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EFFECT OF 4-AMINOPYRIDINE ON RELEASE OF NORADRENALINE FROM THE PERFUSED CAT SPLEEN BY NERVE STIMULATION

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SUMMARY

1. 4-aminopyridine (4-AP, 1 mM) increased noradrenaline (NA) output from the perfused cat spleen at 5 Hz by about fivefold. Enhancement of NA release by 4-AP was reversible. Output of NA induced by potassium was not affected.

2. NA output was doubled at low concentrations (0·1–0·3 mM) of 4-AP, but maximal effect was obtained at 1–3 mM. At 10 mM, it induced spontaneous release of NA which was insensitive to calcium.

3. Insignificant outputs obtained at 5 Hz in 0·1 and 0·3 mM calcium-Krebs solution were markedly enhanced by 4-AP. 4-AP enhanced release at all calcium concentrations up to 5 mM, but maximum output was obtained at 2·5 mM.

4. 4-AP at pH 8·5 was more effective in enhancing NA release than at pH 7·4.

5. 4-AP increased the recovery of intra-arterially infused NA from the control 26 to 47 %.

6. 4-AP did not affect release of catecholamines (CA) from the perfused cat adrenal gland by acetylcholine (ACh).

7. It is suggested that 4-AP inactivates potassium current in sympathetic nerves and prolongs the duration of the action potential, thereby allowing a greater influx of calcium ions into the neurone to enhance release of NA.

INTRODUCTION

4-AP reduces the delayed potassium current in the voltage-clamped isolated giant axons of the cockroach (Pelhate & Pichon, 1974), and also in the internally perfused giant axons of the squid (Meves & Pichon, 1975; Yeh, Oxford, Wu & Narahashi, 1976). Gillespie & Hutter (1975) showed

that 4-AP was more effective than tetraethyl-ammonium (TEA) in reducing the delayed potassium current in skeletal muscle fibres. Llinás, Walton & Bohr (1976) also showed that 4-AP reduced potassium conductance in the presynaptic terminal of the giant synapse without blocking transmission.

TEA, which is well known for its ability to block potassium current, has also been previously shown to enhance ACh release from the neuromuscular junction (Koketsu, 1958; Collier & Exley, 1963) and NA release from sympathetic nerves (Thoenen, Haefely & Staehelin, 1967; Kirpekar, Prat, Puig & Wakade, 1972; Kirpekar, Wakade & Prat, 1976). Since TEA blocks nicotinic receptors, it is not possible to use this agent in the study of CA release from sympathetic nerves, or from the adrenal medulla, by stimulation of nicotinic receptors. If 4-AP blocks potassium current and hence prolongs the duration of the action potential of the sympathetic nerve endings, then, like TEA, it should potentiate the release of NA. We report in this communication that 4-AP markedly enhances NA output from the perfused spleen of the cat by electrical stimulation of its sympathetic nerves, and that it does not block CA secretion from the adrenal gland by ACh. A preliminary report on some of the findings has been published (Kirpekar, Kirpekar & Prat, 1976).

METHODS

Perfusion of the cat spleen

Cats (about 2 kg) were anaesthetized by ether induction, followed by chloralose (60 mg/kg). The spleens were isolated, and perfused *in situ* with Krebs-bicarbonate solution at a rate of about 6 ml./min at 35 °C, as previously described (Kirpekar & Misu, 1967). Venous samples were collected for 2 min during nerve stimulation, and control samples without nerve stimulation were taken for 2 min before stimulation. In some experiments the cats were pre-treated with phenoxybenzamine (PBZ, 10 mg/kg) at least 0·5 hr before starting perfusion with Krebs solution.

NA release was evoked by stimulation of the splenic nerves with supramaximal monophasic rectangular pulses of 1 msec duration at 5 or 30 Hz for a total of 210 stimuli, or by rapidly injecting 0·2 ml. potassium chloride solution (3·7 M) into the splenic artery. In PBZ-treated cats, nerves were stimulated at 5 Hz for 100 stimuli.

