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A QUANTITATIVE MICROIONTOPHORETIC ANALYSIS OF THE RESPONSES OF CENTRAL NEURONES TO NORADRENALINE: INTERACTIONS WITH COBALT, MANGANESE, VERAPAMIL AND DICHLOROISOPRENALINE

R. FREEDMAN, B.J. HOFFER & D.J. WOODWARD*

Laboratory of Neuropharmacology, National Institute of Mental Health, St. Elizabeth's Hospital, Washington D.C. 20032 and *Department of Physiology, University of Rochester, Rochester, New York 14642

1 A new experimental procedure has been devised for the study of pharmacological antagonism in the central nervous system using automated microiontophoresis to deliver pulses of agonists and computer-generated histograms to quantify the neuronal response. The system allows study of potential antagonists having direct depressant effects and also of neurones with irregular or slow discharge rates.

2 The histogram analysis reveals the necessity for regular, periodic delivery of agonists during the assessment of agonist-antagonist interactions. Without regular repetitive delivery, many agonists, such as noradrenaline, exhibit an apparent but artifactual decrease in inhibitory potency after an interruption of agonist pulses. Examples of this phenomenon are shown, using cerebellar Punkinje cells and cerebral cortical neurones in rats anaesthetized with halothane.

3 Preliminary results with these computer-generated drug response histograms revealed manganese, cobalt, and verapamil to be generally ineffective as antagonists of noradrenaline, despite their direct depressant effects.

4 Conversely, dichloroisoprenaline (DCI), a β-adrenoceptor antagonist, was effective in blocking noradrenaline-induced depressions of firing in the cerebral cortex at doses which caused over 50% decrease in spontaneous discharge.

Introduction

Direct demonstration of responses to noradrenaline in central neurones and their antagonism has been made possible by application of drugs directly to the neurones by microiontophoresis. Design of experiments and analysis of the data has been complicated, however, by the many biological and technical difficulties inherent in this technique (Bloom, 1974). The present study examines three problems in the testing of antagonists of noradrenaline (NA) and demonstrates some possible solutions.

The first problem is the establishment of reproducible NA effects, so that the absence of such effects during the application of another substance can properly be interpreted as an antagonism. Clarke, Hill & Simmonds (1973) and Bradshaw, Roberts & Szabadi (1973a) showed that iontophoretic release of radio-labelled substances *in vitro* is affected by the strength and duration of previous retention currents. Work from these same laboratories has shown that the size of NA (Bradshaw, Szabadi & Roberts, 1973b) and γ-aminobutyric acid (GABA)-induced (Hill, Simmonds & Straughan, 1973) depressions in central neurones is a function of the magnitude and duration of the retaining currents. In confirmation of their findings, we report here that regular applications of uniform NA pulses are necessary for reproducible inhibitions and that interruption of these periodic applications alone is sufficient to decrease the apparent inhibitory potency of NA when the pulses are resumed. This effect would cause an apparent, but artifactual antagonism if NA pulses were interrupted during application of an antagonist.

A second problem is the evaluation of potential antagonism by an agent having additional direct depressant effects on activity. When spontaneous discharge rate drops to levels of less than one per second, it may not be possible to judge NA efficacy, and hence antagonism, by simple visual inspection of ratemeter records or spike trains. Computer-generated drug response histograms, used first by Gottesfeld, Kelly & Renaud (1972) to quantitate GABA effects, allow the technique of peri-stimulus histograms to be applied to the NA responses of these slowly discharging cells. Because the histogram sums spikes recorded during

multiple, repeated trials of NA, quantitative comparisons of adrenergic inhibition can be made before, during, and after an antagonist trial. We show here how several reported adrenoceptor antagonists can be tested by this method.

Neurones which fire irregularly, such as cerebral cortical neurones in anaesthetized animals, present a third problem similar to that produced by depressant antagonists. The random bursts and long pauses characteristic of the control discharge pattern of many of these cells make it difficult to evaluate inhibition. The drug response histogram emphasizes drug related changes in firing, while unrelated bursts and pauses are randomized.

Methods

Ten male Sprague-Dawley rats, weight 200-250 gm, were used in these experiments. The animals were anaesthetized with 0.5-0.75% halothane, intubated, and allowed to breathe spontaneously. Body temperature was maintained at 35-36° C. After cisternal drainage, the skull was removed over the posterior cerebellar vermis or one of the cerebral hemispheres, posterior to the bregma. The dura was excised under a drip of warm Earle's balanced salt solution (Grand Island Biological Co.) after which 2% agar in saline was applied to the surface of the brain.

