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MECHANISM OF NICOTINE-INDUCED RELEASE OF NORADRENALINE FROM ADRENERGIC NERVE ENDINGS

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1 A study of the mechanism of release of [^{3}H]-noradrenaline ([^{3}H]-NA) by nicotine from isolated vas deferens of the rat was made using incubation media of different ionic composition.

2 Nicotine (20 μg/ml)-induced release of [^{3}H]-NA was significantly potentiated in K^+-free Krebs solution as compared to that in normal Krebs-Ringer solution.

3 Nicotine-induced release of [^{3}H]-NA was significantly reduced in Na^+-deficient Krebs solution (containing only 11 mM Na^+) and was abolished in Na^+-free Krebs solution.

4 In totally depolarized tissues, nicotine failed to cause an outflow of [^{3}H]-NA but Ca^{2+} (5 mM) did so.

5 Nicotine required the presence of Ca^{2+} in the incubation medium to cause release of [^{3}H]-NA from adrenergic nerve terminals, the magnitude of release being dependent upon the concentration of Ca^{2+}.

6 Nicotine-induced release of [^{3}H]-NA was demonstrated in high Ca^{2+}, Na^+-free Krebs solution in which all Na^+ had been replaced with Ca^{2+}.

7 It is concluded that nicotine increases the membrane permeability to both Na^+ and Ca^{2+}. It is also suggested that the increase in permeability to Ca^{2+} alone is not sufficient but a local depolarizing action of nicotine is necessary to cause release of noradrenaline from adrenergic nerve endings.

Introduction

Nicotinic agents can generate and propagate impulses at the adrenergic nerve terminals (Ferry, 1963; Cabrera & Torrance, 1964), but it has been contended that nicotine-induced release of noradrenaline (NA) is not caused by these impulses (Jayasundar & Vohra, 1977). Bevan & Su (1972) using an isotope and a frozen section technique demonstrated that nicotine is concentrated, probably via a NA uptake process, in the nerve-containing layer of rabbit aortic strips. However, Westfall & Brasted (1972) reported that nicotine did not have to be taken up into the adrenergic neurone before being able to release NA. The exact mode of action of nicotinic agents on the adrenergic nerve terminals remains unknown. The present work was undertaken to investigate whether the release of NA from adrenergic nerve terminals by nicotine requires the presence of Na^+, K^+ and Ca^{2+} in the incubation medium and to determine whether nicotine causes local depolarization and/or increase in permeability to Ca^{2+} at adrenergic nerve terminals.

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Methods

Male Wistar rats (200–300 g) were killed by cervical dislocation and the vasa deferentia were dissected out.

Release of [^{3}H]-noradrenaline ([^{3}H]-NA) by nicotine

Both vasa deferentia were slit open longitudinally and were placed together in a culture tube containing 3 ml Krebs-Ringer bicarbonate solution. The incubation medium was continuously gassed with a mixture of 95% O_2 and 5% CO_2 and its temperature was maintained at 36 ± 1°C. After equilibration for 1 h, the tissues were incubated for 15 min in a tube containing 10 μCi of [^{3}H]-NA ((±)-[7-^{3}H]-noradrenaline, New England Nuclear Corporation, Boston, Specific activity 13.6 Ci/mmol) in 3 ml normal Krebs solution. The incubation was terminated after 15 min by washing the tissues rapidly in a tube containing 3 ml of fresh Krebs solution and repeating the procedure. The tissues were washed at 10 min intervals for 90 min to ensure the elimination of extra-neuronally bound [^{3}H]-NA. The tissues were then incubated in fresh Krebs solution every 5 min and these solutions were retained for determination of [^{3}H]-NA. When the tissues were to be exposed to nicotine (20 μg/ml) for 5 min, the alkaloid contained in 0.1 ml of Krebs solution was added to the tube.