Experiments using high potassium were also done using isolated spleen slices. Spleens were quickly removed and cut into transverse sections of about 0·5 mm thickness with a tissue slicer. Slices were washed 3 times with cold Krebs solution over a 30 min period, and then divided into three groups. One group was incubated in Krebs solution, the second group in high-potassium (140 mM) Krebs solution, and the third group in high-potassium solution containing 4-AP (1 mM). All incubations were carried out at 37 °C for 5 min, and the volume of the incubation medium was 20 ml.

In order to study the effect of 4-AP on NA uptake, NA was infused at a constant rate of 510 ng/min for a period of 22 min. Samples of venous effluents were taken for 1 min before NA infusion began, and at the 3rd, 6th, 10th, 15th and 20th min of the infusion period. After a rest period of 30 min, NA was infused in the presence of 4-AP.

Perfusion of the cat adrenal gland

The left adrenal gland was perfused with Krebs-bicarbonate solution at room temperature, as previously described by Douglas & Rubin (1961). Secretion of CA was evoked by injecting 0·4 ml. ACh (10^{-4} g/ml.) into the perfusion cannula.

Perfusion solutions

The standard perfusion solution was Krebs-bicarbonate solution having the following composition (mM): NaCl, 119; KCl, 4·7; $CaCl_2$, 2·5; $MgSO_4$, 1·2; $NaHCO_3$, 25; KH_2PO_4, 1·2; glucose, 11. This solution was bubbled with 95% O_2 + 5% CO_2, and its final pH was 7·4. In some experiments the pH of the perfusion solution was adjusted to 9·0 by adding Tris buffer (5 mM). In this solution $NaHCO_3$ was replaced by a corresponding amount of NaCl, and KH_2PO_4 by KCl. The solution was bubbled with 100% O_2. High-potassium solution was prepared by adding 140 mM potassium (as K_2SO_4) and proportionately reducing NaCl to maintain isotonicity. Low calcium solutions were made by adding the required amount of $CaCl_2$ to a calcium-free Krebs solution.

Perfusion procedure

The spleen or the adrenal was initially perfused with Krebs-bicarbonate solution for about 30 min, and then with test solutions for 15 min each, and finally with normal Krebs solution for 20–30 min. During different perfusion periods the nerves were stimulated, or high potassium was injected during the last minute of the perfusion interval.

Assay of NA

NA contents of the venous samples or incubation media were determined by the trihydroxyindole method described by Anton & Sayre (1962). Standard solutions of NA were analysed concurrently, with recoveries ranging from 70 to 90%, and all values were corrected for recovery. CA contents of perfusates from the adrenals were assayed by the same method, without the purification procedure using alumina. CA values are expressed as NA equivalents.

All measures of variations of means are standard errors. To evaluate the significance of difference between means, *t* test on paired data was used.

RESULTS

Effect of 4-AP on NA release

The background release of NA without nerve stimulation was usually undetectable. 4-AP (0·1–3 mM) did not increase the background release of NA. However, as the concentration of 4-AP was increased to 10 mM there was a very large increase in the background release of NA, and also in the vascular resistance. In three experiments, background release was increased to 54 ± 12 ng/min during perfusion with 4-AP (10 mM), and removal of calcium from the perfusion medium did not prevent release.

In contrast to the lack of direct effect of 4-AP (1 mM) on release, it potentiated release induced by low-frequency stimulation of splenic nerves. Fig. 1 shows that when splenic nerves were stimulated at 5 and 30 Hz

the mean NA outputs were $0{\cdot}21 \pm 0{\cdot}02$ and $0{\cdot}93 \pm 0{\cdot}14$ ng/stimulus, respectively. During perfusion with Krebs solution containing 4-AP (1 mM), the output at the two frequencies increased to $1{\cdot}24 \pm 0{\cdot}25$ ($P < 0{\cdot}007$) and $1{\cdot}32 \pm 0{\cdot}19$ ($P < 0{\cdot}005$) ng/stimulus, respectively. Control outputs of NA due to nerve stimulation varied considerably in different experiments. Because of this variation, the outputs during perfusion of the spleen with