Five barrel micropipettes were prepared as described previously (Salmoiraghi & Bloom, 1964). The barrels were filled initially by boiling in distilled water, which was aspirated subsequently and replaced by drug or electrolyte. After centrifugation to accelerate drug movement into the tip, the pipettes were broken to 5-7 μm diameter under microscopic observation, a size normally used during physiological experiments. Occasionally, larger tips (22-25 μm) were produced. Pipettes with the larger tips were used to control for the possibility that the drug-time interactions noted below were a function of only a certain range of tip size. Tips larger than 25 μm could not be studied because of difficulty in recording from neurones. Resistance of all barrels before insertion into the brain was less than 200 MΩ. The centre barrel, used for recording, was filled with 5 M NaCl and generally had a resistance of 1.5-3 MΩ. Three of the four side barrels were filled with drugs; the fourth contained 3 M NaCl for current controls and to balance drug ejection and holding currents, as noted below. Holding currents of -15 nA were used whenever drugs were not ejected. Drug solutions used were: 0.5 M (−)-noradrenaline HCl, pH 4.5 (NA, Aldrich); 1.0 M γ-aminobutyric acid, pH 4.0 (GABA, Calbiochem.): 0.1 M manganese chloride (Mn), pH 5.3; 0.2 M cobalt chloride (Co); 0.06 M verapamil hydrochloride in 165 mM NaCl, pH 5.2 (a gift of Dr John Daly, NIAMD); 0.5 M dichlorophenylisopropylaminoethanol (DCI, Aldrich).

Purkinje cells recorded extracellularly in the cerebellar cortex were identified by their characteristic climbing fibre and simple spike discharge (Eccles, Ito & Szentagothai, 1967). Cells with regular discharge rates between 20 and 50 Hz were considered to be uninjured and suitable for study (Murphy & Sabah, 1970). Neurones encountered in penetrations in the parietal cerebral cortex were noted to fire more slowly and irregularly. Cortical neurones chosen for study had uniform action potential amplitudes and no obvious injury bursts.

A crystal clock-regulated stimulator (Digitimer, Devices Ltd.) controlled the iontophoresis circuit (Geller & Woodward, 1972) so that constant current pulses of uniform duration and magnitude could be passed at equal intervals through agonist barrels. An operational amplifier balancing circuit was employed in conjuction with the 3 M NaCl barrel to eject a current equal and opposite to the sum of the currents in the other three barrels at any instant (Geller & Woodward, 1972).

Extracellular action potentials were amplified by conventional means, monitored on an oscilloscope and converted to uniform voltage pulses by a window discriminator. The pulses were fed to a ratemeter which integrated them over one second intervals. The ratemeter output and the iontophoresis currents were displayed together on a polygraph.

The window discriminator output was also led to a digital computer (PDP-12, Digital Equipment Corp.) to generate an on-line drug response histogram, which summed the unit responses during several agonist pulses. Average agonist responses could thereby be computed before, during and at intervals after antagonist application. The computer was used to quantitate the agonist responses by determining the average counts per address during the response period and comparing it with control periods, expressing the difference as percent inhibition. Two control periods, one before and one after the drug ejection, were arbitrarily selected. Counts per bin were calculated and required to be within 10% of each other as criteria for stability, i.e. constancy of neuronal discharge rate. The period of drug response was selected to begin at the particular bin where counts fell significantly below baseline and terminate at the bin where counts re-approached the baseline. Once the bins corresponding to the onset and termination of drug response were chosen in the control histogram, the identical epoch was used for comparison of percent changes in all subsequent

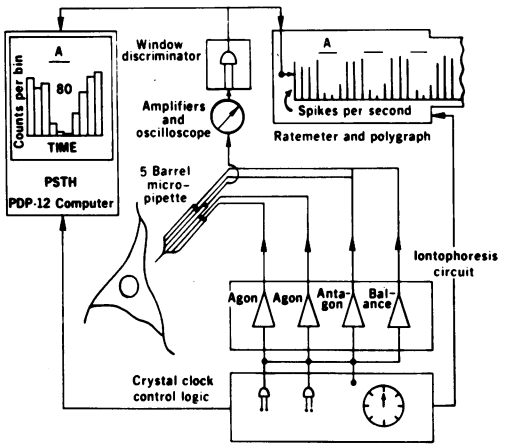

Figure 1 Summary diagram of the experimental apparatus. A logic circuit timed by a crystal clock sends control voltage pulses to an iontophoresis circuit employing operational amplifiers (lower right). The three constant current outputs apply agonists (Agon) and antagonists (Antagon) through three barrels of the five barreled micropipette (centre). Zero net iontophoretic current is maintained through an automatic balancing circuit and a fourth barrel. The neuronal action potentials are recorded extracellularly by the fifth barrel. Action potentials are amplified and converted into constant voltage pulses by a window discriminator. These pulses are either integrated over 1 s intervals by a ratemeter and displayed on a polygraph or led to a PDP-12 computer. Using the PSTH programme, the computer sums several drug responses into a histogram, as shown for agonist A. Timing pulses are provided from the clock logic. The computer calculates the percent inhibition caused by the agonist (see Methods). Responses to agonists can thus be computed before, during, and after iontophoresis of an antagonist.

histograms. The computer programme (PSTH, Woodward, 1972) uses a double precision, 24 bit clock subroutine, so that the sweep can be extended to accommodate the 'slow' responses seen with monoamine iontophoresis while maintaining clock sampling accuracy (1 KHz).

