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RELATIONSHIP BETWEEN ACCUMULATION, STORAGE AND OVERFLOW OF NORADRENALINE IN THE RAT SALIVARY GLAND AFTER CHRONIC TREATMENT WITH GUANETHIDINE

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1 The effect of guanethidine on the endogenous noradrenaline (NA) content, accumulation and overflow of [^{3}H]-noradrenaline ([^{3}H]-NA) in the rat salivary gland was examined at various times after drug administration.

2 Twenty-four h after a single injection of guanethidine (1 or 10 mg/kg s.c.), the respective values for the endogenous NA content and for the accumulation and overflow of [^{3}H]-NA were approximately 55, 85, 30%, and 15, 55, 10% of the controls.

3 Although [^{3}H]-NA accumulation had returned to control levels within 48 h after the dose of 10 mg/kg guanethidine, the overflow of [^{3}H]-NA evoked by electrical stimulation or excess potassium (K^+) remained depressed.

4 After the low or the high dose of guanethidine, the NA content of the salivary gland was restored to about 50% of the normal value between 4 to 24 and 48 to 72 h, respectively.

5 The accumulation of [^{3}H]-NA was inhibited by about 75% by cocaine. The same degree of inhibition was obtained 4 h after 10 mg/kg guanethidine. In these experiments phenoxybenzamine did not reduce the residual (25%) uptake.

6 The reasons for differential rates of recovery of the endogenous NA content and the storage of [^{3}H]-NA after guanethidine are discussed.

Introduction

Administration of guanethidine to experimental animals produces three prominent effects. First, guanethidine interferes with the stimulation-induced release of noradrenaline (NA) or [^{3}H]-NA (Gaffney, Chidsey & Braunwald, 1963; Kirpekar & Furchgott, 1972; Khan & Malik, 1978). Second, it blocks neuronal uptake of exogenous NA (Hertting, Axelrod & Whitby, 1961; Lundborg & Stitzel, 1968); and finally, it reduces the endogenous NA content of various peripheral organs (Cass & Spriggs, 1961; Chang, Costa & Brodie, 1965; Fielden & Green, 1967). In contrast to the bulk of literature available on the long-term effects of reserpine, very few studies deal with the long-term effect of guanethidine on storage, accumulation and release of NA in sympathetic neuroeffector organs. Therefore, in the present study guanethidine was injected into rats, and their salivary glands were removed at various times for the analysis of endogenous NA and the accumulation and overflow of [^{3}H]-NA. A preliminary report of these findings has already appeared (Khan & Wakade, 1977).

Methods

Male albino rats weighing 250 to 300 g were injected with a single dose of guanethidine (1 or 10 mg/kg), subcutaneously. They were killed by a blow on the head at various times (4, 24, 48, 72 and 144 h) after the guanethidine injection and the submaxillary glands were removed. In all experiments the sublingual gland, which is located on top of the submandibular gland and contains insignificant amounts of NA (A.R. Wakade, unpublished observations), was separated, and the submandibular gland was used and is referred to as the salivary gland in this study.

Incubation with [^{3}H]-noradrenaline

Salivary glands were incubated in 5 ml of oxygenated (95% O_2 and 5% CO_2) Krebs solution containing 100 ng/ml of [^{3}H]-NA (specific activity, 7.9 Ci/mmol) at 37°C for 30 min. The composition of the Krebs solution was as follows (mmol/l): NaCl 119, KCl 4.7,

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$CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11. After incubation, the salivary glands were washed four times with Krebs solution over a period of 40 min. One salivary gland from each rat was used to study the accumulation of [^{3}H]-NA and tissue NA content, and the contralateral gland from the same animal was used to study overflow of [^{3}H]-NA.

Transmural stimulation and excess K^+

For the purpose of transmural stimulation, tissues were mounted in a holder between two platinum-plate electrodes. Each tissue was secured to the bottom hook of the holder by means of a cotton thread. Another thread was tied to the upper end of the tissue and to the upper hook to hold the tissue firmly between the two platinum-plate electrodes. The electrodes, along with the tissue, were placed in a cylindrical tissue chamber containing 12 ml of Krebs solution at 37°C, through which 95% O_2 and 5% CO_2 was continuously bubbled. The solution contained 10 μg/ml of disodium ethylenediaminetetraacetic acid and 100 μg/ml of ascorbic acid, to prevent oxidation of NA.