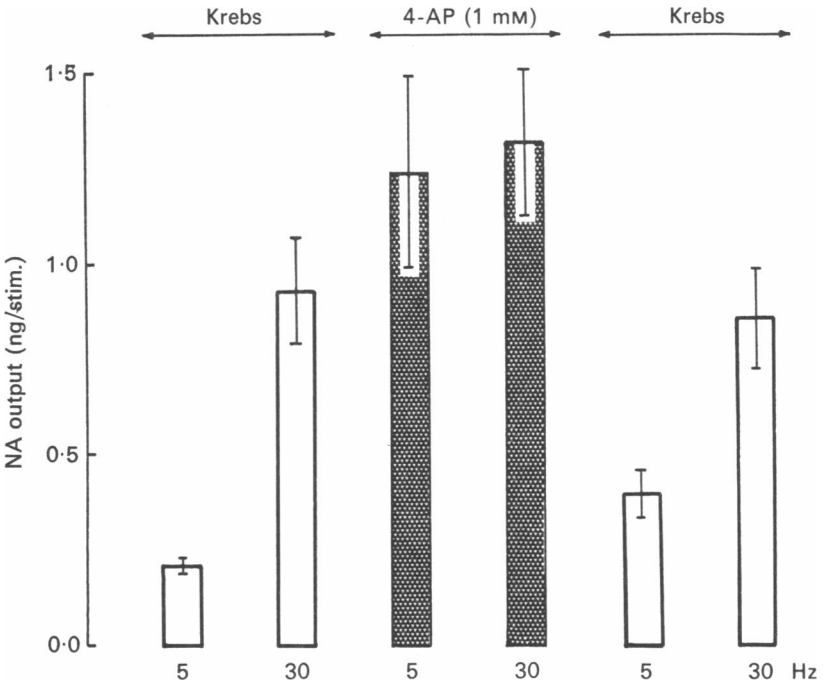

Fig. 1. Effect of 4-AP (1 mM) on NA output from the perfused spleen of the cat. After obtaining control NA outputs at 5 and 30 Hz in normal Krebs solution, the spleen was perfused with 4-AP-Krebs solution for about 20 min. Time interval between 5 and 30 Hz stimulation samples was about 15 min. Vertical lines are the S.E. of mean of seven experiments. The data for the last column (Krebs, 30 Hz) were obtained from five experiments.

4-AP, when expressed as a percentage of the initial output in each experiment, give a better indication of the ability of 4-AP to modify release. 4-AP increased the output at 5 and 30 Hz to 677 ± 169 and $144 \pm 4\%$, respectively, over the corresponding control outputs. On reperfusion with normal Krebs solution for about 30 min, the outputs at 5 Hz were still twice the control values.

Effect of 4-AP on NA release from PBZ-treated spleens

In order to study the effect of 4-AP on release, uncomplicated by either reuptake or by activation of presynaptic alpha-receptors, six experiments were done in cats pre-treated with PBZ. Since PBZ treatment itself markedly increases release at 5 Hz, the enhancing effect of 4-AP was not as pronounced as in untreated spleens. 4-AP increased the control output of $3{\cdot}26 \pm 1{\cdot}27$ to $4{\cdot}79 \pm 1{\cdot}32$ ng/stimulus ($P < 0{\cdot}006$). Expressing the output after 4-AP treatment in each experiment as a percentage of the initial control output, 4-AP significantly increased it to $178 \pm 30\%$. On reperfusion with normal Krebs solution for about 20 min, the outputs were $114 \pm 18\%$ of the initial control outputs.

To exclude the possibility that the nerves had reached the limit of their ability to release transmitter per impulse when PBZ-treated spleens were perfused with 4-AP, four experiments were done in low-calcium solution. During perfusion with low-calcium solution (0·5 mM) the output of NA at 5 Hz was reduced to $0{\cdot}42 \pm 0{\cdot}04$ ng/stimulus. However, after addition of 4-AP (1 mM) to the low-calcium Krebs solution, the output increased to $1{\cdot}52 \pm 0{\cdot}23$ ng/stimulus ($P < 0{\cdot}004$, t test on two sets; $365 \pm 30\%$ of the control). This experiment suggests that 4-AP can also mobilize calcium in PBZ-treated spleens to enhance NA release.