Stable agonist responses were obtained from each cell before recording the first control histogram. Particularly with NA, the response to a pulse of a given current increases markedly with the first few pulses. Residual effects of this 'warm up' phenomenon may be seen as long as 30 min after the beginning of a continuous series of NA pulses. Each antagonist was tested at least twice per cell, with control, antagonist and recovery histograms computed each time. The number of sweeps was adjusted to provide approximately equal numbers of counts in the control addresses of each histogram. Thus, more sweeps were required in histograms recorded while neuronal firing was slowed by an antagonist with direct depressant effects. Generally, cells in this study were held for 1 to 2 h and a regular cycle of agonist pulses was maintained throughout. Data from an experiment was not accepted if the agonist response failed to show recovery from antagonism. To summarize the data in tabular form, a blockade was arbitrarily defined as a reproducible reduction of at least 40% of the agonist response. A diagram of the experimental apparatus is shown in Figure 1.

Results

Timing of agonist pulses

Iontophoresis of NA resulted in inhibition of nearly all Purkinje cells and cerebral cortical neurones observed (Hoffer, Siggins & Bloom, 1971; Stone, 1973). A 2 to 5 min 'warm up' period was generally found, during which the efficacy of inhibition increased markedly without change in iontophoresis parameters. In many cases an initial NA pulse would be almost completely ineffective, but inhibition would increase consistently with the next 5 to 10 pulses. Further increases of 5 to 10% usually occurred over the next 30 min with continual periodic NA application. When a particular interval between pulses was lengthened (Figure 2), a marked decrease in the efficacy of inhibition was noted in the subsequent pulses. Interruptions of as little as 1 or 2 min markedly decreased NA evoked inhibition. Recovery of inhibition was usually not seen until at least the fourth pulse after the interruption.

Experiments testing the effect of interruption of regular, periodic NA applications were performed on 11 cells. The time for recovery of the response to its initial value was directly proportional to the length of interruption ($P < 0.01$, Figure 3B). No difference was noted between cerebral cortical neurones and Purkinje cells. The percentage decrease in inhibition was determined by calculations from drug response histograms. Responses to three pulses of NA were summed from before and after the interruption and the percent inhibition obtained in the two histograms was compared. Most neurones, recorded with pipettes of overall tip diameters of 5-7 μm, showed a 70-95% decrement in inhibition (Figure 3A). The pipettes which did not show as marked an effect generally had very large tip diameters (25 μm), but even these showed decrements of 25-50%. Because three pulses were summed to form the histogram, the magnitude of the decrease in inhibition is

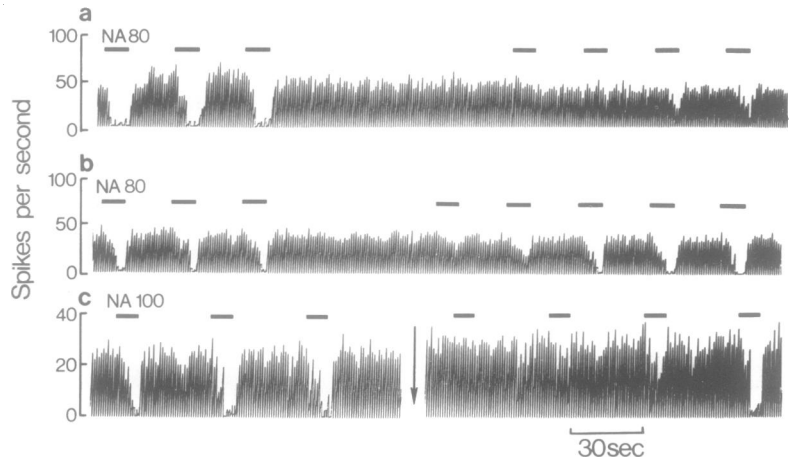

Figure 2 Effects of prolonging interpulse time on the response of Purkinje cells to iontophoresis of NA. (a) and (b) represent continuous records from one cell. (c) shows a different cell in which the arrow indicates a 2 min pause before resuming pulses. In this and all subsequent ratemeter records, the ordinate shows spikes/second. The letters and numbers over the lines indicate the drug and its ejection current (nA). The length of the lines shows the duration of drug ejection. Note the profound reversible decrement in response produced by pause of several minutes between 10 s NA pulses given at 40 s intervals.

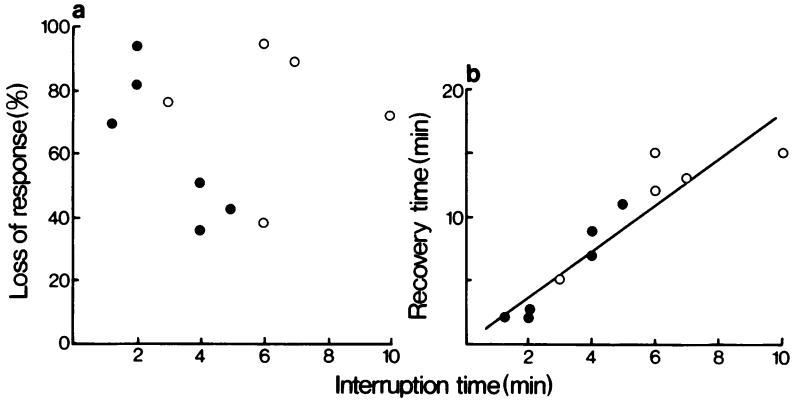

Figure 3 Effects of interruption of NA pulses on NA-induced inhibition. (a) Degree of loss of inhibition of the neuron to NA pulses as a function of pulse interruption time, calculated as the difference between NA response histograms which summed the three pulses before and the three pulses after the interruption. (b) Time for recovery of inhibitory NA responses to at least 80% of control as a function of pulse interruption time. Note linear relationship ($P < 0.01$). Most cells were given a 15 s NA pulse repeated every 60 seconds. • Purkinje cell. ○ Cortical neuron.

