Br. J. Pharmac. (1986), **88**, 847–856

Non-cholinergic synaptic excitation in neostriatum: pharmacological evidence for mediation by a glutamate-like transmitter

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1 We studied the synaptic pharmacology of an excitatory pathway in the neostriatum using electrophysiological techniques in tissue slices from rats.

2 In response to single electrical stimuli, two negative, extracellular potentials (N-1 and N-2) were recorded through micropipette electrodes within 150–450 μm of the stimulating cathode. N-2 was reversibly reduced or abolished by reducing the concentration of calcium in the bathing medium, while N-1 was unaffected. Both N-1 and N-2 were reversibly abolished by the local anaesthetic procaine.

3 Single-unit, extracellular action potentials were, at times, associated with either N-1 or N-2. Intracellular recordings showed action potentials at N-2 latency arising from graded, monophasic, depolarizing potentials.

4 Bath-applied cholinoceptor and dopamine receptor antagonists failed to reduce N-2. By contrast, antagonists of excitatory amino acid transmitters reversibly reduced or abolished N-2. γ-D-Glutamylglycine (GG), (±)-*cis*-2,3-piperidine dicarboxylic acid (PDA) and DL-2-amino-4-phosphonobutyric acid (APB) blocked N-2 with ED_{50}s of 0.79 mM, 1.0 mM and 1.1 mM, respectively. (−)-Baclofen reversibly blocked N-2 with an ED_{50} of 0.79 μM; (+)-baclofen was 330 times less potent.

5 The results suggest that N-1 results from direct activation of fibre tracts or cell bodies, while N-2 is a population spike mediated by excitatory synapses whose natural transmitter pharmacologically resembles glutamate.

Introduction

In vivo pharmacological studies in the neostriatum have been encumbered by the presence of the blood-brain barrier, the lack of control over drug concentration, and the usual presence of anaesthetic drugs. Yamamoto (1973) first showed that electrophysiological techniques could be applied to the *in vitro* neostriatal slice preparation. Exploiting the pharmacological advantages of this preparation, Misgeld *et al.* (1979, 1980) demonstrated an excitatory, cholinergic pathway in slices of the neostriatum by testing the effect of bath-applied neurotransmitter antagonists against the action of endogenously-released transmitter. Using similar techniques in neostriatal slices from rats, we have found an electrophysiologically excitatory pathway whose natural transmitter has the pharmacological attributes of an excitatory amino acid.

Some of these data were published in a preliminary communication (Cordingley & Weight, 1982).

Methods

Sprague-Dawley rats of 200–300 g were stunned by a blow to the head and decapitated. The brain was rapidly excised and placed within 60–90 s of decapitation in 2–6°C artificial cerebrospinal fluid (CSF) saturated with a mixture of 95% O_2:5% CO_2, and allowed to cool for 1 min. The artificial CSF was composed of (mM): NaCl 124, KCl 3.3, NaH_2PO_4 1.25, $CaCl_2$ 2.4, $MgSO_4$ 1.2, D-glucose 10, and $NaHCO_3$ 25. A coronal slab was prepared by inverting the brain, placing it on a platform covered by moistened filter paper, and, using a hand-held razor blade, making one

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cut 1–1.5 mm anterior to the optic chiasm and a second cut approximately 3 mm posterior to the first. The slab was re-submerged in artificial CSF for 20 s, removed, placed anterior surface up, and hand-trimmed with three successive cuts: the first, horizontal and tangential to the superior border of the caudate-putamen; the second, sagittal through the midline; and the third, horizontal 2–3 mm below the first trimming cut. After another 20 s cooling period in artificial CSF, the resultant block of tissue was placed, posterior surface down, on a McIlwain tissue chopper.

Parasagittal slices 400–450 μm in thickness were made from medial to lateral. The slices were put onto a nylon mesh in the oxygenated artificial CSF, the last placement being within 7 min of decapitation. After incubation at room temperature or below for at least 1 h, individual slices were transferred by pipette to the recording chamber as needed.

The recording chamber was a trough milled through two sheets of Plexiglass, and has been described previously (Zbicz & Weight, 1985). Briefly, the slice rested on a nylon mesh which was stretched across the trough, secured by silicone rubber, and sandwiched between the two sheets. Standard and drug-containing solutions were bubbled with O_2:CO_2 and in some experiments pre-heated in reservoirs. Intravenous tubing connected the reservoirs to a switching solenoid. Between the solenoid and chamber, solution flowed through a coil of polyethylene tubing submerged in paraffin oil, which in some experiments was heated. Fluid entered the recording chamber from the side and flowed transversely across the completely submerged slice on the nylon mesh. The height of the fluid in the chamber was determined by a fixed outflow barrier over which the solution spilled, assisted by a thread wick and gentle suction.

