Biochemical Pharmacology, Vol. 17, pp. 2399–2406. Pergamon Press. 1968. Printed in Great Britain

RELATIONSHIPS BETWEEN *N*-ACETYLNEURAMINIC ACID, SEROTONIN AND AMPHETAMINES ON THE ISOLATED RAT STOMACH PREPARATION

ANDREA VACCARI* and FRANCO CUGURRA*

Institute of Pharmacology, University of Trieste, 34100 Trieste, Italy

(*Received* 17 *June* 1968; *accepted* 24 *July* 1968)

Abstract—Differences have been demonstrated between amphetamine and 5-hydroxytryptamine (5-HT) towards the serotonin-receptor *substance*, *N*-acetylneuraminic acid (NANA), on the isolated rat fundal smooth muscle. Selective destruction of the receptor by incubation with neuraminidase plus EDTA, does not affect the extent of the contraction induced by dexamphetamine. Preincubation with NANA, as with 5-HT, increases the maximal contraction induced by some amphetamines, but not by a cholinergic drug. The increasing effect is quantitatively similar with amphetamine and 5-HT: it suggests a physical component common to both amines in the origin of the effect itself. Since the increase in contraction does not seem to be related to a *receptor reserve* for amphetamine, it supports the hypothesis for an *effector reserve*.

ACCORDING to several authors, amphetamine acts on the 5-HT receptor. This applies to isolated guinea-pig ileum[1] and other smooth muscle preparations, particularly to rat gastric fundal strips.[2] Further, amphetamine had an action on the 5-HT receptor of the liver fluke (*Fasciola hepatica*) preparation, as suggested by Mansour.[3]

On the nature of the serotonin-receptor, there are constantly increasing data suggesting the role of gangliosides and also of acetylneuraminic acid in the structure or biosynthesis of the receptor itself.[4–12]

The reversible destruction of the 5-HT receptor with neuraminidase plus EDTA was also proposed by Woolley and Gommi,[8] as a method for the direct measurement of serotonin receptors.

On the basis of the relation 5-HT-NANA, Wesemann and Zilliken[9] showed evidence, on the isolated rat fundus strip preparation, that preincubation with gangliosides and NANA increases the maximal contraction induced by 5-HT. A similar effect occurs on the *Fasciola hepatica* preparation.[10] Moreover, the same authors[9] have shown how inhibitors of the NANA biosynthesis lower the maximal contraction induced by 5-HT.

From all the data reported, some analogies between amphetamine and 5-HT, both in their action on smooth muscle and in their behaviour towards NANA, are to be expected.

Considering the first point, a difference in neonatal sensitivity was however found, on the isolated gastric fundus of the newborn rat.[13, 14] This preparation showed

Abbreviations: 5-HT = 5-hydroxytryptamine (serotonin); NANA = *N*-acetylneuraminic acid; EDTA = ethylenediaminetetracetate disodium salt; HFur = furtrethonium.

* Present address: Institute of Pharmacology and Pharmacognosy, Phaculty of Pharmacy, Via Capo S. Chiara 5, 16146 Genova, Italy.

indeed a marked neonatal hyposensitivity to 5-HT, but only a slight decrease in affinity towards *d*- and *dl*-amphetamine. Such difference raised a doubt on the assumption that 5-HT receptors are concerned in the action of amphetamine.

This led us to investigate, on the rat gastric fundus, whether amphetamine and serotonin behave differently, at level of the receptor substance for 5-HT.

A first series of experiments was then undertaken, to determine whether selective destruction of 5-HT receptors by incubation with neuraminidase plus EDTA, may also affect the amphetamine-induced contraction.

In a second series the effects of preincubation with NANA were studied, on the maximal contraction caused by some amphetamines, in comparison with the cholinergic drug, furtrethonium.

A last series of experiments was finally carried out to examine the possibility of a receptor reserve for amphetamine, in view of the increasing effect on the maximal contraction, after NANA preincubation. This effect was indeed shown by the second series of experiments.

