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RECEPTORS OF NEUROTRANSMITTERS—II

SIALIC ACID METABOLISM AND THE SEROTONIN INDUCED CONTRACTION OF SMOOTH MUSCLE

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Abstract—A number of different *N*-acylneuraminic acids, gangliosides, neuraminidases and synthetic inhibitors of sialic acid biosynthesis have been tested on the serotonin induced contraction of the rat stomach fundus. Gangliosides and *N*-acylneuraminic acids were found to increase sensitivity and maximal contraction height of the fundus preparation. In contrast the inhibitors decreased the rate of contraction. The serotonin receptor and its complex with serotonin were broken down upon incubation with neuraminidases. There was found a certain relationship between the inhibitory action of sialic acid biosynthesis in the cell-free system and the regeneration of serotonin receptors in the muscle preparation.

WOOLLEY and Gommi[1] presented evidence for the chemical nature of serotonin receptors in smooth muscle. According to these authors the serotonin receptors of uterus and stomach fundus of rats are selectively destroyed by the action of neuraminidase in the presence of EDTA. The neuraminidases (*N*-acetylneuraminate glycohydrolase 3.21.18) were derived from *clostridium perfringens* and *vibrio cholerae*.

The receptors of both organ preparations could be resensitized for 5-HT* by the addition of gangliosides. The most active of them, di-sialobiose-ganglioside, exhibited similar chromatographic properties as G'lact (=*N*—acylsphingosinyl-*N*-acetylsialyl-(2→ 8)-*N*-acetylsialyl-(2→3Gal)-lactoside) which has been isolated and identified by Kuhn and Wiegandt[2]. On the basis of *in vitro* dialysis studies, Gielen[3] has recently reported that this ganglioside possesses high binding capacity for serotonin. When inhibits of the NANA-biosynthesis were made available by the studies of Boschman† it became possible to gain more insight in the relationship between NANA-metabolism and the receptor function. In this paper we wish to present evidence that sialic acid is an integral part of serotonin receptors.

MATERIALS AND METHODS

Preparation and incubation of rat fundus strip

Male rats, strain Wistar II, 160 –180 g, starved for 48 hr, water *ad libitum*, have been used for the fundus strip preparation according to Vane.[4] The mucosa was

* Abbreviations used: 5-HT = 5-hydroxytryptamine (serotonin); NANA = *N*-acetylneuraminic acid, NANA-9-P = *N*-acetylneuraminic acid-9-phosphate, CMP-NANA = cytidine-5'-monophospho-*N*- acetylneuraminic acid, Ac Mm = *N*-acetylmannosamine, AcMm-6-P = *N*-acetylmannosamine-6-phosphate, PEP = phosphoenolpyruvate, Pi = inorganic orthophosphate, PPi = inorganic pyrophosphate.

† Th. A. C. Boschman; in press.

carefully removed to facilitate the washing out of drugs. The muscle strip was incubated in 10 ml oxygenated Tyrode at 37°. The strip was fixed at one end of a lightly loaded isotonic lever giving about 20 times magnification. Cumulative dose-response curves were obtained by gradually increasing the dose without washing out (Ariens and de Groot,[5] van Rossum and van den Brink,[6] van Rossum[7]). Between two cumulative dose-response curves obtained with serotonin, one maximal contraction was produced with furthretonium.

Chemicals

Serotonin-creatinin sulphate H_2O was obtained from Fluka, purified beef brain gangliosides Type II from Sigma Chemical Company, clostridium perfringens neuraminidase (activity 0·2 i.u./mg) from Worthington Company, vibrio cholerae neuraminidase, RDE and purified enzyme (activity 0·1 i.u./ml) from Behring–Werke. Drs. Boschman, Philips-Duphar, kindly provided influenza virus neuraminidase (activity 0·24 i,u./ml) and bis-(2,4,5-trichlor-phenoxy)-acetic acid. Synthetic *N*-glycolylneuraminic acid was a gift from Dr. H. Faillard, furthretonium from A. M. Simonis, *N*-salicylidene-D-glucosamine and *N*-D,L-α-propionic acid-ethylester-salicylidene)-D-glucosomine from Dr. H. Rudy. *N*-acetylneuraminic acid was prepared according to Kuhn and Baschang.[8] *N*-carboethoxy-, *N*-benzoyl- and *N*-cbo-neuraminic acid were synthesized according to a similar method of Wesemann and Zilliken[9].

