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PREVENTION BY GUANETHIDINE ANALOGUES OF OUTPUT OF NORADRENALINE INDUCED BY SODIUM REDUCTION IN RABBIT VENTRICULAR SLICES

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1 The prevention by guanethidine and related agents of the output of noradrenaline induced by low sodium was investigated in rabbit ventricular slices. When external NaCl was reduced, the output of noradrenaline into the medium collected at 30 min intervals, increased and the endogenous levels decreased. These changes induced by replacing sodium with sucrose or choline were not affected either by the omission of calcium and addition of 0.5 mM ethylene glycol-bis(aminoethyl-ether)N,N,N′,N′ tetra-acetic acid (EGTA) or by an increase in the calcium concentration to 10 mM 30 min before sodium deprivation.

2 Guanethidine 4×10^{-6} and 4×10^{-5} M and 4-7-exo-methylene-hexahydroisoindoline-ethyl guanidine (No. 865-123) 4×10^{-5} to 8×10^{-4} M inhibited, in a dose-dependent manner, increases in output of noradrenaline induced by reduction of sodium to 18 mM, while guanethidine 8×10^{-5} M and high doses of bretylium produced no inhibition: the latter two released noradrenaline.

3 The inhibitory actions of guanethidine 4×10^{-5} M and No. 865-123 4×10^{-4} M were prevented by tetracaine 3.3×10^{-4} M, which *per se* did not modify the output of noradrenaline induced by 18 mM sodium.

4 Accumulation of guanethidine and No. 865-123 in ventricular slices was greater than that noted in striated muscle slices and was dose-, time- and temperature-dependent. Tetracaine 3.3×10^{-4} M did not prevent the accumulation of guanethidine 4×10^{-5} M and No. 865-123 1.1×10^{-6} to 4×10^{-4} M.

5 The guanidine derivatives appear to increase the permeability of adrenergic nerve endings to sodium ions.

Introduction

Many hypotheses have been put forward concerning the mechanism by which adrenergic neurone blocking agents prevent impulse-evoked noradrenaline release (Boura & Green, 1965; Chang, Costa & Brodie, 1965; Burn & Welsh, 1967; Kirpekar, Wakade, Dixon & Prat, 1969; Haeusler, Haefely & Huerlimann, 1969; Kubo & Misu, 1974; Abbs & Dott, 1974; Giachetti & Hollenbeck, 1976). Kubo & Misu (1974) demonstrated that guanethidine-induced blockade of adrenergic transmission in rabbit hearts is attenuated when nerves are stimulated during perfusion with a low sodium solution and accentuated by a high sodium solution. They proposed that the drug increases the permeability of adrenergic nerve endings to sodium ions, thereby leading to blockade. Furthermore, Misu & Nishio (1978) demonstrated that guanethidine 4×10^{-4} M produced a gradual irreversible membrane depolarization after transient increases in the maximum upstroke velocity of action potentials without causing hyperpolarization and these changes of action and resting potentials were antagonized by tetrodotoxin in rabbit atria. Although there is no direct evidence that the drug increases the permeability of adrenergic nerve endings to ionized sodium, if it were the case, guanethidine might prevent the effect on nerve terminals induced by reduction of the exter-

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nal sodium concentration. Preliminary findings revealed that in rabbit atria, guanethidine inhibits the output of noradrenaline induced by low sodium (Misu, Hosotani, Nakashima & Nishio, 1977). In the present experiments, we investigated in detail the mechanism responsible for this action of guanethidine and related adrenergic neurone blockers on the output of noradrenaline induced by sodium reduction in rabbit ventricular slices.

