THE INTERACTIONS OF OPIOIDS WITH ENTEROCYTE MEMBRANES: EVIDENCE FOR ENZYME-CATALYSED COVALENT BINDING

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The binding of the synthetic opioid hexapeptide Tyr-D-Ala-Gly-Phe-Leu-Arg (dalargin) to rat enterocyte membranes has been studied and compared to its interaction with central opioid receptors. The binding was apparently low affinity, time- and temperature-dependent, sensitive to NaF and N-ethylmaleimide, and esentially irreversible even after incubation with trichloracetic acid. It was inhibited by ascorbate and substances containing hydroxybenzol moieties or halogen-substituted aromatic rings. The binding of [^{14}C]phenol and [^{3}H]tyrosine to rat enterocyte membranes was very similar to the binding of dalargin. The interaction of dalargin with rat enterocyte membranes differed in many ways from its binding to opioid receptors, but it was similar to the covalent binding of hydroxybenzol derivatives to proteins catalyzed by cytochrome P-450 enzyme complex.

The antiulcer activity of exogeneously administered opioids is now well documented[1,2], though the exact mechanism of this effect remains rather obscure. One important point is the possibility of direct action of opiates and opioid peptides on gastric and duodenal mucosa. It is known that these drugs can influence intestinal ion and fluid transport[3-5] and mucus production[1]. Immunohistochemical approach revealed some enkephalin-containing endocrine cells[6,7] and nerve fibers[8-11] within the mucosa. Using the autoradiographic method, Nishimura et al.[12,13] demonstrated the presence of specific binding sites for opiates and opioid peptides in the mucosal layer throughout the gastrointestinal tract. Studies with isolated cells and enterocyte membranes gave somewhat conflicting results. Thus, Gaginella et al.[14] failed to find any specific binding of [^{3}H]naloxone or [^{3}H][D-Ala^{2}, Met^{5}]enkephalinamide to rat ileal mucosa membranes. On the other hand, Lopez-Ruiz et al.[15] were able to demonstrate specific though rather low affinity binding of [^{3}H]Leu^{5}-enkephalin to isolated guinea-pig enterocytes.

In an attempt to better characterize the putative opioid receptors expressed by enterocytes we investigated the binding of an enkephalin analogue, [D-Ala^{2},Leu^{5}, Arg^{6}]enkephalin (dalargin), to rat and human enterocyte membranes and compared its parameters to those of dalargin interaction with central opioid receptors. This

work was prompted by experimental and clinical evidence demonstrating high antiulcer activity of dalargin upon peripheral administration in low doses[16].

Data concerning the binding of dalargin to central opioid receptors are summarized in Fig. 1 and Table I. The binding of 0·2–10·0 nmol l^{-1} of [^{3}H]dalargin to rat brain membranes was inhibited by 85–90% in the presence of 1 μmol l^{-1} of the unlabelled hexapeptide or the active opiate antagonist levallorphan, but ot after the addition of dextrorphan, the inactive stereoisomer of the opiate levorphanol. This means that 85–90% of dalargin total binding to rat brain membranes represent

TABLE I

Competition between dalargin and selective ligands of opioid mu-, delta- and kappa-receptors for specific binding sites on membranes isolated from rat brain

Receptor type	[^{3}H]Ligand	K_i, nmol l^{-1}
Delta	3·05 nmol l^{-1} of [^{3}H]DADLE in the presence of 60 nmol l^{-1} of DAGO	0·584 ± 0·181
Mu	0·406 nmol l^{-1} of [^{3}H]DAGO	6·69 ± 3·04
Kappa	0·812 nmol l^{-1} of [^{3}H]ethylketo-cyclazocine in the presence of DAGO and DADLE (300 nmol l^{-1} each)	>1·000

