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Functional significance of synthesis of noradrenaline in adrenergic nerves of rat salivary glands

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The secretory response and the noradrenaline content of the submaxillary gland due to continuous electrical stimulation of the cervical sympathetic trunk with 5 Hz (5 V, 2 ms) were determined in five differently treated groups of rats. Stimulation during 5 h in untreated control rats reduced the noradrenaline content to about 40% while the secretory response remained unchanged. Another group received DL-α-methyl-*m*-tyrosine 400 mg kg^{-1} i.p. 26 and 18 h before the stimulation and 200 mg kg^{-1} 2 h before. This treatment displaced 93% of the noradrenaline content of the glands, and the secretory response stabilized after an initial decrease, at a level of about two-thirds of the normal response, possibly indicating a reduced rate of transmitter synthesis compared to normal rats. Thus, a nerve with greatly reduced amounts of active transmitter in the store can satisfy rather high demands of transmitter release for long time periods, provided that the synthesis continues. A third group of rats received DL-α-methyl-*m*-tyrosine in the same doses as above but 50, 42 and 26 h before the experiments, respectively. After this treatment, the noradrenaline level was reduced to about 35% of normal, yet the secretory response was even higher than normal. Probably the decreased transmitter synthesis supposed to occur following treatment with α-methyl-*m*-tyrosine has been reversed at this time interval into an increased synthesis rate. To a fourth group of rats 500 mg kg^{-1} DL-α-methyl-*p*-tyrosine methylester HCl (H 44/68) was injected i.p. 15 min before stimulation, resulting in a virtually complete inhibition of the tyrosine hydroxylase. Three h of stimulation reduced noradrenaline to 15%, and the secretory response disappeared. When treatment with both drugs was given, a very rapid disappearance of an initially normal response was seen. In conclusion, synthesis of new transmitter appears to be essential for a normal function of the nerve.

A functional subdivision of the transmitter store into one small, easily available fraction and one larger, more firmly bound storage pool was proposed for cholinergic preganglionic nerves by Perry in 1953. Trendelenburg (1961) achieved experimental evidence indicating that such an organization might also be true for the adrenergic system. In addition, Hillarp (1960) proposed the existence of different pools of catecholamines in the adrenal medulla. Strong evidence in favour of this hypothesis of a compartmentalization of the transmitter store in the noradrenaline nerves resulted from experiments with various agents (*e.g.* (+)-adrenaline, metaraminol, α-methyl-*m*-tyrosine) capable of producing displacement of the major part of the total noradrenaline store by weaker analogues without apparently disturbing nerve function (Andén, 1964a, b, 1965; Andén & Magnusson, 1964; Carlsson, 1964). Apparently the transmitter synthesis alone is of enough magnitude to cope with the

demand. In addition, Kopin, Breese & others (1968) reported a preferential release of newly synthesized transmitter. On the other hand, when transmitter synthesis is blocked, nerve function cannot be kept at a normal level even if part of the transmitter store is intact (Almgren, 1971). Thus it was of interest to study, in one and the same organ, transmitter levels and nerve function after either replacement of the transmitter store by metaraminol (i.e. after treatment with α-methyl-*m*-tyrosine) or inhibition of transmitter synthesis (using α-methyl-*p*-tyrosine, an inhibitor of tyrosine hydroxylase) or after a combination of both these drug regimens. The objective was to assess the importance of the transmitter synthesis *versus* the transmitter store for keeping the function of the adrenergic nerves intact.

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing about 250 g, were used, one group as untreated controls; another group received DL-α-methyl-*m*-tyrosine (α-MmT, Regis Chemical Co. Ltd., 400 mg kg^{-1}, i.p. 26 and 18 h before and 200 mg kg^{-1}, i.p. 2 h before the start of the stimulation) and a third group had the same treatment 50, 42 and 26 h before the start of the stimulation; a fourth group received DL-α-methyl-*p*-tyrosine methylester hydrochloride (α-MpT, H 44/68, AB Hässle, Mölndal, 500 mg kg^{-1}, i.p.) 15 min before the stimulation. A fifth group was pretreated with a combination of these two drugs in the same doses and according to the same time schedules as in the second and the fourth groups. The rats were subsequently anaesthetized with urethane (1 g kg^{-1}, i.p.). The excretory duct of the submaxillary gland on one side was isolated near its entrance into the mouth, and a fine glass cannula (o.d. 0·5 mm) was inserted into the duct (Ohlin, 1965). The cervical sympathetic trunk of one side was isolated and electrically stimulated preganglionically as described earlier (Almgren, 1971), using supramaximal, monophasic pulses (5V, 2ms) at a frequency of 5 Hz. The secretory response of the submaxillary gland to the sympathetic stimulation was recorded by collecting the saliva from the cannula in tared small plastic tubes during consecutive periods of 30 min and reweighing them. Error due to evaporation was ascertained to be too small to affect the recorded values.