Methods

Rabbits of either sex, weighing 1.5 to 2.2 kg, were bled from the carotid arteries. The heart was isolated and the ventricle sliced transversely with a tissue slicer (Natsume, KN-822) into sections approximately 400 mg in weight and 0.5 mm in thickness. These slices were incubated in 10 ml Tris buffer Krebs solution, bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. The composition of the solution was as follows (mM): NaCl 143.4, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.18, glucose 11.1, Tris buffer 2.0 and the disodium salt of ethylene-diaminetetra-acetic acid (EDTA) 0.03. A 30 min stabilization period was allowed before exposure of the slices to the test solutions. Sodium-deficient medium was prepared by replacing NaCl with an equiosmotic amount of sucrose or choline chloride. Calcium-free solution was made by omission of $CaCl_2$ and addition of ethylene glycol-bis (aminoethylether) N,N,N',N' tetra-acetic acid (EGTA) 0.5 mM. A high calcium solution was prepared by the addition of $CaCl_2$ without any compensation being made for changes in tonicity. The final pH of solutions was 7.1 to 7.4.

Incubation medium was collected at intervals of 30 min and was immediately cooled in ice and acidified with 0.5 ml of concentrated perchloric acid. Media and the supernatant from the tissue homogenate were mixed with aluminium oxide 400 mg, sodium metabisulphite 10 mg and EDTA 200 mg. Except for the use of a Tris buffer to adjust the pH of the mixture to 8.0, adsorption, elution and assay of noradrenaline in the media and the final endogenous content were carried out by the method of Anton & Sayre (1962). The recovery of added noradrenaline was 71.5 ± 1.1% (n = 22). Measurements were corrected for recovery and are expressed as μg/g wet weight tissue.

Measurement of guanidine derivatives accumulated into ventricular and striated muscle slices

Ventricular slices and slices made from M. biceps brachii were incubated at 37°C in Krebs solution containing guanidine derivatives for 30 and 120 min. Slices were washed 3 times with fresh solution and then homogenized 10 to 20 times in 7 ml of 0.4 N perchloric acid with a glass homogenizer (Iwaki Code 7740) kept at 0°C. The homogenization was repeated after 60 min. The fluorescence of compounds of guanidine with ninhydrin formed in strongly alkaline solution was measured by the method of Schanker & Morrison (1965). Four ml of the homogenate was pipetted into a 50 ml glass-stoppered centrifuge tube containing 1 ml of 2.5 N NaOH solution and 30 ml of chloroform. The mixture was shaken for 30 min, and then centrifuged for 5 min in a refrigerated centrifuge (Kubota, KR-66), usually at 10,000 g. After removing the aqueous phase, 5 ml of 0.1 N NaOH was added, the tube was shaken for 10 min to extract creatine from the organic phase, and then centrifuged for 5 min; 25 ml of the organic phase was transferred to a 50 ml centrifuge tube containing 3 ml of 0.1 N HCl solution. The tube was shaken for 15 min, and centrifuged for 30 min. The aqueous phase was transferred to a spitz roll and centrifuged for 30 min; 2 ml of the clear aqueous phase was transferred to a test tube for chemical estimation, 1 ml of 1% solution of ninhydrin in 95% ethanol being added to the 2 ml aqueous sample, followed immediately by the addition, with mixing, of 1 ml of a 10% solution of KOH in 90% ethanol. The fluorescence of the reaction mixture was measured after 30 min with a spectrofluorophotometer (Hitachi, MPF-2A). Activation and fluorescence wavelength were 395 and 495 mμ. Tissue recoveries of guanethidine and 4-7-exo-methylene-hexahydroisoindoline-ethyl guanidine hemisulphate (No. 865-123) in rabbit ventricular slices were 88 ± 3 (n = 5) and 91 ± 4% (n = 6) and the value of guanethidine is consistent with that obtained in rat heart slices by Schanker & Morrison (1965). The concentrations of guanidine derivatives accumulated in slices are expressed as the difference between values in drug-treated slices and in drug-free slices (regarded as the blank).

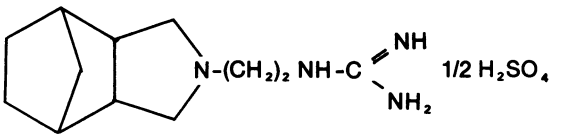

Figure 1 Structural formula of 4-7-exo-methylene-hexahydroisoindoline-ethyl guanidine hemisulphate (No. 865-123).