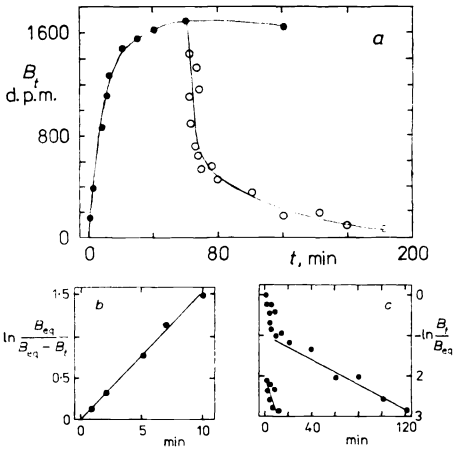

FIG. 1

Kinetics of [^{3}H]dalargin (3·01 nmol l^{-1}) association with rat brain opioid receptors (*a*: ●; *b*) and kinetics of dissociation of [^{3}H]dalargin–opioid receptors complexes (*a*: ○; *c*). Dissociation was induced by the introduction of the 2 000-fold excess of unlabelled dalargin in the incubation medium. The association and dissociation rate constants were evaluated using the linearization procedure described by Simon et al.[22]. B_{eq} [^{3}H]dalargin binding at equilibrium; B_t [^{3}H]dalargin binding at the moment *t*

its interaction with opioid receptors (specific binding). Fig. 1 shows the kinetics of specific association of [^{3}H]dalargin with rat brain membranes and the kinetics of the dissociation of the ligand–receptor complex. The specific binding of dalargin reached the maximum after 80 min of incubation. The estimate of the association rate constant k_1 in the experiment presented in Fig. 1 was $2{\cdot}54 \cdot 10^7$ mol^{-1} l min^{-1}. Dissociation of the ligand–receptor complex was biphasic suggesting either the binding to two types of specific binding sites or negative cooperative interaction of the binding sites. The first suggestion seems to be the correct one since dissociation induced by the dilution of the incubation medium was undistinguishable from that induced by the addition of the excess of unlabelled dalargin (data not shown). In case of the negative cooperativity the initial rate of dissociation should be higher in the presence of the unlabelled ligand[17]. The estimates of the two dissociation rate constants were as follows: $k_{-1} = 0{\cdot}122$ min^{-1} (slow phase) and $k_{-2} = 0{\cdot}0154$ min^{-1} (fast phase). The estimates for the two corresponding dissociation constants of ligand–receptor complex were $K_{d1} = k_{-1}/k_1 = 0{\cdot}606$ nmol l^{-1} and $K_{d2} = k_{-2}/k_1 = 4{\cdot}80$ nmol l^{-1}. Corresponding means for three experiments were $0{\cdot}648 \pm 0{\cdot}399$ nmol l^{-1} and $5{\cdot}34 \pm 2{\cdot}01$ nmol l^{-1}. As it follows from Table I, these dissociation constants reflect the interaction of the hexapeptide with delta and mu opioid receptors, respectively. Indeed, dalargin inhibited the binding of the relatively selective delta-receptor ligand [^{3}H]DADLE* and the binding of the selective mu-receptor ligand [^{3}H]DAGO, but did not influence the interaction of specific ligands with kappa-receptors. The K_i values for inhibition of [^{3}H]DADLE and [^{3}H]DAGO binding by dalargin were $0{\cdot}584 \pm 0{\cdot}181$ nmol l^{-1} and $6{\cdot}69 \pm 3{\cdot}04$ nmol l^{-1}, respectively. These values correspond to K_d's calculated from kinetic data. As far as enkephalin analogues compete for identical binding centers of opioid receptors, the K_i values give reasonable estimates of dissociation constants of the unlabelled dalargin–delta-receptor and dalargin–mu-receptor complexes. Thus, both kinetic studies with tritiated dalargin and competition experiments at equilibrium suggest the binding of the hexapeptide to opioid delta- and mu-receptors. The affinity of dalargin binding to the former is about 10 times higher than to the latter.

The interaction of dalargin with rat enterocyte membranes differed from its binding to central opioid receptors. First, its affinity was apparently much lower. Unlabelled dalargin added to the incubation mixture in the concentration of 0·1 mmol l^{-1} inhibited the binding of 15 nmol l^{-1} of [^{3}H]dalargin by 80–85% indicating the saturability of the process. The half-maximal inhibition of the binding occurred only in the presence of micromolar concentrations of the unlabelled hexapeptide which are at least 2 orders of magnitude higher than in the experiments with brain membranes.

* Abbreviations: DADLE [D-Ala^2, D-Leu^5]enkephalin; DAGO [D-Ala^2, (Me)Phe^4, Gly-ol^5]-enkephalin; ATEE N-acetyltyrosine ethyl ester.

