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^{14}C-5-HYDROXYTRYPTAMINE AND ^{3}H-D-AMPHETAMINE: UPTAKE AND CONTRACTION BY THE RAT STOMACH FUNDUS *IN VITRO**

ANDREA VACCARI and RODOLFO VERTUA†

University Institute of Pharmacology, Faculty of Pharmacy,
Via Capo S. Chiara 5, Genova, Italy

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Abstract—Binding of radioactively labeled 5-hydroxytryptamine (5-HT) and D-amphetamine (DA) by the isolated rat fundal smooth muscle is independent of contraction. After a short incubation period the two amines bind to extrareceptor sites which are not related to smooth muscle contraction. The uptake of both amines by normal tissues is similar after treatment with neuraminidase plus EDTA (5-HT receptor destruction) and after preincubation with lysergic acid diethylamide (LSD). This may support the hypothesis of a common receptor system for both 5-HT and DA. Neuraminidase also has a relatively aspecific effect on the cell membrane.

KNOWLEDGE of the structure of receptors and the identification of their components is essential to understanding the action of drugs. That membrane lipids may be part of the receptor and may be essential for drug action is of particular interest. A review has recently been published on this aspect.[1]

Woolley *et al.*[2] have shown that joint treatment of rat fundal strips with neuraminidase and EDTA is able to induce selective inhibition of the response to 5-HT, suggesting that gangliosides play a functional role in the 5-HT receptor.

Recently Fleisch and Ehrenpreis,[3, 4] using physicochemical methods suggested that the 5-HT receptor is fully or partially composed of protein molecules.

The specificity of the 5-HT receptor has been challenged. Amphetamine can interact with the 5-HT receptor on several preparations: isolated guinea pig ileum,[5, 6] rat fundal strips,[6] and on the liver fluke (*Fasciola hepatica*).[7, 8] Moreover a series of sympathomimetic amines tested on the guinea pig ileum seems to act on the 5-HT receptor.[9]

Moving from these data and in agreement with Woolley's hypothesis on the role of gangliosides in the 5-HT receptor, Vaccari *et al.*[10, 11] have tried to evaluate whether the receptor, after destruction of the "receptorial" ganglioside (or gangliosides) can still selectively respond to 5-HT and DA. Almost complete inhibition of the 5-HT contraction was observed under these conditions, while the DA contractions were less affected.

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† University Institute of Pharmacology, Trieste, Italy.

Abbreviations: 5-HT = 5-hydroxytryptamine (serotonin); DA = D-amphetamine (dexamphetamine); EDTA = ethylenediaminetetracetate disodium salt.

In this paper we have measured the uptake of radioactive 5-HT and DA after enzymatic alteration of the receptors by neuraminidase and EDTA and after receptor blockade by LSD.

MATERIALS AND METHODS

Rat gastric fundus strip preparation

Rat fundal strips were prepared by Vane's method[12] as modified by Offermeier.[13] The tissues, mucosa ablated, were set up in 10 ml modified Ringer solution, and bubbled with pure O_2. The organ bath temperature was 37°. The composition of the modified Ringer solution was the following (g/l.): NaCl 9·0–KCl 0·42–$NaHCO_3$ 0·5–glucose 0·5–$CaCl_2$ 60 mg. The magnification by the writing lever was about 1:30. Before testing, the fundus strips were allowed to relax for 60 min and the bathing fluid was changed every 15 min. This waiting period led to a good tissue stabilization with constant response to drugs.

Radioisotope experiments and radioactivity detection

5-hydroxytryptamine-3-^{14}C creatinine sulphate (Radiochemical Centre, Amersham, England: specific activity 56 mc/mM) and D-amphetamine-^{3}H (G) sulphate (New England Nuclear Co., Boston, U.S.A.: specific activity 89 c/mM), both dissolved in 0·1 N HCl, were used. Small amounts of the radioactive solutions were added to solutions of the corresponding cold drugs, so that the effective final concentration of the two agonists in the isolated organs bath were 1×10^{-4} m-moles/ml as base. The corresponding radioactivity was 0·056 μc/ml for 5-HT and 0·10 μc/ml for DA. Before each experiment the strips were carefully blotted and weighed. At the end of each incubation time the strips were rapidly removed from the radioactive bathing medium, rinsed in unlabeled Ringer and blotted. Incubation times were used which corresponded, under our experimental conditions, to the times needed to induce: (1) a maximal contraction after both 5-HT and DA (2 min) (2) a slow contracture, presumably independent of the drug–receptor interaction (8 min), and (3) a well stabilized contracture (30 min).