For pharmacological experiments, bathing solutions were switched at the solenoid. At the usual flow rate of 1 ml min^{-1}, about 15 min were required to replace completely the fluid in the chamber, as judged by the change in conductance when saline in the chamber was replaced by water.

In early experiments, fluid in the chamber was maintained at 33–35°C, but a higher success rate was obtained in later experiments at 24–28°C (room temperature). Bipolar, 40 μm diameter, platinum-iridium stimulating electrodes with 80–150 μm tip separation (centre-to-centre) and 200–400 μm exposed metal were advanced into the tissue, almost to the insulating hub. Cellular regions of the slice were more easily penetrated by the stimulating electrodes, and were usually used. A horizontal wire acted in concert with the stimulating electrodes to pin the slice against the nylon mesh. Extracellular potentials were recorded with glass microelectrodes containing 3 M NaCl (0.5–3 megohms). A second microelectrode made in the same way was positioned so that its tip was in the bathing solution just above the surface of the slice; this electrode served as the 'indifferent' lead for differential recording experiments. Intracellular potentials were recorded with glass microelectrodes containing 3 M KCl (50–80 megohms). Stimuli were voltage pulses 0.1 ms in duration, delivered at a frequency of 0.5 Hz. Stimulus voltages are listed in Table 1. Extracellular and intracellular evoked potentials were amplified in standard fashion and filtered at 20 Hz and 10 kHz. Resting membrane potentials were recorded with d.c.-coupled amplifiers. Recorded waveforms were digitized and stored on floppy disks with a PDP 11/03 or 11/23 computer for later averaging on a PDP 11/34. In most experiments, averaged waveforms were derived from 64 single sweeps. Oscilloscope tracings were photographed with a Grass camera.

Since recordings were obtained within 0.5 mm of the stimulating cathode, a large stimulus artifact after-positivity coincided in time with, and introduced possible error in measurements of the first evoked negative potential (N-1). In order to minimize the effect of the stimulus artifact on the potentials evoked in the tissue, a second lead was placed in the bath immediately above the tissue. The stimulus artifact recorded by this 'indifferent' electrode was electronically subtracted from the waveform in the tissue, by differential recording, to produce a 'nulled' tracing. Most potentials reported in this investigation were obtained by this differential recording method, with a few recorded in single-ended fashion.

In pharmacological experiments, an averaged waveform was obtained in drug-free medium. After equilibration of the slice with drug-containing solution, another averaged waveform was obtained in otherwise identical conditions, followed by a third such waveform after the slice had re-equilibrated with drug-free solution. Amplitudes of potentials obtained during drug application were compared with those obtained during the preceding and following control periods. An effect from most active drugs was first apparent at 4–6 min after solution changes; the drug-induced effect usually reached a plateau by 9–12 min. We were guided in the choice of drug concentrations by reports in the literature of known pharmacological ranges in other tissue preparations. In some cases, solubility was the limiting factor (e.g. primidone and phenylethylmalonic acid diamide).

Baclofen, 5-chloro-4-(2-imidazolin-2-YL-amino)-2,1,3-benzothiadazole (DS103–282), ethosuximide, fluphenazine hydrochloride, flurazepam dihydrochloride, primidone and sulpiride were obtained from the drug companies indicated in the Acknowledgements. (+)-Bicuculline methobromide was synthesized from (+)-bicuculline according to the method of Collins & Cryer (1978). The remaining chemicals described in this study were obtained commercially.

Results

A series of two, negative, extracellular potentials (N-1 and N-2) was recorded in response to a single stimulus (Figures 1–3, 5, 7). N-1 and N-2 appeared at similar stimulus thresholds (Figure 1a, first trace). With increasing stimulus intensities, N-1 and N-2 grew in amplitude and followed the stimulus pulses at progressively shorter latencies. N-2 became maximal at stimulus intensities that were still sub-maximal for N-1 (Figure 1a, third trace). With further increase in stimulus intensity, N-2 amplitude often appeared to decrease, although this may have resulted from the appearance of a simultaneous superimposed N-1 after-positivity (Figure 1a, fourth trace). Pharmacological experiments were conducted at stimulus intensities which were just-maximal for N-2. Stimulus frequency also affected latencies and amplitudes of the waveforms (Figure 1b). N-2 amplitude decreased at stimulus frequencies of 2–5 Hz, as did N-1 amplitude. At the usual stimulus frequency of 0.5 Hz, there was no effect on N-2 amplitude and minimal change in N-2 latency, in comparison to waveforms obtained at lower stimulus frequencies.