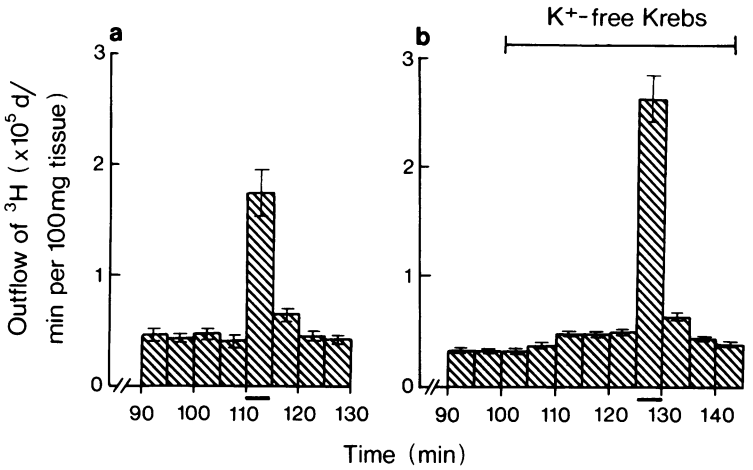

Figure 1 The effect of nicotine on the outflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from isolated vas deferens of the rat incubated in normal and K^+-free Krebs solution. The tissues were incubated with [^{3}H]-NA for a period of 15 minutes. After the outflow of tritium attained a steady state (90 min), the tritium outflow per 5 min fraction was determined. (a) The tissues were exposed for 5 min to nicotine (20 μg/ml, indicated by a bar); (b) before exposure to nicotine, the tissues were incubated in K^+-free Krebs solution for 25 minutes. The results are mean of 6 experiments. Vertical lines show s.e. mean.

Aliquots (0.5 ml) of each of the retained solutions were used to determine the total radioactivity. Counts per min (ct/min) were determined by the use of a Unilux II scintillation spectrometer and Bray's solution (Bray, 1960). The degree of quenching was determined by the dual channel ratio counting procedure.

Composition of incubation media

Normal Krebs-Ringer solution had the following composition (mM): NaCl 138.56, KCl 4.63, $CaCl_2$ 2.47, $MgCl_2$ 1.16, dextrose 11, disodium edetate 0.01 and ascorbic acid 0.005. This and the following modified Krebs solutions were buffered with Tris base (1 mM) and the pH was adjusted to 7.4–7.6. The composition of the following solutions remained the same as in normal Krebs-Ringer solution except for the modifications indicated below.

Na^+-free Krebs solution: all NaCl was replaced with an osmotically equivalent amount of sucrose (246.2 mM).

Na^+-deficient Krebs solution: all but 11 mM of NaCl was replaced with an osmotically equivalent amount of sucrose (226.7 mM).

K^+-free Krebs solution: KCl (4.63 mM) was omitted.

High K^+ Krebs solution: all but 45 mM of NaCl was replaced with 115 mM of KCl and $CaCl_2$ was reduced to 0.25 mM. This solution has a $Ca^{2+}/[Na^+]^2$ ratio which is the same as in normal Krebs solution.

Ca^{2+}-free Krebs solution: $CaCl_2$ (2.47 mM) was omitted.

Low and high Ca^{2+}-Krebs solution: $CaCl_2$ was either reduced to 0.25 mM or increased to 5 to 10 mM respectively. Since this involved an alteration of only small amounts of salts (up to 10 mM), a corresponding adjustment in tonicity of the solution was not made.

High Ca^{2+}, Na^+-free Krebs solution: all NaCl was replaced with 110 mM of $CaCl_2$.

Statistical analysis

Statistical differences were analysed by Student's t test. A value of $P < 0.05$ was considered significant.

Results

Effect of nicotine on the outflow of [^{3}H]-noradrenaline from tissues incubated in normal and K^+-free Krebs solution

As shown in Figure 1a, nicotine (20 μg/ml) increased the resting outflow of [^{3}H]-NA from tissues incubated in normal Krebs solution. The nicotine-induced outflow of [^{3}H]-NA was 420 ± 52% of the control resting outflow.