Radioactivity of the fundal tissues was measured according to the method of Petroff, Patt and Nair[14] using a Packard Tri-Carb Liquid Scintillation Spectrometer (model 2211). Strips were individually treated with 2N methanolic KOH (1 ml/25 mg fresh tissue), placed in a preheated water bath (70°) and continuously shaken for 30 min to ensure dissolution. After 30 min all samples were brought to room temperature and 0·5 ml aliquots (2 per tissue) were pipetted into counting vials, each containing 10 ml of scintillator solution consisting of 850 ml of toluene, 150 ml of ethyleneglycol monoethyl ether, 8 g of 2,5-diphenyl-oxazole (PPO) and 110 mg of 1,4-*bis*-2-(4-methyl-5-phenyl-oxazolyl)-benzene (dimethyl-POPOP). Counts were made on samples without tissue and on samples from control tissues, to determine the background and counting efficiency (established as 65–72 for ^{14}C and as 12–13 for ^{3}H).

The counts (counts per minute per sample: cpm/sample) for each strip were corrected according to the efficiencies, and expressed as disintegrations per minute per mg of fresh tissue (dpm/mg).

Treatment with neuraminidase plus EDTA (receptor destruction)

The method used was based on that of Woolley and Gommi.[2] The destroying

enzyme, purified neuraminidase derived from *Clostridium perfringens*, was twice applied to the tissue. The method was as follows: 0·5 ml of neuraminidase solution (1 mg in 5 ml of modified Ringer solution at 4°) was mixed with 4·5 ml of a solution of ethylenediaminetetracetate disodium salt (22 mg/ml). The EDTA solution was previously adjusted to pH 7·3 and cooled to 4°. The mixture (0·5 ml) was immediately added to the tissue bath (10 ml). The enzyme–EDTA mixture was allowed to act for 10 min, and was then washed out. Five min after washing, calcium chloride (2 mg) was added for 90 sec and the tissue was washed for 60 min. Neuraminidase plus EDTA (0·5 ml freshly mixed) was added a second time for 10 min, the tissue was then washed for 5 min, $CaCl_2$ (2 mg) added for 90 sec, and again washed for 10 min. The tissue was then ready for the assay.

LSD preincubation

The strips were incubated for 1 hr with 0·5 μg/ml of LSD, a dose which assured a maximal blocking effect. After washing with fresh Ringer for 5 min, the radioactive amines were added.

Chemicals

The following drugs were used: 5-hydroxytryptamine creatinine sulphate (Merck-Germany); D-amphetamine sulphate (Merck-Germany). The enzyme, purified neuraminidase type V (*Clostridium perfringens*) was purchased from Sigma, U.S.A.—All drugs are expressed as their bases.

RESULTS

Uptake of 5-HT-3-^{14}C and D-amphetamine-^{3}H (G)

In normal fundal strips incubated with either 5-HT-3-^{14}C or DA-^{3}H (G), the radioactivity levels increased linearly with time during the three selected incubation periods (2, 8 and 30 min) (Table 1).

In the neuraminidase + EDTA treated tissue (5-HT receptor destruction), the uptake of both drugs was altered (Table 2). There was no significant variations of the tissue radioactivity levels at 2 min. However, a significant increase in the uptake of both drugs after 8 min (average increase 52·0 per cent for DA and 39·0 per cent for

TABLE 1. UPTAKE OF ^{14}C-5-HYDROXYTRYPTAMINE AND ^{3}H-D-AMPHETAMINE BY NORMAL RAT FUNDAL STRIPS

Agonist* (m-moles/ml)	Incubation time (min)	Fresh tissue (dpm/mg ± S.E.)	No. of strips
^{14}C-5-HT 1×10^{-4}	2	28·5 (± 1·78)	12
	8	53·0 (± 3·28)	8
	30	106·8 (± 8·94)	6
^{3}H-D-Amphetamine 1×10^{-4}	2	76·1 (± 1·97)	9
	8	154·4 (± 5·53)	8
	30	457·2 (±29·67)	8

* Final radioactivity: ^{14}C-5-HT 0·056 μc/ml.
^{3}H-DA 0·100 μc/ml.

