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Kinetics of the release of noradrenaline from micropipettes: interaction between ejecting and retaining currents

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Summary

1. The role of ejecting and retaining currents in determining the kinetics of the release of [^{14}C]-noradrenaline (NA) from micropipettes of the type used in microelectrophoresis experiments has been investigated by the liquid scintillation counting technique.

2. In the absence of any electrophoretic current a constant rate of release of NA was established.

3. All retaining currents examined gradually reduced the spontaneous release to zero. Higher retaining currents abolished spontaneous release more quickly.

4. A linear relationship was identified between the rate of electrophoretic release of NA and the intensity of the ejecting current. The mean transport number of NA was found to be 0·17.

5. All retaining currents studied reduced the amount of NA released during a subsequent application of an ejecting current. This was due to a prolongation of the time necessary to establish a steady-state rate of release. The magnitude of this effect was related to both the intensity and the duration of application of the retaining current.

6. The results are discussed in terms of a theoretical model of ion movements within the tip of the micropipette.

Introduction

The technique of microelectrophoresis involves the use of electrical currents to control the release of drugs from micropipettes. It has been suggested on theoretical grounds that there is a close relationship between rate of drug release from the micropipette and the concentration of drug molecules achieved at receptor sites. If there is a constant rate of release, the concentration at receptor sites will gradually increase until, after some time, a plateau concentration is achieved (Curtis, Perrin & Watkins, 1960). Since the concentration of drug molecules at receptor sites at any time depends on the rate of release, it is important to know how the latter changes with time during the application of an ejecting current.

We have examined the shape of the release curve (rate of release plotted against time) by measuring the release of [^{14}C]-noradrenaline (NA). There have been previous reports concerning the release of NA from micropipettes, in which either a fluorimetric assay of NA (Krnjević, Laverty & Sharman, 1963) or, more recently the liquid scintillation technique to assay [^{3}H]-NA were used (Bradley & Candy,

1970; Hoffer, Neff & Siggins, 1971). The latter technique is more sensitive but less specific than the fluorimetric method since it has been reported that as much as 50% of the radioactivity in samples of [^{3}H]-NA may not be carried by NA molecules (Offerman & Merrills, 1968). Therefore, [^{14}C]-NA was used in the present experiments.

Methods

Five barrelled micropipettes were constructed from Pyrex glass tubing of external diameter 1·5 mm and internal diameter 1·0 mm (Herz, Wickelmaier & Nacimiento, 1965), and were filled by boiling in distilled water under reduced pressure. The water in the barrels was replaced by the appropriate solutions by means of a thin Portex catheter. After filling, the pipettes were stored in the dark at 4° C in an atmosphere of nitrogen for at least 36 h before use. Immediately before use, the tip of the micropipette was carefully broken in order to obtain an overall tip diameter of 4·0–6·5 μm.

Four barrels of each micropipette were filled with radioactive noradrenaline (NA) solution (0·02 M, specific activity 5 mCi/mmol). In some preliminary experiments in which [^{3}H]-NA was used, it was found that more radioactivity was released during the passage of retaining currents than when no current was passed. Subsequent chromatographic analysis of the samples collected during the passage of retaining currents showed that the radioactivity released was not associated with NA (see also Offerman & Merrills, 1968). Therefore, because of its greater radiochemical stability, we decided to use [^{14}C]-NA rather than the ^{3}H-labelled compound, in our experiments. The lower specific activity of the [^{14}C]-NA, however, made it necessary to dilute the radioactivity as little as possible with unlabelled NA when preparing the final solution. Accordingly, we used a 0·02 M solution, rather than the 0·2 M solution which we use in our *in vivo* experiments. In order to increase the resolution of the method when very small outputs were measured, as in the presence of retaining currents, four barrels of each micropipette were filled with the radioactive NA solution and the electrophoretic currents were applied to all of them simultaneously. Errors were further reduced by the use of long sampling periods (4–128 minutes).