Figure 1b shows the effect of nicotine (20 μg/ml) in K^+-free Krebs solution. The total withdrawal of K^+ caused a gradual increase in the resting outflow of [^{3}H]-NA which was well sustained for at least 15 minutes. An exposure to nicotine at this time resulted in a marked increase in the outflow, which was 555 ± 44% of the control resting outflow of [^{3}H]-

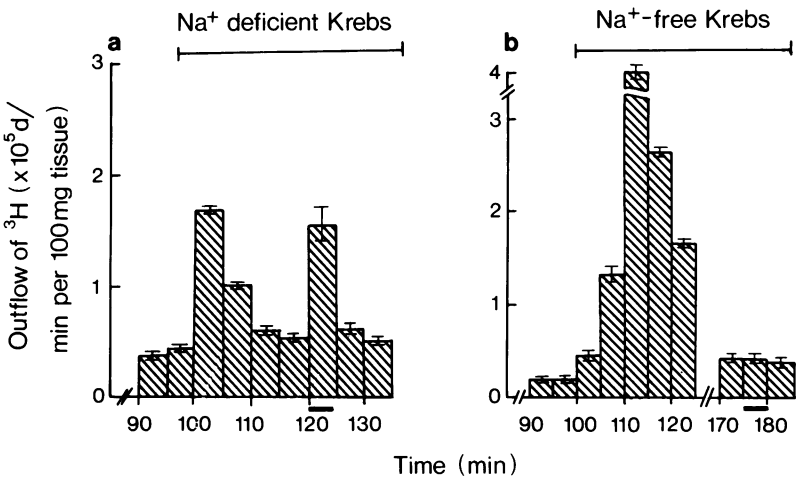

Figure 2 The effect of nicotine on the outflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from the isolated vas deferens of the rat incubated in Na^+ deficient and Na^+-free Krebs solution. The tissues were incubated with [^{3}H]-NA for 15 minutes. After the outflow of tritium attained a steady state (90 min), the tritium outflow per 5 min fraction was determined. The tissues were incubated (a) in Na^+ deficient Krebs solution and (b) in Na^+-free Krebs solution. The tissues were exposed for 5 min to nicotine (20 μg/ml, indicated by a bar). The results are mean of at least 5 experiments. Vertical lines show s.e. mean.

NA. This is significantly ($P<0.05$) higher than the response obtained in normal Krebs solution.

Effect of nicotine in Na^+-deficient and Na^+-free Krebs solution

When tissues were exposed to a medium containing Na^+ 11 mM, the resting outflow of [^{3}H]-NA was enhanced but declined within 15 min almost to the initial level. Exposure to nicotine at this time resulted in an enhanced (Figure 2a) outflow which was $294 \pm 27\%$ of the control resting outflow of [^{3}H]-NA. This response is significantly ($P<0.05$) lower than that obtained in normal Krebs solution.

The total withdrawal of Na^+ from the incubation medium caused a progressive increase in the resting

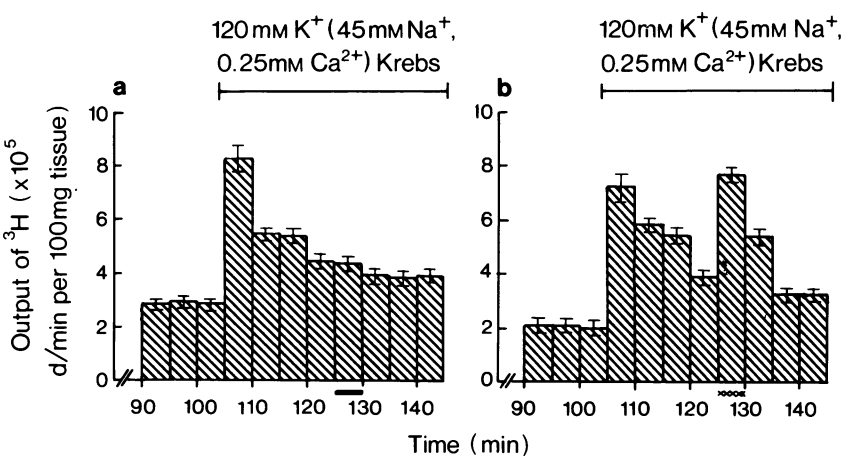

Figure 3 The effect of nicotine and calcium on the outflow of [^{3}H]-noradrenaline ([^{3}H])-NA) from isolated vas deferens of the rat incubated in high K^+-Krebs (120 mM K^+, 45 mM Na^+, 0.25 mM Ca^{2+}) solution. The tissues were incubated with [^{3}H]-NA for 15 minutes. After the outflow of tritium attained a steady state (90 min), the tritium outflow per 5 min fraction was determined. (a) The tissues were exposed to nicotine (20 μg/ml, indicated by a bar); (b) the concentration of Ca^{2+} in the incubation medium was raised to 5 mM (indicated by xxxx). The results presented are mean of at least 5 experiments. Vertical lines show s.e. mean.

