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A method for comparing the potencies of γ-aminobutyric acid antagonists on single cortical neurones using micro-iontophoretic techniques

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Summary

1. By the use of micro-iontophoretic techniques, quantitative estimates of the depressant effect of γ-aminobutyric acid (GABA) have been obtained from single neurones in the middle suprasylvian gyrus of cat cerebral cortex.

2. The progressive reduction in firing rate of the neurone during each micro-iontophoretic application of GABA was followed until inhibition was complete. The resultant time-response curves represented cumulative concentration-response relationships which could be characterized by measuring the time taken to achieve 50% inhibition (T50) of neuronal firing.

3. The time-response curves for GABA could be displaced along the time axis by micro-iontophoretic application of picrotoxin, bicuculline or strychnine. A displaced curve was more nearly parallel to the control curve when responses were plotted against linear rather than log time.

4. Picrotoxin usually increased T50 values for GABA, bicuculline could both increase and decrease them and strychnine usually decreased them.

5. When displacements of GABA response curves were expressed as difference between T50 (test−control)/T50 (control), the values obtained were minimally influenced by the size of the current applying GABA and were unaffected by changes in the retaining current passed through the GABA barrel between applications.

6. The use of this method to compare the micro-iontophoretic potencies of different GABA antagonists is discussed.

Introduction

Micro-iontophoretic application of substances onto single neurones in the C.N.S. is a well established technique (Curtis, 1964), but the results obtained have been of a predominantly qualitative nature. For the technique to be used to compare the potencies of different drugs on central neurones, a more quantitative approach is required.

Previously published dose-response curves have used the firing rate of a cell as the response and the iontophoretic current expelling the drug as a measure of the dose of drug applied (Johnson, Roberts & Straughan, 1970; Obata, Takeda & Shinozaki, 1970a; Godfraind, Krnjević & Pumain, 1970; Engberg & Thaller, 1970; ten Bruggencate & Engberg, 1971; Curtis, Duggan & Johnston, 1971). Certain problems attending this form of dose-response relationship have been de-

scribed by Curtis *et al.* (1971). We have, therefore, investigated an alternative form of dose-response relationship which may overcome some of the difficulties. The procedure is based on the theoretical method described by Cuthbert & Dunant (1970) and involves the construction of cumulative dose-response curves within single iontophoretic applications of a drug. Specifically, we have determined dose-response relationships to micro-iontophoretic applications of γ-aminobutyric acid (GABA) and the effects on these of picrotoxin, bicuculline and strychnine.

Methods

Experimental procedures

Nineteen adult cats were anaesthetized with a mixture of 50% nitrous oxide in oxygen containing 1% halothane. A circular area (diameter 8 mm) of skull and dura was removed to expose the middle of one suprasylvian gyrus of the cerebral cortex. Respiratory and circulatory pulsation of the exposed surface of the brain was reduced by means of a thin celluloid disc fixed just under the inner margin of the skull. Holes in the disc allowed the passage of a micropipette. Blood pressure, E.E.G. and breathing rate were monitored continuously and the animal's rectal temperature was maintained at $38{\cdot}0 \pm 1{\cdot}0°$ C by means of a thermostatically controlled d.c. heating pad.

Seven-barrelled glass micropipettes, with tip diameters 5–7 μm, were filled by centrifugation. The barrels contained sodium L-glutamate (BDH) 0·2 M pH 8·0, GABA (Sigma Chemicals) 0·2 M pH 3·5, picrotoxin (Sigma Chemicals) 0·005 M in 0·15 M NaCl at neutral pH, bicuculline (K & K Laboratories) 0·005 M in 0·15 M NaCl at pH 4, strychnine sulphate (Hopkin & Williams) 0·005 M in 0·15 M NaCl at pH 4; the remaining barrels contained NaCl 3 M. The d.c. resistances of the drug-containing barrels were 35–85 MΩ and those of the NaCl-containing barrels 2–6 MΩ. Retaining currents of 25 nA were used routinely for all ionized drugs. One of the NaCl-containing barrels was used to pass a balancing current so that the net current applied to the neurone was zero. The second NaCl-containing barrel was used to record extracellular spike potentials via a Grass P16 microelectrode amplifier. The potentials were viewed on an oscilloscope and electronically counted, the counting epochs being successive periods of 2, 5 or 10 s, depending on the firing rate of the cell.