under-estimated, since there was some increase in efficacy in successive pulses after the interruption. The decrease in inhibition assessed in this way was not linearly related to the length of interruption (Figure 3A).

To test whether this loss of response depended solely on the release of drug from the micropipette or whether biological factors, such as reuptake or a change in the sensitivity of the membrane, might also be important, NA was applied alternately from two barrels of the same pipette in some experiments. When the second pulse was interrupted, as shown by the drug response histograms in Figure 4, the effectiveness of the first pulse was

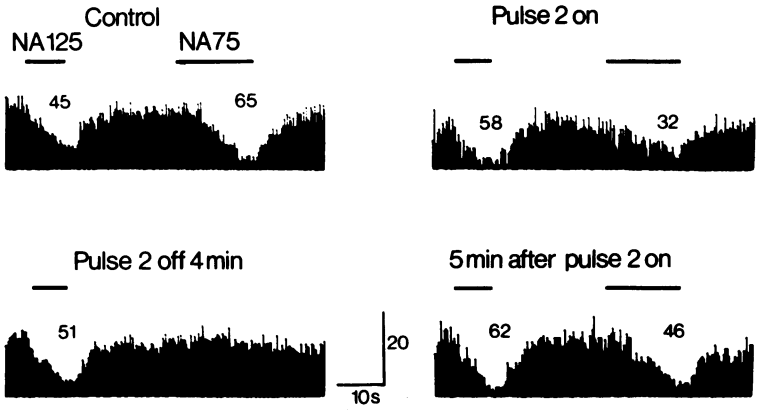

Figure 4 Effects of interruption of NA pulses on inhibition of Purkinje cell spike activity. Four drug responses histograms are illustrated, each with a total sweep time of 50 seconds. Two pulses of NA were applied through different barrels during each sweep at the times indicated by the horizontal bars in the control histogram (upper left). Cessation of the second pulse does not reduce the efficacy of the first pulse (lower left). However, the second pulse is much less effective when resumed after a 4 min interruption (upper right). Recovery is still not complete 5 min later (lower right). The continued improvement in the control response was seen frequently with periodic application of NA. Numbers below the bar on this and subsequent histograms are the % inhibition of spontaneous activity. Calibration bars are: ordinate 20 counts per address and abscissa 10 seconds.

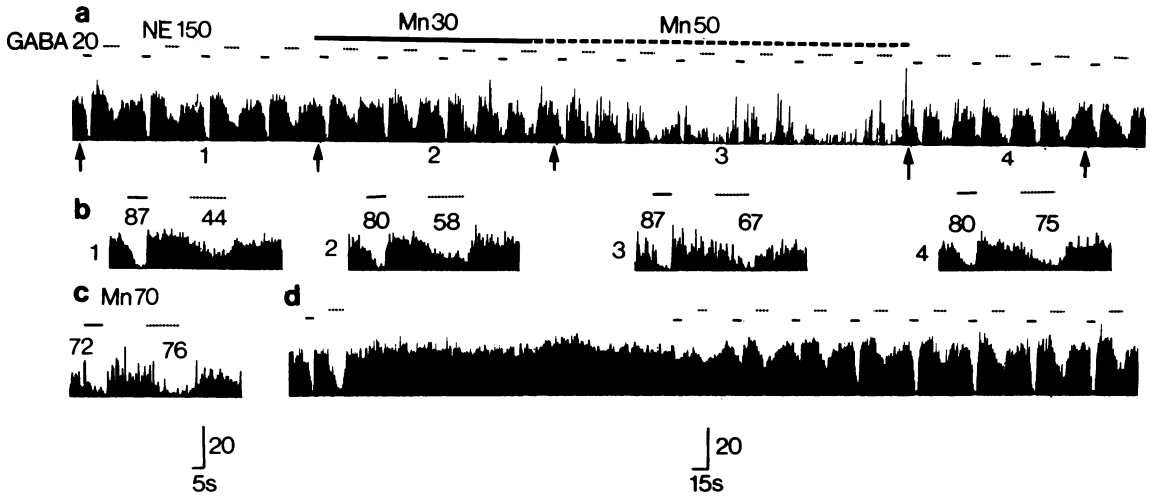

Figure 5 Ratemeter and drug response histograms showing effect of manganese (Mn) and interruption of agonist pulses on inhibitory responses to GABA (solid line) and NA (dotted line). (a), (b), (c) and (d) are all from the same Purkinje cell, recorded over a period of 6 hours. (a) shows the continuous ratemeter record of the effects of periodic application of GABA and NA. The numbers and arrows beneath the record show the regions used to construct the associated drug response histograms in (b). Note that despite the large reduction of spontaneous activity produced by 50 nA of manganese, there is no decrement in noradrenaline-induced inhibition during (b3) or after (b4) manganese. An even larger dose of 70 nA of manganese (c) reduced spontaneous discharge rate to 0.3 spikes/s but did not reduce NA-induced inhibition. (d) shows the differential effect of pulse interruption. At the resumption of pulsing, both GABA and NA-induced inhibition are diminished. However, the GABA response recovers by the 4th cycle whereas the NA inhibition is still only 50% recovered by the 8th cycle. The calibrations bars under (c) and (d) are for histogram (counts per address) and ratemeter (spikes/s) records, respectively.