Freeze-dried (±)-noradrenaline [carbinol-^{14}C] (±)-bitartrate (specific activity 22, 52 and 54 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England. A solution of (±)-noradrenaline bitartrate was prepared by mixing the required amount of radioactive and non-radioactive NA to give the final solution to be used in the micropipettes (0·02 M, specific activity 5 mCi/mmol). The pH of this solution was 3·0.

The microelectrophoretic circuit used in these experiments was described by Roberts & Straughan (1967). The electrophoretic current was continuously monitored with a Pye Scalamp Galvanometer.

The micropipette was held vertically in a micromanipulator and the tip was lowered into a small glass vial containing 1·0 ml 0·165 M NaCl solution. A silver wire, immersed into the saline solution, served as an earth electrode. Electrophoretic currents of identical intensities were applied to each of the four NA-containing barrels. Since the NA ion is a cation, positive currents were used to eject and negative currents to retain the drug ions. (In this paper, values of

current intensity will always refer to the intensity of the electrophoretic current applied to each of the four NA-containing barrels). The outer surface of the micropipette was washed with distilled water before the collection of each sample.

At the end of each period of collection, the contents of the vial were transferred into a glass scintillation vial. The collecting vial was washed repeatedly with the scintillator and the washings were added to the contents of the scintillation vial. A total of 10 ml of scintillator was added to each sample. The scintillator contained 0·267% PPO (2,5-diphenoxyloxazole), 0·0067% POPOP (1,2-bis-(5-phenyloxazol-2-yl)-benzene) in toluene with 33% Triton X-100.

Disintegrations for each sample were counted for 10 min in a Packard Tricarb liquid scintillation spectrometer. Disintegrations per minute (d/min) values were determined for each sample (after subtraction of the background), and these were converted into pmol values on the basis of the specific activity of the NA solution contained in the micropipette. Background counts per min were approximately equivalent to 2·5–3·0 pmol NA.

Results

Release in the absence of an electrophoretic current

In fourteen micropipettes (Nos. 1–14, see Table 1) the spontaneous release of [^{14}C]-NA was measured before any electrophoretic current had been applied. Before the first sample was collected, the tip of the micropipette was broken in order to obtain the desired tip diameter (4·0–6·5 μm). Then, samples were collected during successively increasing periods of time (8–128 minute). With most micropipettes, the rate of spontaneous release fell from an initially high level, until a steady-state rate of release was attained 8–64 min after the start of the experiment (Figure 1).

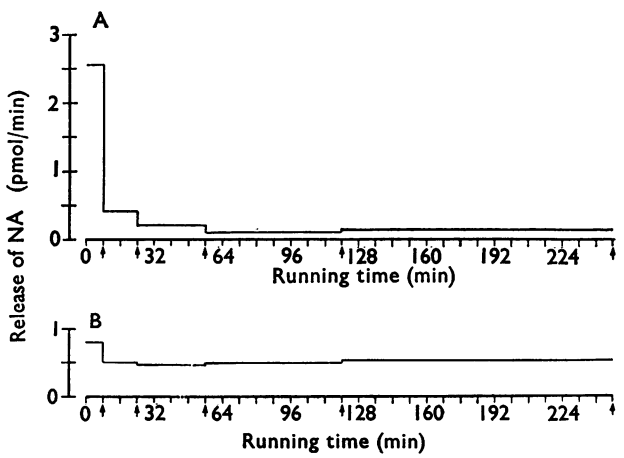

FIG. 1. The rate of spontaneous release of noradrenaline (NA) from two micropipettes. (Mean rate of release from all 4 barrels for each collection period.) The times at which samples were collected are indicated by arrows below the time base. The rate of spontaneous release, measured before any current had been applied to the barrels, declined from an initially high level, until a steady-state rate was attained. A: micropipette No. 9 ; B: micropipette No. 4 (see Table 1).