Table 1 gives the range of latencies and amplitudes of recorded N-1 and N-2 waveforms at two temperature ranges. The effect of temperature on the extracellular waveforms was studied in detail in 4

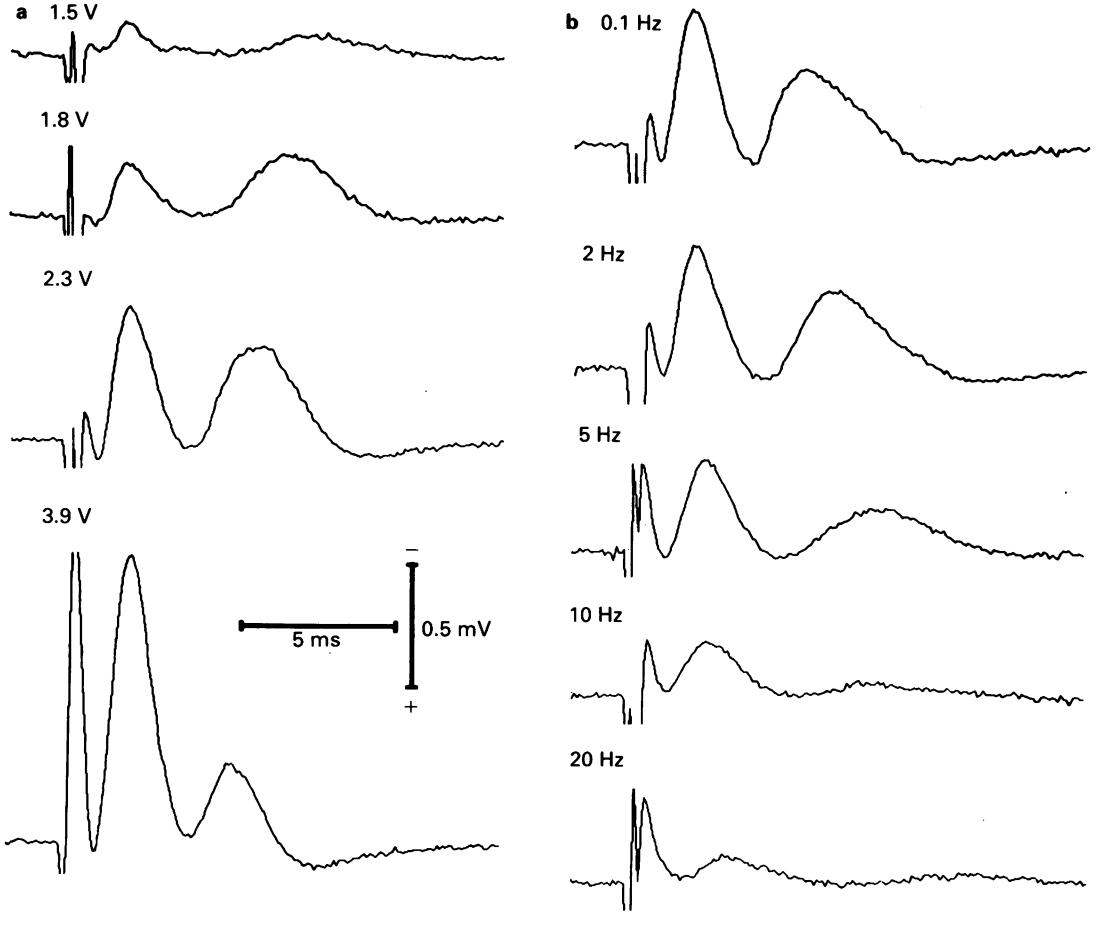

Figure 1 Effect of stimulus intensity and frequency on N-1 and N-2 waveform averages in rat neostriatum. (a) Waveforms obtained at the indicated stimulus intensities with a stimulus frequency of 0.5 Hz. (b) Waveforms obtained at the indicated stimulus frequencies with a stimulus intensity of 2.3 V.