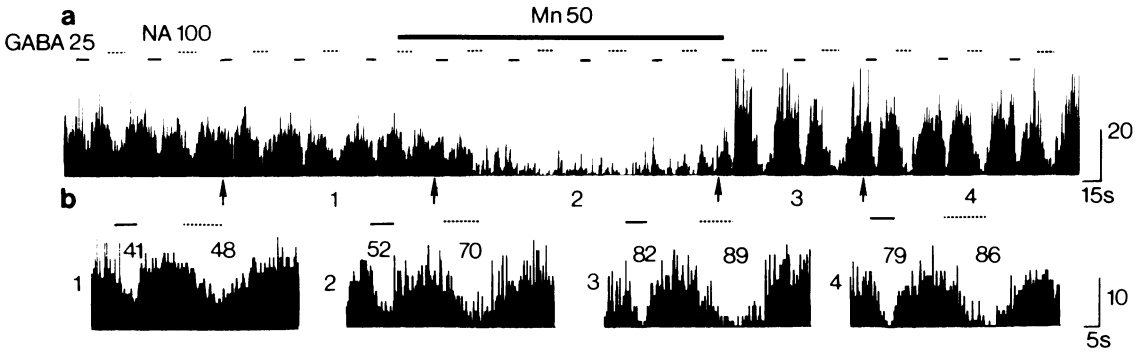

Figure 6 Effects of manganese on NA and GABA-induced inhibitions in a cerebellar Purkinje cell. Continuous ratemeter recording shown in (a) and histograms in (b). Periodic application of GABA 25 nA (solid line) and NA 100 nA (dotted line) produced approximately equal inhibition. Note the profound depressant effect of manganese 50 nA (solid bar). Numbers and arrows beneath (a) show the regions used to construct associated histograms in (b). Note that there is no decrement in either NA- or GABA-induced inhibition during (b2) or after (b3, b4) this dose of manganese. Calibration bars at right in (a) are for ratemeter (spikes/s), and in (b), are for histograms (counts per address).

not diminished. When the second pulse resumed, it had a decrease in efficiency similar to that shown in the examples of Figure 2. Note that the first barrel shows continued slight improvement, presumably reflecting a continuing, residual 'warm up'.

Although the effect of pulse interruption was more pronounced with NA, a similar decrease in effectiveness was noted when GABA pulses were interrupted (Figure 5D). Recovery time for GABA inhibition was generally less than one half that required for NA.

Analysis of depressant antagonists on cerebellar Purkinje neurones

The effects of lengthening a single agonist pulse interval detailed above become important in the design of experiments to test depressant antagonists. If pulses of agonist are interrupted during the time when a putative antagonist produces depression of neuronal firing, an artifactual blockade, due to the interruption, might be observed. By using computer-generated drug response histograms, however, the agonist effects can be evaluated even during severe depression of spontaneous discharge. Several calcium antagonists, manganese, cobalt and verapamil, previously reported to be cortical adrenoceptor antagonists (Yarbrough, Lake & Phillis, 1974; Phillis & Limacher, 1974), were tested as examples of antagonists with direct depressant effects.

Figure 5 shows an experiment in which manganese was tested on cerebellar Purkinje cells using alternate NA and GABA pulses. Agonist response histograms were used to evaluate possible antagonism of the inhibitions of NA and GABA during manganese iontophoresis (Figure 5A). The histograms (Figure 5B) reveal that consistent inhibitions were produced by GABA and NA throughout the manganese application, despite the fact that iontophoresis of manganese usually produced slowing of discharge from 20 spikes per second to less than 1 per second and converted the firing pattern to irregular bursts of 5-15 spikes. Inhibitory responses to agonists during this period of direct depression are ordinarily impossible to evaluate using only ratemeter analysis (Figure 5A). Even upon cessation of manganese applications, when spontaneous rate and pattern returned to control levels, NA and GABA inhibitions remained unaffected. Not only was NA inhibition not antagonized by manganese (58 and 67% inhibitions versus control of 44%), it exhibited the same continual increase in efficiency shown in normal prolonged periods of repeated testing (Figure 4). Reapplication of manganese at a higher dose (Figure 5C) again produced no antagonism of either agonist. On the other hand, had both agonist pulses been interrupted for the duration of manganese iontophoresis (Figure 5D), an apparent blockage of both agonists would have resulted; in fact, NA efficacy was still only about 50% recovered eight pulses after the interruption. Figure 6 shows similar results in another trial of manganese on a different cell. Computer generated histogram analysis of the period of direct depression shows no antagonism of GABA or NA. Manganese, cobalt and verapamil were similarly tested on a total of six cerebellar Purkinje cells;