TABLE 1. *Rate of steady-state spontaneous release and transport number of noradrenaline (NA) obtained from each of the micropipettes.*

Micropipette number	Tip diameter (μm)	Rate of spontaneous release from four barrels (pmol/min)	Transport number (mean ± standard deviation)
1	5·0	0·37	0·11±0·04
2	5·0	1·36	0·14±0·02
3	5·0	1·40	0·03±0·00
4	4·0	0·50	0·19±0·01
5	5·0	0·63	0·11±0·03
6	5·0	0·88	0·14±0·04
7	5·0	3·00	0·13±0·02
8	5·0	1·30	0·29±0·06
9	5·0	0·16	0·19±0·02
10	6·5	3·30	0·17±0·00
11	5·0	0·80	0·22±0·02
12	6·0	1·91	0·33±0·05
13	5·5	2·56	0·18±0·01
14	6·0	2·05	not measured

Transport numbers were calculated individually for each of the samples collected from each micropipette. Four barrels of each micropipette contained [^{14}C]-NA (0·02 M, specific activity 5 mCi/mmol). Mean value of transport number for 13 micropipettes=0·17.

The mean rate of spontaneous release from four NA-containing barrels in 14 micropipettes measured under steady state conditions was 1·44 pmol/min (see Table 1).

Release in the presence of a retaining current

1. When the application of a retaining current is not preceded by an ejecting pulse

The effectiveness of various retaining currents in counteracting the spontaneous release of [^{14}C]-NA was examined with two micropipettes (nos. 10 and 14). The spontaneous output of NA during a 16 min period, during which no current was applied, was compared with the output measured during a 16 min period when

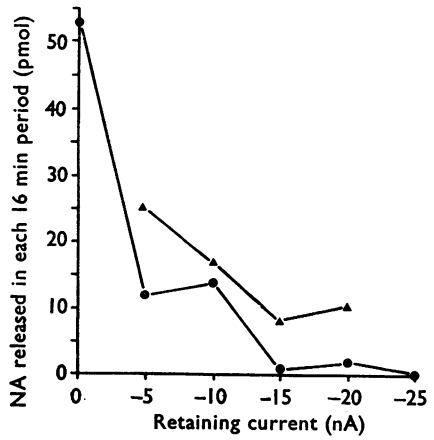

FIG. 2. The relationship between the intensity of the retaining current and the release of noradrenaline (NA) from 4 barrels of micropipette No. 10 (see Table 1). ●——●: release in the presence of a retaining current following a period (32 min) when no current was applied. ▲——▲: release in the presence of a retaining current following an ejecting pulse (+100 nA, 16 min). The release of NA declined as higher retaining currents were applied. A retaining current was less effective following an ejecting pulse.

a retaining current was applied. The retaining currents used were −5, −10, −15, −20 and −25 nA. Each application of a retaining current was preceded by two successive 16 min periods of spontaneous release. From both micropipettes, a retaining current of −5 nA reduced the output to about 25% of the spontaneous release. A further reduction in the spontaneous release was observed when higher retaining currents were used; no spontaneous release could be detected when −25 nA was applied. The results obtained from micropipette No. 10 are displayed in Figure 2.

2. When the application of a retaining current is preceded by an ejecting pulse

A higher output was measured during the application of a retaining current if the retention period was preceded by an ejecting pulse. The results obtained from micropipette No. 10 are shown in Figure 2. Similar observations were made with micropipettes Nos. 8 and 9, with which retaining currents of up to −100 nA were tested. The output decreased with higher currents but could not be eliminated completely within the first collection period, even when −100 nA was used.

The time-course of the action of retaining currents was measured by applying a retaining current of given intensity continuously and measuring the output at regular intervals of 5 minutes. Every retaining current tested (−5 to −100 nA) invariably became effective if it was applied for a long enough time. This was true even of weak retaining currents (Figure 5C).

Release during the application of an ejecting current

1. When the application of an ejecting current is not preceded by a retaining current

The electrophoretic release of [^{14}C]-NA was measured with a wide range of ejecting current intensities (+25 to +200 nA) and a wide range of collection times (4–128 minutes). Since the prior application of a retaining current can influence subsequent drug release (see below), it was important to ensure that measurements of the rate of electrophoretic release were not subject to this distortion. Therefore retention periods or periods of spontaneous release were not interspersed between ejection periods.