At appropriate intervals before electrical stimulation the solution in the tissue chamber was exchanged for fresh fluid. The tissue was then stimulated transmurally by a train of 300 impulses (5 Hz, 80 V, 1 ms) from a Grass stimulator, Model S-88, and the solution present during stimulation was replaced by fresh solution. To obtain the net overflow produced by stimulation, the amount of radioactivity spontaneously overflowing within the immediately preceding equivalent time period was subtracted from the total amount overflowing during the stimulation period. In most instances, overflow is expressed as ct/min per impulse by dividing the total net overflow by the number of shocks (300) given during the stimulation period. K^+-induced overflow was achieved by adding 0.95 ml of 1.8 M KCl solution to 12 ml of tissue chamber fluid for 2 min. At 15 min intervals the muscle chamber solution was removed to obtain background and K^+-induced overflow.

Extraction and analysis

After the last wash, salivary glands were rapidly blotted, weighed, and transferred to a plastic tube containing 3 ml of ice-cold 0.4 N perchloric acid. Tissues were homogenized in a Polytron homogenizer (Brinkman Instruments, Westbury, N.Y.). The homogenate was transferred to a graduated centrifuge tube and the homogenizer tube was rinsed with 2 ml of 0.4 N perchloric acid, which was then added to the tube to bring the final volume to 5 ml. After centrifugation of the homogenate, an aliquot from the supernatant was analyzed for total endogenous NA content and accumulation of [^{3}H]-NA. Aliquots (1 ml) of the solution from the tissue chamber, before and after transmural stimulation or excess K^+, were also acidified with 1.0 ml of 0.8 N perchloric acid for the analysis of [^{3}H]-NA and its ^{3}H-metabolites. Endogenous NA, [^{3}H]-NA and ^{3}H-metabolites were separated by the alumina adsorption method described by Shellenberger & Gordon (1971). In all cases, a standard solution of NA was analyzed concurrently, with recoveries ranging from 75 to 85%. In this method there may be small amounts of ^{3}H-deaminated catechols eluted from alumina along with [^{3}H]-NA. Therefore, in some experiments ^{3}H-metabolites were separated from [^{3}H]-NA of the alumina eluated by the extraction method described by Crout (1964). Radioactivity was measured in a 3-channel Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3314). Appropriate corrections for recovery of NA in alumina eluate, dilutions and quenching effects have been made.

Drugs

The following drugs were used: guanethidine sulphate (Ciba Pharmaceutical Co., Summit, N.J.); (−)-noradrenaline bitartrate (Nutritional Biochemicals Corporation, Cleveland, Ohio); (−)-[^{3}H]-noradrenaline (New England Nuclear, Boston, Mass.); phenoxybenzamine hydrochloride (Smith, Kline & French Laboratories, Philadelphia, Pa.); cocaine hydrochloride (Merck, Sharp & Dohme, Westpoint, Pa.).

Results

Effect of guanethidine on noradrenaline content and accumulation of [^{3}H]-noradrenaline in the salivary gland

The endogenous NA content of the salivary gland of the normal rat was 1.48 ± 0.05 μg/g. Four, 24, 72 and 144 h after guanethidine (1 mg/kg), it was 0.57 ± 0.05, 0.85 ± 0.03; 1.16 ± 0.01, and 1.67 ± 0.09 μg/g, respectively (Figure 1). The accumulation of [^{3}H]-NA by the salivary glands of untreated rats was 879 ± 41 ct $min^{-1}mg^{-1}$. Guanethidine 1 mg/kg had no significant inhibitory effect on the accumulation of [^{3}H]-NA in the salivary gland (Figure 1). The high dose (10 mg/kg) of guanethidine caused a greater depletion of endogenous NA content compared with that caused by the lower dose. A maximum reduction of about 85% of the endogenous NA stores was attained at 4 and 24 h after the high dose of guanethidine. A gradual recovery in the NA content of the salivary gland was seen after a single dose of guanethidine, and by about 72 to 144 h the values were near normal levels. The accumulation of [^{3}H]-NA after