MATERIALS AND METHODS

Rat gastric fundus strip preparation

The rat gastric fundus was cut according to Offermeier.[15] The tissues were set up in 10 ml Tyrode solution and bubbled with pure O_2. The organ bath temperature was 37°. The magnification by the writing lever was about 1 : 30. Before testing, the fundus strips were allowed to relax for 60–90 min; the bathing fluid was changed every 15 min. This waiting period led to a good tissue stabilization with constant response to drugs.

NANA preincubation

The strips were preincubated for 15 min with NANA 1×10^{-2} or 1×10^{-1} mM/ml; NANA was then washed away. The cumulative curve with the agonist was effected 15 min after washing. Cumulative log dose-response curves were obtained according to Van Rossum.[16] Affinity (pD_2) and variations in maximal contraction following the preincubation with NANA, were referred to at least two equal dose-response curves.

Selective destruction of 5-HT receptors

The method used was based on that of Woolley and Gommi.[8] The destroying enzyme, purified neuraminidase derived from *Clostridium perfringens* (0·05 micromolar units per mg), was applied two times to the tissue. The method briefly involves the following procedures. Neuraminidase solution (1 mg) in 5 ml of Tyrode solution at 4°. Immediate mixing of the enzyme (0·5 ml) with 4·5 ml of a solution of EDTA (22 mg/ml). The EDTA solution was previously adjusted to pH 7·3 and cooled to 4°. After mixing, 0·5 ml of the mixture were immediately applied to the tissue bath (10 ml). The enzyme-EDTA mixture was allowed to act for 10 min, and was then washed away for 5 min. After washing, 2 mg of $CaCl_2$ were added, for 90 sec, then the tissue was washed for 60 min. Finally, a second application of 0·5 ml of neuraminidase plus EDTA (freshly mixed): incubation time 10 min. Then washing for 5 min, addition of $CaCl_2$ (2 mg) to the tissue, for 90 sec, washing for 10 min again. The tissue was ready for the assay, and the agonist was assayed.

Receptor reserve

Cumulative dose-response curves were obtained with *d*-amphetamine, after repeated incubations with the irreversible blocker dibenamine 1×10^{-2} mM/ml. The blocker was allowed in tissue contact for 10 min and was then washed away for 5 min. Receptor reserve is indicated by shifting of the dose-response curve to the right (to higher concentrations); the curve declines, when reserve is totally exhausted by the receptor blocking agent.

Chemicals

The following drugs were used: *d*-amphetamine sulphate (Merck, Germany), *dl*-amphetamine sulphate (Merck), L(+)-*N*-methyl-amphetamine HCl (Merck). Furtrethonium jodide and *N*,*N*-dibenzyl-β-chloroethylamine HCl (Dibenamine) were kindly supplied by Dr. H. Green and Dr. E. A. Kimes, S.K. & F., Philadelphia, U.S.A. *N*-acetylneuraminic acid (synthetic crystalline type IV) and purified neuraminidase type V (*Clostridium perfringens*) were purchased from Sigma, U.S.A. Doses are expressed as base.

RESULTS

Destruction of the serotonin receptors

Incubation with neuraminidase plus EDTA does not affect the height of contraction caused by *d*-amphetamine 1×10^{-1} mM (sub-maximal dose). The response was unchanged in all 6 experiments performed.*

Preincubation with NANA

Preincubation with *N*-acetylneuraminic acid elicits a net increase in maximal response (contraction) induced by *dl*-amphetamine, *d*-amphetamine and also by a substituted in lateral chain amphetamine as methyl-amphetamine (Table 1 and Figs. 1–2). In presence of *d*-amphetamine, the increasing effect is well clear, after the smaller dose of NANA. In fact we obtain 100 per cent of positive responses (increased maximal contraction), after NANA 1×10^{-2} mM preincubation, in presence of *d*-amphetamine, but only 66·7 per cent after NANA 1×10^{-1} mM. Preincubation with NANA 1×10^{-2} mM gives respectively 71·4 and 61·1 per cent of positive responses on the maximal contractions. In presence of *dl*-amphetamine and methyl-amphetamine, after NANA 1×10^{-1} mM, the percentage values are further lowered.