RESULTS

Rat fundus strips exhibit an increase in response to 5-HT after preincubation with 10^{-5} M solutions of sialic acids or gangliosides for 15′. This effect was maximal after 60′ meanwhile the fundus strip was washed with tyrode solution. Fig. 1 shows

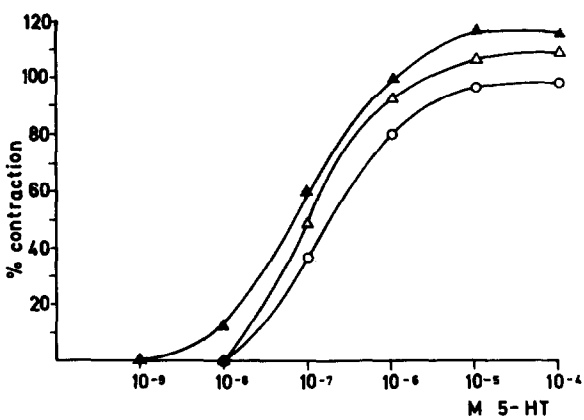

FIG. 1. The effect of NANA on the 5-HT log dose-response curve of the rat stomach fundus. The fundus preparation was incubated with 10^{-5} M NANA for 15 min. 5-HT log dose-response curve of the untreated preparation ○—○, 5 min △—△ and 60 min ▲—▲ after washing out the NANA.

a parallel shift of the 5-HT log-dose-response curve towards lower concentrations and an increase in maximal height after preincubation with NANA. The shift of the 5-HT log dose-response curve is expressed as a factor of sensitisation and not as a change of affinity (Table 1).

The increase in sensitisation is maximal 60′ after incubation with sialic acids or the ganglioside mixture and of about the same order of magnitude, while pyruvate and butyrate show no or almost no effect after this time. The maximal height of the 5-HT contraction though increased by all substances if the 5-HT dose-response curve is registered immediately after washing out shows a further increase only after pre-treatment with sialic acids and gangliosides.

TABLE 1. INFLUENCE OF 15′ PREINCUBATION WITH SIALIC ACIDS, GANGLIOSIDES, BUTYRIC ACID, AND PYRUVIC ACID ON THE 5-HT LOG DOSE-RESPONSE CURVE OF THE RAT FUNDUS

Compounds*	5′ after incubation sensitisation†	5′ after incubation maximal contraction‡	60′ after incubation sensitisation†	60′ after incubation max. contract. contraction‡	No. strips
N-acetyl-NA	6–8	110–115	7·5–8·5	115–120	32
N-cbo-NA	3–6	110–115	7–9	120–130	26
N-carboethoxy-Na	7–9	125–135	9–11	130–140	12
N-benzoyl-NA	2–5	125–130	8–12	135–145	8
N-glycolyl-NA	6–7	110–115	7·5–8·5	115–120	14
Gangliosides (mixture)	0	115–120	4–6	125–135	18
Pyruvate	2–5	130–140	0–2	120–125	6
Butyrate	1–2	105–115	0	100	8

* Each compound was tested in a concentration of 10^{-5} M.

† The sensitisation is expressed as factor of the shift of the log dose-response curve towards lower concentrations with the 5-HT contraction of the untreated strip as reference.

‡ The maximal 5-HT contraction of the untreated strip was set 100 per cent.