Second, the kinetics of [^{3}H]dalargin binding to enterocyte and brain membranes differed. The rate of the association of the hexapeptide with enterocyte membranes and the maximal binding depended upon the temperature (Fig. 2). Decrease of the temperature of incubation resulted in the reduction of both the initial rate of association and the amount of the membrane-bound tracer. The maximal binding at 25°C was 850 ± 180 pmol/mg protein. Apparent steady-state was reached after 40 min of incubation. The most important difference of [^{3}H]dalargin binding to enterocyte membranes from the peptide's binding to central opioid receptors was its essential irreversibility. This was proved by the lack of the tracer dissociation following addition of 10 000-fold excess of the unlabelled peptide (shown in Fig. 2), 100-fold dilution of the incubation mixture with either 50 mM Tris-HCl buffer pH 7·4 or distilled water and even after 30 min incubation of the samples in the presence of 10% of trichloracethic acid. In the latter case the label was almost quantitavely precipitated with the proteins.

Third, the concentration of the binding sites for dalargin in enterocyte membranes was much higher than that of the opioid recpetors in brain membranes: about 1 nmol per 1 mg protein and 100–400 fmol per 1 mg protein, respectively.

Fourth, the specificity of the saturable dalrgin binding to enterocyte and brain membranes was quite different. The binding of [^{3}H]dalargin to enterocyte mem-

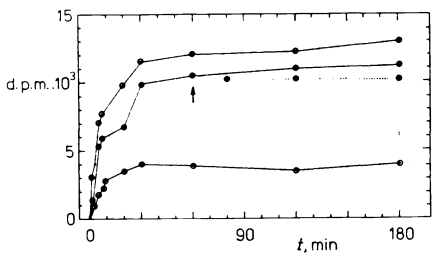

FIG. 2

Kinetics of [^{3}H]dalargin association at 0°C (○), 25°C (●) and 37°C (◑) and kinetics of its dissociation at 25°C (⊗). Dissociation was induced by the addition of the 10 000-fold excess of the unlabelled dalargin after 60 min of incubation at 25°C (shown with the arrow). d.p.m. .10^3 [^{3}H]dalargin binding per tube

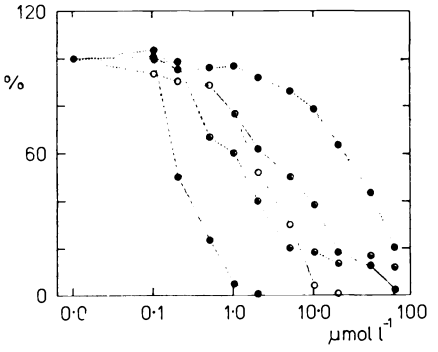

FIG. 3

Inhibition of the saturable [^{3}H]dalargin binding by dalargin (○), dalargin (1–5) (●), naloxone (◑), serotonin (⊗) and adrenaline (○). Inhibition concentration, μmol l^{-1}; residual binding of dalargin, %

branes could be inhibited not only by opiates and opioid peptides, but also by quite a lot of bioactive compounds with different pharmacological characteristics (Fig. 3 and Table II). The inhibition was dose-dependent (Fig. 3). We did not find any change of the binding in the presence of peptides lacking the tyrosine residue (including dalargin fragments), several amino acids (Phe, Trp, Leu, Lys, Arg, Gly), propranolol, cyproheptadine, methysergide, haloperidol, atropine, imipramine, sucrose, sorbitol, mannitol and a number of other substances. The comparison of the structures of the compounds inhibiting [^{3}H]dalargin binding revealed one common feature shared by the majority of them, namely, the presence of the hydroxybenzol moiety which

TABLE II

Inhibition of [^{3}H]dalargin binding to enterocyte membranes by different substances. [^{3}H]dalargin concentration was 17·3 nmol l^{-1}. The tested substance were added in the following concentrations: 0·1; 0·2; 0·5; 1; 2; 5; 10; 20; 50; 100; 200 (μmol l^{-1}). The table shows concentration inervals containing IC_{50} values