In all the micropipettes tested (Nos. 1–13) the rate of release of NA was linearly related to the intensity of the ejecting current (see Figure 3). The transport number of NA was calculated individually for each sample collected from each micropipette, by substitution in the following equation (Curtis, 1964):

$$n = \frac{R_i z F}{i}$$

where n is the transport number, z is the valency (in the case of NA, z=1), F (coulombs) is Faraday's constant, i (amps) is the total ejecting current applied to all four barrels and R_i (mol/s) is the rate of electrophoretic release of NA (total rate of release minus steady-state rate of spontaneous release). Mean values of the transport numbers obtained from each of the micropipettes are shown in Table 1; the average value for the 13 micropipettes was 0·17. It can be seen that the transport number of NA measured in most of the micropipettes was between 0·1 and 0·25. No correlation could be found between the transport

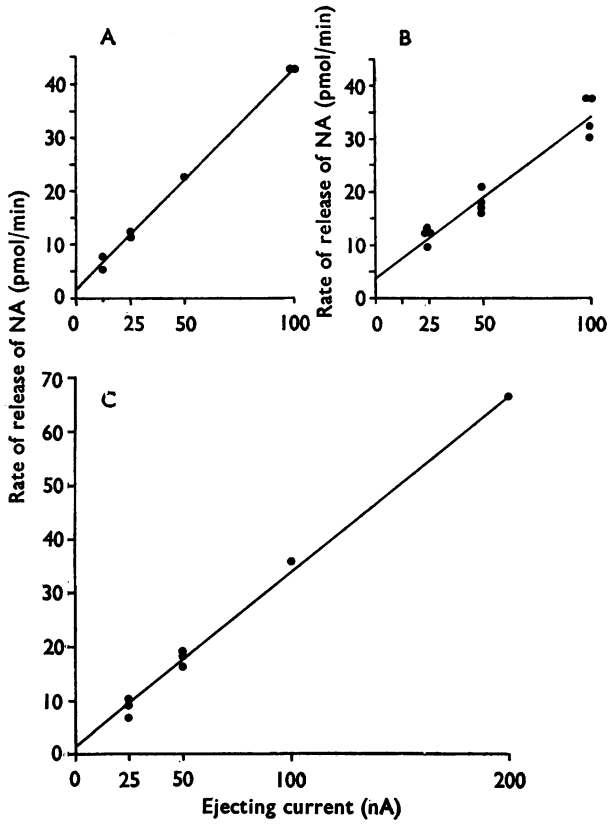

FIG. 3. The relationship between the intensity of the ejecting current and the rate of release of noradrenaline (NA) from three micropipettes (all four barrels). The rate of release was linearly related to current intensity over a wide range of ejecting current intensities (+12·5 to +200 nA). A: micropipette No. 10 (see Table 1); calculated regression line: $y=1{\cdot}623+0{\cdot}414$ x. B: micropipette No. 7; calculated regression line; $y=3{\cdot}690+0{\cdot}308$ x. C: micropipette No. 2; calculated regression line: $y=0{\cdot}832$ x.

number measured with different micropipettes and tip diameter, electrical resistance or steady-state rate of spontaneous release (product moment correlation test).

2. When the application of an ejecting current is preceded by a retaining pulse

In microelectrophoresis experiments, the application of an ejecting current is normally preceded by the application of a retaining current. We, therefore, examined the release of NA during ejecting periods following retaining pulses of various intensities and durations using five micropipettes (8, 9, 12, 13 and 14).

When standard ejecting pulses were used, an increase in the intensity of the retaining current reduced the amount of NA released during the subsequent ejecting pulses. Restoration of the original retaining current resulted in a progressive increase in the output of NA over the course of the next few ejecting pulses (Figure 4).