Effect of 4-AP on NA release induced by high potassium

To compare the effect of 4-AP on NA release induced by intermittent as against maintained depolarization, NA was released by injecting 0·2 ml. potassium chloride solution (3·7 M) into the splenic artery. In six experiments, the mean control output was 68 ± 14 ng, and after 4-AP (1 mM) it was $79 \pm 19{\cdot}7$ ng. Outputs after 4-AP were $120 \pm 20\%$ of the initial control output, and on reperfusion with normal Krebs solution they were $107 \pm 21\%$ of the initial value. The outputs after 4-AP were not significantly different from controls.

In four experiments, the effect of 4-AP on potassium-induced release was also studied in isolated spleen slices. When slices were incubated in 140 mM potassium solution, the NA output was 118 ± 21 ng/g. 5 min, and in the presence of 4-AP it was 132 ± 36 ng/g. 5 min ($107 \pm 11\%$ of the control).

Effect of graded concentrations of 4-AP on NA release

Fig. 2 shows the relationship between the concentration of 4-AP and the output of NA evoked by stimulation of the splenic nerves at 5 Hz. In these experiments the control output without 4-AP was $0{\cdot}39 \pm 0{\cdot}03$ ng/stimulus. As the Figure shows, the enhancing effect of 4-AP on NA release

was observed at a concentration as low as 0·1 mM, and the output was enhanced by about 60 %. The maximum enhancing effect was observed at 1–3 mM, and at 10 mM the output was diminished. On reperfusion of the spleen for about 20 min, following exposure to 10 mM-4-AP, the outputs were still twice as large as the initial controls.

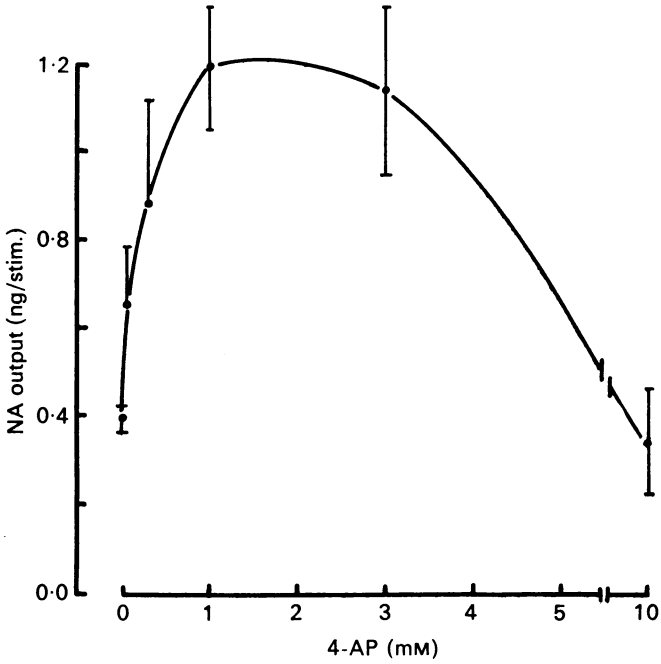

Fig. 2. Relationship between 4-AP concentration and output of NA from the perfused spleen of the cat. After obtaining the initial control output in normal Krebs solution, the spleen was consecutively perfused with Krebs solution containing 0·1, 0·3, 1, 3, and 10 mM-4-AP for 15 min each, and then again with normal Krebs solution. Splenic nerves were stimulated at 5 Hz during the last 2 min perfusion with normal Krebs solution and during perfusion with each concentration of 4-AP. Vertical lines are the S.E. of mean of five experiments. Release at 10 mM was obtained in three experiments. Curve is fitted by eye.