The affinity values (pD_2) after each of the two NANA doses, does not differ significantly from the controls: this implies absence of sensitization in the dose-response curve. At the same time peculiar is the increasing effect (Table 1).

Repeated increases of the maximal contraction are generally not possible, mostly after preincubation with the higher dose of NANA (Fig. 2).

With the cholinergic drug, furtrethonium, no variations in the maximal contraction have been induced, after preincubation with the usual doses of NANA (Table 2).

Receptor reserve

Fig. 3 shows that after repeated incubations for consecutive equal periods (10 min), with 1×10^{-2} mM dibenamine, the log dose-response curves for *d*-amphetamine decline immediately, without previous shift to higher doses. This indicates the probable absence of a receptor reserve for amphetamine.

* In low Calcium Ringer solution[8], treatment with neuraminidase plus EDTA was able to delay the onset of amphetamine-induced contractions; the maximal extent was unchanged.

TABLE 1. INFLUENCE OF 15 min PREINCUBATION WITH NANA ON THE *d*-AMPHETAMINE, *dl*-AMPHETAMINE AND METHYL-AMPHETAMINE DOSE-RESPONSE CURVES OF THE RAT FUNDUS STRIP PREPARATION

Agonist	NANA mM/ml	Minutes after incubation	Increase of the maximal contraction (%)*	Positive responses (%)‡	pD_2	α§	No. of strips/ no. of curves
d-Amphetamine	0				4·63	1·00	15/54
d-Amphetamine	1×10^{-2}	15	+19·8 (±4·62)†	100·0	4·77	~1·20	12/12
d-Amphetamine	1×10^{-1}	15	+16·8 (±13·51)	66·7	4·73	~1·17	12/12
dl-Amphetamine	0				4·73	1·00	50/122
dl-Amphetamine	1×10^{-2}	15	+17·9 (± 6·92)	71·4	4·70	~1·18	15/15
dl-Amphetamine	1×10^{-1}	15	+20·1 (±17·30)	50·0	4·73	~1·20	14/14
Methyl-amphetamine	0				4·00	0·60	21/41
Methyl-amphetamine	1×10^{-2}	15	+18·8 (±10·21)	61·1	3·97	~0·79	18/18
Methyl-amphetamine	1×10^{-1}	15	+16·5 (±15·51)	38·5	4·10	~0·76	13/13

* Maximal contraction of the untreated preparation = 100 per cent.
† (±) : 95 per cent confidence limits for the mean.
‡ Percentage experiments with increase of the maximal contraction.
§ Relative intrinsic activity.

TABLE 2. INFLUENCE OF 15 min PREINCUBATION WITH NANA ON THE FURTRETHONIUM DOSE-RESPONSE CURVES OF THE RAT FUNDUS STRIP PREPARATION

NANA mM/ml	Minutes after incubation	Variation of the maximal contraction (%)*	no. of strips/ no. of curves
1×10^{-2}	15	0·0	6/6
1×10^{-2}	15	+4·8	4/4
1×10^{-2}	15	−3·1	1/1
1×10^{-1}	15	0·0	1/1
1×10^{-1}	15	+3·6	7/7
1×10^{-1}	15	−10·6	4/4

* Maximal contraction of the untreated preparation = 100 per cent.