Table 1 Amplitude and latency characteristics of evoked, extracellular waveforms at two temperature ranges

Temperature	n	*Stimulus voltage*	*N-1 amplitude*	*N-1 latency*	*N-2 amplitude*	*N-2 latency*
33–35°C	20	6–86 V	0.3–1.2 mV	1.1–2.2 ms	0.1–0.9 mV	2.8–4.4 ms
24–28°C	116	2–30 V	0.1–1.5 mV	1.3–3.2 ms	0.2–0.6 mV	4.6–8.2 ms

The sample consists of 'best' waveforms obtained from individual neostriatal slices considered stable enough for subsequent pharmacological study. The indicated voltage ranges are those required to elicit maximal N-2 amplitude, with an associated, usually sub-maximal N-1 amplitude. Latencies were measured from the beginning of the stimulus artifact to the waveform peak. Results are expressed as a range of values.

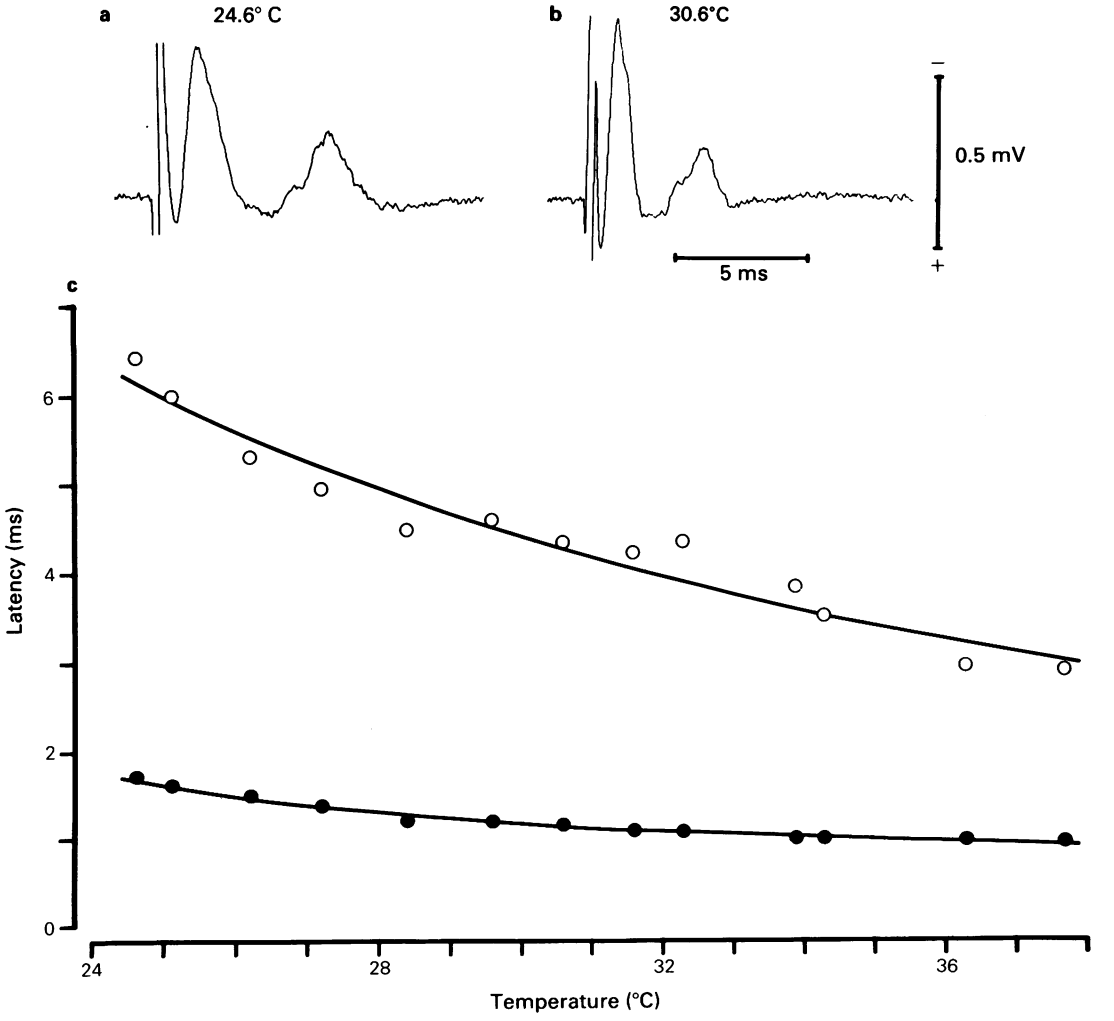

Figure 2 Effect of bathing medium temperature on N-1 and N-2 waveform averages. (a and b) Sample waveform averages at the indicated temperatures. (c) Sequential measurements of N-1 (●) and N-2 (○) latency in a progressively warmed chamber, from the same experiment as records in (a) and (b).

slices. As temperature increased, N-1 and N-2 latencies progressively shortened in graded fashion, with the latency of N-2 more temperature-sensitive than that of N-1 (Figure 2).