The majority of cells studied did not fire spontaneously and were, therefore, driven with continuous minimal iontophoretic application of L-glutamate. Responses to GABA were obtained with rates of iontophoretic application adjusted to cause complete inhibition of cell firing after 20–80 seconds. Repeated applications of GABA were made with an interval of 1 min between the end of one application and the beginning of the next. Once reproducible responses were achieved, a continuous iontophoretic application of picrotoxin, bicuculline or strychnine was made whilst the repeated applications of GABA were continued as before. Each GABA application was maintained until cell firing was completely inhibited. Following termination of the iontophoresis of picrotoxin, bicuculline or strychnine, the applications of GABA were repeated until the responses had returned to control values.

Analysis of the responses to γ-aminobutyric acid

If, initially, it is assumed that the passage of a constant expelling current causes

ionized drugs contained in a micropipette to be released at a constant rate (Bradley & Candy, 1970 ; Obata, Takeda & Shinozaki, 1970b ; Hoffer, Neff & Siggins, 1971), the concentration of drug in the tissue will rise asymptotically. Since GABA is a highly potent depressant of neuronal firing, its iontophoresis at a sufficiently high rate should allow the maximum effect on cell firing rate to be reached while the concentration is still rising in an approximately linear fashion. In this situation, the concentration of GABA at any point on the neurone will be proportional to the duration of the GABA application.

It is possible, however, that during the initial stages of an iontophoretic release of a substance the rate of release increases with time (Curtis, 1964 ; Clarke, Hill & Simmonds, 1973). The time-course of this increase depends on both the size of the expelling current and the size and duration of the previous retaining current, but appears to extend over the period in which the response is normally completed. If this progressive increase in the rate of release is approximately exponential, then the log concentration of GABA at any point on the neurone will be approximately proportional to the duration of the GABA application.

In order to test these alternative possibilities, the percentage inhibition of cell firing rate was plotted against the duration of GABA application on both linear and logarithmic scales. The lateral displacement of a log concentration-response curve for a potent agonist in the presence of an antagonist would be expected to be approximately parallel, whether the antagonist is competitive or non-competitive

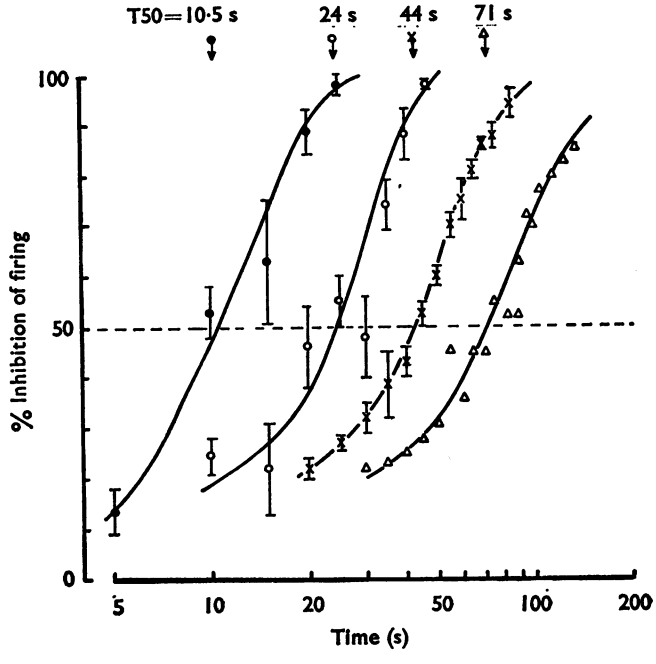

FIG. 1. Time-courses of inhibition of neurone firing due to micro-iontophoretic application of γ-aminobutyric acid (GABA) with four different currents, 20 nA (●), 10 nA (○), 5 nA (×) and 2 nA (△). Each curve was obtained from the same neurone at a depth of 957 μm in the middle suprasylvian gyrus of cat cortex. The neurone was driven by continuous micro-iontophoretic application of L-glutamate 20 nA. Each of the points for the 20 nA, 10 nA and 5 nA applications of GABA is the mean ±S.E.M. of 3 values obtained from three separate applications of the same current of GABA. The values of T50 shown are the times taken to achieve 50% inhibition of neurone firing.