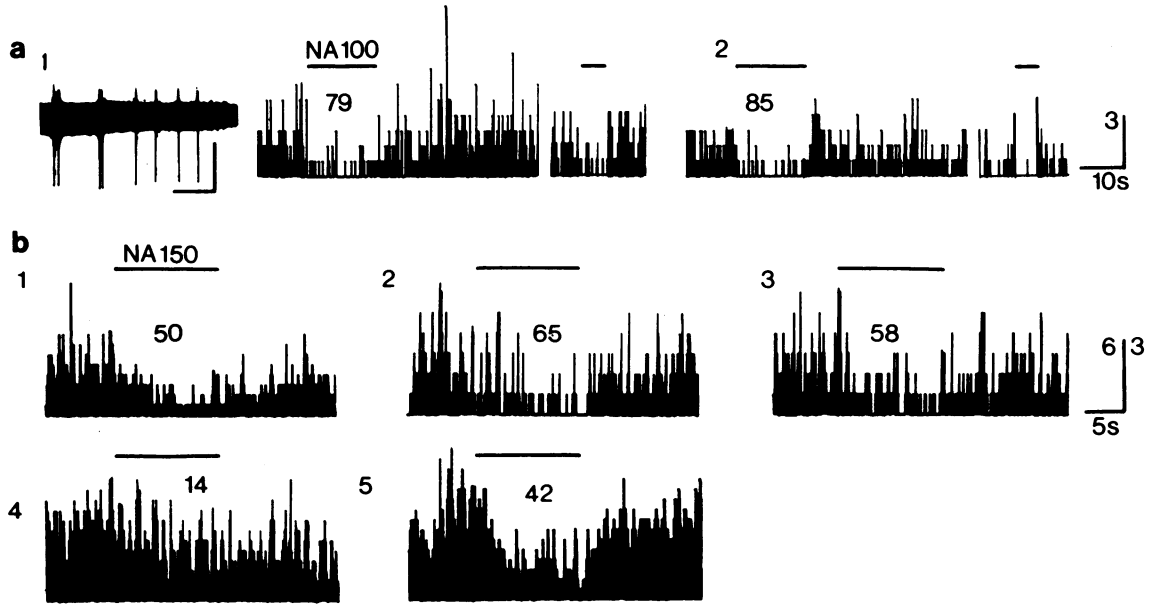

Figure 7 Effects of iontophoresis of cobalt (a) and verapamil (b) on NA-induced inhibition of cortical neurones. Two different neurones were studied in (a) and (b). A1. Control records showing action potentials (left), histogram (middle) and ratemeter (right). (a2). During 10 nA of Co iontophoresis, despite the large reduction in discharge rate shown in the ratemeter record, there is little change in the NA-induced inhibition in either the histogram (left) or the ratemeter (right). Calibration bars at right refer to (a1) and (a2) histograms only. Action potential calibration bar = 400 μV, 500 ms. (b) Effects of verapamil, 1. Control, 2. During 200 nA of verapamil. This was accompanied by a decrease in discharge rate to 10% of control, 3. Immediately after Verapamil, 4. Effects of 6 min interruption of the NA pulse, 5. Recovery of NA-induced inhibition 10 min after restoration of regular NA pulses. Horizontal calibration bar in (b3) refers to all (b) histograms Vertical bar = 6 counts per address for b1, 4, 5 and 3 counts per address for b2, 3.

partial antagonism of NA was found in only one case, with manganese (Table I).

Analysis of cerebral cortical neurones

Many cerebral cortical neurones in anaesthetized animals fire in slow, irregular discharge patterns (Figure 7A-1). The drug response histogram, by summing many agonist responses, effectively randomizes the bursts and pauses and allows quantitative determination of drug effect. Initial investigations (Yarbrough *et al.*, 1974) with manganese, cobalt and verapamil were done on cerebral cortical neurones. These agents were

Table 1 Summary of noradrenaline antagonists

	Cobalt, Manganese, Verapamil		*β-adrenoceptor antagonists**	
	Block	*No block*	*Block*	*No block*
Cerebellar Purkinje cells	1	5	18	5
Cerebral Cortical Neurones	1	7	4	0

Blockade is arbitrarily defined as a reproducible reduction of greater than 40% of NA effect. However, the block of NA by cobalt and manganese seen with 2 cells was, in fact, only partial antagonism, since the blockade was less than 40% in several of the trials.

* Data on cerebellar cells (with sotalol) from Hoffer *et al.* (1971), and Woodward, Hoffer & Altman (1974).