In the control rats and in the rats treated with α-MmT alone stimulation was continued for 5 h. In the two groups of rats treated with α-MpT stimulation was continued throughout the 30 min period during which the secretory response disappeared.

Immediately after stimulation the submaxillary plus sublingual glands of both sides were taken out, weighed, placed on dry-ice and stored at −25° for analysis of the noradrenaline content. The amount of saliva secreted should relate to the secretory cell mass of the glands; responses recorded were therefore expressed as mg saliva g^{-1} salivary gland. As the stimulation affected the weight of the glands in some but not all of the groups, the weight of the contralateral unstimulated gland was used as reference.

The effect of metaraminol on salivary secretion was also tested. Three rats, pretreated with protriptyline 10 mg kg^{-1} 30 min before the experiment, received (±)-metaraminol in cumulative doses by a catheter inserted into a femoral vein. The saliva appearing during 15 s following each injection was collected and weighed, dose-response curves were drawn and the dose needed to give a secretion of 10 mg saliva g^{-1} salivary gland was estimated from each dose-response curve.

Two glands were required for each analysis of noradrenaline. It was extracted with 10 ml 0·4N perchloric acid, isolated and purified on strong cation exchange columns (Dowex 50W-X4, 4 × 128 mm at pH 0) and assayed spectrophotofluorimetrically (Bertler, Carlsson & Rosengren, 1958). The recovery through the entire procedure was in the range 75–80% and was not corrected.

RESULTS

Weight of the salivary glands

The mean weight of the unstimulated submaxillary plus sublingual glands from the untreated rats was 0·239 ± 0·0107 g. Stimulation reduced the glandular weight to 0·228 ± 0·0096 in this group (n = 8; $P<0.01$, *t*-test, process of pairing (Davies, 1949). Also after treatment with α-MmT alone stimulation reduced the weight of the glands from 0·227 ± 0·0097 to 0·215 ± 0·0115 (n = 10; $P<0.025$, *t*-test, process of pairing). In the other groups no significant difference in glandular weight was seen after stimulation.

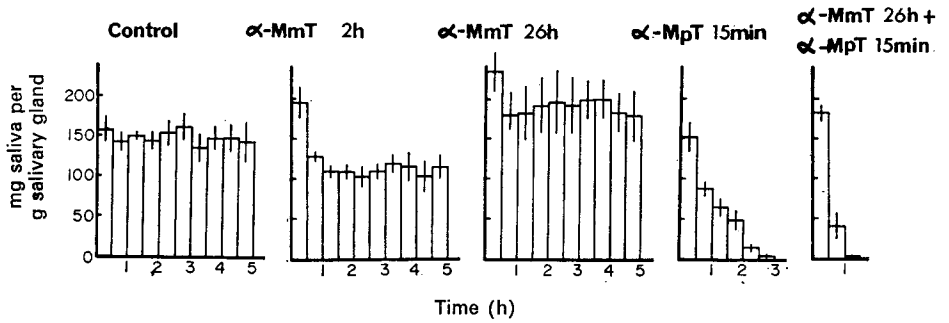

FIG. 1. Secretory response of rat submaxillary glands to stimulation of the cervical sympathetic with 5 Hz. Rats were either untreated (n = 8), treated with DL-α-methyl-*m*-tyrosine [latest dose 2 h (n = 5) or 26 h (n = 5) before the stimulation], with DL-α-methyl-*p*-tyrosine methylester HCl 15 min before stimulation (n = 8), or with a combinaton of α-methyl-*m*-tyrosine and α-methyl-*p*-tyrosine (n = 3). For details see Methods. Each bar represents the mean value of the amount of saliva collected during a period of 30 min expressed as mg per g of salivary gland. The vertical lines are s.e. Statistical comparison was made by the Mann-Whitney U-test (Siegel, 1956).

Secretory responses

No spontaneous secretion was seen from the submaxillary glands. The response to continuous stimulation is shown in Fig. 1. In the untreated control rats the response to sympathetic stimulation with 5 Hz was steady at about 150 mg saliva per g salivary gland in 30 min, i.e. about 5 mg g^{-1} min^{-1}. As about one-third of the glandular weight represents the sublingual gland, which is devoid of sympathetic secretory innervation (Andén, Norberg & Olson, 1966), and from which saliva was not collected, it means that the submaxillary gland of the rat at an impulse frequency in the sympathetic supply of 5 Hz continuously produces an amount of saliva equal to its own weight in about 2 h.