(Stephenson, 1956). Therefore, the particular form of the GABA response-time relationship which results in a more nearly parallel displacement of the relationship in the presence of an antagonist is likely to be the more correct representation of the true log concentration-response curve.

Results

Inhibition versus log time

Percentage inhibition of cell firing in successive counting epochs during the application of GABA was plotted against log duration of the GABA application. Figure 1 shows the curves obtained by applying four different currents of GABA to the same cell. The concentration of GABA in the biophase would be expected to rise more slowly with low than with high currents, so that the response develops more slowly with a low current. For any given current, however, the time course of the response is very reproducible, as shown by the standard errors in Figure 1.

Fully reversible shifts in the GABA response curves were obtained by iontophoretic application of picrotoxin (50–75 nA), bicuculline (50–75 nA) or strychnine (25–50 nA). An example of the effect of picrotoxin is shown in Figure 2. The slope of the linear portion of each curve was calculated by the method of least

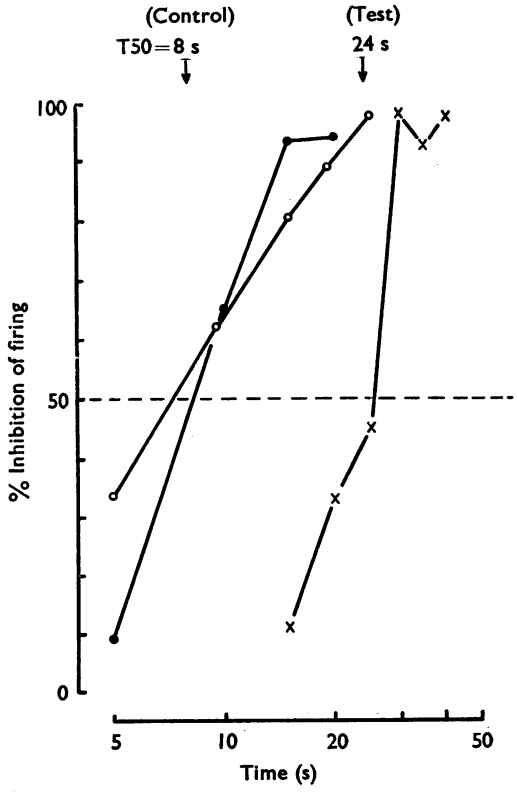

FIG. 2. Effect of micro-iontophoretic application of picrotoxin on the time-course of inhibition of neurone firing due to micro-iontophoretic application of γ-aminobutyric acid (GABA). Each curve is the response to GABA 20 nA of a neurone at a depth of 1,848 μm in the middle suprasylvian gyrus, being driven by continuous application of L-glutamate 5 nA. ●, Mean of 2 control responses; ×, single response 2·5 min after the start of picrotoxin 50 nA; ○, mean of 2 recovery responses.

squares and the time to 50% inhibition (T50) was also calculated. The ratio of T50 in the presence of picrotoxin to the mean T50 for control and recovery responses was used to measure the effect of picrotoxin.

In contrast to picrotoxin, strychnine often shifted GABA response curves to the left (Fig. 3), the effect being measured in the same way as for picrotoxin. Bicuculline sometimes shifted the GABA response curves to the right and sometimes to the left. Examples of these effects have already been published (Straughan, Neal, Simmonds, Collins & Hill, 1971).