Effect of calcium on the enhancement of NA release by 4-AP

Fig. 3 shows that 4-AP increases release of NA in low- as well as in high-calcium Krebs solution. During perfusion of the spleen with Krebs solution containing 0·1 and 0·3 mM calcium, the release of NA at 5 Hz was usually undetectable. The NA output in 0·1 mM calcium-Krebs solution containing 4-AP was about equal to the control output obtained in normal Krebs solution without 4-AP, and in 0·3 mM calcium the output

was nearly doubled. In 2·5 and 5 mM calcium, the NA output was enhanced by nearly four to fivefold over the control output of 0·28 ± 0·06 ng/stimulus. It is difficult to compare the increase of NA output by 4-AP in 0·1 and 0·3 mM calcium-Krebs solution, since the control outputs at the respective calcium concentrations were below the sensitivity of the assay procedure. Even though 4-AP potentiated release at all calcium concentrations, maximum release was still obtained in 2·5 mM calcium.

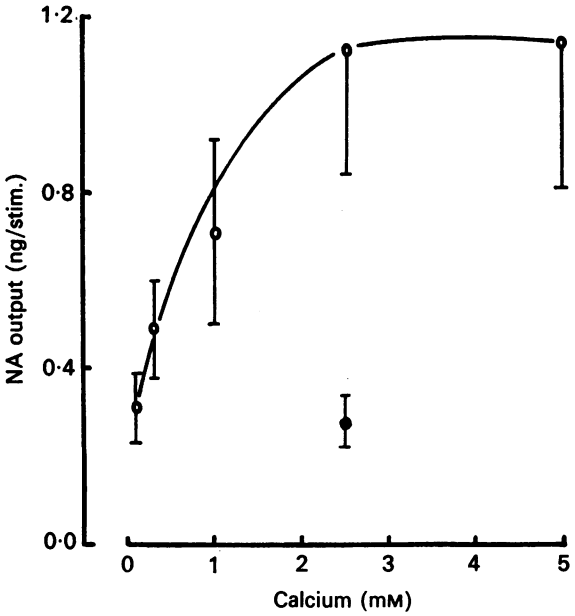

Fig. 3. Relationship between 4-AP and calcium on the output of NA from the perfused spleen of the cat. After obtaining the initial control NA output (0·28 ± 0·06 ng/stimulus; represented by ●) in normal Krebs solution, the spleen was consecutively perfused with 4-AP (1 mM)-Krebs solution containing 0·1, 0·3, 1·0, 2·5, and 5 mM calcium for 15 min each. Splenic nerves were stimulated at 5 Hz during the last 2 min perfusion with normal Krebs solution, or during perfusion with 4-AP-Krebs solution containing different calcium concentrations. Vertical lines are the S.E. of mean of four experiments. Curve is fitted by eye.

Effect of pH on the enhancement of NA release by 4-AP

Since 4-AP has a pKa of 9·17, it would mostly remain in an uncharged form in alkaline solutions (about pH 10). It was not possible, however, to perfuse the spleen with solutions having a pH greater than 9·0, since such solutions had a deleterious effect on spleen. Stimulation of the splenic nerves at 5 Hz during perfusion of the spleen with a solution at pH 8·5 (when the spleen was perfused with a solution at pH 9·0, the pH

of the venous effluent was about 8·5) released 0·39 ± 0·08 ng/stimulus of NA. Fig. 4 shows that 0·1 mM-4-AP at pH 8·5 enhances release of NA maximally to the same extent as 1 mM at pH 7·4. At 0·3 mM, 4-AP did not further enhance release, but at 1 mM the output was diminished. It should be pointed out that during perfusion with 0·3 and 1 mM-4-AP and stimulation, the spleen contracted and the flow rates were reduced by about 50%. Background release of NA did not increase at the lower concentrations (0·1 and 0·3 mM) of 4-AP, but at 1 mM the release was increased to 26 ± 10 ng/min and was comparable to the release obtained with 10 mM in normal Krebs solution.