TABLE 2. UPTAKE OF ^{14}C-5-HYDROXYTRYPTAMINE AND ^{3}H-D-AMPHETAMINE AFTER 5-HT RECEPTOR DESTRUCTION (NEURAMINIDASE PLUS EDTA)

Agonist* (m-moles/ml)	Incubation time (min)	Fresh tissue (dpm/mg ± S.E.)	No. of strips	Significance of differences† vs. normal tissue	Significance of differences† vs. L S D preincubation
^{14}C-5-HT 1×10^{-4}	2	28·5 (± 3·34)	6	n.s.	n.s.
	8	73·5 (± 6·67)	6	$P < 0.020$	n.s.
	30	93·3 (± 2·76)	6	n.s.	$P < 0.001$
^{3}H-D-Amphetamine 1×10^{-4}	2	72·6 (± 6·76)	6	n.s.	n.s.
	8	234·5 (± 6·19)	6	$P < 0.001$	$P < 0.025$
	30	410·0 (±19·00)	6	n.s.	$P < 0.001$

* Final radioactivity: ^{14}C-5-HT 0·056 μc/ml ^{3}H-DA 0·100 μc/ml.
† Student's *t*-test.

TABLE 3. UPTAKE OF ^{14}C-5-HYDROXYTRYPTAMINE AND ^{3}H-D-AMPHETAMINE AFTER PREINCUBATION WITH LSD*

Agonist† (m-moles/ml)	Incubation time (min)	Fresh tissue (dpm/mg ± S.E.)	No. of strips	Significance of differences‡ vs. normal tissue	Significance of differences‡ vs. neuraminidase plus EDTA
^{14}C-5-HT 1×10^{-4}	2	30·3 (± 1·33)	6	n.s.	n.s.
	8	79·0 (± 3·17)	6	$P < 0.001$	n.s.
	30	168·5 (±11·09)	6	$P < 0.005$	$P < 0.001$
^{3}H-D-Amphetamine 1×10^{-4}	2	75·5 (± 2·79)	6	n.s.	n.s.
	8	278·2 (±15·32)	6	$P < 0.001$	$P < 0.025$
	30	694·8 (±22·52)	5	$P < 0.001$	$P < 0.001$

* L S D: 1 hr of preincubation with LSD 0·5 μg/ml.
† Final radioactivity: ^{14}C-5-HT 0·056 μc/ml ^{3}H-DA 0·100 μc/ml.
‡ Student's *t*-test.

5-HT) was observed. Finally, after 30 min, the tissue radioactivity showed a slight, but not significant decrease (— 10·0 per cent for DA and — 13·0 per cent for 5-HT).

The results of the uptake of both amines after LSD preincubation, is shown in Table 3. LSD was unable to induce significant variations of amine uptake after 2 min; after 8 min, the radioactivity levels sharply increased (DA: + 80·0% and 5-HT: + 49·0%). At 30 min such increase was still evident although less pronounced (DA: + 52·0% and 5-HT: + 58·0%).

Effects on contraction and contracture by the various treatments.

In normal conditions (Figs. 1 and 2), the 5-HT and DA induced contractions developed and were completed in 1–2 min. DA took slightly longer than 5-HT to reach peak heights.

Leaving the tissue in contact with the agonists for a longer period of time, generally caused an initial, moderate relaxation which was followed by a slow increase in tone

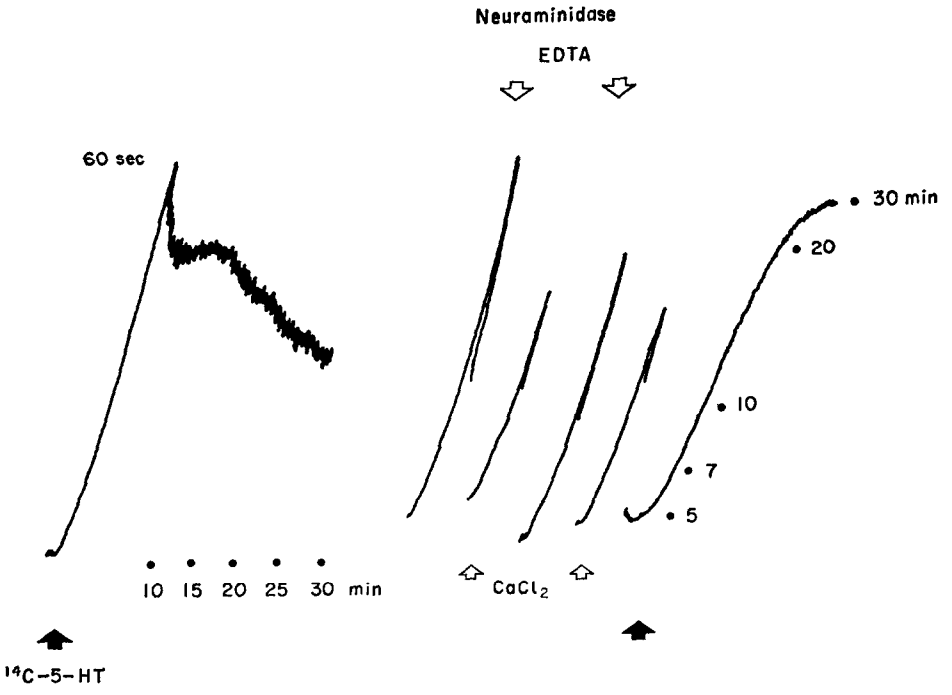

FIG. 1. Recording showing complete and prolonged blockade of the 5-HT induced contractions by neuraminidase + EDTA treatment (receptor destruction).