Drugs used were guanethidine sulphate (Ciba-Geigy), bretylium tosylate (Wellcome), tetracaine hydrochloride (Kyorin) and No. 865-123 (Eisai); the latter is a white crystalline powder with a molecular weight of 271 and is soluble as a 1% solution in 0.9% w/v NaCl solution (saline) at the usual pH and temperature. Figure 1 shows the structural formula. All drugs were dissolved in glass-distilled water and the concentrations are expressed as M. Student's t-test was used to evaluate data.

Results

Effects of graded sodium concentrations on the output of noradrenaline from rabbit ventricular slices

When external sodium concentrations were reduced from 143 to 72, 36 or 18 mM with the addition of sucrose to maintain osmolarity, the output of noradrenaline at each incubation period increased and the amount of endogenous noradrenaline remaining after a 2 h incubation period decreased (Table 1). The sum of the output of noradrenaline into the media and final contents was not modified by the different sodium concentrations in the external medium. The results suggest that endogenous noradrenaline and not a metabolite was released by low sodium.

Effects of calcium on the output of noradrenaline induced by low sodium

The output of noradrenaline induced by replacing sodium with sucrose was affected neither by omission of $CaCl_2$ and addition of 0.5 mM EGTA nor by an increase in calcium concentration to 10 mM 30 min before sodium deprivation (Table 2). Decreases in final endogenous noradrenaline content were also not modified by these procedures. Even when NaCl was replaced with choline chloride, the parameters remained the same, regardless of the calcium deprivation.

Effects of adrenergic neurone blocking agents on the output of noradrenaline induced by low sodium

Guanethidine. The addition of guanethidine 4×10^{-6} and 4×10^{-5} M produced no modifications of the resting output of noradrenaline in normal sodium medium and inhibited, in a dose-dependent manner, increases in the output of noradrenaline induced by reduction of sodium concentrations to 18 mM during the 0 to 60 min incubation periods (Figure 2). However, guanethidine 8×10^{-5} M produced no inhibition: the drug itself released noradrenaline. It seems likely that the noradrenaline releasing action cancels the characteristic inhibitory action obtained with the lower doses.

Bretylium tosylate. At 4×10^{-5} ($n = 6$) and 4×10^{-4} M ($n = 9$) bretylium alone also caused a considerable release of noradrenaline, approximately 2 to 3 times that of the resting output ($n = 12$) and did not inhibit the output of noradrenaline induced by low sodium. At 4×10^{-6} M ($n = 3$) bretylium produced no modifications of these parameters. It is not clear whether bretylium has the same inhibitory action as guanethidine.

No. 865-123. This drug shows no local anaesthetic activity when tested by the guinea-pig weal method. It has a slightly less potent adrenergic neurone blocking action with a slower time course than guanethidine, a similar noradrenaline-depleting action to guanethidine and exhibits little sympathomimetic activity in rabbit atria (Misu, Nishio, Hosotani & Hamano, 1976). Alone it caused only a slight release of noradrenaline and inhibited, in a dose-dependent manner, the output of noradrenaline induced by 18 mM sodium media during the 0 to 90 min incubation periods (Figure 3). The highest dose of No. 865-123 did not produce sufficient release of noradrenaline to cancel its inhibitory effect on low sodium release, as did guanethidine. No. 865-123 proved to be a more effective tool for the following studies.