We examined whether the reduction in output brought about by a prior retaining current was due to a change in the time-course of drug release. In these experiments, the ejecting current was applied for a prolonged period and samples were

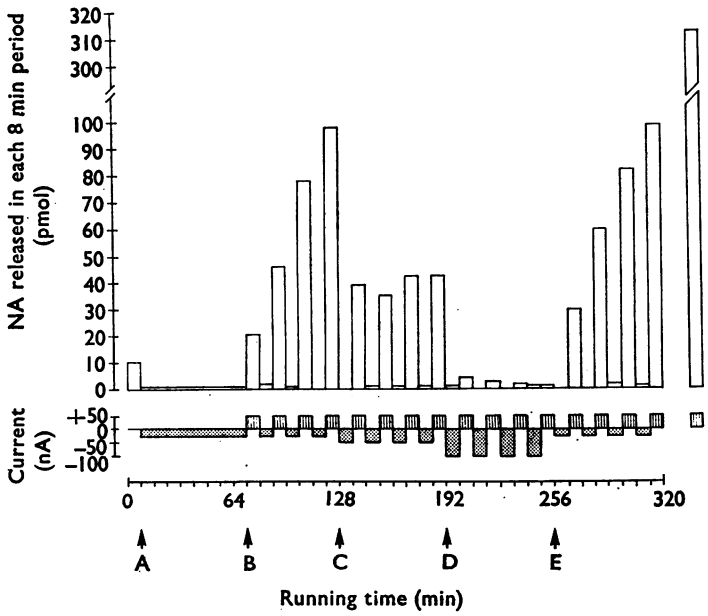

FIG. 4. The effect of various retaining currents upon the electrophoretic release of noradrenaline (NA) from 4 barrels of micropipette No. 8 (see Table 1). Lower graph: electrophoretic current applied to each of the four NA-containing barrels. ⁝⁝⁝, retaining current; ||||, ejecting current. Upper graph: release of NA during each 8 min period of sample collection. Ejecting and retaining currents were applied alternately: the parameters (intensity and duration) of the ejecting pulse were kept constant throughout the study. Changes in the parameters of the applied currents are indicated by capital letters under the time base. After a prolonged application of a retaining current (A–B), successive ejecting pulses (B–C) evoked progressively greater outputs. Increases in the intensity of the retaining current reduced the amount of NA released by the ejecting pulse (C–D, D–E). Restoration of the original retaining pulse (E) was followed by progressively increasing outputs. Control (output during an ejecting pulse not preceded by a retaining pulse) is shown at the right of the figure.

collected at regular intervals of 5 min (Figure 5). When no retaining current had been applied previously, the application of the ejecting current resulted in a rectangular release curve. The effect of the prior application of a retaining current was to prolong the rising phase of the release curve without affecting the plateau rate of release. Increases in either the intensity (Fig. 5 B) or the duration (Fig. 5 A) of the retaining current magnified this distortion of the release curve. It can be seen that, after a high retaining current or a long retention time, the rate of release during the ejection period gradually increased before a steady-state rate of release was achieved. Even weak retaining currents, when applied for prolonged periods, distorted the rising phase of the release curve. In the experiment shown in Fig. 5 C, a retaining current of −5 nA was not effective for the initial 20 min of its application, but, after it had been applied for a further 60 min, it caused a marked distortion of the release curve during the next ejection period.

Discussion

The release of a drug from a micropipette consists of spontaneous release (resulting from diffusion and hydrodynamic outflow of the solution) and electrophoretic release (resulting from iontophoresis and electroosmosis) (Curtis, 1964).