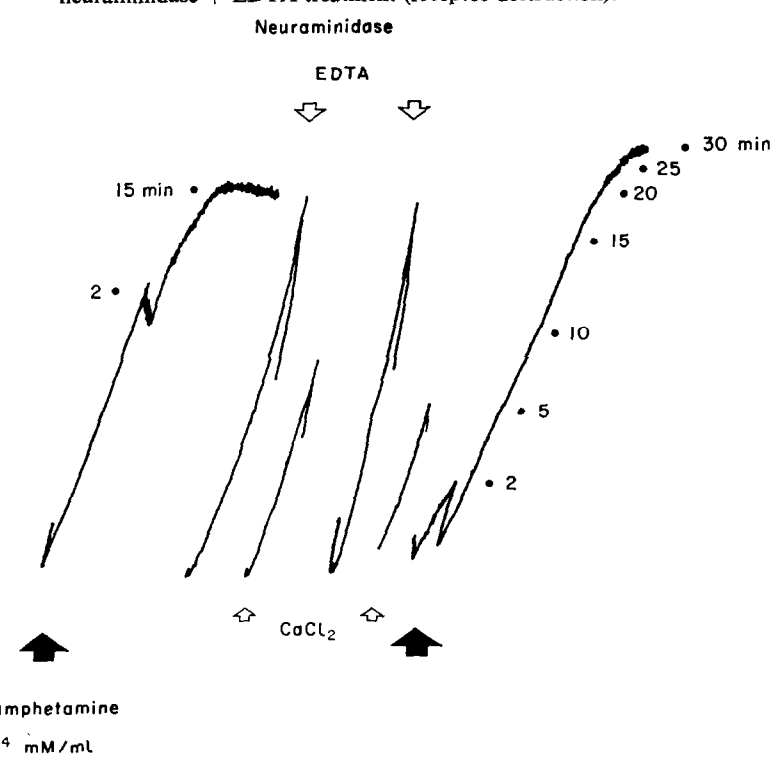

FIG. 2. Recording showing the partial inhibition of the D-amphetamine induced contraction by neuraminidase + EDTA treatment (receptor destruction).

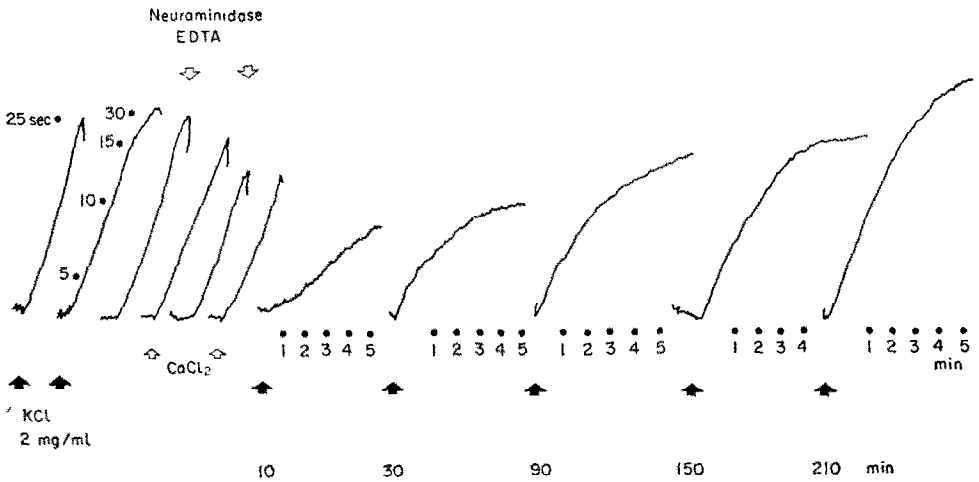

FIG. 3. Recording showing the net inhibitory effect by neuraminidase + EDTA treatment (5-HT receptor destruction) on the KCl induced contraction. At 210 min after the enzyme treatment, the onset of contraction is still delayed.