After pretreatment with α-MmT (400 + 400 + 200 mg kg^{-1} 26, 18 and 2 h, respectively before stimulation) the secretory response initially declined during the first hour and was then steady at about 100 mg saliva per g salivary gland in 30 min. This difference compared to the control rats was statistically significant after 1 h ($P<0.025$, Mann-Whitney U-test, Siegel, 1956).

When the experiments were started 26 h after the last administration of α-MmT, the salivary secretion in response to sympathetic nerve stimulation with 5 Hz was increased to around 190 mg saliva per g of gland in 30 min after the first 30 min, which is significantly higher than in the control rats ($P<0{\cdot}05$), at two intervals between 3 and 4 h after the start of the stimulation (Mann-Whitney U-test).

Treatment with α-MpT, 500 mg kg^{-1} 15 min before stimulation, did not affect the initial secretory response, but thereafter a gradual decrease was recorded. After 3 h of stimulation the secretion had stopped.

Also after the combined treatment with α-MmT and α-MpT the initial secretory response was unchanged, but then disappeared within 1 h.

The mean dose of (±)-metaraminol required to give a salivary secretion of 10 mg g^{-1} salivary gland in 15 s in rats pretreated with protriptyline, an inhibitor of neuronal uptake, amounted to 96·4 ± 19·79 μg (n = 3).

The corresponding dose for noradrenaline (in protriptyline-treated rats) has been obtained in other experiments, performed in parallel (Almgren & Jonason, 1973), and amounts to 6·3 ± 2·59 μg (n = 5), i.e. metaraminol seems to be about 15 times weaker as an agonist in the rat salivary gland.

Content of noradrenaline

The results of the noradrenaline analyses are presented in Table 1. Continuous stimulation for 5 h reduced the content of the glands in untreated animals to 36%, and in the animals treated with α-MpT 3 h of stimulation reduced the noradrenaline

Table 1. *Noradrenaline concentration (μg g^{-1}) of rat salivary glands after treatment with α-methyl-*m*-tyrosine, α-methyl-*p*-tyrosine or both.* Rats were either untreated, or treated with DL-α-methyl-*m*-tyrosine (α-MmT, 400 + 400 + 200 mg kg^{-1} i.p., 26, 18 and 2 h, respectively, before the start of the stimulation or the corresponding doses 50, 42 and 26 h, respectively, before stimulation), or with DL-α-methyl-*p*-tyrosine methylester HCl (α-MpT, 500 mg kg^{-1} i.p., 15 min before the start of the stimulation) or with the combination of both drug regimens, α-MmT given 26, 18 and 2 h before stimulation (for details see methods). Assays were made on samples of two identically treated glands. The values are given in μg g^{-1} and are means ± s.e. Figures within brackets are number of samples.

Treatment (time after last dose)	Untreated	α-MmT (2 h)	α-MmT (7 h)	α-MmT (26 h)	α-MmT (31 h)	αMpT (3 h 15 min)	α-MmT (3·5 h) + α-MpT (1 h 45 min)
Time of stim. (h)	5	0	5	0	5	3	1·5
Unstimulated glands	1·16 ±0·082 (4)	0·08** ±0·037 (3)	0·01** ±0·010 (3)	0·40** ±0·019 (3)	0·18** ±0·029 (3)	1·06 ±0·059 (4)	0·02** ±0·015 (2)
Stimulated glands	0·42 ±0·014 (4)		0·03** ±0·015 (3)		0·26* ±0·068 (3)	0·14** ±0·017 (4)	0·05** ±0·015 (2)

* differs from corresponding untreated control, $P<0{\cdot}025$

** differs from corresponding untreated control $P<0{\cdot}001$

(*t*-test after one-way analyses of variance).

content to 13% of that on the unstimulated side ($P<0{\cdot}001$, *t*-test after one-way analysis of variance, Davies, 1949, and this reduction was significantly greater than in the untreated animals, $P<0{\cdot}001$).

Two h after the last injection of α-MmT only about 7% of the normal noradrenaline content of the salivary glands remained. 24 h later the noradrenaline content had increased to approximately 35%. When these rats were anaesthetized and stimulated for 5 h, the content was reduced both in the stimulated and in the unstimulated gland. When the rats were treated with α-MmT and α-MpT together and stimulated on one side for 1·5 h, the noradrenaline levels of both the stimulated and the unstimulated glands were near zero.

