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ACTION OF GUANETHIDINE ON RABBIT ATRIAL MEMBRANES

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1 Intracellular potentials were recorded in driven left atria from reserpine-treated rabbits. Guanethidine 2×10^{-5} M slightly increased V_{max} and shortened the total duration (TD) of the action potential (AP) without causing hyperpolarization. For the first 30 min after 4×10^{-4} M, V_{max} increased without hyperpolarization and AP height increased slightly. Thereafter, V_{max} and height decreased with a slight and gradual depolarization. This depolarization was irreversible. TD was increased after 15 minutes. Guanethidine 2×10^{-3} M initially decreased V_{max} and height before causing depolarization.

2 Pretreatment with tetrodotoxin (TTX) 1.6×10^{-7} M prevented or reversed the initial increases in V_{max}, height and TD induced by guanethidine (4×10^{-4} M).

3 TTX 3.1 to 6.2×10^{-6} M, added 15 or 30 min after guanethidine 4×10^{-4} M, delayed or prevented depolarization by guanethidine.

4 Ouabain 10^{-5} M incubated for 20 and 90 min greatly inhibited Na^+, K^+-adenosine triphosphatase and K^+-phosphatase activities; guanethidine was without effect.

5 Guanethidine probably increases resting sodium permeability after the promotion of increases in sodium permeability during the AP. High doses of the drug decrease sodium permeability during the AP.

Introduction

Many hypotheses have been advanced concerning the mechanism by which adrenergic neurone blocking drugs prevent the release of noradrenaline (Boura & Green, 1965; Brodie, Chang & Costa, 1965; Burn & Welsh, 1967; Abbs & Dodd, 1974; Kubo & Misu, 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 during perfusion with a high sodium solution. They proposed that the drug increases the permeability of adrenergic nerve endings to sodium ions, thereby leading to blockade. In the present work, we investigated the effects of guanethidine on membrane function of rabbit atria, keeping in mind the antiarrhythmic properties of the drug (Leveque, 1964; Wellens & Wauters, 1972).

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Methods

Experimental arrangements for electrophysiological observations

Rabbits of either sex weighing 1.6 to 2.1 kg were bled from the carotid arteries. The heart was isolated and the left atrium was excised in an O_2 saturated solution. The preparation was mounted by means of stainless-steel pins on a cork plate in a shallow open plastic chamber filled with modified Tyrode solution of the following composition (mM): NaCl 147.2, KCl 2.7, $CaCl_2 2H_2O$ 1.4, $MgCl_2$ $6H_2O$ 0.25, NaH_2PO_4 $2H_2O$ 1.3, Na_2HPO_4 $12H_2O$ 4.5 and glucose 5.6. The solution was constantly gassed, stirred with 5% CO_2 in O_2 and maintained at 30°C. The final pH was 7.4. The septal side of the preparation was electrically driven by means of a shielded bipolar platinum electrode with 1 Hz rectangular pulses of 1 ms and 3 to 5 times threshold voltage generated by an electronic stimulator MSE-3R (Nihon Kohden). The stabilization period before recording potentials was 1 hour. The threshold was determined as an average of voltages required for firing and for the disappearance of action potentials induced by gradually increasing and decreasing stimulus strength. Glass microelectrodes filled with 3 M KCl had a resistance

of 10 to 25 megohms. Intracellular endocardial potentials were displayed on one beam of a dual-beam oscilloscope VC-7 (Nihon Kohden). The maximum rate of rise of action potentials (V_{max}) was obtained by an electronic differentiator circuit (time constant 10 μs) and displayed on the other beam. The potential changes were monitored with a paper oscilloscope and were photographed through a cathode oscilloscope by use of a continuous recording camera PC-18 (Nihon Kohden). Zero level was always adjusted to a horizontal line of the scale of the oscilloscope before every insertion of a recording electrode, which was displaced immediately after taking a photograph. The drift (if any) of the beam from 0 mV was photographed, in order to correct values of the resting potentials.