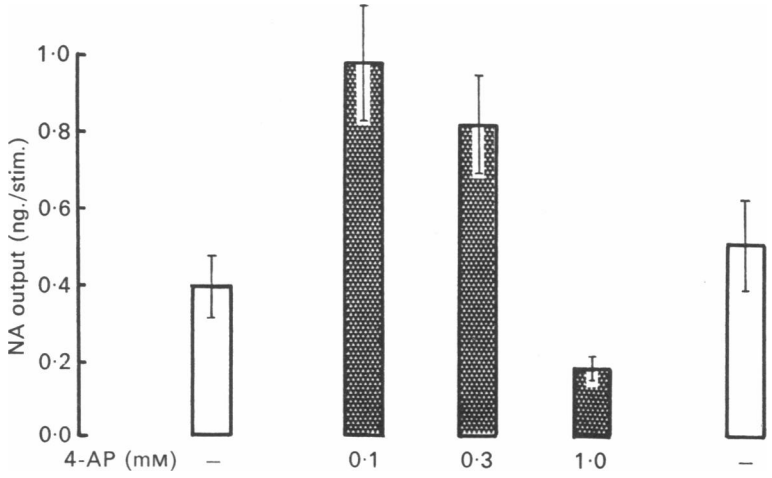

Fig. 4. Effect of 4-AP on NA output during perfusion of the spleen with a solution at pH 8·5. After obtaining the control output at pH 8·5, the spleen was consecutively perfused with this solution containing 0·1, 0·3, and 1·0 mM-4-AP, and then finally without 4-AP, for 15 min each. Splenic nerves were stimulated at 5 Hz during the last 2 min perfusion with Krebs solution (pH 8·5), or during perfusion with 4-AP solution. Vertical lines are the S.E. of mean of seven experiments. Release at 1 mM was obtained in three experiments, and recovery in five experiments.

Effect of 4-AP on the recovery of infused NA

Since NA is principally inactivated by uptake into the splenic nerves (Gillespie & Kirpekar, 1965), it was of interest to study the effect of 4-AP on NA uptake. Recovery of NA from the control spleen was first obtained, and then after a rest period of 30 min, recovery in the presence of 4-AP was determined. The mean NA recoveries during the 10th, 15th and 20th min periods in control and 4-AP treated spleens were 26 and 47%, respectively (Table 1). Even though 4-AP partially inhibits the uptake of

NA, its ability to prevent uptake is far less than the classical uptake blockers such as cocaine and PBZ (Gillespie & Kirpekar, 1965).

Effect of 4-AP on CA release from the cat adrenal gland by ACh

In three control experiments the CA output in response to repeated ACh injections was first determined. Injection of 0·4 ml. ACh (10^{-4} g/ml.) into the adrenal circulation released $3{\cdot}33 \pm 0{\cdot}6$ μg CA. When ACh was injected for the 2nd and 3rd time, the release was $2{\cdot}22 \pm 0{\cdot}4$ and $1{\cdot}54 \pm 0{\cdot}17$ μg, respectively. Expressing the outputs, in each experiment, after

TABLE 1. Effect of 4-AP on the recovery of NA in the splenic venous perfusate during arterial infusion of NA

Treatment	*n*	NA infused in 60 sec (ng)	NA recovered in 60 sec (ng ± S.E.) 10 min	15 min	20 min	Mean % recovery in 10, 15 and 20 min samples
Control	3	510	122 ± 23	134 ± 5	153 ± 20	27
4-AP (1 mM)	3	510	206 ± 46	267 ± 37	253 ± 24	47

NA was infused at a rate of 510 ng/min and venous samples were taken at 10, 15 and 20 min of infusion.

the 2nd and 3rd ACh injection as a percentage of the output during the 1st injection, they were $66 \pm 1{\cdot}7$ and $47 \pm 3{\cdot}2$ %, respectively. 4-AP did not appreciably affect release of CA by ACh. In six experiments the control CA output in response to the 1st ACh injection was $3{\cdot}02 \pm 0{\cdot}58$ μg. During perfusion of the gland with 4-AP (1 mM), the output was $1{\cdot}84 \pm 0{\cdot}22$ μg ($67 \pm 6{\cdot}7$ % of the control output), while during reperfusion with Krebs solution without 4-AP the output was $1{\cdot}65 \pm 0{\cdot}29$ μg ($58 \pm 5{\cdot}9$ % of the control output).