Table 1 Output of noradrenaline induced by sodium reduction in rabbit ventricular slices

External [*Na*] (mM)	*No. of estimations*	*Output of noradrenaline (μg/g tissue) into media* *Incubation periods (min)* 0 to 30	30 to 60	60 to 90	90 to 120	*Final endogenous noradrenaline (μg/g tissue)*
143 Control	3	0.06 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.00	1.25 ± 0.36
72	3	0.11 ± 0.01†	0.05 ± 0.00	0.05 ± 0.01	0.06 ± 0.01	1.10 ± 0.36
36	3	0.17 ± 0.02††	0.12 ± 0.00††	0.14 ± 0.02††	0.14 ± 0.02	0.84 ± 0.47
18	8	0.26 ± 0.04††	0.31 ± 0.05††	0.28 ± 0.06†	0.16 ± 0.02†	0.47 ± 0.15†

Slices were incubated for periods of 30 min during 120 min in Krebs solution with modified concentration of sodium. Values are mean ± s.e.mean.
Statistically significantly different from control: † $P < 0.05$; †† $P < 0.01$.

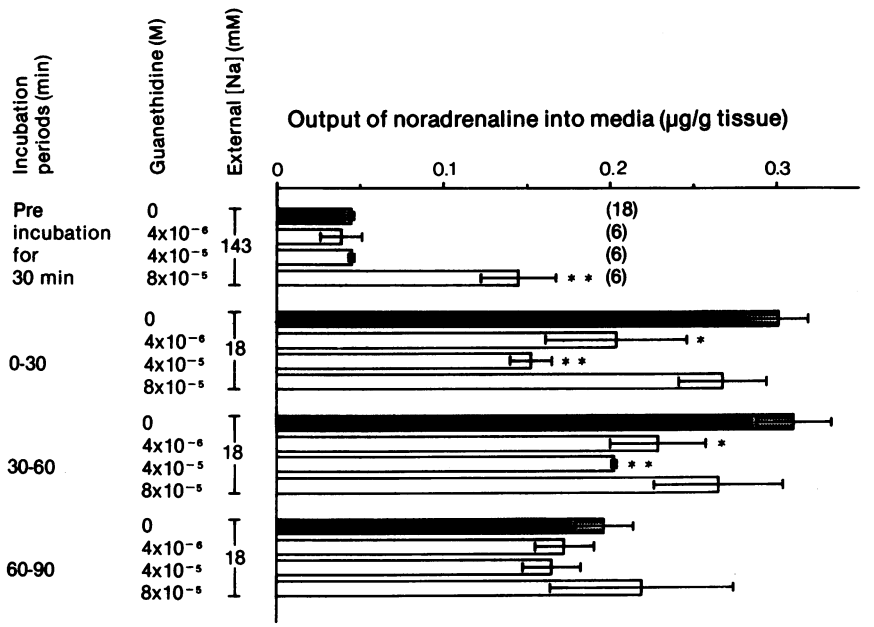

Figure 2 Effects of guanethidine on output of noradrenaline induced by low sodium in rabbit ventricular slices. After a pre-incubation period of 30 min, slices were incubated in 18 mM sodium solution (with sucrose) for intervals of 30 min. Guanethidine was added throughout incubation periods and concentrations are shown on the ordinate scale. Abscissa scale indicates output of noradrenaline, hatched columns show control output, horizontal bars indicate standard errors and the number of estimations is given in parentheses. *: $P < 0.05$ and **: $P < 0.01$, compared with control.

Table 2 Effects of calcium on output of noradrenaline induced by sodium deprivation in rabbit ventricular slices

External [*Ca*] (mM)	*Na substitute*	*No. of estimations*	*Output of noradrenaline into media (μg/g tissue)* *Preincubation** (*30 min*)	*Incubation periods (min)* 0 to 30	30 to 60	60 to 90	*Final endogenous noradrenaline (μg/g tissue)*
10		5	0.05 ± 0.01	0.41 ± 0.06	0.24 ± 0.05	0.10 ± 0.03	0.09 ± 0.02
2.5 Control	Sucrose	11	0.04 ± 0.00	0.45 ± 0.04	0.25 ± 0.03	0.11 ± 0.01	0.11 ± 0.02
0 (+EGTA 0.5)		6	0.05 ± 0.01	0.49 ± 0.08	0.26 ± 0.04	0.14 ± 0.02	0.15 ± 0.03
2.5 Control	Choline chloride	3	0.03 ± 0.00	0.22 ± 0.03	0.19 ± 0.02	0.15 ± 0.04	0.24 ± 0.08
0 (+EGTA 0.5)		3	0.05 ± 0.00	0.22 ± 0.02	0.17 ± 0.04	0.16 ± 0.05	0.20 ± 0.07

* After a preincubation period of 30 min in 143 mM external [Na], slices were incubated in sodium-free media (with sucrose or choline chloride to maintain osmolarity) for periods of 30 min. Calcium was removed or added 30 min before sodium deprivation throughout the incubation periods. Values are mean ± s.e.mean.