Reserpine (1 mg/kg i.p.) was injected into all rabbits 18 to 24 h before the experiments. The reserpine reduced endogenous noradrenaline content (assayed fluorometrically by the method of Anton & Sayre, 1962) in atria and ventricles (2.77 ± 0.15 and 2.03 ± 0.24 μg/g tissue respectively, $n = 3$) to a negligible amount (0.04 ± 0.01 and 0.03 ± 0.01 μg/g tissue, $n = 3$). Guanethidine (4×10^{-4} M) produced a prolonged positive chronotropic response in spontaneously beating atria and the peak increase was 28.7 ± 11.2% ($n = 6$) 30 min after addition. This response was negligible after reserpinization.

Determination of Na^+, K^+-dependent adenosine triphosphatase (ATPase) and K^+-dependent phosphatase activities

Enzyme preparation The hearts from untreated rabbits were homogenized for 45 s in 4.5 volumes of an ice cold 0.25 M sucrose solution containing disodium edetate (EDTA) 1 mM. After repeated homogenization and filtration through gauze, the filtrate was centrifuged at 10,000 *g* for 15 minutes. The precipitate was again homogenized for 5 min in 8 volumes of the above solution to which was added 1% deoxycholate. Centrifugation at 10,000 *g* was carried out for a further 15 minutes. The microsomal and soluble fractions were then separated by re-centrifugation of the supernatant at 63,000 *g* for 60 minutes. The precipitate was suspended in distilled water and used for enzyme assay.

Na^+, K^+-dependent ATPase activity The reaction mixture, 1 ml of enzyme preparation (with or without drugs) and 1 ml of solution containing (mM) NaCl 200, KCl 20, $MgSO_4$ 10 and Tris buffer 80 (pH 7.4), was preincubated at 37°C for 20 or 90 min and then 1 ml of adenosine triphosphate (ATP) 10 mM solution was added. After incubation at 37°C for 20 min, 0.5 ml of 50% trichloroacetic acid was added to deproteinize the mixture. The amount of inorganic orthophosphate in the supernatant after centrifugation at 10,000 *g* for

Table 1 Effects of guanethidine (2×10^{-5} M) on transmembrane potentials in driven left atria from reserpine-treated rabbits ($n = 3$)

Time after application (min)	No. of recordings	Resting potential (mV) (% change)	Maximum rate of rise (V/s) (% change)	Action potential Height (mV) (% change)	Total duration at 50% repolarized level (ms) (% change)	Total duration at 90% repolarized level (ms) (% change)
Control	29	86.9 ± 1.1†	239.7 ± 13.0	108.3 ± 1.5	53.4 ± 5.0	161.4 ± 5.1
0 to 15	34	86.5 ± 1.0 (−0.4)	265.9 ± 11.8 (+10.9)	109.2 ± 1.5 (+0.8)	48.3 ± 4.9 (−9.6)	156.6 ± 5.5 (−3.0)
15 to 30	33	88.4 ± 1.2 (+1.7)	255.5 ± 13.9 (+6.6)	109.2 ± 1.3 (+0.8)	42.3 ± 3.8 (−20.8)	153.6 ± 3.1 (−4.8)
30 to 60	65	88.4 ± 0.6 (+1.7)	247.9 ± 7.3 (+3.4)	108.9 ± 0.9 (+0.6)	41.1 ± 3.6* (−23.0)	163.7 ± 3.7 (+1.4)
60 to 90	62	86.1 ± 0.7 (−0.9)	249.7 ± 8.1 (+4.2)	107.8 ± 1.0 (−0.5)	32.5 ± 2.6** (−39.1)	150.2 ± 2.6* (−6.9)
90 to 120	57	87.7 ± 0.9 (+0.9)	273.5 ± 7.9* (+14.1)	111.2 ± 0.8 (+2.7)	29.9 ± 2.5** (−44.0)	149.2 ± 3.4* (−8.2)

†Mean ± s.e. mean; *significantly different from control, $P < 0.05$; **significantly different from control, $P < 0.01$.

10 min was determined by the method of Martin & Doty (1949). Na^+ and K^+-free mixture was used for estimation of the basic ATPase activity and the increase produced by addition of both monovalent cations was expressed as Na^+, K^+-dependent ATPase activity.