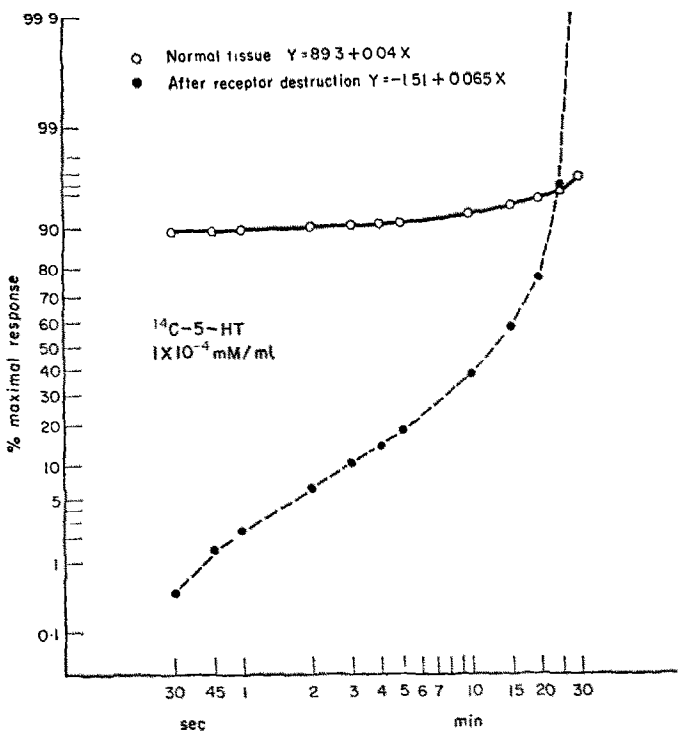

FIG. 4. Regression lines for the 5-HT induced contraction and contracture on normal (○——○) and neuraminidase + EDTA-treated fundal smooth muscle (●——●). Note the net blockade of the contraction and the smaller effect on the contracture. (Maximal extent of the contracture set = 100.)

(contracture). The contracture sometimes exceeded the maximal height obtained during the initial contraction, but was usually stabilized at the maximum height reached during the true contraction.

For comparison, we have studied KCl induced contractions, which do not act through a receptor system. The KCl induced contraction was very fast, reaching its maximum in 25–30 sec (Fig. 3).

5-HT activity was completely blocked in neuraminidase + EDTA -treated tissue (Figs. 1 and 4); such inhibition lasted for 5–6 min and was followed by a slow contracture. With DA (Figs. 2 and 5), the enzyme treatment induced a less evident inhibitory effect on the contraction. More precisely, a clear partial inhibition of the true contraction was observed that was followed by a contracture similar to that elicited by 5-HT.

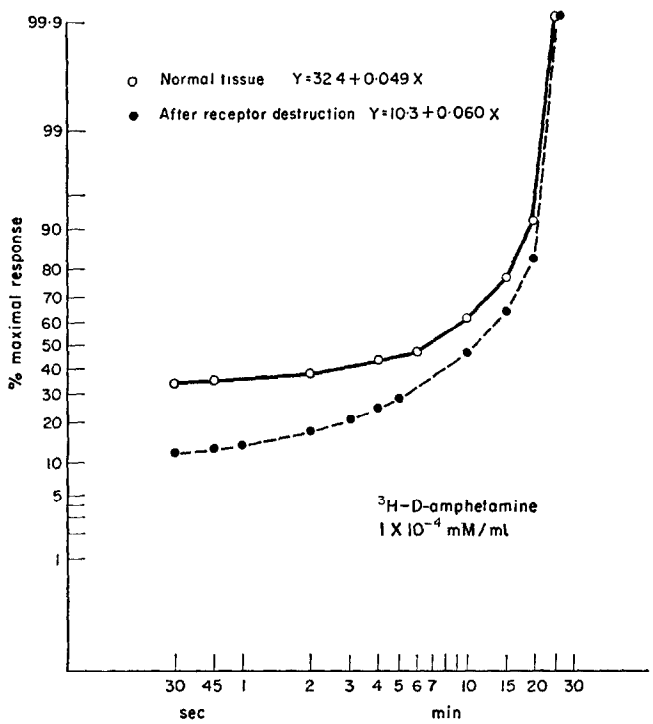

FIG. 5. Regression lines for the D-amphetamine induced contraction and contracture on normal (○——○) and neuraminidase + EDTA-treated (●——●) fundal smooth muscle. Partial inhibition only on the contraction; contracture scarcely affected by the enzyme treatment. (Maximal extent of the contracture set = 100.)