In experiments where Ca^{2+} concentration was reduced 8 fold to 0.3 mM, N-2 was greatly reduced or abolished, while N-1 was either little affected or increased in amplitude ($n = 5$) (Figure 3a–c). This effect of low concentrations of calcium was confirmed at both temperature ranges. The amplitude of N-2 was also greatly reduced or abolished when Mg^{2+} concentration was increased 6.7 fold to 8 mM; however, a decrease was observed in N-1 as well ($n = 5$) (Figure 3c–e). The local anaesthetic procaine greatly reduced or abolished both N-1 and N-2 in reversible fashion, when bath-applied at 1 mM ($n = 6$) (Figure 3e–g). Although both N-1 and N-2 were susceptible to the action of procaine, and were diminished with similar ED_{50}s, the shapes of the respective dose-response curves were different. A steeper relationship between blocking action and dose was found with N-2 than with N-1 in concentrations

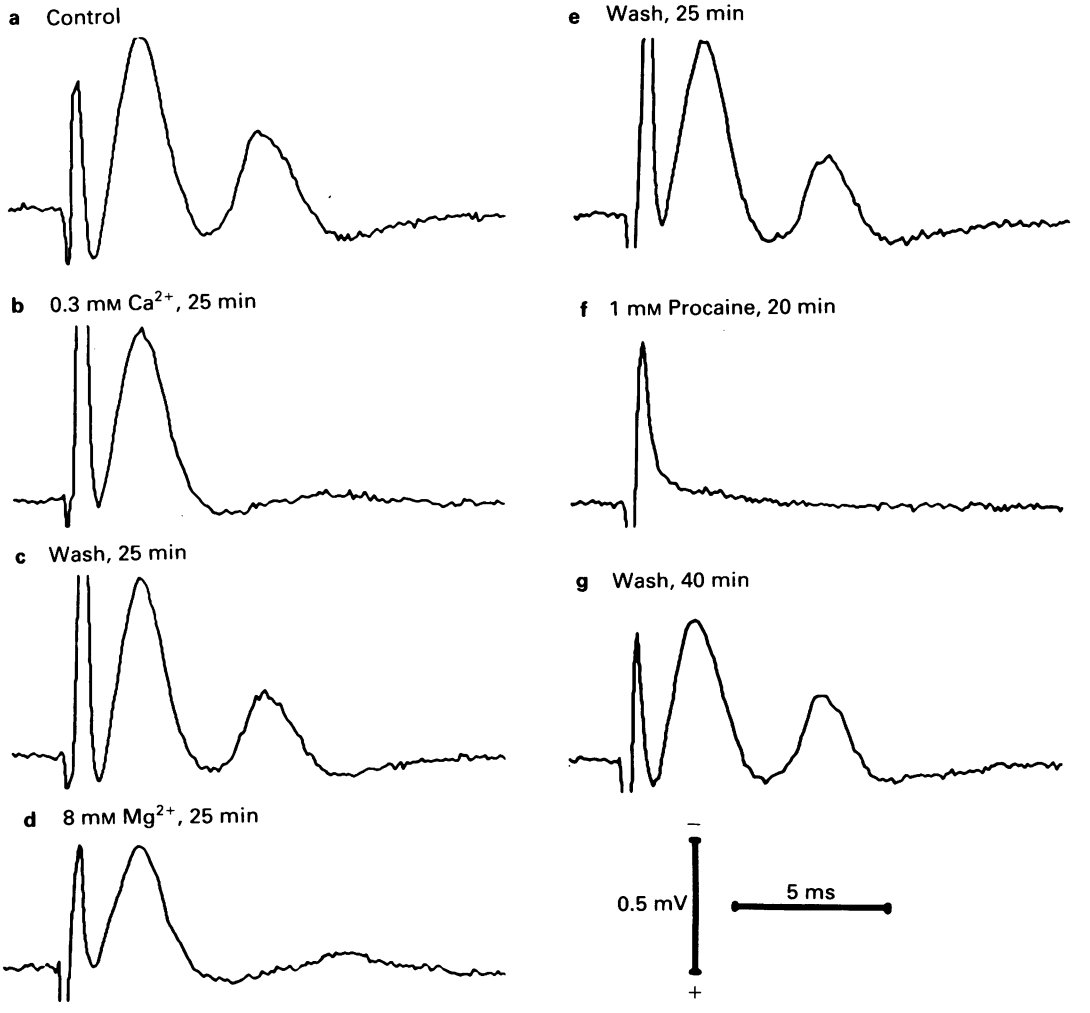

Figure 3 Effect of a low concentration of Ca^{2+}, a high concentration of Mg^{2+}, and procaine on N-1 and N-2 waveform averages. (a–g) Tracings obtained in the sequence indicated, illustrating the reversibility of the ion and drug effects. The control Ca^{2+} and Mg^{2+} concentrations were 2.4 and 1.2 mM, respectively.

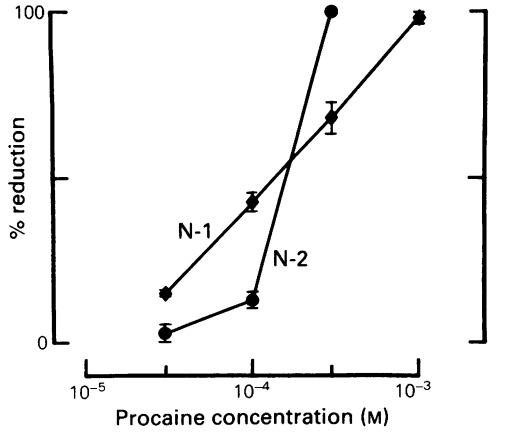