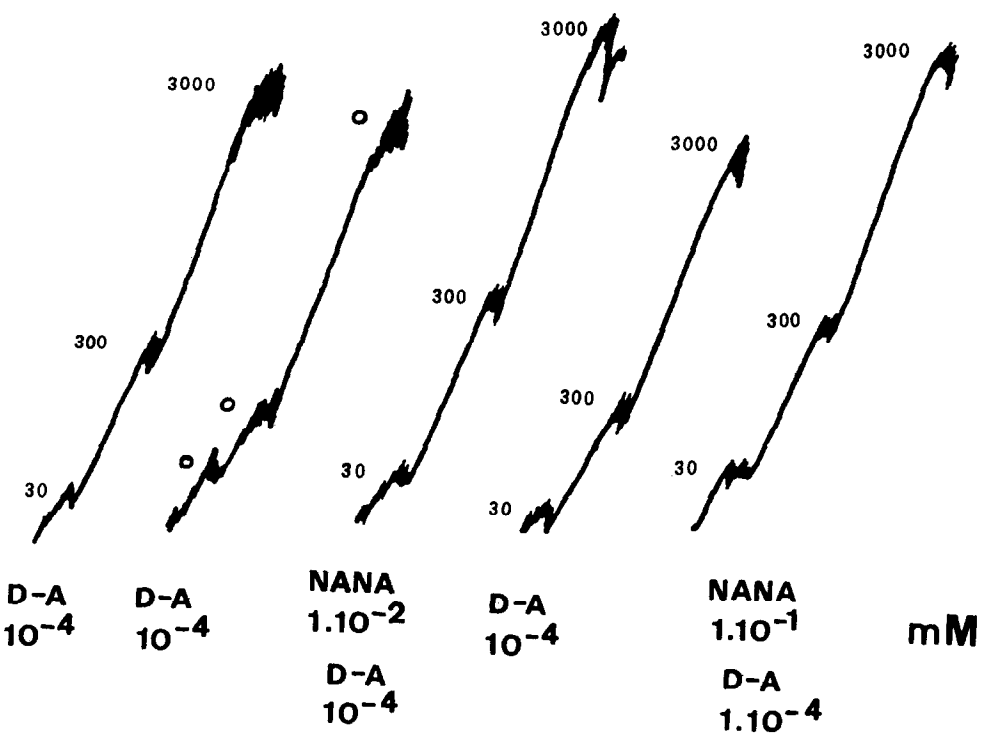

FIG. 1. Registrogram showing the increase in the maximal contraction of *d*-amphetamine (D-A), after incubation with NANA 1×10^{-2} and 1×10^{-1} mM (incubation time 10 min). O = 30,300 and 3000×10^{-4} mM.

DISCUSSION

The assumption that 5-HT and amphetamine act on the same receptor, and the participation of NANA in the receptor structure, should imply inhibition or decrease of the amphetamine induced contraction, after destruction of the receptor itself.