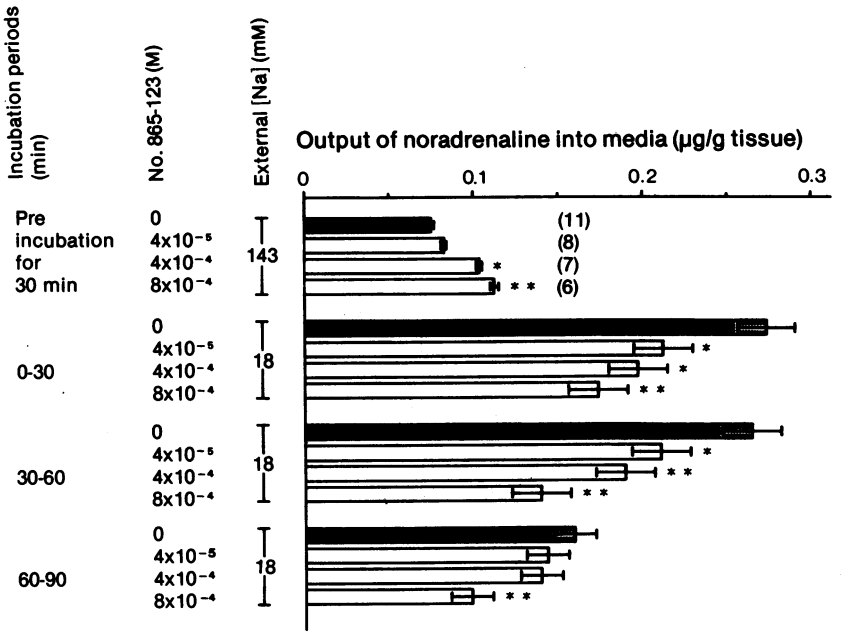

Figure 3 Effects of No. 865-123 on output of noradrenaline induced by low sodium in rabbit ventricular slices. Details are as in Figure 2.