DISCUSSION

4-AP enhanced the amount of NA which appeared in the venous effluent during stimulation of the splenic nerves at 5 Hz. Since 4-AP also enhanced release from the spleen treated with PBZ, which blocks neuronal and extraneuronal uptake of NA and also blocks presynaptic alpha-receptors (see Kirpekar, 1975), it appears that the increase of NA output by 4-AP is probably due to a greater release of NA from sympathetic nerves. Even though 4-AP blocked the uptake of NA to a certain extent, this property alone is not sufficient to account for a nearly fivefold increase in output at the lower frequency of stimulation. The classical uptake blockers, such as cocaine and desmethylimpramine, do not appreciably enhance release on nerve stimulation (Kirpekar & Cervoni, 1963; Geffen, 1965). Even

though 4-AP increased NA release at 30 Hz, the enhancement was much less in comparison with its effect on release at 5 Hz.

Failure to increase release at 30 Hz as compared to 5 Hz is difficult to explain, but it may be related in some way to the proportion of time the nerve membrane remains depolarized. Nerve stimulation causes short-lived periods of nerve membrane depolarization, and as the frequency of stimulation increases, the proportion of time the membrane is depolarized also increases; high potassium could represent the extreme situation in which the membrane is permanently depolarized for the duration of potassium application. If 4-AP loses the ability to increase output by the time a frequency of 30 Hz is reached, it is not surprising that it also fails to increase output in high potassium. Like 4-AP, TEA is also more effective in enhancing the responses of the anococcygeus muscle to field stimulation of the motor adrenergic nerves at low frequencies of stimulation than at high frequencies (Gillespie & Tilmisany, 1976), and it does not enhance NA release at 30 Hz (Kirpekar *et al.* 1976). Alternatively, if facilitation of release with increase in frequency reflects a greater accumulation of calcium in the terminals on the time average during a train of pulses at a high frequency, as compared to a low frequency, then 4-AP conceivably may be less effective in further enhancing NA release at the higher frequency of stimulation.

Enhancement of NA release by 4-AP occurred in low as well as in high extracellular concentrations of calcium. In low-calcium solutions (0·1 and 0·3 mM), the release of NA at 5 Hz must have been markedly increased by 4-AP, since NA release is virtually undetectable in the absence of this agent in low-calcium solutions. Maximum output was obtained in 2·5 mM calcium. Since 4-AP increases release, it was hoped that maximal release would perhaps be obtained at an extracellular concentration of calcium much lower than 2·5 mM.

Since 4-AP facilitates release, it is possible that it may also facilitate calcium entry, which is essential for the evoked release of NA. However, the fact that this facilitatory effect is specific to release of NA induced by nerve stimulation and not by potassium, suggests that 4-AP does not have a direct action facilitating calcium entry into the sympathetic nerves. The selective enhancement of nerve-induced release by 4-AP may be explained on the property of this agent to inactivate potassium current in nerves (Pelhate & Pichon, 1974), and thereby prolong the duration of the action potential in the sympathetic nerve, which would allow the calcium channels to open longer and more calcium to enter.

4-AP was more effective in enhancing release at extracellular pH of 8·5 than 7·4. This probably is understandable, since 4-AP, which has a pKa of 9·17, would remain mostly in an unionized form at an external pH of

about 9·5, and would penetrate the nerve endings more readily in a solution with high pH value. These findings suggest that 4-AP may act intracellularly to produce its characteristic effects on potassium inactivation.

4-AP, unlike TEA, did not block the release of CA from the perfused cat adrenal gland by ACh. Because of its lack of effect on ganglionic transmission, it may prove useful in the study of CA release from the adrenal gland and the sympathetic nerves by nicotinic stimulation.

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