The KCl induced contraction (Fig. 3) was clearly inhibited by the enzyme treatment for about 1–2 min; the usual contracture followed which reached a maximum of 70 per cent in comparison with the controls. The effect of time after the enzyme treatment on KCl contraction was also studied (irreversible membrane damage). Figure 6 shows time–response curves performed at various time intervals (10–30–90–150 min) after the neuraminidase + EDTA treatment. These response

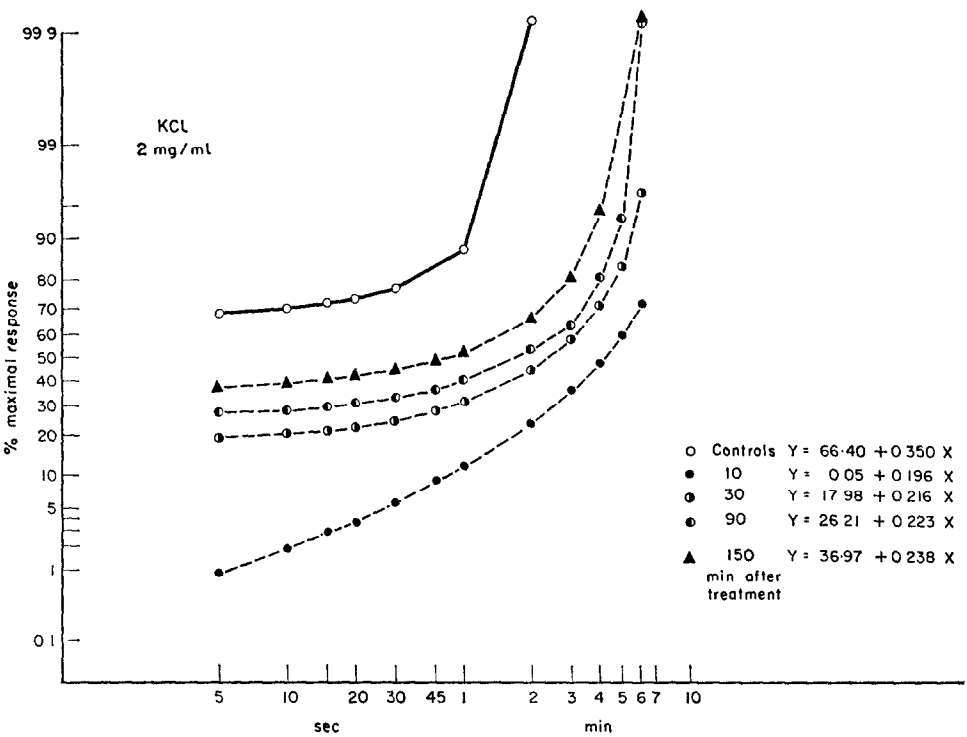

FIG. 6. Regression lines for the KCl induced contraction and contracture on normal (○——○) and neuraminidase + EDTA-treated (●——●) fundal smooth muscle at various times after treatment. Prolonged and not fully reversible inhibition of both contraction and contracture: membrane alteration by the enzyme treatment. (Maximal extent of the contracture set = 100.)

increased progressively but never reached the control level; at 150 min after the treatment a marked inhibitory effect was still evident on both contraction and contracture.

A complete blockade of both 5-HT and DA-induced contractions was seen after LSD preincubation (Fig. 7). Often the addition of the two drugs to the bath was followed by an initial relaxation of the tissue lasting 8–10 min; then it slowly returned to the initial tone. The LSD preincubation had no effect on the KCl induced contraction (Fig. 7).

DISCUSSION

The data shows that the uptake of both 5-HT and DA by normal smooth muscle is very similar both after receptor destruction and after LSD preincubation.

After 2 min, the time usually required for normal smooth muscle to develop and complete a contraction, the uptake of both amines by normal and LSD pretreated tissue were not significantly different in comparison with controls. For such a short incubation time, during which catabolic processes are unlikely to be very advanced, the following hypothesis may be suggested. The two drugs can normally be bound to non-gangliosidic receptors or extrareceptor sites in the cell membrane which are not affected by neuraminidase + EDTA and not reached by LSD. It is clear that in this