K^+-dependent phosphatase activity After the same preincubation procedure of the reaction mixture without NaCl, 1 ml of *p*-nitrophenyl phosphate 20 mM solution was added and incubation was carried out at 37°C for 20 minutes. The reaction was then stopped by addition of 2 ml of 1 N NaOH at 0°C. *p*-Nitrophenol released was measured at 410 mμ in a spectrophotometer by the absorbance of the clear supernatant obtained by centrifugation at 900 *g* for 10 minutes. The K^+-dependent phosphatase activity was expressed as the difference between activities in the presence and absence of K^+ in the reaction mixture. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Drugs used were guanethidine sulphate (Ciba-Geigy), ouabain octahydrate (Merck) and tetrodotoxin (TTX, Sankyo). Concentrations of drugs are expressed as M. Student's *t*-test was used to evaluate data.

Results

Effects of guanethidine on transmembrane potentials

In each preparation, approximately 10 potentials were recorded as control, drugs were then added to the bath and potentials were recorded usually for 2 h (Table 1). The dose of 2×10^{-5} M guanethidine is within the upper limit of the usual concentrations required to produce adrenergic neurone blockade (Misu, Nishio, Hosotani & Hamano, 1976). Guanethidine slightly increased V_{max} in the whole time course up to 2 hours. V_{max} is a manifestation of early inward Na^+ current and is dominantly affected by resting potentials (Hodgkin & Huxley, 1952; Weidmann, 1955). However, guanethidine did not produce a hyperpolarization. The height of action potentials was slightly increased or not modified. The total duration of action potentials was shortened 30 min after the addition.

Guanethidine 4×10^{-4} M produced biphasic effects on the action potential (Figure 1). V_{max} increased without hyperpolarization up to 30 min after drug addition. Over the initial 30 min contact period the action potential height was hardly modified (Figure 1). After 30 min of contact V_{max} and the height of the action potential progressively decreased and the duration of the repolarization phase increased. These changes in the action potential are attributed in part to the slight, gradually occurring resting depolarization. At this time one third of the preparations failed to respond to stimuli at 3 times threshold voltage. These effects of guanethidine were not reversible over a 3 h period even after repeated washing. Consistent increases in threshold voltage for firing of action potentials were not observed until 60 min after the application of guanethidine (Figure 2).

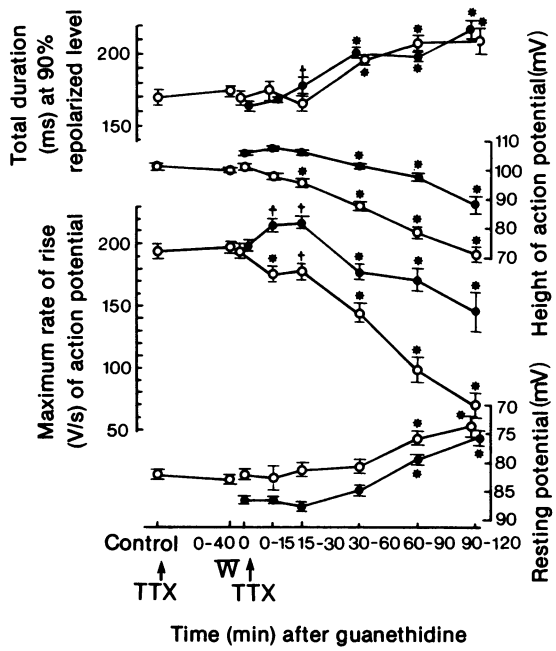

Figure 1 Effects of pretreatment with a low dose of tetrodotoxin (TTX, O, $n = 6$ to 47) on guanethidine-induced changes in transmembrane potentials (● $n = 29$ to 118) in driven left atria isolated from reserpine-treated rabbits. Each point is mean of parameters (shown on ordinate scale) of potentials recorded within time intervals shown on abscissa scale. Vertical lines show s.e. means. TTX 1.6×10^{-7} M was added at upward arrows twice, 40 min before washing-out organ bath at W and 10 min before the application of guanethidine 4×10^{-4} M. †$P < 0.05$ and *$P < 0.01$, compared with control.