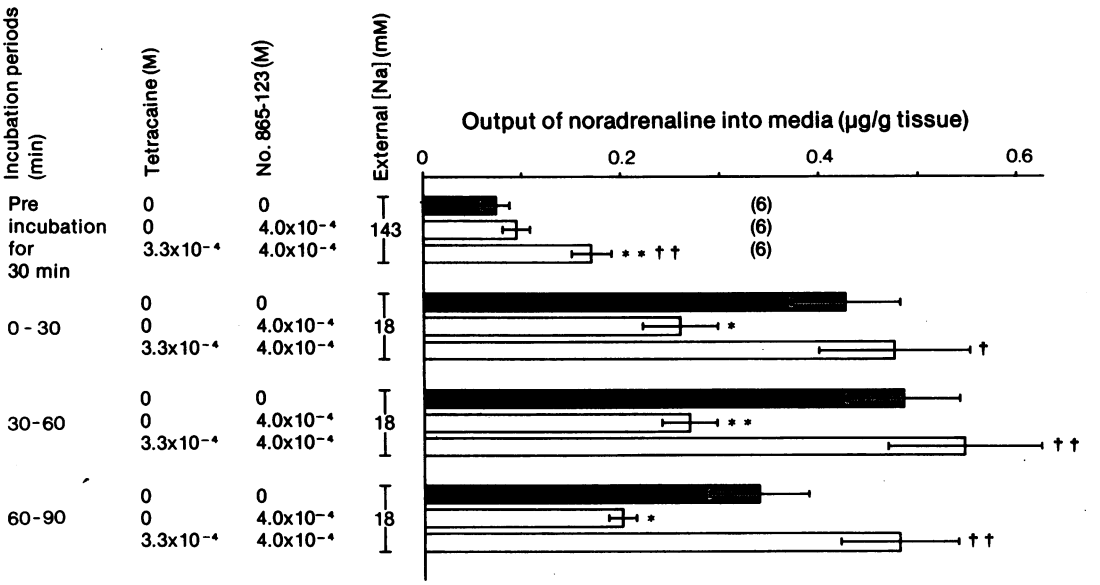

Figure 4 Effects of tetracaine on No. 865-123-induced inhibition of noradrenaline output by low sodium in rabbit ventricular slices. Tetracaine was applied 10 min before the addition of No. 865-123 throughout incubation periods. †: $P < 0.05$ and ††: $P < 0.01$, compared with values modified by No. 865-123 alone. Other details as in Figure 2.

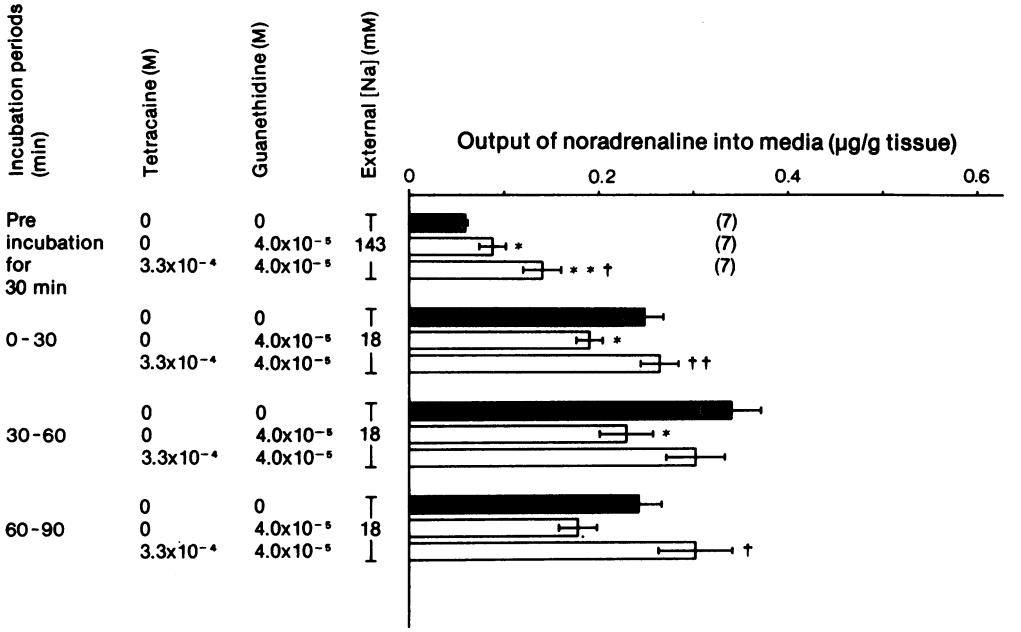

Figure 5 Effects of tetracaine on guanethidine-induced inhibition of noradrenaline output by low sodium in rabbit ventricular slices. Significance of difference shown as in Figure 4; other details as in Figure 2.

Effects of pretreatment with tetracaine on No. 865-123- and guanethidine-induced inhibition of the output of noradrenaline induced by low sodium

Tetracaine 3.3×10^{-4} M itself to some extent increased the resting output of noradrenaline, from 0.08 ± 0.00 ($n = 6$) to 0.15 ± 0.03 ($n = 6$) μg/g tissue. However, tetracaine did not modify the output of noradrenaline induced by low sodium: the amount produced during the 0 to 30, 30 to 60 and 60 to 90 min incubation periods after reduction of the sodium concentration to 18 mM was 0.34 ± 0.06 (control) vs.