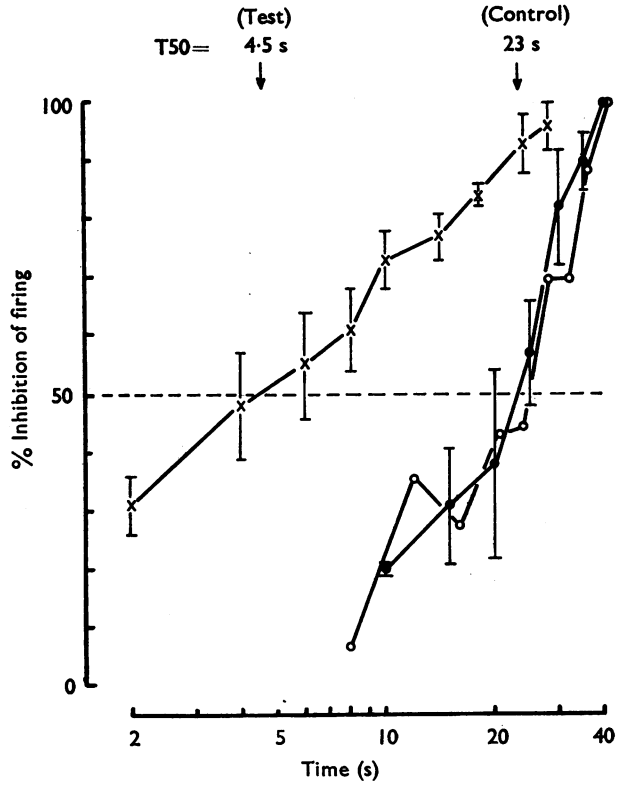

FIG. 3. Effect of micro-iontophoretic application of strychnine on the time-course of inhibition of neurone firing due to micro-iontophoretic application of γ-aminobutyric acid (GABA). Each curve is the response to GABA 5 nA of a neurone at a depth of 1,351 μm in the middle suprasylvian gyrus being driven by continuous application of L-glutamate 15 nA. ●, Mean ±S.E.M. of 3 control responses; ×, mean ±S.E.M. of 3 responses 6–10 min after the start of strychnine 50 nA; ○, mean of 2 recovery responses.

To determine whether the GABA response curves in the presence of picrotoxin, strychnine or bicuculline were parallel to their respective control curves, the ratios of the slopes of the curves were plotted against the ratios of the T50 values (Fig. 4A). Values obtained for the effects of each of the three substances showed positive correlations between slope ratio and T50 ratio. The combined plot shown in Fig. 4A has a regression coefficient of 0·745 and a correlation coefficient of 0·830, both highly significant ($P<0·001$). Thus, independent of the nature of the substance causing the shift of a GABA response-log time curve, a shift to the right involves an increase in slope and a shift to the left a decrease in slope.

Inhibition versus linear time

All the GABA responses contributing to the data presented above were replotted as % inhibition of cell firing versus the duration of GABA application on a linear scale. Lateral displacements of these curves were expressed both as differences in T50 values and as T50 ratios. When either of these expressions was plotted against slope ratio (Fig. 4B), neither the regression coefficients nor the correlation coefficients were significant. Thus, GABA response curves plotted as % inhibition versus linear time showed no consistent change in slope when displaced either to the right or to the left by picrotoxin, strychnine or bicuculline.