At a higher concentration (2×10^{-3} M) guanethidine decreased V_{max} (182.9 ± 7.8, $n = 57$ from 206.9 ± 8.5 V/s, $n = 51$) and the height of the action potential (98.8 ± 1.3 from 103.0 ± 1.1 mV) without membrane depolarization (resting membrane potential 83.0 ± 0.8 from 83.6 ± 1.0 mV). The total duration of the action potential after 15 min contact was not modified (154.9 ± 5.8 from 151.0 ± 6.2 ms). After this time these parameters changed in a similar

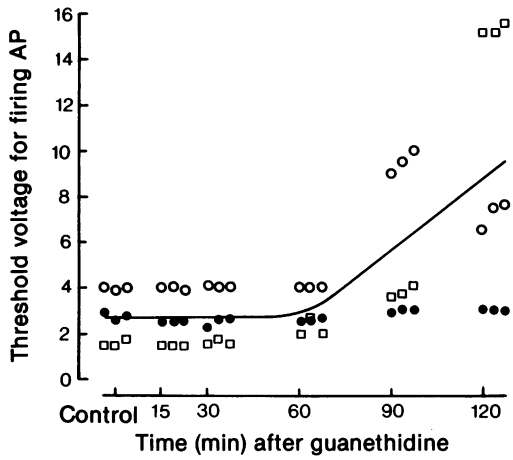

Figure 2 Effects of guanethidine (4×10^{-4} M) on threshold voltage for firing of action potentials (AP) in driven left atria isolated from reserpine-treated rabbits. Each point represents the threshold determined as an average of voltages for the firing and for the disappearance of action potentials induced by gradually increasing and decreasing stimulus strength at time shown on abscissa scale.

manner to that described for the lower drug concentration (see Figure 1).

Effects of pretreatment with a low dose of tetrodotoxin on guanethidine-induced changes in transmembrane potentials

The maximum dose of TTX, which *per se* did not modify either the resting membrane or action potentials, was 1.6×10^{-7} M. In the presence of TTX, guanethidine reduced V_{max} at 0 to 30 min without modifying the resting potential. A similar tendency was also observed in changes in the height of action potential. There was no prolongation of total duration before depolarization, such as was induced by guanethidine alone after 15 to 30 min contact time. TTX accelerated the subsequent decreases in V_{max} and action potential height induced by guanethidine alone (Figure 1). Conduction block occurred in 3 out of 5 atria at 3 times threshold voltage. There were no consistent changes in action potential duration.

Effects of subsequent addition of higher doses of tetrodotoxin on guanethidine-induced depolarization

In doses higher than 1.6×10^{-7} M, TTX itself markedly decreased V_{max} and the action potential duration and slightly decreased the height of the action potential. These results are consistent with the fact that TTX predominantly inhibits the early inward sodium current and has no effect on potassium conductance during spike activation (Kao, 1966). Resting potentials were not significantly modified. After the application of TTX 6.2×10^{-6} M, the changes in resting membrane potential and in V_{max} were + 1.4% ($n = 47$) and −59% respectively after 30 min and +2.7% ($n = 13$) and −81.4% after 30 to 60 min as compared with control levels which were 78.0 ± 1.2 mV and 164.5 ± 7.0 V/s ($n = 54$). The slight degree of hyperpolarization induced by this relatively high concentration of TTX is consistent with the idea that this toxin reduces resting sodium permeability (Freeman, 1969; Narahashi, Deguchi & Albuquerque, 1971; Hogan & Albuquerque, 1971). As shown in Figure 3, TTX 3.1×10^{-6} M delayed the onset and extent of depolarization induced by guanethidine. TTX 6.2×10^{-6} M added 15 min after guanethidine application almost completely prevented guanethidine-induced depolarization. In these atria, guanethidine alone in the initial phase at 0 to 30 min replicated increases in V_{max} without hyperpolarization.