Table 3 Effects of low temperature and tetracaine on accumulation of No. 865-123 and guanethidine in rabbit ventricular slices

		Accumulation of guanidine derivatives (μg/g tissue)			
		Control			*Tetracaine*
	M	*37°C*	*0°C*	*Control*	*3.3 × 10⁻⁴ M*
No. 865-123	1.1×10^{-6}	1.45 ± 0.30	0.55 ± 0.19†	1.14 ± 0.41	0.99 ± 0.45
	4.0×10^{-6}	2.77 ± 0.12	1.12 ± 0.21†	2.79 ± 0.11	2.61 ± 0.24
	3.7×10^{-5}	20.0 ± 0.6	10.6 ± 1.1†		
	4.0×10^{-4}			232 ± 18	226 ± 24
Guanethidine	4.0×10^{-6}	3.82 ± 0.40	0.94 ± 0.09†		
	4.0×10^{-5}			22.9 ± 2.1	19.9 ± 1.1

Slices were incubated in media containing each concentration of drug for 120 min. Temperature was reduced or tetracaine applied 10 min before addition of guanidine derivatives.
Values are mean ± s.e. mean ($n = 6$);
† Statistically significantly different from control, $P < 0.01$.

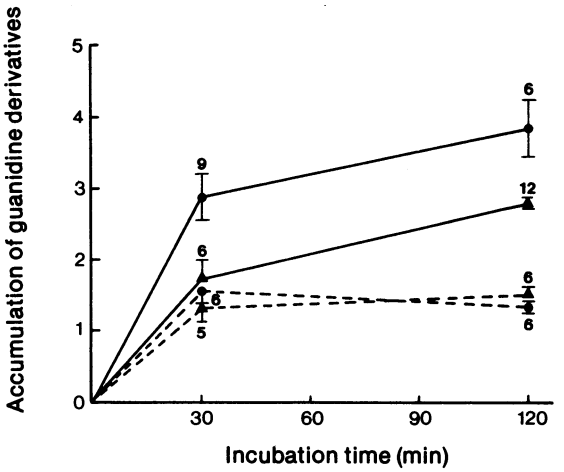

Figure 6 Accumulation of No. 865-123 and guanethidine in ventricular (continuous line) and striated muscle slices (broken line). Slices were incubated in normal solution containing No. 865-1234 $\times 10^{-6}$ M (▲) or guanethidine 4×10^{-6} M (●) at time intervals shown on abscissa scale. Vertical bars show standard errors and numbers of estimations are given beside each point.

0.33 ± 0.06 (tetracaine-treated), 0.35 ± 0.03 vs. 0.35 ± 0.07 and 0.27 ± 0.05 vs. 0.32 ± 0.03 µg/g tissue, respectively. When tetracaine was added 10 min before the application of No. 865-123 (Figure 4) the resting output of noradrenaline was increased compared to the output with No. 865-123 alone. At each incubation period, tetracaine significantly antagonized the inhibitory action of No. 865-123 on the output of noradrenaline induced by low sodium. This was the case even when comparison was made between the values from which the resting output of noradrenaline had been subtracted.

The inhibitory action of 4×10^{-5} M guanethidine on the output of noradrenaline induced by low sodium was also prevented by tetracaine 3.3×10^{-4} M (Figure 5). However, the use of both agents together resulted in a relatively marked release of noradrenaline in normal sodium media.

Effects of low temperature and tetracaine on the accumulation of No. 865-123 and guanethidine into ventricular slices

The accumulation of both guanidine derivatives in ventricular slices abundantly innervated with sympathetic nerves was greater than that found in striated muscle slices and was dependent upon incubation times (Figure 6) and dose (Table 3). The accumulation of guanethidine was more marked than that of No. 865-123 in ventricular slices. When ventricular slices were incubated at 0°C, the accumulation of both substances was greatly reduced.

Tetracaine 3.3×10^{-4} M produced no inhibition of the accumulation of guanidine derivatives in ventricular slices.