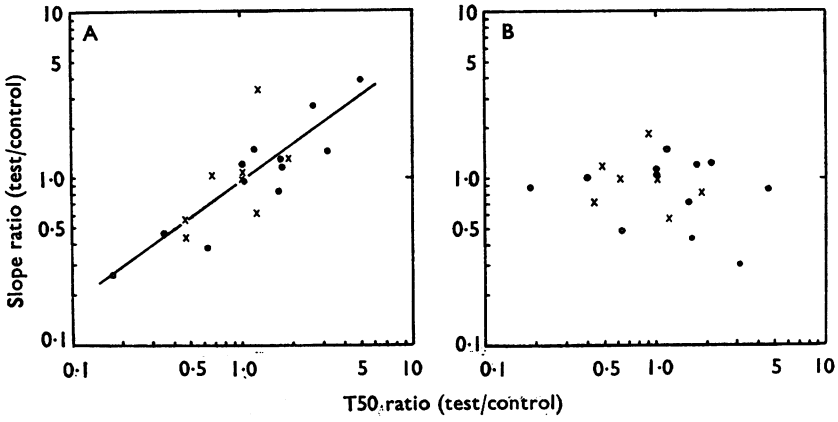

FIG. 4. Relationship between the change in slope (slope ratio) and displacement of T50 (T50 ratio) for responses to micro-iontophoretically applied γ-aminobutyric acid (GABA) in the presence of micro-iontophoretically applied picrotoxin (●), bicuculline (×) and strychnine (○) when (A), response was plotted versus log time and (B), response was plotted versus linear time. Each point is an individual experiment and each was obtained with a different neurone. A. Correlation coefficient between slope ratio and T50 ratio=0·83 which is significant ($P<0·001$). Gradient of the line=0·75±0·12 which is significant ($P<0·001$). B. Correlation coefficient between slope ratio and T50 ratio=−0·16 which is not significant ($P>0·1$).

The lateral displacement of a GABA response-linear time curve should be expressed as the difference between T50 values since the answer obtained is proportional to the classical log concentration ratio at 50% inhibition and can be equated to it by a factor which reflects the time-course of the rise in GABA concentration at the neurone. In order to compare the effects of different antagonists and potentiators on the different time-courses of GABA responses obtained with different micropipettes and neurones, we expressed the change in response caused by antagonist or potentiator as a proportion of the control T50 value, i.e. degree of antagonism (or potentiation)=T50 (test)−T50 (control)/T50 (control). Thus, the reciprocal of the T50 value for the control response has been taken as a measure of the rate of rise of GABA concentration at the neurone.

Experiments were performed to determine whether this quantitative expression of the degree of potentiation or antagonism of a response to GABA was independent of the size of both the expelling and retaining currents applied to the GABA barrel. In one series of experiments, two different expelling currents of GABA were applied alternately, the higher current being two or three times the lower, When the effects of picrotoxin, bicuculline and strychnine were determined as

before, the numerical expression of the potency of these drugs was approximately 20% greater when tested against a high current than when tested against a low current of GABA (Fig. 5). This discrepancy was significant ($P<0{\cdot}01$) but was of little practical concern since we have found that the variation in potency of an antagonist in different experiments is considerably greater than 20%. Such a large variation probably represents the innate difficulty in exactly reproducing pipette release characteristics and neurone to pipette distances from experiment to experiment.