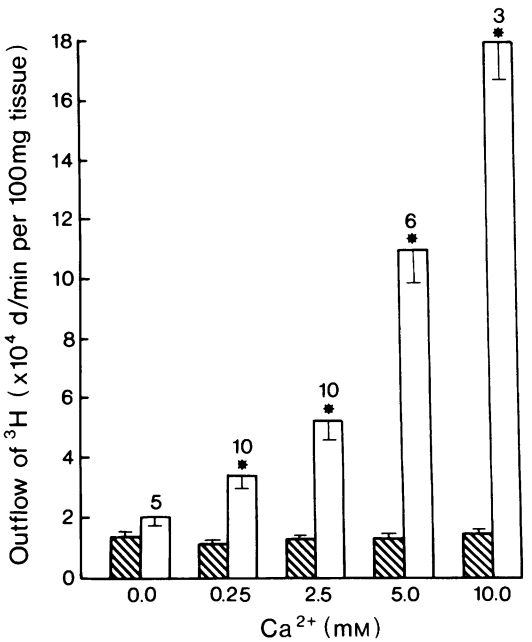

Figure 4 The effect of various concentrations of calcium on the nicotine-induced outflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from isolated vas deferens of the rat. The tissues were exposed to the Krebs solution containing different concentrations of Ca^{2+} for 20 min before the addition of nicotine (20 μg/ml). Control outflow—hatched columns; nicotine-induced outflow—open columns. The results presented are the mean of the number of experiments shown above the pairs of columns. Vertical lines show s.e. mean.
* Significantly different from the control resting outflow ($P < 0.01$).

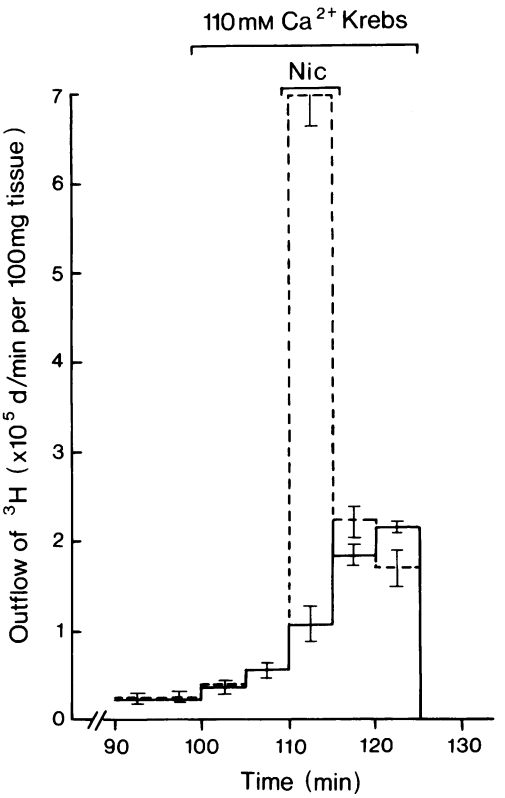

Figure 5 The effect of nicotine (Nic) on the outflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from isolated vas deferens of rat incubated in high Ca^{2+}, Na^+-free Krebs solution. The tissues were incubated with [^{3}H]-NA for 15 minutes. After the outflow of tritium attained a steady state (90 min), the tritium outflow per 5 min fraction was determined. The tissues were exposed to high Ca^{2+} (110 mM), Na^+-free Krebs solution indicated by a bar at the top of the figure. The solid line represents mean ± s.e. (6 control experiments). The broken line superimposed on the solid line represents mean ± s.e. (6 experiments) in which the tissues were exposed for 5 min to nicotine (20 μg/ml).

outflow of [^{3}H]-NA reaching a maximum in 15 minutes. The enhanced rate of release returned to the initial level in about 60 minutes. The addition of nicotine then failed to cause an increase in the resting outflow (Figure 2b).