Discussion

Our results demonstrate that in rabbit ventricular slices No. 865-123 and the lower doses of guanethidine prevent in a dose-dependent manner increases in the output of noradrenaline induced by low sodium. These actions are antagonized by tetracaine. Our findings are consistent with the hypothesis that guanethidine increases the permeability of adrenergic nerve endings to sodium ions (Kubo & Misu, 1974).

The output of noradrenaline increased and the final endogenous content decreased, effects that were inversely related to the external sodium concentration. It has been established that sodium ions play an essential role in the uptake, retention and storage of noradrenaline in adrenergic nerve endings (Iversen & Kravitz, 1966; Gillis & Paton, 1967; Kirpekar & Wakade, 1968; Bogdanski & Brodie, 1969). The major mechanism involved in this increase in the output of noradrenaline seems to be an inhibition of the retention rather than that of the reuptake, because guanethidine, a potent inhibitor of the uptake (Hertting, Axelrod & Patrick, 1962), did not potentiate the efflux induced by low sodium. Garcia & Kirpekar (1973) demonstrated that the various procedures which are known to inhibit Na^+, K^+-activated ATPase or the sodium potassium pump, induce the efflux of noradrenaline. However, the present result, showing that guanethidine inhibits the output of noradrenaline induced by low sodium, is not explicable in terms of the activation of this enzyme in the adrenergic nerve endings because the drug does not modify the myocardial transport ATPase activity (Misu & Nishio, 1978).

The output of noradrenaline induced by sodium deprivation was not dependent upon external calcium concentrations. This result is consistent with the data from cat spleen (Garcia & Kirpekar, 1973). On the other hand, it has been reported that in the rat heart, increases in the release and decreases in the stores of [^{3}H]-noradrenaline induced by sodium deprivation, especially when sodium is replaced with choline, depend on calcium concentrations (Keen & Bogdanski, 1970; Blaszkowski & Bogdanski, 1971). However, in rabbit atria (Misu *et al.*, 1977) and in the ventricular slices used in the experiment described here, noradrenaline efflux induced by replacing sodium with choline was not inhibited by calcium

omission and EGTA addition 30 min before sodium deprivation. These results demonstrate that the efflux of noradrenaline induced by low sodium is qualitatively different from the calcium-dependent release of the transmitter evoked by sympathetic nerve stimulation (Hukovic & Muscholl, 1962; Kirpekar & Misu, 1967; Boullin, 1967).

The inhibitory actions of No. 865-123 and guanethidine on increases in the output of noradrenaline induced by low sodium were antagonized by the 'non-effective' dose of tetracaine. Tetracaine may produce this antagonism by inhibiting the accumulation of the guanidine derivatives into adrenergic neurones, because guanethidine (Schanker & Morrison, 1965; Brodie, Chang & Costa, 1965; Chang *et al.*, 1965) and bretylium (Garcia & Sánchez-Garcia, 1975; Hosotani & Misu, 1977) are selectively taken up, probably via the same active process as that involved in the uptake of noradrenaline and tetracaine can inhibit to some extent the uptake of noradrenaline (Starke, Wagner & Schümann, 1972). However, this possibility is evidently excluded, because the accumulation of guanidine derivatives is not prevented by tetracaine. In the case of tetracaine and guanethidine, their combined use induced relatively marked increases in the resting output of noradrenaline: it might be possible that such increases are a component part of this antagonism.

Results obtained in the present experiments support the possibility that the 2 guanidine derivatives increase the permeability of adrenergic nerve endings to sodium ions. This is consistent with the data from the rabbit atria that guanethidine (4×10^{-4} M) produced a gradual but irreversible depolarization and the depolarization was prevented by relatively high doses of tetrodotoxin (Misu & Nishio, 1978). Guanethidine appears to produce a persistent depolarization in adrenergic nerve endings, thereby leading to the blockade. This idea is consistent with the hypothesis of Chang *et al.* (1965) and Brodie *et al.* (1965).

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