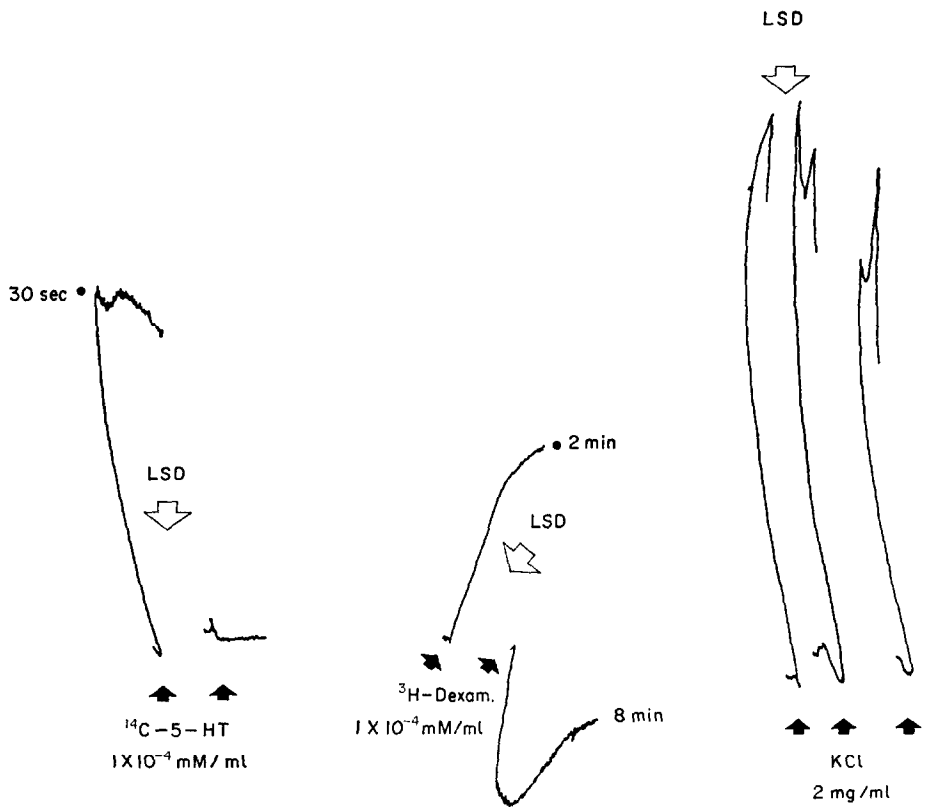

FIG. 7. Preincubation with LSD (0·5 μg/ml for 1 hr). Net blockade of the 5-HT— and D-amphetamine. No effect on the KCl—induced contractions.

case the relatively small amount of agonist fixed to the ganglioside-containing receptor could be a negligible fraction of the total amount of drug bound to the tissue.

The fact that after neuraminidase + EDTA treatment or LSD preincubation, the 5-HT and DA induced contractions are partially or fully inhibited suggests that the binding of the amines to the smooth muscle and especially to the cell membrane is independent of the integrity of the receptor gangliosides and of the availability of the receptors. The gangliosidic fraction in the receptors may, on the other hand, play an essential role in the initiation of the processes between the stimulus and the response and therefore may be essential for production of contractions.

The neuraminidase plus EDTA treatment, besides possibly selectively modifying the 5-HT and DA receptors, also induces changes in the cell membrane, as shown by the blockade of the KCl contraction after enzyme treatment. KCl induces smooth muscle contraction by a pure membrane mechanism, not involving pharmacologically demonstrable receptor sites. Membrane alteration is also supported by the well known fact that some hydrolytic enzymes induce selective alterations of the cell membrane of several structures such as red blood cells,[15] protozoa[16] and sea urchin eggs.[17]

The enzyme induced membrane alteration can help to explain the strong increase in the uptake of 5-HT and DA, after 8 min of incubation. This incubation time is long

enough to allow membrane modifications to become more important than the receptor inactivation. The significant increase in the uptake from 5-HT (or its metabolites), and the even greater uptake of DA could therefore be due to a simple binding to extrareceptor membrane sites, and also to a possible diffusion of the amines across the altered membrane. In addition, a portion of the radioactive amines probably diffuses into extracellular spaces, connective tissue fibers etc.

Clearly, LSD pretreatment further increases amine uptake by the tissue. Here too, blockade of the receptors cannot prevent binding of drugs to non-specific sites on the membrane even if contractions are blocked. It remains difficult to explain the reason for the relatively higher uptake of DA in comparison with 5-HT.

Increase in the uptake after LSD becomes even more evident after 30 min of incubation. On the contrary, 30 min after the neuraminidase plus EDTA treatment, a reduced uptake of 5-HT and particularly DA appeared. It cannot be excluded that membrane modifications favour a loss of radioactivity towards the bathing solution after such a long incubation time. Also in *Taenia coli* a loss of radioactivity corresponding to a large amount of 5-HT degradation products and to intact 5-HT was observed under normal conditions.[18]

In conclusion, similar kinetics for the uptake of both 5-HT-3-^{14}C and DA-^{3}H were observed under all experimental conditions. This similarity may give experimental support to the hypothesis that a common receptor for both 5-HT and DA exists, but mainly it clearly shows that the mechanisms regulating the 5-HT and DA binding to the receptor or to the cell membrane are very similar.