Effect of nicotine in high K^+ Krebs solution

In preliminary experiments, it was seen that nicotine failed to cause an increase in the outflow of [^{3}H]-NA from tissues soaked in high K^+ Krebs solution. To test whether this failure to respond to nicotine in high K^+ medium was due to tissue damage, a response to calcium was chosen as the criterion, as it has been shown (Douglas & Rubin, 1963) that the addition of Ca^{2+} caused release of adrenaline from adrenal gland perfused in high K^+ medium. Calcium (up to 20 mM) failed to release [^{3}H]-NA from tissues immersed in high K^+ (containing 0 or 25 mM Na^+ and 2.5 mM Ca^{2+}) Krebs solution. When tissues were soaked in high K^+ Krebs solution in which the $Ca^{2+}/[Na^+]^2$ ratio was kept the same as in normal Krebs solution, the resting outflow of [^{3}H]-NA abruptly increased then declined but remained at a level higher than initially for at least 60 minutes. In this solution, addition of Ca^{2+} (5 mM) caused an increase in the outflow of [^{3}H]-NA (Figure 3b) which was 198 ± 8% of the control resting outflow. Nicotine (20 μg/ml) still failed to cause an increase in the outflow of [^{3}H]-NA (Figure 3a).

Effect of various concentrations of Ca^{2+} on nicotine-induced outflow of [^{3}H]-noradrenaline

The tissues were exposed to various concentrations of Ca^{2+} for 20 min before nicotine (20 μg/ml) was added.

The changes in the concentration of Ca^{2+} in the incubation medium did not alter significantly the rate of resting outflow of [^{3}H]-NA. The outflow of [^{3}H]-NA obtained before and in the presence of nicotine at various concentrations of Ca^{2+} is shown in Figure 4. In normal calcium medium (2.5 mM), the nicotine-induced release was $419 \pm 45\%$ of the control resting outflow. When Ca^{2+} concentration in the medium was reduced to 0.25 mM, there was a relatively smaller ($299 \pm 38\%$ of control resting outflow) but significant ($P < 0.01$) increase in the outflow of [^{3}H]-NA caused by nicotine. In Ca^{2+} free medium, niotine caused only a small insignificant increase in the outflow of [^{3}H]-NA. When the concentration of Ca^{2+} was raised to 5 and 10 mM, the nicotine-induced outflow of [^{3}H]-NA was enhanced to $842 \pm 97\%$ and $1256 \pm 89\%$ of the control resting outflow respectively.

Effect of nicotine in high Ca^{2+}, Na^+-free Krebs solution

Addition of nicotine 15 min after exposure of tissues to high Ca^{2+}, Na^+-free Krebs solution caused a profound increase in the outflow of [^{3}H]-NA. As may be seen in Figure 5, at the time of exposure to nicotine, the resting outflow in control tissues was 1.07×10^5 d/min per 100 mg tissue wet wt, while the outflow in the presence of nicotine reached a peak of 6.9×10^5 d/min per 100 mg tissue wet weight. The difference between these values is highly significant ($P < 0.01$).

Discussion

It has been shown that nicotine-induced release of [^{3}H]-NA was potentiated in K^+-free Krebs solution as compared to that in normal Krebs solution. This suggests that the presence of K^+ in the extracellular fluid may not be necessary for the action of nicotine. The potentiated response to nicotine in K^+-free medium may be because of reduced reuptake of released noradrenaline (Gillis & Paton, 1967; Bogdanski & Brodie, 1969).

Nicotine-induced outflow of [^{3}H]-NA was significantly reduced in Na^+-deficient medium and was totally abolished in Na^+-free medium. These results suggest that nicotine requires at least a small amount of Na^+ for its action on the adrenergic nerve terminals. In Na^+-deficient and Na^+-free media, the inward movement of Na^+ (and hence depolarization of membrane) by any drug is expected to be impaired (Douglas & Rubin, 1963). Thus, the failing response to nicotine in Na^+-deficient and Na^+-free media observed in this study suggest that in normal Krebs solution, nicotine must increase the permeability of the nerve terminal membrane to Na^+ and consequently cause an influx of Na^+. This would result in local depolarization of the terminal membrane and cause the release of noradrenaline.