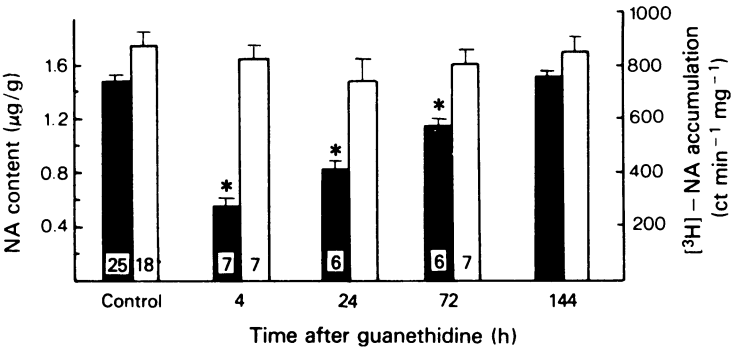

Figure 1 Effect of 1 mg/kg guanethidine on the noradrenaline (NA) content and the accumulation of [^{3}H]-NA in the salivary gland. Solid columns represent NA content, and open columns represent accumulation of [^{3}H]-NA in the salivary gland. Number of experiments is given at the bottom of each column, and vertical bars represent s.e.; * denotes significant difference ($P < 0.005$) between NA content before and after guanethidine treatment.

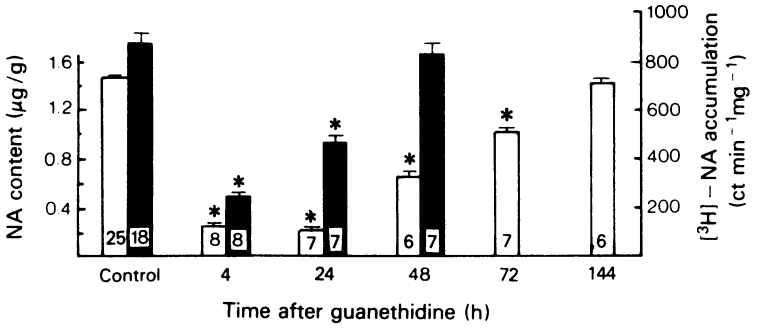

Figure 2 Effect of 10 mg/kg guanethidine on the noradrenaline (NA) content and the accumulation of [^{3}H]-NA in the salivary gland. Solid columns represent accumulation of [^{3}H]-NA, and open columns represent NA content of the salivary gland. Number of experiments is given at the bottom of each column, and vertical bars represent s.e.; * denotes significant difference ($P < 0.005$) between NA content or accumulation of [^{3}H]-NA before and after guanethidine treatment.

guanethidine (10 mg/kg) at 4, 24 and 48 h was 244 ± 5, 474 ± 32 and 827 ± 56 ct min^{-1} mg^{-1} respectively (Figure 2).

Effect of guanethidine on the overflow of [^{3}H]*-noradrenaline in the salivary gland*

Transmural stimulation (5 Hz for 1 min) of the salivary gland resulted in a marked efflux of [^{3}H]-NA. These results are shown in Figure 3. In the normal salivary gland the net overflow averaged 8.76 ± 0.73 ct min per impulse. The overflow of [^{3}H]-NA by transmural stimulation was maximally inhibited 4 h after guanethidine. Partial recovery of stimulation-induced overflow was observed after 24 to 48 h, and overflow after 72 h was almost comparable to that found in the untreated salivary gland.

In another series of experiments, [^{3}H]-NA overflow was evoked by raising the external K^+ concentration. The results of these experiments are also included in Figure 3. Exposure of the untreated salivary gland to K^+ (150 mM for 2 min) caused a 250% increase in [^{3}H]-NA overflow over the background. The overflow of [^{3}H]-NA by excess K^+ was also maximally reduced 4 h after guanethidine (10 mg/kg); 24 h after guanethidine, K^+-induced overflow was markedly depressed. However, the degree of inhibition was comparatively less in the case of K^+-induced overflow than electrically-induced overflow (56.2 and 89.6%, respectively). Almost 67% recovery in the overflow occurred after 48 h, and after 72 h K^+-evoked overflow was practically restored to normal levels.