Incubation with 0·03 i.u. neuraminidase for 15′ results in a 20–30 per cent increase of the contraction and a shift to lower concentrations of a factor of 5 if the dose-response curve is registered immediately after the period of incubation. Sixty minutes after treatment with the enzyme a normal 5-HT dose-response curve was obtained. Fig. 2a illustrates the effect on the contraction obtained with neuraminidase from *vibrio cholerae*.

In order to demonstrate whether or not terminal sialic acid is indeed an integral part of serotonin receptors we added inhibitors of NANA-biosynthesis. Table 2

TABLE 2. INFLUENCE ON THE 5-HT DOSE-RESPONSE CURVE OF THE RAT FUNDUS BY 15′ PREINCUBATION WITH INHIBITORS OF NANA-BIOSYNTHESIS

Compound	conc. M	maximal contraction* %	No. strips
N-salicylidene-D-glucosamine	10^{-6}	128–132	10
	10^{-3}	10–30	
	3×10^{-3}	0	
N-[D,L-α-propionic acid-ethylester-salicylidene]-D-glucosamine	10^{-7}	120–130	7
	10^{-3}	0–5	
Bis-(2,4,5-trichlor-phenoxy)-acetic acid	$1·7 \times 10^{-5}$	40–60	21
	6×10^{-5}	0	

* The maximal 5-HT contraction of the untreated strip was set 100 per cent.

summarizes the effect of three typical inhibitors on the maximal contraction height. The strip preparations were incubated with these compounds for 15 min. The contraction is maximal, but reversibly inhibited 5 min after washing out the compounds. Fig. 2b shows the inhibition of the 5-HT contraction after incubation with a $1{\cdot}5 \times 10^{-5}$ M solution of *bis*-(2,4,5-trichlor-phenoxy)-acetic acid. An absolute and reversible

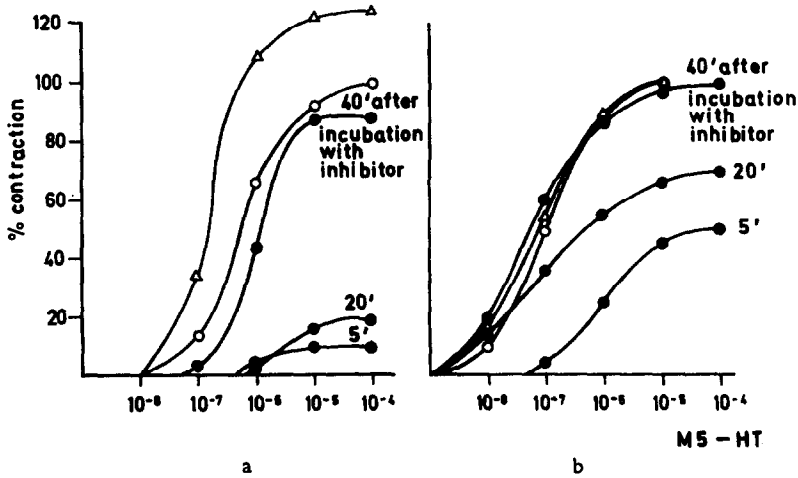

FIG. 2. The effect of 0·03 i.u. neuraminidase from *vibrio cholerae* and *bis*-[2,4,5-trichlor-phenoxyl]-acetic acid on the 5-HT log dose-response curve of the rat stomach fundus (a) 5-HT log dose-response curve before ○—○ and after △—△ an incubation period of 15 min with 0·03 i.u. neuraminidase from clostridium perfringens. ●—● 5-HT curves obtained 5, 20, and 40 min after incubation with 0·03 i.u. neuraminidase for 15 min followed by treatment with $1{\cdot}5 \times 10^{-5}$ M *bis*-[2,4,5-trichlor-phenoxy]-acetic acid for another 15 min. (b) 5-HT log dose-response curve before ○—○ and after △—△ an incubation period of 15 min with 0·005 per cent $CaCl_2$ ●—● 5-HT curves obtained 5, 20, and 40 min after incubation with 0·005 % $CaCl_2$ for 15 min followed by treatment with a $1{\cdot}5 \times 10^{-5}$ M solution of the inhibitor *bis*-[2,4,5-trichlor-phenoxyl]-acetic acid for another 15 min.