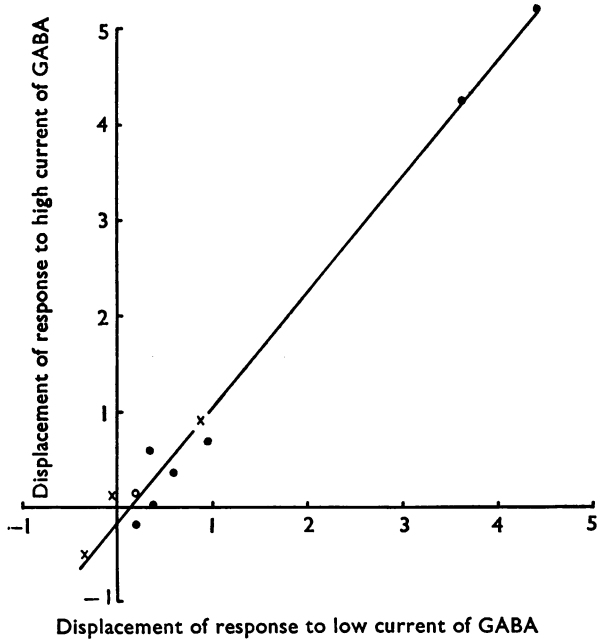

FIG. 5. Effect of high and low currents of γ-aminobutyric acid (GABA) application on the size of the displacement of the GABA response-linear time relationship in the presence of micro-iontophoretically applied picrotoxin (●), bicuculline (×) and strychnine (○). The higher current of GABA was 2 to 3 times the lower and the displacements of the GABA responses were expressed as T50 (test-control)/T50 (control). The gradient of the line is $1{\cdot}217 \pm 0{\cdot}049$ (mean ± S.E.M.) which is significantly ($P<0{\cdot}01$) greater than 1, the value which would be expected if the expression for the displacement of the GABA response was independent of the applying current of GABA. Values of T50 for the control responses to the higher current of GABA were less than the values for the lower current of GABA by a factor of $0{\cdot}578 \pm 0{\cdot}066$ (mean ± S.E.M. of 11 results).

In the experiments described so far, GABA was held in the micropipettes between ejections by a negative retaining current of 25 nA. The values of T50 for the responses to a given expelling current of GABA are, however, markedly affected by the size of the retaining current used (Figure 6). In a further series of experiments (Fig. 7), repeated applications of the same current of GABA were made from a retaining current which alternated between 25 nA and 5 or 10 nA from one inter-ejection period to the next. Figure 7 shows that when the GABA response was displaced by a drug, the magnitude of the displacement expressed as T50 (test−control)/T50 (control) was independent of the retaining current passed between ejections of GABA.

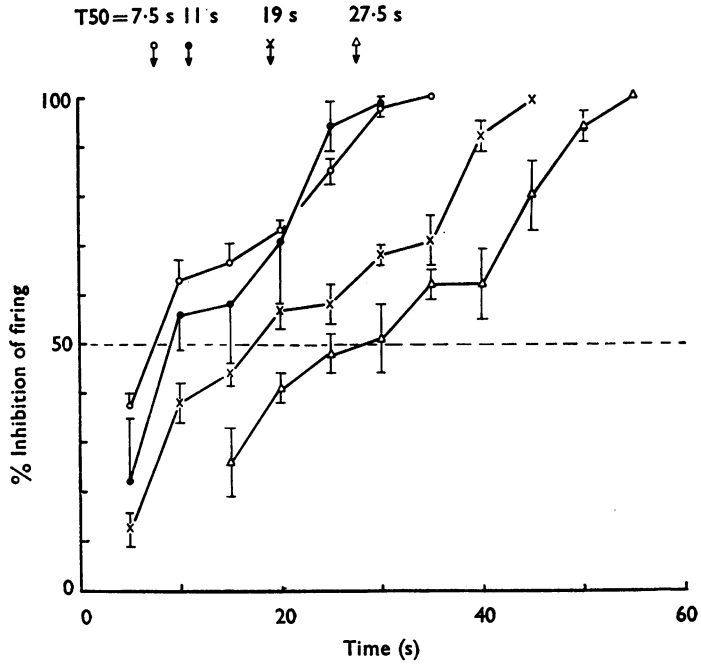

FIG. 6. Effect on the γ-aminobutyric acid (GABA) response-linear time relationships of 4 different retaining currents applied to the GABA barrel between expulsions. Each point is the mean ± S.E.M. obtained from four separate applications of GABA 10 nA following passage of a retaining current of 5 nA (●), 10 nA (○), 25 nA (×) or 50 nA (△). The responses were obtained in a 4×4 latin square sequence from a single neurone driven by continuous application of L-glutamate 15 nA at a depth of 1062 μm in the middle suprasylvian gyrus. The control firing rates of the neurone (spikes/s) during passage of a retaining current through the GABA barrel were 42±2·8 at 5 nA, 48±2·9 at 10 nA, 53±3·7 at 25 nA and 59±3·1 at 50 nA (means ± S.E.M. of 4 values).