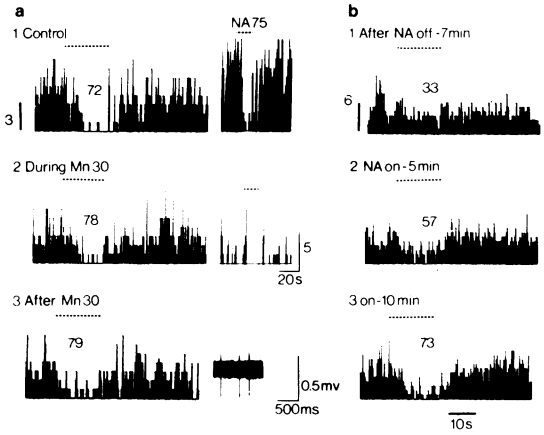

Figure 8 Effects of manganese (a) and pulse interruption (b) on NA-induced inhibitions of a cortical neuron. The same neuron was studied for both (a) and (b). (a1) Control response is shown with both histogram (left) and ratemeter (right). (a2) During iontophoresis of manganese (30 nA), NA-induced inhibition is intact as shown by the histogram (left), despite a large reduction in spontaneous discharge shown on the ratemeter (right). (a3) Histogram immediately after manganese shows NA inhibition (left). Action potential record of this cell is shown on right. (b1) Effects of a 7 min NA pulse interruption. Note large decrement in inhibitory response. (b2-3) Gradual recovery of inhibitory response over the next 10 min of continued pulsing. Histogram vertical calibrations are shown in (a1) and (b1). Note that (b) histograms are half the vertical gain of (a) histograms. Histogram time calibration shown in (b3). Ratemeter and specimen record calibrations are to the right of the lines in (a2) and (a3), respectively.

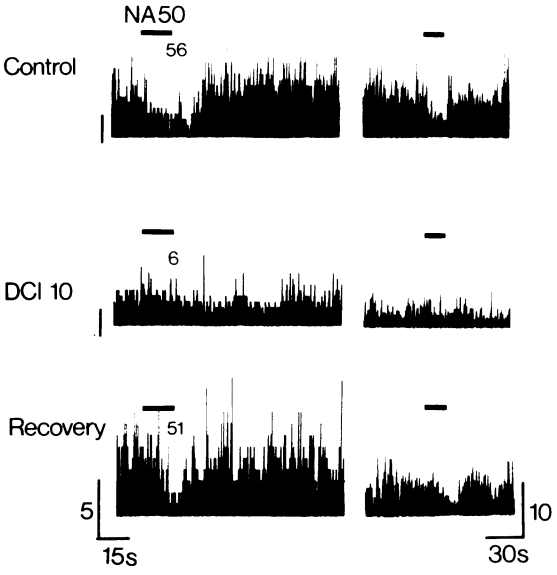

Figure 9 Blockade of NA-induced inhibition of a cerebral cortical neuron by Dichloroisoproterenol (DCI). Ratemeter records of typical single drug responses are shown the right of each NA histogram. 1. Control. 2. During 10 nA of DCI. This dose of DCI slowed discharge rate to a level similar to that seen with cobalt and manganese, as shown by the ratemeter.

studied in the cortex using the histogram technique.

As in cerebellum, cobalt, verapamil and manganese caused marked depression of spontaneous activity in cerebral cortex (ratemeter records in Figures 7A, 8A). Noradrenaline inhibitions, tested repeatedly during application of these substances and analyzed by the histograms, were not usually antagonized. Examples are shown for cobalt (Figure 7A), verapamil (Figure 7B1-3) and manganese (Figure 8). Note that during manganese iontophoresis, for instance, spontaneous rate fell to less than one spike per second (Figure 8A-2), while NA inhibition remained at 78%, compared to a control of 72%. If NA applications were interrupted for a time equal to the length of manganese application, however, inhibition fell to 33% and did not recover for 10 min (Figure 8B). The same effect is shown in Figure 7B-4, 5. Manganese, cobalt and verapamil were tested on eight cortical neurones. A slight antagonism (20%) of NA inhibition was seen in only one case, with cobalt (Table I).

For purposes of comparison, DCI was investigated as an example of β-adrenoceptor antagonist with depressant side effects. Iontophoresis of DCI nearly abolished spontaneous discharge of cerebral cortical neurones, an effect comparable to that seen with manganese, cobalt or verapamil. However, NA-induced depression was also blocked. Inhibition was reduced from 55% to 6% in one example (Figure 9). Recovery to 51% was seen 3 min later. DCI blockade of NA inhibitions was observed in all four cells studied (Table I).

Discussion

The strategy of the automated drug delivery and drug response analysis system presented here is an approach to the problems encountered in studying adrenoceptor inhibition. This system has made possible the demonstration of effective inhibition in cells firing too slowly, either inherently or due to depressant antagonists, for critical ratemeter analysis.

Problems with direct depression have been circumvented in previous studies by the selection of antagonists with which such an effect is minimal. Thus, sotalol (Hoffer *et al.*, 1971) rather than propranolol was employed to demonstrate β-adrenoceptor blockade on cerebellar Purkinje cells. Although sotalol is a less potent antagonist, it is also a less potent anaesthetic. Similarly, fluphenazine (Freedman & Hoffer, 1975) was chosen rather than chlorpromazine to demonstrate phenothiazine antagonism of NA, because of a more favourable ratio of antagonistic to direct depressant properties. The use of computer-generated histograms should now widen the range of drugs which can be tested, as well as making more quantitative analyses possible.