inhibition was obtained after pretreatment with 6×10^{-5} M *bis*-(2,4,5-trichlor-phenoxy)-acetic acid. Comparison of Figs. 2a and 2b shows the effect of neuraminidase and inhibitor alone as well as a combination of neuraminidase plus inhibitor treatment on the contraction. The contraction height drops to about 10 per cent if after 15′ pretreatment with neuraminidase the strip is incubated for another 15′ with the inhibitor. There also exists a significant difference in the recovery time between the strip incubated with neuraminidase plus inhibitor and the strip incubated with inhibitor and 0·005 % $CaCl_2$ instead of neuraminidase.

The fundus strips of eight rats, treated 12 days with 2×50 mg *bis*-(2,4,5-trichlor-phenoxy)-acetic acid per kg per die i.p., killed on the 14th day, showed quite normal 5-HT log dose-response curves. Another three rats were treated for 12 days with 100 mg inhibitor per kg/perdie peros. The response of the fundus strip towards furtheretonium was normal, but since it was impossible to wash out this drug no 5-H contraction could be obtained*.

* These experiments have been performed in collaboration with Doctors Boschman and Ockenfels.

DISCUSSION

Fig. 3 shows a diagram of the sialic acid metabolism based upon the work of Warren, Felsenfeld[10], and Roseman.[11]

It is still unknown whether NANA is incorporated as CMP-NANA into the glycoproteins and gangliosides. Warren[10] discusses the possibility that NANA-9-P is converted by a mutase to a labile NANA-2-P, an intermediate activated at the proper

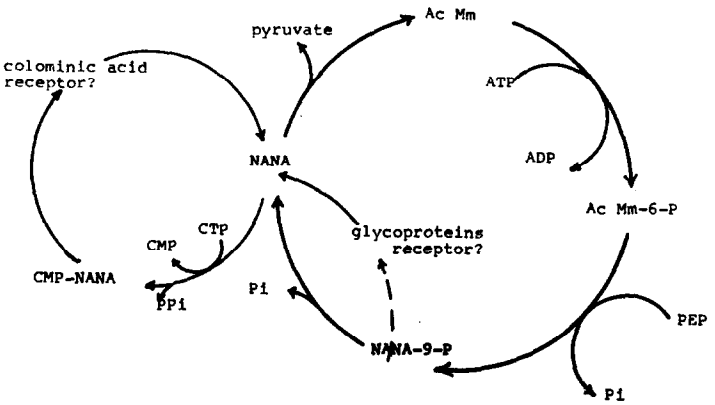

FIG. 3. Metabolism of *N*-acetylneuraminic acid.

site for insertion of the NANA moiety into mucopolysaccharides or glycoproteins. The diagram includes the 5-HT receptor to indicate the hypothesis that the receptor can be synthezised from a sialic acid free receptor precursor X and sialic acid (reaction 2). It has been shown that the 5-HT log dose-response curve is influenced by treating the fundus strip preparation with sialic acid and gangliosides, inhibitors of NANA biosynthesis and neuraminidases. These results can be interpreted with the reaction sequence:

(1) *N*-acetyl-D-mannosamine-6-P + PEP $\xrightarrow{\text{condensing enzyme}}$ NANA-9-P + P_i.

(2) $X + \boxed{\text{NANA}} \rightleftharpoons \boxed{\text{X-NANA}}$ (=receptor)

(3) $\boxed{\text{X-NANA}} + 5 - \text{HT} \rightarrow \boxed{\dfrac{\text{X} - \text{NANA}}{\text{5-HT}}}$ (=receptor complex)

(4) $\boxed{\dfrac{\text{X} - \text{NANA}}{\text{5-HT}}} \rightarrow X + \text{NANA} + \text{5-HT}$