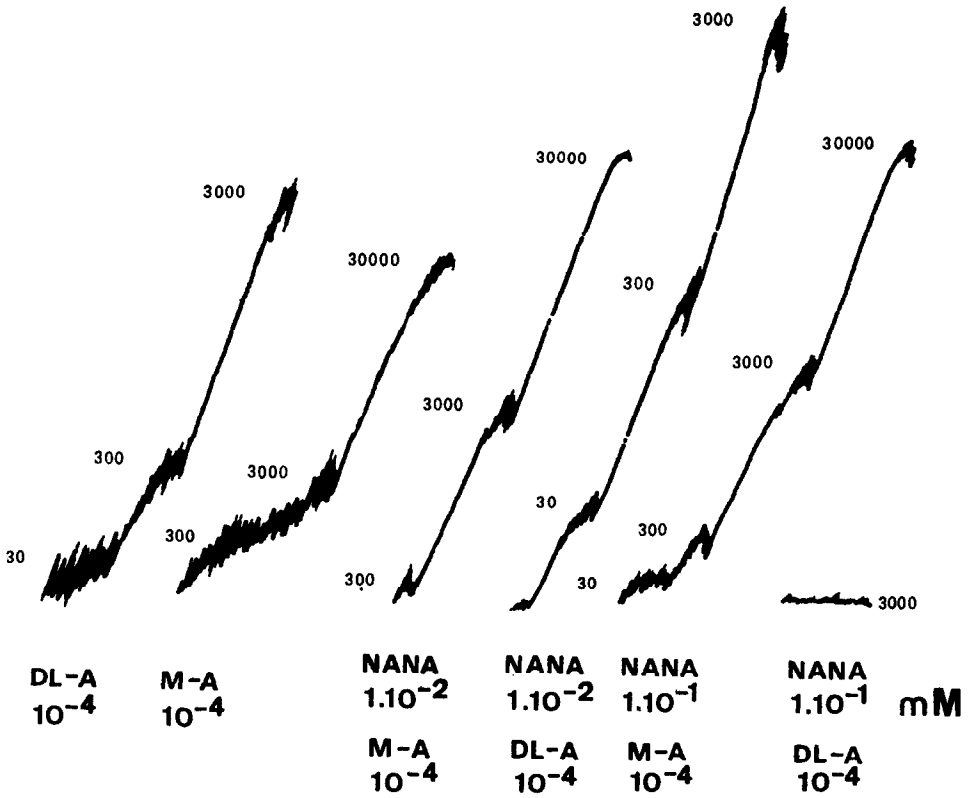

FIG. 2. Registrogram showing the increase in the maximal contraction of *dl*-amphetamine (DL-A) and methylamphetamine (M-A), following to 10 min incubation with NANA 1×10^{-2} and 1×10^{-1}mM. Increase does not occur after repeated incubations with the higher dose of NANA.

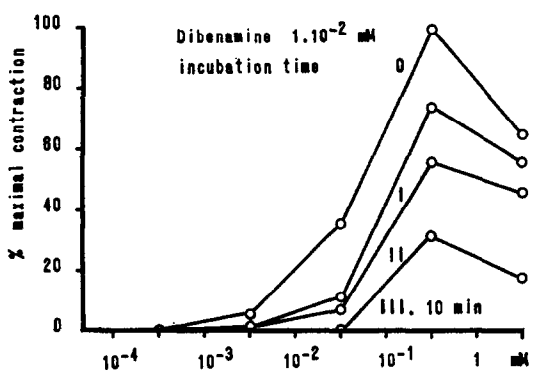

FIG. 3. Cumulative dose-response curves for *d*-amphetamine, after repeated incubations (10 min) with constant concentrations of dibenamine. The immediate decline of the curves and the absence of a net shift to higher concentrations of *d*-amphetamine show absence of a receptor reserve.

Similar effect was indeed observed by Woolley and Gommi,[8] with 5-HT. In the present experiments however, treatment with neuraminidase do not affect the contraction caused by amphetamine. It may be argued, therefore, that amphetamine, in contrast with 5-H T, do not need NANA for the contraction of the fundus smooth muscle.

As far the the interaction amphetamine-NANA is concerned again, there is an apparent behavioral analogy with 5-HT, after NANA preincubation. The increase in maximal contraction by amphetamine is however not concomitant with increased affinity, i.e. sensitization: Wesemann and Zilliken[9] have on the contrary found also an increase in sensitivity to 5-HT, after NANA or gangliosides preincubation.

It must be further noted that the increasing effect is quantitatively similar for the two amphetamine isomers and methyl-amphetamine. The percent increases are +17·9; +19·8 and +18·8 for *dl*-, *d*- and methyl-amphetamine respectively. These values are similar to those obtained with 5-HT by Wesemann and Zilliken[9] and confirmed by us (+15% to +20% about).

The non-selective effect of NANA on drugs having different phisico-chemical characters, beside the fact that the potentiating effect cannot generally be obtained after repeated preincubations with NANA, suggest that also some physical, beside biological component, may be present.

Furthermore, if we admit that NANA or some of its metabolites or the whole metabolism of gangliosides are part of the 5-HT and amphetamine receptor, it is difficult to explain how addition of NANA to the tissue preparation may induce an increase in the maximal contraction obtainable. Incubation with NANA should indeed correspond to an increase in the receptor "pool" for 5-HT and amphetamine.

With isolated organs a direct relationship is admitted[18, 19] between:

number of occupied receptors——stimulus——effect

Except where a receptor reserve exists,[20, 21] when saturation of the whole receptor pool by the agonist has occurred, there corresponds the maximal effect. As regards 5-HT, a receptor reserve is excluded by Offermeier and Ariëns.[22] We exclude also, from the referred experiments, a receptor reserve for *d*-amphetamine. From this it can be suggested that the incubation with receptor material (NANA or gangliosides), must imply the occurrence of an effector-reserve, i.e. effectors unemployed in the contraction, under physiological conditions.

The agonist (5-HT or amphetamine) in excess in the organ bath could bind the added NANA; this could elicit a supplementary quantity of stimulus that would find available the rate of usually unemployed effectors. When also the effector-reserve is exhausted, it would be no longer possible to induce increases in contraction, in spite of further addition of receptor substance.