Comparison of the inhibitory effects of cocaine, phenoxybenzamine and guanethidine on the accumulation of [^{3}H]*-noradrenaline*

The results of these experiments are shown in Figure 4. Fifteen-minute exposure of the normal salivary gland

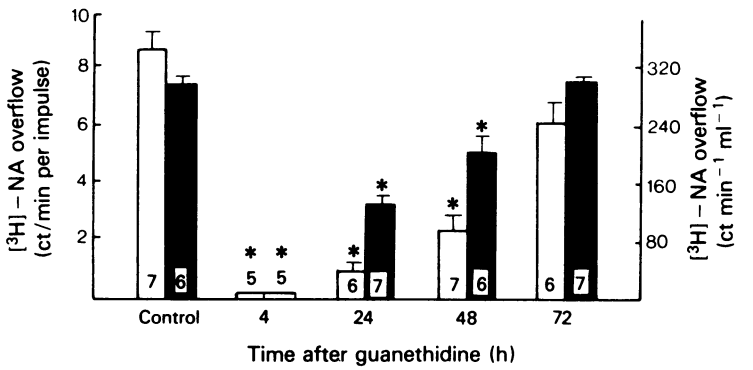

Figure 3 Effect of guanethidine on the overflow of [^{3}H]-noradrenaline ([^{3}H]-NA) by electrical stimulation or by excess K^+. Overflow of [^{3}H]-NA was evoked by transmural stimulation (300 impulses at 5 Hz, open columns), or by excess K^+ (150 mM for 2 min, solid columns). Number of experiments is given at the bottom of each column, and vertical bars represent s.e.; * denotes significant difference ($P < 0.005$) between the overflow of [^{3}H]-NA caused by transmural stimulation or excess K^+ before and after guanethidine treatment.

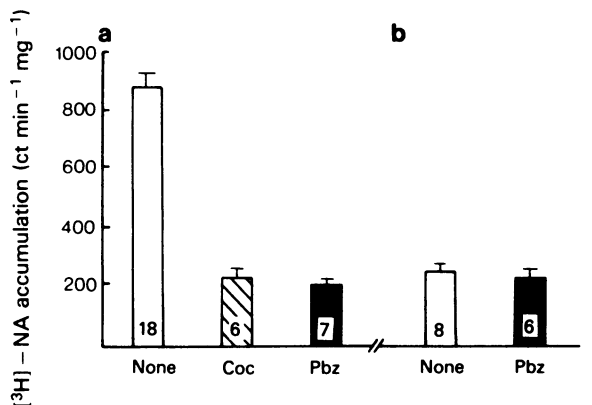

Figure 4 Effect of cocaine and phenoxybenzamine on the accumulation of [^{3}H]-noradrenaline ([^{3}H]-NA) by normal and guanethidine-treated salivary glands. Salivary glands were obtained from normal (a) and from rats given guanethidine (10 mg/kg, s.c.) 4 h previously (b). The glands were incubated with cocaine (Coc) (10 μg/ml) for 15 min, or with phenoxybenzamine (Pbz) (10 μg/ml) for 20 min, and then incubated with [^{3}H]-NA (100 ng/ml) for 30 min, followed by a 40 min wash period. The number of experiments is given at the bottom of each column, and the vertical bars represent s.e.

to cocaine (10 μg/ml) led to approximately 75% reduction in the accumulation of [^{3}H]-NA. Although not shown, higher concentrations of cocaine (30 μg/ml) had no additional effect on the accumulation. In another group, salivary glands from normal rats were incubated in Krebs solution containing phenoxybenzamine (10 μg/ml) for 20 min, and then in the continued presence of phenoxybenzamine the tissue was incubated with [^{3}H]-NA for 30 min. As shown in Figure 4, accumulation of [^{3}H]-NA was reduced by about 75%. As described earlier, 4 h after the injection of guanethidine (10 mg/kg), [^{3}H]-NA accumulation by the isolated salivary gland was depressed by about 72%, which was not further reduced by *in vitro* treatment of the salivary gland with phenoxybenzamine.