Substance	IC_{50}, μmol l^{-1}	Substance	IC_{50}, μmol l^{-1}
Dalargin	4·0 ± 1·5	Glycine	No activity
Dalargin(1—5)	2—5	Leucine	No activity
Dalargin(1—4)	2—5	Arginine	No activity
Dalargin(1—3)	5—10	Tryptophan	No activity
Dalargin(2—5)	No activity	Serotonin	0·2—0·5
[Val^2]Dalargin	1—2	Propranolol	No activity
[Leu^5]Enkephalin	1—2	Apomorphine	2—5
Tyr-Pro-Arg	10—20	Haloperidol	No activity
Thr-Pro-Arg	No activity	Ketanserine	No activity
Phe-Pro-Arg	No activity	Methysergide	No activity
Naloxone	1—2	Imipramine	No activity
Dextrorphan	50—100	Atropine	No activity
Levallorphan	5—10	*d*-Tubocurarine	2—5
Morphine	20—50	Histamine	No activity
SKF 10,047	20—50	Muscomol	No activity
Promedol	No activity	Reserpine	No activity
L-Tyrosine	1—2	Cinnarizine	50—100
D-Tyrosine	2—5	Flunarizine	50—100
ATEE	1—2	Verapamil	No activity
Adrenaline	2—5	Ascorbic acid	0·5—1
Noradrenaline	1—2	Sorbitol	No activity
Dopamine	2—5	Mannitol	No activity
L-DOPA	1—2	Sucrose	No activity
Isoproterenol	0·2—0·5	Pyridoxal phosphate	No activity
Phenylalanine	No activity	Glycogen	No activity

seems to be crucial for interfering with the tracer binding. All opiates contain this group. It is present in tyrosine, which is essential for the biological activity of opioid peptides, and in tyrosine derivatives including biogenic neurotransmitter amines. It is also present in a number of other substances listed in Table II, including indolamines and a number of pharmacologically active alkaloids. The importance of the hydroxybenzol moiety can be illustrated by the following examples. Tyrosine (used in the form of ATEE because of low solubility of the amino acid itself) was a good inhibitor of dalargin binding, while phenylalanine had no inhibiting potency. Serotonin possessing a hydroxybenzol moiety condensed with a heterocyclic structure was active unlike tryptophan lacking the OH-group at the aromatic ring. Besides hydroxybenzols, substances containing halogen-substituted aromatic rings (e.g. cinnarizine and flunarizine) also exhibited a certain inhibitory activity. Finally, ascorbate which does not contain aromatic structures was one of the most potent inhibitors.

Fifth, dalargin binding to enterocyte membranes unlike the binding of opioids to opioid receptors[18] was not sensitive to sodium, but was inhibited in the presence of potassium and bivalent cations (Table III).

TABLE III

Influence of ions, sodium fluoride and N-ethylmaleimide on the saturable binding of [^{3}H]dalargin to rat enterocyte membranes; [^{3}H]dalargin was added in the concentration of 17·5 nmol l^{-1}

Substance	Concentration nmol l^{-1}	Inhibition, %
NaCl	20	8 ± 4
	100	12 ± 3
	200	17 ± 5
KCl	20	10 ± 5
	100	62 ± 12
	200	70 ± 11
$MgCl_2$	1	40 ± 8
	10	56 ± 10
	50	79 ± 9
$CaCl_2$	1	40 ± 7
	10	57 ± 10
	50	83 ± 14
NaF	10	53 ± 7
N-Ethylmaleimide	0·5	46 ± 18

The binding of dalargin to enterocyte membranes was also attenuated by the addition of sodium azide and the SH-alkylating agent N-ethylmaleimide (Table III) suggesting its dependence upon energy supply and upon the activity of SH-protein(s) or SH-peptide(s). It is noteworthy, that sodium azide and low temperature (see Fig. 2) caused equivalent attenuation of the binding. One may speculate that the process is only partly energy-dependent.

We used reversed-phase chromatography at Sep-Pak C_{18} cartridges to check the degradation of dalargin during incubation. The incubations of 15 nmol l^{-1} of [^{3}H]dalargin with enterocyte membranes were carried out in an ordinary way. The samples were filtered and the filtrates gathered. [^{3}H]Dalargin itself and labelled dalargin(1–5) and dalargin(1–4) constituted 73 ± 8% of total non-membrane-bound radioactivity (radioactivity in the filtrates). The remaining radioactivity was associated with tyrosine (about 15%) and N-terminal tri- and dipeptides of dalargin. It is not known in what form the label is bound to the membranes. It may be the whole hexapeptide or its tritiumcontaining fragment. Another possibility is the isotope exchange of tritium in the molecule of dalargin for hydrogen of membrane proteins. To exclude this possibility we studied the binding of [^{14}C]phenol to rat enterocyte membranes. It was demonstrated that the binding of this simplest hydroxybenzol had characteristics essentially identical to those of dalargin binding being saturable, temperature-dependent and irreversible even in the presence of trichloracetic acid. The binding was inhibited by dalargin and ATEE (Table IV) but was not affected by phenylalanine suggesting that it represents the same process as the interaction of dalargin with enterocyte membranes. The ^{14}C of the aromatic ring cannot be exchanged for unlabelled carbogen of a membrane protein. Therefore it can be concluded that at least the aromatic ring of Tyr^1 of dalargin is bound to the membrane. We also studied the binding of [^{3}H]tyrosine to rat enterocyte membranes.