Figure 4 Effect of procaine concentration on N-1 (◆) and N-2 (●) amplitude. Experiments were performed as illustrated in Figure 3e–g. Each data point is the average of 3–5 determinations, with the error bars showing the standard error of the mean. Marks on the abscissa scale between the decade marks represent decade values multiplied by 3.

greater than 10^{-4} M (Figure 4).

The results of the preceding experiments suggest that N-1 results from the activation of fibre tracts or cell bodies while N-2 may be a synaptically-mediated population spike. Single unit studies provided observations consistent with this interpretation. Extracellular, single-unit action potentials were frequently recorded at or near the peak of N-2, but only rarely recorded at or near the peak of N-1. Driven discharges were usually single. Units were very seldom spontaneously active. Preliminary intracellular studies revealed stimulus-elicited, graded, monophasic depolarizing potentials (presumed e.p.s.ps). At higher stimulus intensities, intracellular action potentials were triggered by these graded depolarizations, at latencies corresponding to those for extracellular N-2.

In an attempt to characterize the neurotransmitter released at the excitatory synapse mediating the N-2 response, several neurotransmitter antagonists were bath-applied and their actions studied. The cholinoceptor antagonists (+)-tubocurarine chloride (30 μM), mecamylamine (0.1 mM), and atropine sulphate (0.1 mM) did not diminish N-2. The putative