Nicotine failed to cause a release of [^{3}H]-NA in high K^+ Krebs solution, but the solution used in this study was considerably deficient in Na^+ (45 mM) and Ca^{2+} (0.25 mM). However, the failure of nicotine to release [^{3}H]-NA in this solution is unlikely to be due to the deficiency of Na^+ or Ca^{2+} because it was demonstrated that nicotine caused a significant increase in the outflow of [^{3}H]-NA in incubation media containing only 11 mM Na^+ (Figure 2a) or 0.25 mM Ca^{2+} (Figure 4). Incubation media containing high K^+ have been used to depolarize totally various isolated tissues such as cat adrenal gland (Douglas & Rubin, 1961; 1963), mammalian non-myelinated nerve fibres (Armett & Ritchie, 1963) and smooth muscles (Evans, Schild & Thesleff, 1958). If nicotine causes release of noradrenaline by depolarization of nerve terminal membrane, it would be expected to fail in totally depolarized tissues (i.e. in high K^+ Krebs solution). Since in the present study, nicotine did not cause release of [^{3}H]-NA in high K^+ Krebs solution, this further supports the contention that nicotine causes a local depolarization of nerve terminal membrane to bring about release of noradrenaline.

It has been demonstrated earlier that the release of noradrenaline following depolarization of adrenergic nerve endings was calcium-dependent and the magnitude of release was directly proportional to extracellular calcium concentration (Kirpekar & Wakade, 1968). Since depolarization of the cell membrane increases the influx of calcium ions in skeletal and cardiac muscles, adrenal medulla and squid axon (Hodgkin & Keynes, 1957; Bianchi & Shanes, 1959; Douglas & Poisner, 1962; Grossman & Furchgott, 1964), it was suggested that the depolarization of postganglionic adrenergic fibres may also lead to an increased influx of calcium (Kirpekar & Misu, 1967). In the present study, it was shown that the outflow of [^{3}H]-NA caused by nicotine is dependent upon the presence of Ca^{2+} in the medium and that the magnitude of the response is directly related to the extracellular concentration of Ca^{2+}. Thus nicotine may induce local depolarization which in turn causes influx of calcium, resulting in the release of noradrenaline.

The demonstration (Figure 5) that nicotine in high Ca^{2+}, Na^+-free Krebs solution causes a large outflow of [^{3}H]-NA, when local depolarization by nicotine is not possible suggests that nicotine is also capable of increasing permeability of membrane to Ca^{2+} to release [^{3}H]-NA. On the other hand, nicotine failed to cause a release of [^{3}H]-NA in Na^+-free and in high K^+ media, although these media contained enough Ca^{2+} to support nicotine-induced release of [^{3}H]-NA. Therefore, it seems that in a physiological ionic environment (normal Krebs solution), an increase in permeability to Ca^{2+} alone may not be sufficient to cause the release of noradrenaline. Thus a

stronger stimulus by nicotine, i.e. the depolarization of nerve terminals seems to be necessary to bring about the release of noradrenaline.

It is concluded that nicotine increases the membrane permeability to both Na^+ and Ca^{2+}. It is also suggested that a mere increase in permeability to Ca^{2+} by nicotine is not sufficient but a local depolarizing effect of nicotine is necessary to cause release of noradrenaline from adrenergic nerve terminals. Nicotinic agents have earlier been shown to cause increase in permeability to Na^+ as well as Ca^{2+} at various nicotinic sites such as mammalian non-myelinited nerve fibres (Armett & Ritchie, 1963), neuromuscular junction (Del Castillo & Katz, 1955; Jenkinson & Nicholls, 1961) and adrenal medulla (Douglas & Rubin, 1961; 1963). Thus, the mode of action of nicotine on adrenergic nerve terminals resembles its action at other sites.

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