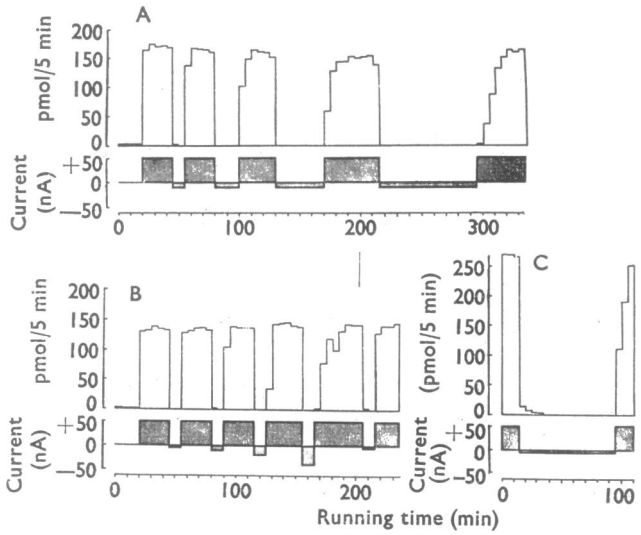

FIG. 5. The effect of retaining currents on the kinetics of electrophoretic release of noradrenaline (NA). Lower graphs: electrophoretic currents applied (see Fig. 4). Upper graphs: release curves for NA. A: micropipette No. 11 (see Table 1): increases in the retention time prolonged the rising phase of the release curve; B: micropipette No. 13: increases in the intensity of the retaining current prolonged the rising phase of the release curve; C: micropipette No. 12: a weak retaining current (−5 nA) did not abolish spontaneous release immediately, but prolonged the rising phase of a subsequent release curve.

When a micropipette of small tip diameter is filled with a drug solution of low molarity, the contribution of hydrodynamic outflow to spontaneous release is likely to be very small (Curtis, 1964), and therefore will be ignored in our discussion. In our experiments it was not possible to differentiate between iontophoretic and electroosmotic release and, thus, electroosmotic release will not be discussed further.

The time-course of spontaneous release is illustrated in Figure 1. After an initial period during which the rate of spontaneous release gradually declined, a constant rate of release was established. When the tip of the micropipette is first immersed in the external medium, drug ions diffuse out of the pipette. This may result in the development of an 'interphase layer' within the terminal part of the micropipette in which the drug concentration gradually decreases towards the tip orifice (Bradshaw, Roberts & Szabadi, 1973a). The initial period of declining release probably reflects the development of this interphase layer, whereas the establishment of a steady-state rate of release reflects an interphase layer of unchanging thickness. This latter condition is described by Fick's first law of diffusion (see Bockris & Reddy, 1970). It follows from Fick's first law that the rate of spontaneous release should be linearly proportional to the concentration gradient across the tip orifice. In our experiments, with a 0·02 M NA solution, the steady-state rate of spontaneous release represents a relatively small contribution to the total rate of release during the passage of an ejecting current. However, it would be expected that when a 0·2 M solution is used (as is often the case in *in vivo* experiments), the rate of spontaneous release would contribute to the total rate of release to a much greater extent. It may be advisable, therefore, to use weaker drug solutions in *in vivo* experiments if it is intended to construct dose-

response curves in which the intensity of the ejecting current is used as the measure of dose (Davidoff, Aprison & Werman, 1969; Johnson, Roberts & Straughan, 1970; Curtis, Duggan & Johnston, 1971).

Figure 5C shows that, in the presence of a weak retaining current, the rate of spontaneous release gradually declines to zero. This is in keeping with the theoretical prediction that drug retention is a time-dependent process (Bradshaw *et al.*, 1973a). In order to determine the efficacy of different retaining currents, we measured the output during standard collection periods. When no output was measured during the whole collection period, we could conclude that the retaining current had become effective instantaneously. However, as the rate of spontaneous release depends upon the concentration of the drug solution, it may be expected that higher retaining currents need to be applied to counteract spontaneous release instantaneously when more concentrated solutions are used. Our finding that the efficacy of a retaining current is reduced after an ejecting pulse (Fig. 2) confirms the suggestion of Castillo & Katz (1957) that there is an enhanced diffusional release after the application of an ejecting current ('after-diffusion').

As a preceding retaining pulse can interfere with the release during an ejection period (see below), it was necessary to examine electrophoretic release when no retaining current had been applied previously. In these experiments, there was a linear relationship between the intensity of the ejecting current and the rate of drug release (Figure 3). This shows that drug release, under these circumstances, can be described by Faraday's law. The data obtained from these experiments were used to calculate the transport number of NA. When transport numbers are calculated, it is necessary to know the contribution of spontaneous release to total release. Our practical procedure was to subtract the steady-state rate of spontaneous release from the total rate of release during an ejection period. This was, however, a necessary simplification, since the application of the ejecting current may result in local changes in the concentration of drug ions inside and outside the tip, which in turn could alter the rate of diffusional release.