Incubation with NANA (reaction 2) will increase the number of receptors and thus sensitize the 5-HT contraction. The symbol NANA shall indicate that the sialic acid most probably will react as an activated intermediate of *N*-acetyl or *N*-glycolylneuraminic acid. Table 1 shows that substitution at the amino-N of sialic acid does not influence the sensitisation but that a more lipophilic substituent as the benzoyl-group results in a higher increase of the maximal contraction. Since sialic acids stimulate the

5-HT contractions themselves the sensitizing effect of gangliosides can be explained in two ways: either the receptor is a ganglioside or the ganglioside just functions as a 'donator' for sialic acid. The ganglioside $G'_{lact.}$ first described by Kuhn and Wiegandt[2] and regarded as the 5-HT receptor by Woolley, Gommi[12] and Gielen,[3] suffices for both explanations. It gives the best restoration on the sialidase plus EDTA treated muscle preparation (Woolley and Gommi[12]) of all gangliosides so far tested and it also shows the maximal binding capacity towards 5-HT (Gielen[2]). On the other hand it is very easily hydrolyzed by sialidase thus yielding sialic acid which can be incorporated into the precursor of the receptor (= X).

Since it is most likely that the receptor consists of a hydrophilic acid group and a lypophylic part, butyrate was tested and found to be inactive. This demonstrated the specific response of the muscle strip towards the sialic acids and gangliosides. Even pyruvate—an intermediate of sialic acid biosynthesis—caused 1 hr after incubation almost no sensitisation, but well a high increase of the contraction.

Since it is not known how the sialic acids cause a sensitisation and an increase in maximal height, we prefer to express the shift of the 5-HT log dose-response curve as a factor of sensitisation rather than as a change in affinity 1/KA (the value KA being the dissociation constant of the drug-receptor complex) and the increase in maximal height as the percentage of the maximal contraction obtained with 10^{-4} M 5-HT at the untreated muscle strip.

Dr. Boschman* demonstrated in a cell free system that NANA-biosynthesis is inhibited by *N*-salicylidene-D-glucosamine, *N*-[D,L-α-propionic acid—ethylester--salicylidene]-D-glucosamine, and bis-[2,4,5-trichlorphenoxy-]-acetic acid. The latter compound specifically inhibits the condensing enzyme synthesizing NANA-9-P from *N*-acetyl-mannosamine-6-P and phosphoenolpyruvate (reaction 1). On the fundus preparation the inhibition of NANA-synthesis decreases the number of receptors (reaction 2). Thus the synthesis of the 5-HT receptor complex (reaction 3) will be diminished. In Fig. 2b it is shown that pretreatment with *bis*-[2.4.5-trichlor-phenoxy]-acetic acid decreases the contraction.

The 5-HT receptor complex (reaction 4) is split into precursor, NANA, and 5-HT. By incubation with neuraminidase the receptor (reaction 2, dotted line) and the 5-HT receptor complex (reaction 4) will be destroyed. The sensitizing effect of neuraminidase (Fig. 2a) may be explained in two ways: either treatment with neuraminidase enhances the whole sialic acid metabolism at the receptor site or it is not the synthesis but rather the breakdown of the 5-HT receptor complex into precursor X, NANA, and 5-HT which will create the stimulus. Incubation with neuraminidase and inhibitor should give a greater inhibition than incubation with the inhibitor alone for the number of receptors available for 5-HT binding is decreased by neuraminidase and the resynthesis of receptors is inhibited since there is less NANA synthesized after incubation with *bis*-(2.4.5-trichlor-phenoxy)-acetic acid. Though at first the contraction increases after sialidase treatment the contraction height drops strongly after incubation with the inhibitor (Fig. 2a) and even more than after incubation with inhibitor alone (Fig. 2b).

Though only a few steps of the complete reaction sequence for the synthesis and breakdown of the 5-HT receptor could be given, these experiments support the idea that sialic acid is an integral part of the 5-HT receptor. Furthermore there is a sialic

* Unpublished.

acid metabolism in the rat fundus which is closely related to the receptor function for 5-HT.

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