This working hypothesis still needs further supports, but it is able to explain also the non-repetition of the increasing effect, after consecutive incubations with NANA

As a conclusion, a possible explanation for the increasing effect after incubation with NANA, could follow this schema:

5-HT and amphetamines —exogenous— NANA —supplementary— stimulus — effector reserve

|

increase in maximal contraction

All these observations suggest the general conclusion that, on the basis of NANA as common receptor substance for 5-HT and amphetamine, the two amines do not

act on the same receptor in the rat stomach fundus. This fact is supported also by preliminary data obtained in experiments done to evaluate the calcium content of tissue, during the 5-HT induced contraction. As regards the relationship Calcium —5-HT, it must be remembered that a role of gangliosides as calcium-carrier through the cell-membrane was proposed by Woolley and Campbell,[17] for the 5-HT induced smooth muscle contraction.

In our conditions, using ^{45}Calcium, a net increase in tissue radioactivity was obtained, during the 5-HT induced contraction. In amphetamine-induced contraction, no increase in the tissue radioactivity was found.

Finally it is interesting that also on other tissues than rat gastric fundus (rabbit aorta), doubts are recently raised on the action of *d*-amphetamine on 5-HT receptors.[23]

REFERENCES

1. 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).
2. I. R. INNES, *Br. J. Pharmac.* **21**, 427 (1963).
3. T. E. MANSOUR, *Br. J. Pharmac.* **12**, 406 (1957).
4. D. W. WOOLLEY, *Proc. natn. Acad Sci. U.S.A.* **44**, 1202 (1958).
5. D. W. WOOLLEY and B. W. GOMMI, *Proc. natn. Acad. Sci. U.S.A.* **52**, 14 (1964).
6. D. W. WOOLLEY and B. W. GOMMI, *Nature, Lond.* **202**, 1074 (1964).
7. D. W. WOOLLEY and B. W. GOMMI, *Proc. natn. Acad. Sci. U.S.A.* **53**, 959 (1965).
8. D. W. WOOLLEY and B. W. GOMMI, *Archs. int. Pharmacodyn. Thér.* **159**, 8 (1966).
9. W. WESEMANN and F. ZILLIKEN, *Biochem. Pharmac.* **16**, 1773 (1967).
10. W. WESEMANN and F. ZILLIKEN, *Biochem. Pharmac.* **17**, 471 (1968).
11. W. GIELEN, *Z. Naturforsch.* **23b**, 117 (1968).
12. P. M. CARROLL and D. D. SEREDA, *Nature, Lond.* **217**, 667 (1968).
13. A. VACCARI and F. CUGURRA, *Biochem. Pharmac.* **17**, 824 (1968).
14. A. VACCARI, *Biochem. Pharmac.* **17**, 2493 (1968).
15. J. OFFERMEINER, Serotonin and its derivatives, a study on structure-activity relations, Thesis, 45 Thoben Offset, Nijmegen (1965).
16. J. M. VAN ROSSUM, *Archs. int. Pharmacodyn. Thér.* **143**, 299 (1963).
17. D. W. WOOLLEY and N. K. CAMPBELL, *Biochim. biophys. Acta* **57**, 384 (1962).
18. A. J. CLARK, in *Heffter: Handbuch der experimentellen Pharmakologie*, Ergänzungswerk, Bd. 4. Springer, Berlin (1937).
19. E. J. ARIËNS, A. M. SIMONIS and J. M. VAN ROSSUM, in *Molecular Pharmacology I.* (Ed. E. J. ARIËNS). Academic Press, New York and London (1964).
20. R. P. STEPHENSON, *Br. J. Pharmac.* **11**, 379 (1956).
21. M. NICKERSON, *Nature, Lond.* **178**, 697 (1956).
22. J. OFFERMEIER and E. J. ARIËNS, *Archs. int. Pharmacodyn. Thér.* **164**, 198 (1966).
23. J. D. KOHLI, *Br. J. Pharmac.* **32**, 273 (1968).