TABLE IV

Inhibition of [^{14}C]phenol and [^{3}H]tyrosine binding to rat enterocyte membranes by dalargin, acetyl tyrosine ethyl ester (ATEE) and phenylalanine

Labelled ligand	Unlabelled ligand	IC_{50}, μmol l^{-1}
[^{14}C]Phenol, 50 nmol l^{-1}	ATEE	8·6 ± 4·0
	Dalargin	12·8 ± 5·2
	Phenylalanine	≫ 200
[^{3}H]Tyrosine 15 nmol l^{-1}	ATEE	2·1 ± 0·5
	Dalargin	6·2 ± 1·4
	Phenylalanine	≫ 200

The binding of the amino acid was similar to that of dalargin and phenol and could be inhibited by dalargin and ATEE, but not by phenylalanine (Table IV).

It is clear that the above-described saturable and irreversible binding of dalargin to rat enterocyte membranes does not represent the interaction of the hexapeptide with opioid receptors. Moreover, it has little in common with the binding of the mediators of intercellullar communication to specific receptors on the target cells Really, ligand–receptor interactions are usually reversible and more selective. Besides that, the concentrations of most receptors are very low (less than 1 pmol per mg of membrane protein) while enterocyte membranes bind about 1 nmol of the ligand per mg protein. The physico-chemical nature of ligand–receptor interactions is poorly understood. Still there is no doubt that as a rule they are non-covalent.

Our data argue against the presence of opioid receptors in rat enterocyte membranes. In an attempt to reconcile our results with those of Nishimura et al.[12,13] who demonstrated the binding of opioids to rat intestinal mucosa on tissue sections we studied the binding of [^{3}H]DADLE to sections of rat brain and duodenum using the same extraordinary high concentrations of the labelled and unlabelled opioid ligands as those indicated in the cited references[12,13]. We also checked the influence of non-opioid hydroxybenzols on the binding of the tracer. Our data are shown in Table V. In agreement with Nishimura et al.[12,13] we found saturable [^{3}H]DADLE binding to brain and duodenum slices. In contrast to brain slices the binding to the slices of duodenum was inhibited only by very high concentrations of unlabelled opioids and also by norepinephrine suggesting that in fact Nishimura

TABLE V
Binding of [^{3}H]DADLE to slices of rat brain and duodenum

Tissue	[^{3}H]DADLE concentration nmol l^{-1}	Unlabelled ligand, μmol l^{-1}	Binding, c.p.m.
Brain	3	—	737 ± 122
	3	DADLE, 5	98 ± 14
	20	—	1 684 ± 212
	20	DADLE, 100	186 ± 16
	20	Norepinephrine, 100	1 640 ± 196
Duodenum	10	—	159 ± 36
	10	DADLE, 5	168 ± 40
	20	—	247 ± 29
	20	DADLE, 100	114 ± 16
	20	Norepinephrine, 100	129 ± 18

and co-workers[12,13] have not demonstrated the presence of opioid receptors in rat small intestine mucosa but rather dealt with the binding of hydroxybenzols described above. This does not mean that opioid receptors cannot be expressed by some minor cellular population within the mucosa. The presence of enkephalin-containing cells[6,7] and nerve fibers[8-11] in the mucosal layer makes this possibility quite likely. But there is no evidence of the expression of opioid receptors by the cells of the predominant type in this tissue.