Table 2 The effects of ouabain and guanethidine on myocardial ATPase and phosphatase

Drug (M)	*Preincubation time (min)*	*Na^+, K^+-dependent ATPase activity (%)*	*K^+-dependent phosphatase activity (%)*
Control	20	(4) 100 (131.6 ± 4.5†)	(4) 100 (37.6 ± 0.5*)
	90	(4) 100 (136.2 ± 5.6†)	(4) 100 (52.2 ± 0.6*)
Ouabain 10^{-5}	20	(5) 20.8 ± 1.3**	(5) 54.4 ± 1.9**
	90	(5) 27.6 ± 1.4**	(6) 56.5 ± 0.8**
Guanethidine 2×10^{-5}	20		(4) 101.1 ± 5.1
	90		(4) 100.9 ± 4.8
Guanethidine 4×10^{-4}	20	(4) 94.4 ± 3.9	(4) 93.8 ± 3.6
	90	(6) 92.3 ± 4.3	(6) 96.3 ± 2.1

Numbers of estimations are shown in parentheses. †nM inorganic phosphate . mg^{-1} protein . min^{-1} (mean ± s.e. mean); *nM *p*-nitrophenyl phosphate . mg^{-1} protein . $min.^{-1}$; **significantly different from control, $P < 0.01$.

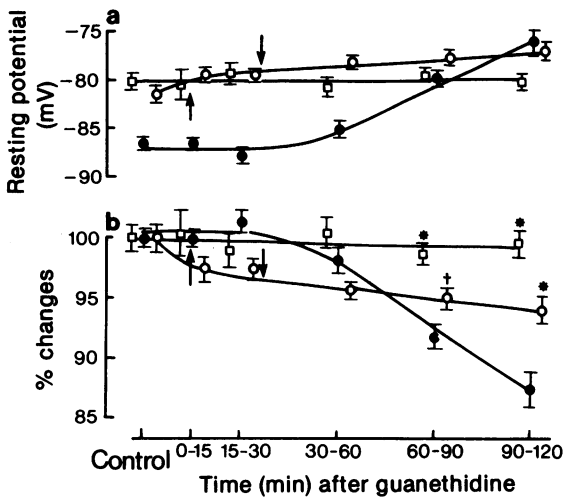

Figure 3 Effects of subsequent additions of tetrodotoxin (TTX 3.1×10^{-6} M, O, $n = 27$ to 55) and 6.2×10^{-6} M (□, $n = 27$ to 52) on guanethidine-induced depolarization (●, $n = 28$ to 118) in driven left atria isolated from reserpine-treated rabbits. Values are means, vertical lines show s.e. means. Each point demonstrates the absolute value (a) and the % change (b). TTX at a concentration of 3.1×10^{-6} M was added 30 min after (downward arrows) and at a concentration of 6.2×10^{-6} M 15 min after (upward arrows) the application of guanethidine (4×10^{-4} M.) †$P < 0.05$; *$P < 0.01$, compared with values modified by guanethidine alone.

However, after the addition of TTX, V_{max} and action potential height markedly decreased and conduction block occurred at 5 times supramaximal voltage in 6 out of 9 atria. This appears to be caused by synergism of the direct inhibitory actions of TTX and guanethidine.

Effects of ouabain and guanethidine on Na^+, K^+-dependent ATPase and K^+-dependent phosphatase activities in rabbit hearts

Ouabain 10^{-5} M markedly inhibited ATPase and phosphatase activities in samples prepared from untreated rabbit hearts (Table 2). However, guanethidine 4×10^{-4} M produced no effect on these enzymes after either 20 or 90 min incubation.