The mean transport number of NA (0·02 M NA bitartrate, pH 3·0), for the 13 micropipettes tested, was 0·17. Other workers have determined the transport number of NA on the basis of electrophoretic release experiments, using a NA hydrochloride solution. Values of 0·34 and 0·37 were obtained from two micropipettes (1·7 M, pH 3·0–4·0) when a fluorimetric method was used for the assay of NA (Krnjević *et al.*, 1963), and values of 0·09 (0·03 M, pH 5·5), 0·19 (0·3 M, pH 5·5) (Bradley & Candy, 1970) and 0·05–0·30 (0·5 M) (Hoffer *et al.*, 1971) were obtained when a liquid scintillation method was used for the assay of [^{3}H]-NA. Our results are within the range of values obtained in the two more recent studies. More precise comparisons, however, are not possible because of the use of different experimental parameters in these studies, e.g. different NA salt, ^{3}H-labelled NA, the application of retaining currents between ejecting pulses.

It has been suggested (Curtis, 1964) that 'the use of an excessively strong retaining current for prolonged periods . . . (may) . . . prevent the substance under test from being ejected from the micropipette during the application of short current pulses'. Our results confirm this suggestion and demonstrate that retaining currents within the range used in *in vivo* experiments can significantly reduce the output during a subsequent ejection period (Figure 4). This reduction in output is

apparently due to a prolongation of the rising phase of the release curve (Figure 5). The gradual rise in the rate of release at the beginning of the ejection period reflects a gradual increase in the transport number of NA until a steady-state rate of release is established. This may be due to a gradual increase in the concentration of NA between the pipette tip and the bulk of the drug solution contained within the micropipette (Bradshaw *et al.*, 1973a). The effect of the retaining current is probably to increase the length of this 'interphase layer' so that during the subsequent ejection period a long time is needed to attain the steady-state rate of release. Both parameters of the retaining current (intensity and duration) are important in determining the distortion of the release curve (Figure 5). Even a relatively weak retaining current, which is not capable of counteracting spontaneous release instantaneously, significantly reduces the output measured during a subsequent ejection period. This finding is in contrast with the suggestion that it is possible to select a 'just adequate' retaining current (Curtis, 1964), which eliminates diffusional leakage without interfering with release during the following ejection period.

The results presented in this paper show that the application of weak retaining currents for periods of 10 min (which is within the range of retention times used in our *in vivo* experiments) can seriously distort the subsequent release curve. This distortion is significant and long-lasting, since it is apparent even when relatively long (5 min) collection periods are used (see Figure 5). The use of a relatively dilute (0·02 M) solution in our experiments would tend to favour the depletion of the pipette tip of drug ions during a retention period (Bradshaw *et al.*, 1973a), and this may prolong the time taken to achieve a steady-state rate of release during an ejection period. There is evidence, however, that retaining currents can have a significant distorting effect on the release curve when more concentrated (0·2 M) drug solutions are used. In a separate series of experiments, we have found that the time-course of neuronal responses can be affected by altering the parameters of ejecting and retaining currents (Bradshaw, Szabadi & Roberts, 1973b). An increase in either parameter (intensity, duration of application) of a pre-ejection retaining current increases the latency to onset of the response and prolongs the time required for the attainment of a plateau firing rate in the presence of an ejecting current (cf. Figure 5). This would suggest that the neuronal response curve closely follows the release curve. The quantitative features of the release curve in a particular *in vivo* experiment are likely to be influenced by such factors as the transport number of the drug ions and the electrical and geometrical characteristics of the micropipette, as well as by the drug concentration and the parameters of the ejecting and retaining currents.

Responses of the same neurone to repetitive microelectrophoretic application of a drug cannot be compared meaningfully unless standard ejecting pulses result in identical pulses of drug release. In order to ensure this, both parameters of the ejecting and retaining pulses should be kept constant throughout the study. As a retaining current is often applied for long time periods (occasionally for hours) before a suitable neuronal unit is found, it may be necessary to repeat the standard ejecting pulse several times before an unchanging drug output, and thus an unchanging neuronal response is achieved. Thus one can avoid drawing misleading conclusions when the size of the response to an agonist changes in the presence of a protagonist or an antagonist.

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