Expression of the extent of inhibition as a percentage change in firing rate, rather than as an absolute difference, would appear to be the most useful formulation, since, as shown in the examples, percentage inhibition remains relatively constant despite wide variations in spontaneous rate. Other parameters have been used as appropriate measures of inhibition. Hill & Simmonds (1973) calculated T_{50}, the time required to reach one-half of maximal inhibition, as a measure of GABA effects. Such a calculation could also be made with the histograms. The log dose-response curves suggested by Kelly & Renaud (1973), also for use with amino acids, are yet another possible response index. We have found, however, that several pulses of NA may be required to reach a new steady state of inhibition whenever ejection currents are changed. The reason for this phenomenon may be that a lower ejection current results in fewer residual drug ions in the tip and, therefore, less inhibition when the next pulse is applied. Thus, a given current might result in less or more inhibition depending on whether the preceding ejection current was lower or higher, an effect similar to that of pulse interruption. These phenomena do not seen to be as marked with GABA and other amino acid transmitters and, therefore, the log dose-response technique might be more applicable to those putative transmitter substances.

Irregularly firing cells, such as cortical or caudate neurones, have previously been studied under the influence of brief pulses of excitatory amino acids (Yarbrough *et al.*, 1974; Feltz & de Champlain, 1972) or low steady levels of these drugs (Nelson, Hoffer, Chu & Bloom, 1973; Connor, 1970). In view of potential interactions which might occur at sites other than the postsynaptic receptor (Nicoll, 1971), caution must be used in interpreting such data. It would be important to determine, for example, if the percentage of inhibition produced by NA and GABA is as independent of the level of amino acid-induced discharge as has been shown here for spontaneous activity. Other more physiological approaches have included stimulation of specific synaptic or sensory input to produce neuronal discharge (Foote, Freedman & Oliver, 1974; Kelly & Renaud, 1973). Such experiments provide, of course, a more detailed view of the effects of inhibition on both spontaneous and evoked activity.

The apparent decrease in inhibition resulting from interruption of a series of ejection pulses can be predicted from the studies of Hill & Simmonds (1973) and Bradshaw *et al.* (1973b) on the relationship between holding currents and drug release. Using radio-labelled NA, Bradshaw *et al.* (1973a) found that application of weak holding currents for as short a period as 10 min could reduce release *in vitro* for over 30 minutes. They proposed that the holding currents lower drug concentration in the tip as ions from the bath are drawn into the pipette. Our experiments using alternate pulses of NA from two barrels also support the hypothesis that reductions in inhibition seen after interruption of periodic drug applications is due to a diminution of iontophoretic release. One would predict that drug pulses from the barrel used without interruption would also diminish in efficacy if one of a variety of biological factors, such as saturation of reuptake sites, were of major importance.

Although we have studied only fourteen neurones in the cerebral and cerebellar cortices, our preliminary results revealed little adrenoceptor antagonism with manganese, cobalt and verapamil. This contrasts with results of Yarbrough *et al.* (1974) who described these agents as being potent antagonists. Their protocol called for interruption of NA testing for periods of up to 5 min while cells were exposed to, and recovered from, the direct depressive effects of the antagonists. Our results indicate that NA pulses would be expected to be only partly effective for several minutes after such an interruption. Tested with uninterrupted periodic pulsing from the NA pipette, these substances are generally not antagonistic even when causing a marked direct depression of the neurone. By contrast, the β-adrenoceptor antagonist DCI, which also has depressant and agonist activity, abolished NA inhibitions in cortex, confirming the report of Stone (1973).

The assumed adrenoceptor antagonism by manganese, cobalt and verapamil was cited as a main support for a theory of calcium mediation of NA effects (Yarbrough *et al.*, 1974), as opposed to mediation by adenosine 3′,5′-monophosphate (cyclic AMP; Siggins, Hoffer & Bloom, 1971). Our failure to find such antagonism by no means

argues against a role for calcium. Calcium has been shown biochemically to be necessary for NA modulation of adenyl cyclase (Rasmussen, 1970) and may be important in other NA or cyclic AMP dependent processes as well. Furthermore, lanthanum, another putative calcium antagonist, effectively antagonizes NA inhibition of cerebellar Purkinje cells (Freedman & Hoffer, unpublished results). However, interpretation of such effects remains complex since lanthanum also blocks the increase in cyclic AMP produced in myometrium by isoprenaline, while incubation in calcium-free medium potentiates the isoprenaline-induced increase (Kroeger & Marshall, 1974). Hence, lanthanum may modify cyclic-AMP mediated events either directly or through calcium sensitive mechanisms which are as yet unresolved.

Techniques based on the peri-stimulus time histogram have been used in this paper to quantify the efficacy of putative adrenoceptor antagonists. With these techniques it has been possible to avoid potential artifacts resulting from inconsistent iontophoretic release of agonists and problems with irregularly discharging cells and depressant antagonists. The employment of these tactics should, thus, enhance the usefulness of microiontophoresis in the analysis of adrenergic mechanisms.

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