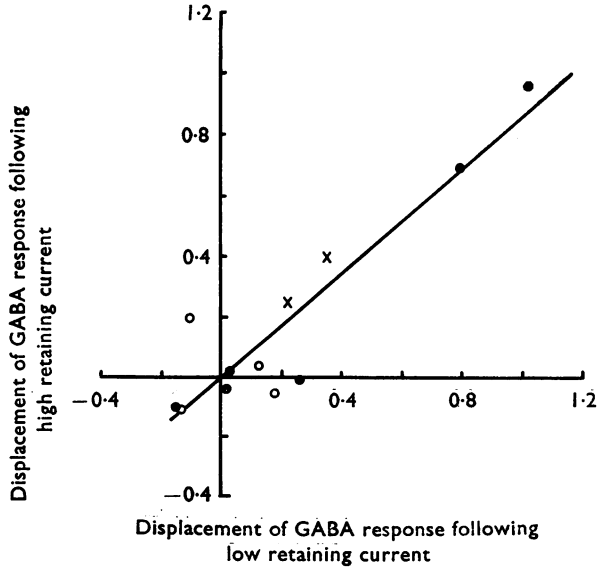

FIG. 7. Effect on displacements of the γ-aminobutyric acid (GABA) response-linear time relationships of high and low retaining currents passed through the GABA barrel between expulsions. GABA responses were displaced by micro-iontophoretic application of picrotoxin (●), bicuculline (×) and strychnine (○). Each point represents a separate experiment in which the expelling current of GABA was kept constant and the retaining current alternated between 25 nA and 5 or 10 nA. The gradient of the line is 0·860±0·120 (mean ± S.E.M.) which is not significantly different from 1 ($P>0·2$). Values of T50 for control GABA responses following the low retaining current were reduced by a factor of 2·65±0·10 (mean ± S.E.M. of 10 results). At the same time, the firing rate between GABA applications was not significantly changed by the low retaining current.

Discussion

A practicable method of obtaining dose-response relationships for the effects of micro-iontophoretically applied GABA required that the responses be easily and accurately quantifiable, reproducible and obtainable over a short period of time. Intracellular recording techniques would permit the measurement of changes in cell membrane conductance in response to GABA but this was considered technically not feasible for the present investigation. Extracellular recordings of neuronal firing rate were, therefore, used as measures of the GABA response and this also had the advantage of being a direct measure of neuronal activity.

The extracellular methods used hitherto, in which various applying currents of GABA were taken to represent dose, involve considerable difficulties. The time taken to obtain a complete dose-response curve is prolonged and the usual measure of the response, the equilibrium depression of firing, we found almost impossible to obtain in a graded fashion with graded applying currents of GABA. For example, in Fig. 1, the response to 2 nA eventually approached 100% although it took over 2 min to do so and gave the appearance of transient plateaux at two or three points during the response. In addition, the relationship between the applying current of GABA and the concentration of GABA achieved near the neurone, either at equilibrium or after a given time, is not known. A simple proportion cannot be assumed (Hoffer *et al.*, 1971 ; Clarke *et al.*, 1973) since the fraction of release due to diffusion, as distinct from iontophoresis, may be substantial.

These difficulties were of little consequence in the method that we adopted, but other problems were encountered. The determination of a dose-response relationship from a single iontophoretic application of GABA depends basically on a knowledge of how the concentration of GABA changes with time during the application. An initial assumption was made that the concentration of GABA in the biophase was linearly related to the duration of its application at high currents. Analysis of the results, however, suggested that this relationship was unlikely to obtain since the slopes of the log time-response curves increased when the curves were displaced to the right and decreased when displaced to the left. Such changes in slope were not readily explicable in terms of drug-receptor theories. It seemed probable, therefore, that the early phase of iontophoretic release of GABA, during which the rate of release was increasing with time, extended throughout the period in which the responses to GABA were obtained. The gradually increasing rate of release during this early phase is probably caused by the prior passage of a retaining current diluting the GABA just inside the tip of the barrel. Upon reversal of the current, the concentration of GABA in the tip gradually increases to an equilibrium (del Castillo & Katz, 1957 ; Curtis, 1964). These changes mainly affect the component of release which is due to diffusion, such that the diffusional release increases with time during the early phase of expulsion. This effect can be shortened by use of a lower retaining current or higher expelling current, but these manoeuvres may have little practical advantage since in both cases the time-courses of the responses will also be considerably shortened. On the assumption that lateral displacements of the true log concentration-response curves for GABA should be parallel, the approximately parallel displacements of the linear time-response relationships indicated that the concentration of GABA in the biophase was increasing approximately exponentially with time over the response range.

