino acid residues are substituted for other chemical groups) which exhibit substantially the same galanin antagonistic effect as said peptide group.

2. A process for the preparation of a peptide according to claim 1, characterized by
a) sequentially incorporating in solid phase the desired amino acid residues one at a time into the growing peptide chain, or

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b) coupling the amino acids stepwise in a solution phase using appropriate derivatized amino acids.

3. A peptide according to claim 1, when obtained by the process according to claim 2.

4. Use of a peptide according to claim 1 or 3 in the preparation of a pharmaceutical composition.

5. A peptide according to claim 1 or 3 in the form of a pharmaceutical preparation.

6. A pharmaceutical composition comprising a peptide according to claim 1 or 3 as an active ingredient, together with a pharmaceutically acceptable additive(s).

7. A method of treating or curing disorders in a mammal which depends on the physiological function of galanin at the galanin receptor, which comprises administering to the mammal a peptide according to claim 1 or 3, or administering to the mammal a pharmaceutical composition according to claim 5 or 6.

DATED this 14th day of August, 1995

AKTIEBOLAGET ASTRA,
By its Patent Attorneys,
E. F. WELLINGTON & CO.,
By:

(B. S. Wellington)

A/JD/3633

AUSTRALIAN PATENT OFFICE

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 92/00316

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 7/08, 7/10, 7/02, 7/18, A 61 K 37/24, 37/42// C 07 K 7/12

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	Proc. Natl. Acad. Sci. USA, Vol. 88, pp. 10961--10965,December 1991, Tamas Bartfai et al: "M-15: High-affinity chimeric peptide that blocks the neuronal actions of galanin in the hippocampus, locus coeruleus, and spinal cord". --	1-7
P,X	Proc. Natl. Acad. Sci. USA, Vol. 89, April 1992 Zsuzsanna Wiesenfeld-Hallin et al: "Galanin-mediated control of pain: Enhanced role after nerve injury", see page 3334 - page 3337 --	1-7
P,X	Chemical Abstracts, volume 116, no. 11, 16 March 1992, (Columbus, Ohio, US), Stefan Lindskog et al: "The novel high-affinity antagonist, galantide, blocks the galanin-mediated inhibition of glucose-induced insulin secretion ", see page 108, abstract 99736t, & Eur. J. Pharmacol. 1992, 210(2), 183- 188 --	1-7

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th August 1992	1992 -08- 21
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Elisabeth Carlborg

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Proc. Natl. Acad. Sci. USA, Vol. 86, December 1989 Gilberto Fisone et al: "N-terminal galanin-(1-16) fragment is an agonist at the hippocampal galanin receptor", see page 9588 - page 9591 --	1-7
A	Chemical Abstracts, volume 112, no. 1, 1 January 1990, (Columbus, Ohio, US), Kjeld Hermansen et al: "On the nature of the galanin action on the endocrine pancreas: studies with six galanin fragments in the perfused dog pancreas ", see page 77, abstract 807z, & Acta Endocrinol. 1989, 121(4), 545- 550 --	1-7
A	Chemical Abstracts, volume 111, no. 23, 4 December 1989, (Columbus, Ohio, US), Lagny-Pourmir I. et al: "Structural requirements for galanin interaction with receptors from pancreatic beta cells and from brain tissue of the rat ", see page 72, abstract 209268y, & Peptides (Fayetteville, N.Y.) 1989, 10(4), 757- 761 --	1-7

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 8, because ~~they~~ it relate/s to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 92/00316

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 01/07/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date