It appears, that while LSD completely blocks contractions of strips of the rat stomach fundus induced by both 5-HT and amphetamine, enzyme treatment brings out a difference between the two amines. In fact, neuraminidase produces a strong inhibition of the 5-HT contractions, while having a rather weak effect on the DA contractions. Enzyme treatment produces also a weak, although quantitatively similar block of contractures induced by both amines. The time–action curves clearly show different behaviour in the responsiveness to the two amines after enzyme treatment. This indicates that the DA receptor may be more complex but not completely different from that of the 5-HT. Certainly ganglioside breakdown does not block the activity of DA as strongly as it does that of 5-HT.

Finally it must be noted that the enzyme treatment does not affect the stimulus-effect sequence: in fact, although after neuraminidase + EDTA, the 5-HT contraction is completely blocked and the DA contraction only partially blocked, other agonists such as the cholinergic drug furtrethonium and the polypeptide eledoisin are still able to induce normal contraction of the fundal strip (unpublished results).

From the present experimental results and related considerations, the following conclusions can be drawn.

(1) The binding of 5-HT-3-^{14}C and DA-^{3}H to the tissue and particularly to the cell membrane and receptor is relatively independent of the contraction, since a blockade of the contraction does not necessarily correspond with a decrease in the uptake of the agonist. This is because the two amines normally must bind not only to the receptors but also to other sites of the membrane: that is, sites of a non-gangliosidic nature and thus not affected by neuraminidase plus EDTA.

(2) The receptor gangliosides may be the cause, up to a point, of the start of the smooth muscle contraction.

(3) Serotonin and amphetamine behave similarly under several experimental conditions; it is therefore conceivable that both drugs bind to the cell membrane and to their receptors by a very similar mechanism. This observation may at least partially support the hypothesis of a common receptor for both drugs.

(4) Neuraminidase, an enzyme which selectively disrupts the integrity of the 5-HT receptor, also has another, less specific effect, i.e. it alters the cell membrane: its effective function as a "pure 5-HT receptor destroying enzyme" is therefore doubtful.

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REFERENCES

1. A. W. CUTHBERT, *Pharmac. Rev.* **19**, 59 (1967).
2. D. W. WOOLLEY and B. W. GOMMI, *Archs. int. Pharmacodyn. Ther.* **159**, 8 (1966).
3. J. H. FLEISCH and S. EHRENPREIS, *J. Pharmac. exp. Ther.* **162**, 21 (1968).
4. J. H. FLEISCH and S. EHRENPREIS, *J. Pharmac. exp. Ther.* **163**, 362 (1968).
5. J. R. VANE, in *Ciba Foundation Symposium on Adrenergic Mechanisms* (Eds. J. R. VANE, G. E. W. WOLSTENHOLME and M. O'CONNOR), p. 356. Churchill, London (1960).
6. I. R. INNES, *Br. J. Pharmac.* **21**, 427 (1963).
7. T. E. MANSOUR, *Br. J. Pharmac.* **12**, 406 (1957).
8. C. BERETTA and A. LOCATELLI, *J. Pharm. Pharmac.* **20**, 744 (1968).
9. I. R. INNES and J. D. KOHLI, *Br. J. Pharmac.* **35**, 383 (1969).
10. A. VACCARI and F. CUGURRA, *Biochem. Pharmac.* **17**, 2399 (1968).
11. A. VACCARI and R. VERTUA, *Br. J. Pharmac.* **34**, 687P (1968).
12. J. R. VANE, *Br. J. Pharmac.* **12**, 344 (1957).
13. J. OFFERMEIER, Serotonin and its derivatives, a study on structure–activity relations, Thesis, 45 Thoben Offset, Nijmegen (1965).
14. C. P. PETROFF, H. H. PATT and P. P. NAIR, *Int. J. appl. Rad. Isotop.* **16**, 599 (1965).
15. R. BALLENTINE and A. K. PARPART, *J. cell. comp. Physiol.* **16**, 49 (1940).
16. J. H. NORTHROP, *J. Gen. Physiol.* **9**, 497 (1926).
17. B. HAGSTRÖM and B. HAGSTRÖM, *Expl Cell Res.* **6**, 532 (1954).
18. G. V. R. BORN, *J. Physiol.* **161**, 160 (1962).