The expression evolved as a measure of the displacement of the time-response curves for GABA, i.e. T50 (test−control)/T50 (control), was shown to be independent of the retaining current passed through the GABA barrel and only slightly influenced by the applying current of GABA. The relationship between this expression and the ratio of the GABA *concentrations* required to cause 50% inhibition, however, is not a simple proportion but depends on the intrinsic sensitivity of the neurone to GABA. So long as the sensitivities of different neurones to GABA are similar, approximate comparisons can be made between the effects of the same or different drugs in displacing the GABA response curves. When the drugs are applied micro-iontophoretically, such comparisons of potency may be considerably influenced by the ease or difficulty with which the drug is expelled from the pipette. In the present experiments, picrotoxin, bicuculline and strychnine were each prepared as 0·005 M solutions in 0·15 M NaCl. It is likely, therefore, that these drugs were expelled from the micropipette principally by electro-osmosis (Curtis, 1964), which depends on the NaCl present, so that the release characteristics were probably similar for each drug. When other means of administration are used, the comparative potencies may be somewhat different.

The most reliable comparisons of the micro-iontophoretic potencies of different substances which affect responses to GABA should be obtained by comparing two or more of these substances on the small cell. This is quite a feasible proposition with our present technique. The repeated administration of the same current of GABA allows the effect of the first substance to be followed until it is constant. Recovery can then be followed until it is complete, whereupon the second substance can be applied. The entire experiment may take 30 to 60 min but this is well within the time over which we can normally obtain very reproducible responses from single cortical neurones to repeated applications of the same current of GABA.

It has not been our present aim to make detailed comparisons of the potencies of picrotoxin, bicuculline and strychnine as antagonists of GABA since this will be the subject of a subsequent paper. Nevertheless, it is apparent from the present results that, as previously reported (Straughan *et al.*, 1971), bicuculline sometimes antagonized and sometimes potentiated GABA. Equal micro-iontophoretic currents of picrotoxin, however, did not potentiate GABA and were capable of causing more marked antagonism than did bicuculline (see also Hill, Simmonds & Straughan, 1972). Strychnine, on the other hand, potentiated rather than antagonized GABA in the present experiments.

There is one further consideration in evaluating the potency of an antagonist, which applies to any form of micro-iontophoretic comparison. If the substance under test influences any of the processes which remove GABA from the biophase then the entire current-time-concentration relationship for GABA is altered and any effects due to an action on the receptors for GABA will be distorted. It is, therefore, necessary to know whether the substance blocks the uptake of GABA by brain tissue and whether the substance can antagonize GABA transaminase. Picrotoxin, bicuculline and strychnine are known not to block the uptake of GABA (Iversen & Johnston, 1971) and the activity of GABA transaminase is little affected by bicuculline (Straughan *et al.*, 1971 ; Beart & Johnston, 1972) and unaffected by picrotoxin and strychnine (M. S. Starr & I. Sutton, unpublished observations).

In conclusion, therefore, we believe that the technique described in this paper is a useful method for making quantitative comparisons between the potencies of

micro-iontophoretically applied substances in the C.N.S. Although the present results refer only to GABA responses in the cerebral cortex, recent experiments on feline brain stem neurones show that similar dose-response curves can be obtained for both GABA and glycine. In addition, strychnine has been found to cause parallel displacements of the glycine dose-response curves in a manner consistent with its known action as a potent antagonist of glycine (Curtis *et al.*, 1971). Preliminary experiments on the excitatory responses of pyramidal tract neurones to acetylcholine and their antagonism by atropine (G. Clarke and P. A. Forrester, unpublished observations) suggest that antagonism of excitatory responses can also be analysed in a similar fashion. Whilst certain difficulties in interpreting the answers remain, the method offers considerable advantages over currently used procedures.

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