Our data fit the following hypothesis. Enterocyte membranes contain enzymes catalysing the covalent binding of hydroxybenzols to membrane proteins. There is evidence that these enzymes catalyse oxidation/reduction reactions (inhibition by ascorbate) and contain SH-groups essential for their activity (inhibition by N-ethylmaleimide). The covalent binding of hydroxybenzols to membrane proteins may serve for inactivation of physiologically active substances from blood or intestinal lumen or for protein modification. Both possibilities may have intriguing implications in further research.

One important task of the future research is the identification of the enzymes catalyzing the hydroxybenzol binding to enterocyte membranes. In this context it is noteworthy that cytochrome P-450-containing microsomes isolated from liver[19] or bone marrow[20] display the activity of ascorbate- and SH-reagentsensitive enzymes catalyzing the covalent binding of the hydroxybenzol derivatives and substances with halogenized aromatic rings to microsomal proteins. In the cited publications only relatively simple hydroxybenzols were studied. Anyhow, cytochrome P-450 enzyme complex is the first candidate for the role of the catalyst of hydroxybenzol covalent binding to rat enterocyte membranes. If this is the case, a previously unknown function of this enzyme complex – inactivation not only of xenobiotics but also of endogenous physiologically active substances such as peptides and catechol- and indolamines will be established.

EXPERIMENTAL

Materials: Synthetic enkephalin analogues and other peptides were provided by prof. M. Titov and Dr Zh. Bespalova (Cardiology Research Center of the U.S.S.R. Academy of Medical Sciences, Moscow). The identity and purity of these substances were checked by NMR, HPLC and amino acid analysis. [^{3}H]Dalargin (specific radioactivity 1·8 TBq/mmol) was prepared by Dr D. Zatsev (Institute of Molecular Genetics, Moscow). [^{3}H]Tyrosine (specific radioactivity 3·0 TBq/mmol) and [^{14}C]phenol (specific radioactivity 4·4 GBq/mmol) were purchased from Amersham International, Aylesbury, U.K. The radiochemical purity of the labelled substances was monitored by HPLC and exceeded 95% in all experiments. Dextrorphan, levallorphan and M-allylnormetazocine (SKF 10047) were kindly gifted by Dr H. Roemer (Sandoz AG, Basel, Switzerland). Morphine and methysergide were from the Institute of Pharmacology, Moscow). All other drugs were purchased from ICN Pharmaceuticals, Costa Mesa, California. Conventional chemicals were from Sigma–Aldrich Corp., St. Louis, Missouri.

Preparation of enterocyte membranes: Rat enterocyte membranes were prepared as in ref.[21] In brief: male Wistar rats weighing 180—220 g after overnight fasting were decapitated and the duodenum was excized, washed with saline, cut longitudinally and placed on a cold plate with the mucosal surface up. The mucosa was scraped off carefully with a razor blade and the scrapings were homogenized using the Polytron homogenizer (setting 7, 20 s, Kinematica GmbH, Luzern, Switzerland) in icecold 50 mM Tris-HCl buffer pH 7·4 (10 ml of the buffer per 1 g of the tissue). The membranes were sedimented by centrifugation at 4°C for 20 min at 15 000 *g*, washed twice with ice-cold 50 mM Tris-HCl buffer pH 7·4, resuspended in the same buffer, frozen in liquid nitrogen and kept at −70°C until use.

Preparation of rat and guinea pig brain membranes: Adult male guinea pigs and Wistar rats were decapitated. Whole brains (without carebellum) were homogenized using the Polytron homogenizer (setting 7, 20 s) in 10 volumes of ice-cold 50 mM Tris-HCl buffer pH 7·4 and centrifuged at 4°C for 20 min at 42 000 *g*. The pellet was resuspended in 20 volumes of the same buffer and incubated for 40 min at 37°C to remove endogenous opioid peptides. After washing the membranes were resuspended in the buffer, frozen in liquid nitrogen and kept at −70°C.

Preparation of tissue sections: Frozen tissue sections (20 μm thick) were prepared as described by Nishimura et al.[13].

Binding assay: The standard binding assays were conducted at 25°C in 0·5 ml of the incubation medium containing 50 mM Tris-HCl buffer pH 7·4, 0·5% (w/w) bovine serum albumin, 0·2 mg/ml bacitracin, an appropriate concentration of [^{3}H]dalargin, [^{14}C]phenol or [^{3}H]tyrosine and, in competition experiments, indicated concentrations of different substances. In some specially indicated cases the temperature of incubation and the volume of the sample were different. The reaction was initiated by the addition of the membranes (50 μg of enterocyte membrane protein or 200 μg of brain membrane protein per sample). After incubation the membrane-bound radioactivity was separated by vacuum filtration of the samples through GF/C glass fiber filters (Whatman, U.K.) and quantified by liquid scintillation counting. The results of the kinetic studies were analysed as in the work of Simon et al.[22].