Discussion

Our results demonstrate that guanethidine produces biphasic effects on transmembrane potentials in driven left atria of rabbits. Up to 2 h after exposure to 2×10^{-5} M (and up to 30 min after a concentration of 4×10^{-4} M) the drug increased V_{max}. This action was not secondary to hyperpolarization and was neither marked nor dose-dependent. Noradrenaline, which is released from the sympathetic nerve endings after the application of high doses of guanethidine (Boura & Green, 1965; Hosotani & Misu, 1976), increases in canine Purkinje fibres the upstroke velocity of the action potential (Hoffman & Singer, 1967). However, all of the rabbits used in our experiments were reserpine-treated and this treatment almost completely depleted cardiac noradrenaline stores and abolished the sympathomimetic action of guanethidine. Furthermore, in atria taken from rabbits given a higher dose of reserpine (3 mg/kg), guanethidine inhibited the decrease in V_{max} and action potential height induced by exposure to sodium-deficient media (Misu & Nishio, 1973). These findings exclude the possibility that the increase in V_{max} is caused by an indirect effect of guanethidine. Pretreatment with a non-effective dose of TTX (1.6×10^{-7} M) reversed the increases in V_{max} and action potential height induced by guanethidine (4×10^{-4} M). Thus, our findings indicate that guanethidine promotes a rapid increase in the sodium permeability during spike activation and prevents a rapid decline to the resting value. On the other hand, guanethidine (2×10^{-5} M) shortened the repolarization phase, which may be a manifestation of an increase in potassium permeability.

From 30 min after exposure to guanethidine (4×10^{-4} M), a slight and gradually developing depolarization occurred and this persisted after washing. It was delayed or prevented at highest concentrations of TTX. This indicates that the factor responsible for the depolarization of the atrial membrane is an increase in resting sodium permeability rather than a decrease in potassium permeability. It has been demonstrated that, in canine Purkinje fibres, low doses of batrachotoxin produce a marked and relatively acute depolarization and that this action is delayed or prevented by similarly high concentrations of TTX (Hogan & Albuquerque, 1971). A similar phenomenon has been observed in squid giant axons (Narahashi *et al.*, 1971). Compared with batrachotoxin, the grade of depolarization induced by guanethidine was probably too small to exclude involvement of an increase in chloride permeability or in leakage permeability in the mechanism.

In certain concentrations guanethidine initially decreased V_{max} and action potential height. The guanethidine molecule is apparently too large to pass through sodium channels in an ionized form, as suggested by Hille's experiments (1971) in myelinated fibres of the frog sciatic nerve. Findings obtained with extremely high doses of this guanidine derivative may be explained by extrapolation of Kao & Nishiyama's hypothesis (1965) that the charged guanidinium of

TTX and saxitoxin may enter the sodium channel whilst the remainder of the molecule may act as a plug and prevent the passage of the toxins through it. This would lead to prevention of impulse generation.

At a concentration of 4×10^{-4} M guanethidine increased threshold voltage for firing of action potentials. Possible factors relevant to this inexcitability of the atrial membrane are the TTX-sensitive persistent depolarization after transient excitation and direct inhibition of the action potential. Such an inexcitability may also occur in adrenergic nerve endings at guanethidine concentrations lower than 2×10^{-5} M, because of selective, active accumulation of the drug (Schanker & Morrison, 1965; Brodie *et al.*, 1965). Membrane depolarization after transient excitation in adrenergic nerve endings seems to be mainly responsible for the blockade, because the adrenergic neurone blocking action in rabbit hearts depends upon the external sodium concentrations (Kubo & Misu, 1974). On the other hand, the direct inhibition of the action potential could be an additional factor. If a TTX-like action were a dominant factor, the grade of the blockade should be reversely related to external sodium concentrations. Results obtained with lower doses of guanethidine in the atrial membrane are consistent with the hypothesis that the drug increases the permeability of adrenergic nerve endings to sodium ions, thereby leading to blockade (Kubo & Misu, 1974).

Doses of guanethidine required to render the atrial membrane inexcitable are probably too high to be involved in the mechanism of the antiarrhythmic action. The adrenergic neurone blocking action may be a specific and major factor for the antiarrhythmic properties (Szekeres & Papp, 1968; Roberts, Kelliher & Lathers, 1976).

In conclusion, guanethidine probably increases resting sodium permeability after the promotion of increases in sodium permeability during spike activation in rabbit atria. High doses of the drug decrease sodium permeability during activation.

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