Discussion

In our studies, the rate of depletion of NA stores produced by guanethidine from the salivary gland is consistent with that reported in rat heart, spleen and intestine (Cass & Spriggs, 1961; Spriggs, 1966), and in mouse heart (Fielden & Green, 1967). In the rat salivary gland we found a recovery of about 45% of the control levels 48 h after guanethidine, and complete restoration was seen after 144 h. Despite the above-mentioned variation in the rates of recovery of NA, it is quite clear that recovery occurs at a much faster rate after guanethidine than it does after a similar degree of depletion by reserpine. In the rat salivary gland, 0.1 mg/kg reserpine produced about 90% loss of NA in 24 h, and even after 8 days NA stores were 50% below normal (Wakade, Rosenberg & Mark, 1976). The reasons for such differences in recovery after depletion by guanethidine and reserpine are not known. One possibility is that guanethidine may produce depletion by displacing NA from storage vesicles of sympathetic nerve terminals, an action analogous to that of tyramine. Previously, Durant, Roe & Green (1970) made a similar suggestion to explain the depletion produced by guanethidine. If physical displacement of stored NA is the main reason for guanethidine-induced depletion, then recovery of depleted stores should largely depend on the rapid

removal of guanethidine from the storage sites. In the case of reserpine, it is felt that depletion is neurogenic in origin; that is, a major portion of stores lose their NA via exocytotic release of the neurohumoral substance (cat salivary gland, Hertting, Potter & Axelrod, 1962; rat adrenal gland, Dixon, Garcia & Kirpekar, 1975).

Accumulation of [^{3}H]-NA was restored to the control level at a time (48 h) when the endogenous NA content of the salivary gland was still 45% of the control. This observation suggests that the effect of guanethidine on neuronal uptake mechanism must have worn off by this time, thus allowing exogenous NA to enter the neurone. A relative delay in restoration of the endogenous NA content compared with the ability to accumulate [^{3}H]-NA may be due to a slow rate of synthesis. Surprisingly, though the ability to accumulate [^{3}H]-NA was almost restored to normal in salivary glands removed 48 h after guanethidine, it was not accompanied by a similar recovery in the overflow of [^{3}H]-NA in response to nerve stimulation. Failure of release can result if accumulated [^{3}H]-NA is in extravesicular sites of the sympathetic neurone and, therefore, unavailable for release by electrical stimulation (Wakade & Kirpekar, 1974). Since cocaine reduced [^{3}H]-NA accumulation by about 75%, it is reasonable to assume that a major portion of [^{3}H]-NA was in the neurone. Further, it is believed that intraneuronal NA, if not stored by the vesicles, is rapidly deaminated within the neurone by monoamine oxidase (Wakade & Furchgott, 1968; Furchgott & Garcia, 1968). We found that accumulation of unmetabolized [^{3}H]-NA was comparable to that in the normal gland, and therefore it must have been within the storage granules. An alternative explanation for the dissociation between accumulation and failure of release could be that the neuronal blocking effect of guanethidine persists for a longer period than its effect on neuronal uptake sites.

Why guanethidine is less effective in blocking K^+-induced release is not clear. Similar findings were reported by Kirpekar, Wakade, Dixon & Prat (1969) in the perfused cat spleen.

Attempts made to block the residual accumulation of [^{3}H]-NA in cocaine- and guanethidine-treated salivary glands by a high concentration of phenoxybenzamine, which blocks extraneuronal uptake (Avakian & Gillespie, 1968), were unsuccessful. The source of this residual accumulation of [^{3}H]-NA remains obscure. In this context it should be noted that even after almost complete degeneration of sympathetic neurones of the salivary gland, about 30% accumulation of exogenous NA was found, and this was not affected by phenoxybenzamine. (Wakade, 1978).

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