DISCUSSION

The secretory response to prolonged continuous stimulation of the rat submaxillary gland with 5 Hz was found to be constant over at least 5 h (*cf.* Emmelin & Engström, 1960). This finding is especially interesting as the noradrenaline level of the stimulated gland was reduced rapidly and markedly during this period (*cf.* also Fredholm & Sedvall, 1966). In the present study 36% of the normal noradrenaline content was found in glands stimulated for 5 h and after 90–120 min of stimulation with 5 Hz a steady-state is reached at a level of about 0·35–0·50 $\mu g\ g^{-1}$, i.e. 30–40% of normal (Almgren, to be published). Previously (Almgren, 1971) a mean noradrenaline content of 25% of control was found in rat salivary glands after 3 h of stimulation with 5 Hz. In those experiments, however, the noradrenaline content of one salivary gland could be used for the assay, increasing the uncertainty at low values. If rate. a steady-state level is reached, transmitter output may be assumed to equal synthesis Apparently the synthesis of transmitter, admittedly higher during stimulation than during neuronal test (see *e.g.* Sedvall, Weise & Kopin, 1968), is of sufficient magnitude to give a full effector response.

The decarboxylation products of the amino-acid α-methyl-*m*-tyrosine, α-methyl-*m*-tyramine and metaraminol (Carlsson & Lindqvist, 1962) effectively reduce the noradrenaline store of the adrenergic nerve terminals in all probability by a stoichiometric displacement (Andén, 1964b; Carlsson, 1964; Shore, Busfield & Alpers, 1964).

In the present study 93% of the noradrenaline store of the rat submaxillary gland was depleted 2 h after the last treatment with α-MmT. The initial secretory response was not significantly greater than that of the untreated control rats. During the first hour of stimulation the response decreased, but was thereafter constant, and persisted in spite of a virtually total absence of noradrenaline from the stores. It must therefore have been maintained by the synthesis and immediate release of new transmitter. As this response was about 70% of that of untreated control rats, it seems unlikely that the transmitter release, and thus the synthesis rate, was the same for these two groups. Transmitter synthesis may have been reduced during this interval following treatment with α-MmT, due to an inhibitory effect on the L-aromatic amino-acid decarboxylase by α-MmT itself (*cf.* Andén, 1964b). The finding that 26 h after the last injection of α-MmT the secretory response to nerve stimulation was again normal or increased may be in favour of such a mechanism. After this time interval only small amounts of α-MmT remain in the tissues (Udenfriend & Zaltzman-Nirenberg, 1962), but the noradrenaline level of the salivary gland is still reduced to 35% of normal.

After treatment with the tyrosine hydroxylase inhibitor α-MpT (Spector, Sjoerdsma & Udenfriend, 1965; Corrodi & Malmfors, 1966) noradrenaline levels and the secretory response of the submaxillary gland were reduced in parallel following nerve stimulation (Fig. 1, *cf.* Almgren, 1971; Fig. 2). In the present study 12% of the normal noradrenaline content of the salivary gland was present in glands stimulated for 3 h after treatment with α-MpT at a time when no secretory response was any longer elicited by the stimulation. In rats treated with α-MmT a lower content of noradrenaline was found, and yet the secretory response was reduced only to 30%. It seems likely that when the transmitter store is not replenished by new noradrenaline synthesis, it decreases exponentially as does fractional release and effector response.

When both α-MmT and α-MpT were administered to the rats, the effector response to nerve stimulation disappeared rapidly. In this case synthesis was blocked and the store of noradrenaline had already been reduced to 7%. This amount of noradrenaline was apparently sufficient to elicit a normal response in the salivary gland, following sympathetic nerve stimulation, during the initial period of 30 min. It is unlikely that the decarboxylation products of α-MmT, mainly metaraminol, contribute much to the response, since metaraminol was found to have a much lower potency on this receptor than noradrenaline.

It may seem paradoxical that the noradrenaline level tended to be more reduced in the unstimulated than in the stimulated glands after treatment with α-MmT. Two factors, which both should tend to give such an effect, may be pointed out, namely (1) stimulation of the gland increases the synthesis rate of noradrenaline over that of the unstimulated gland (see *e.g.* Sedvall & Kopin, 1967), and (2) a greater displacement of noradrenaline occurs in the unstimulated than in the stimulated gland by circulating decarboxylation products of α-MmT.

The results presented here clearly demonstrate that an intact synthesis of transmitter is of profound importance for the normal adrenergic nerve function, and adrenergic nerves with markedly reduced transmitter stores can function for a long time, even under high demands, provided that the synthesis of transmitter is intact.

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