In the competition experiments at equilibrium the concentrations of the unlabelled substances causing the half-maximal inhibition of the tracer specific binding (IC_{50}) were determined as described by Rodbard[23]. The IC_{50} values were used for calculation of inhibition constants (K_i's) using the corrected Cheng-Prusoff equation[24].

Dalargin degradation during its incubation with enterocyte membranes was evaluted using reversed phase chromatography technique described elsewhere[25].

Protein concentration were determited by the method of Lowry et al.[26].

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REFERENCES

1. Flemstrom G., Kivilaakso E., Briden S., Nylander O., Jedstedt G.: Dig. Dis. Sci. *30*, 63s (1985).
2. Olson G. A., Olson R. D., Kastin A. J.: Peptides *7*, 907 (1986).
3. Beubler E., Lembech F.: Naunyn-Schmiedeberg's Arch. Pharmacol. *306*, 113 (1979).
4. Kachur J. F., Miller R. J., Field M.: Proc. Natl. Acad. Sci. U.S.A. *77*, 2753 (1980).
5. Kachur J. F., Miller R. J.: Eur. J. Pharmacol. *81*, 177 (1982).
6. Larsson L. I., Stengaard-Pedersen K.: J. Histochem. Cytochem. *29*, 1088 (1981).
7. Jonsson A.-C.: Cell Tissue Res. *240*, 361 (1985).

8. Jessen K. R., Saffrey M. G., Van Noorden S., Bloom S. R., Polak J. M., Burnstock J.: Neuroscience *5*, 1717 (1980).
9. Schultzberg M., Hokfelt T., Nilsson J., Terenius L., Rehfeld J. F., Brown M., Elde R., Goldstein M., Said S.: Neuroscience *5*, 689 (1980).
10. Feurle G. E., Helmstaedter V., Weber U.: Life Sci. *31*, 2961 (1982).
11. Furness J. B., Costa M., Miller R. J.: Neuroscience *8*, 653 (1983).
12. Nishimura E., Buchan A. M. J., McIntosh C. H. S.: Neurosci. Lett. *50*, 73 (1984).
13. Nishimura E., Buchan A. M. J., McIntosh C. H. S.: Gastroenterology *91*, 1084 (1986).
14. Gaginella T. S., Rimele T. J., Wietecha M.: J. Physiol. *335*, 101 (1983).
15. Lopez-Ruiz M. P., Arilla E., Gomez-Pan A., Prieto J. C.: Biochem. Biophys. Res. Commun. *126*, 404 (1985).
16. Smagin V. G., Vinogradov V. A., Bulgakov S. A.: Ter. Arkhiv *10*, 49 (1984).
17. Varfolomeev S. D., Zaitsev S. V.: *Kineticheskie metody v biochimicheskich issledovaniyakh.* Moscow University Publishers, Moscow 1982.
18. Pert C. B., Pasternak G. W., Snyder S. H.: Science *182*, 4119 (1973).
19. Van Ommen B., Adang A. E. P., Brader L., Posthumus M. A., Miller F., Van Bladeren P. H.: Biochem. Pharmacol. *35*, 3233 (1986).
20. Smart R. C., Zannoni V. G.: Biochem. Pharmacol. *35*, 3180 (1986).
21. Jackson R. J., Stewart H. B., Sachs G.: Cancer *40*, 2487 (1977).
22. Simon E. J., Szucs M., Benuhe S., Borsodi A., Zeman P., Wollemann M.: J. Neurochem. *43*, 957 (1984).
23. Rodbard D. in: *Radioimmunoassay, Clinical Concepts* (O. B. Hunter, Ed.). Searle and Co., Skokie, Illinois 1974.
24. Munson P. J., Rodbard D.: J. Recept. Res. *8*, 533 (1988).
25. Yarigin K. N., Eriksson I., Nyberg F.: Protides Biol. Fluids Proc. Colloq. *34*, 505 (1986).
26. Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).