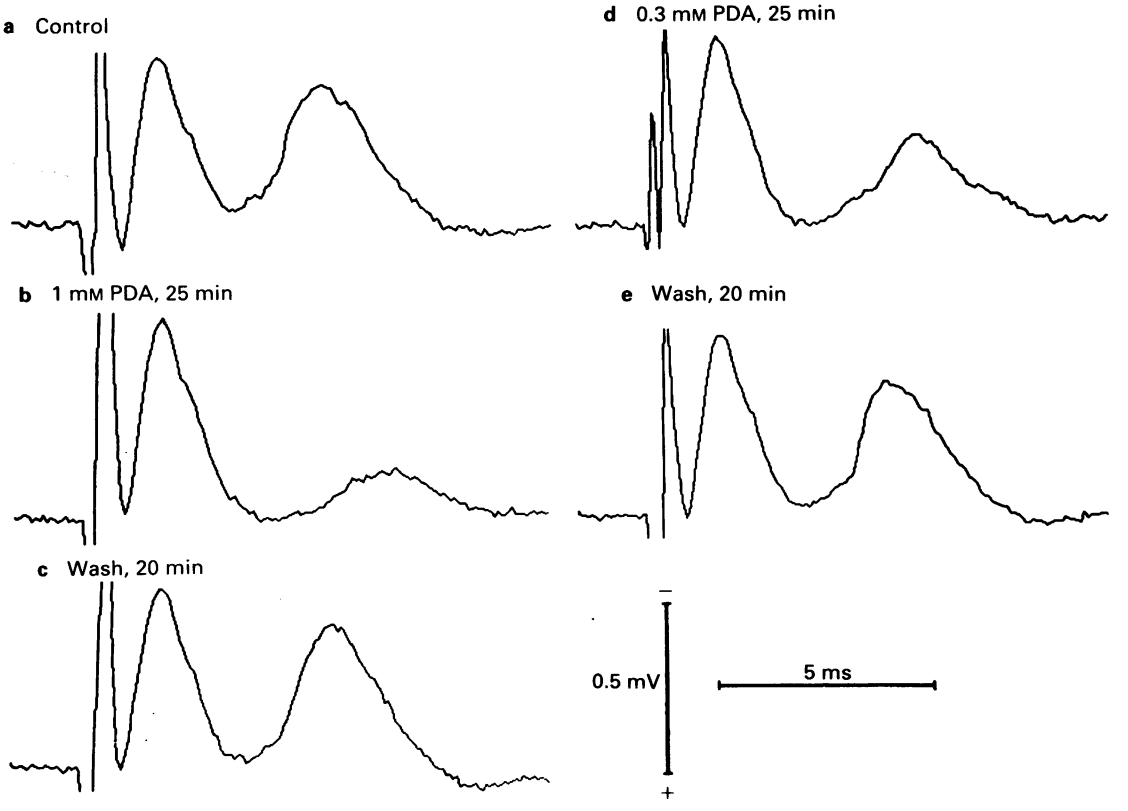

Figure 5 Effect of (±)-*cis*-2,3-piperidine dicarboxylic acid (PDA) on N-2. (a–e) Solution changes performed in the order indicated. The dose-dependency of PDA effects is further illustrated in Figure 6.

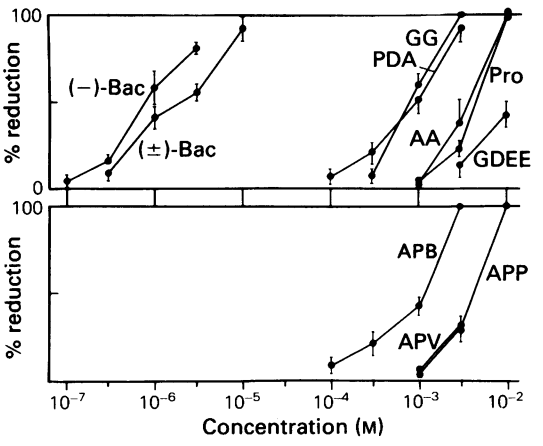

Figure 6 Effects of excitatory amino acid analogues and baclofen on N-2. Each data point is the mean of 3–11 determinations performed as illustrated in Figures 5 and 7. The error bars display the standard error of the mean. Marks between the decade marks on the abscissa scale represent decade values multiplied by 3. AA = α-amino-adipic acid; APB = DL-2-amino-4-phosphonobutyric acid; APP = DL-2-amino-3-phosphonoproprionic acid; APV = DL-2-amino-5-phosphonovaleric acid; Bac = baclofen; GDEE = L-glutamic acid diethyl ester; GG = γ-D-glutamylglycine; PDA = (±)-*cis*-2,3-piperidine dicarboxylic acid; Pro = L-proline.

dopamine receptor antagonists fluphenazine hydrochloride (10 μM) and (−)-sulpiride (0.3 μM) also did not diminish N-2. By contrast, several antagonists of excitatory amino acid receptors reversibly reduced or abolished N-2 (Figures 5–6). γ-D-Glutamylglycine (GG), (±)-*cis*-2,3-piperidine dicarboxylic acid (PDA) and DL-2-amino-4-phosphonobutyric acid (APB) blocked N-2 with ED_{50}s of 0.79 mM, 1.0 mM, and 1.1 mM respectively, and had no apparent effect on N-1. DL-2-Amino-5-phosphonovaleric acid (APV), on the other hand, was effective only in concentrations well above those at which it is believed to act specifically on a sub-class of excitatory amino acid receptors, *viz.* < 50 μM (Davies *et al.*, 1981; Evans *et al.*, 1982; Collins, 1982). Concentrations of glutamic acid diethyl ester (GDEE) which reduced N-2 also diminished N-1, suggesting an extra-synaptic site of action for this agent. All drug effects were reversible except for those with 10 mM DL-2-amino-3-phosphonoproprionic acid (APP) and 10 mM L-proline (Pro) which produced a blockade of N-2 that was poorly or not reversible within 19–60 min.

Of all the substances tested that affect amino acid transmitter function, (−)-baclofen was the most potent (Figures 6–7), the ED_{50} being 7.9×10^{-7} M. (+)-Baclofen was 330 times less potent than (−)-baclofen. Baclofen was first synthesized as a structural analogue of the inhibitory transmitter γ-aminobutyric acid (GABA) (Keberle & Faigle, 1972). In view of this, we tested the effect of the GABA-antagonists picrotoxin and bicuculline methobromide on the evoked response. Neither picrotoxin (10^{-4} M) nor bicuculline (10^{-4} M) (Figure 7) prevented the partial

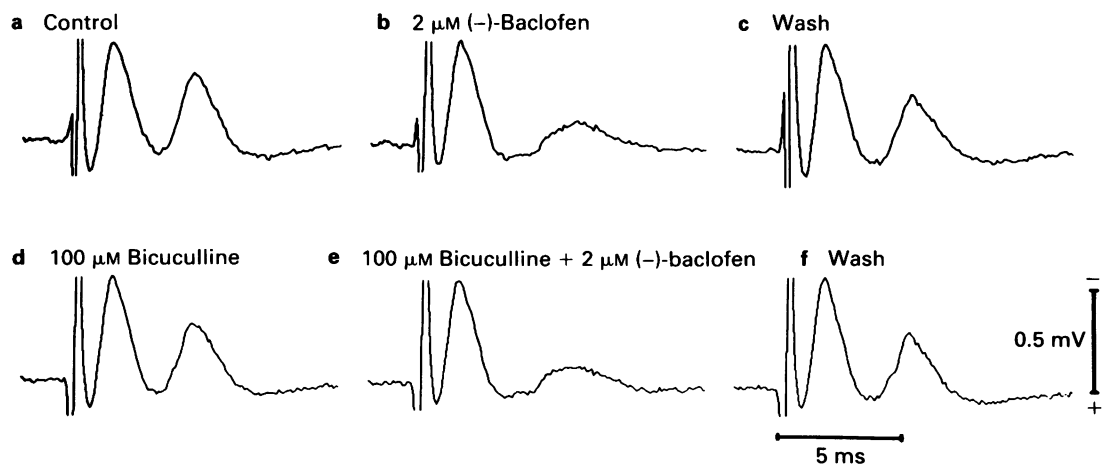

Figure 7 Lack of effect of (+)-bicuculline methobromide on the suppression of N-2 by (−)-baclofen. (a–f) Solution changes were performed in the sequence indicated. In the illustrated experiment, 2 μM (−)-baclofen suppressed N-2 by 57% in the absence of (+)-bicuculline methobromide, and by 61% in the presence of this compound. Note that application of (+)-bicuculline methobromide preceded that of (−)-baclofen in (d) and (e), without intervening washout of (+)-bicuculline methobromide.

blockade of N-2 produced by 2 μM (−)-baclofen ($n = 3$, for each drug). The blocking of N-2 by baclofen and the lack of block by (+)-tubocurarine chloride were confirmed at both temperature ranges.

The action of diverse classes of drugs was also tested against N-2 amplitude (Table 2). In view of the clinical utility of baclofen as a centrally acting muscle relaxant, other such drugs were tested. In contrast to baclofen, chlordiazepoxide (30 μM) and mephenesin (300 μM) did not decrease N-2. Experimental relaxant DS103-282 (Davies, 1982) at 300 μM decreased N-2 by 13–43%, but also decreased N-1 by 34–43%, suggesting a non-specific effect at this concentration.

Several water-soluble anticonvulsants, phenobarbitone, primidone, phenylethylmalonic acid diamide, valproic acid, and ethosuximide, did not diminish N-2 amplitude at tested concentrations (Table 2). However, the anaesthetic barbituate pentobarbitone at 0.3 mM produced a 45–57% decrease in N-2 ($n = 3$), although 0.3 mM phenobarbitone was ineffective.

Table 2 Drugs with no apparent effect on N-2 at concentration indicated

Transmitter antagonists	*Concentration (M)*
(+)-Tubocurarine chloride	3×10^{-5}
Mecamylamine	1×10^{-4}
Atropine sulphate	1×10^{-4}
Fluphenazine hydrochloride	1×10^{-5}
(−)-Sulpiride	3×10^{-7}
Picrotoxin	1×10^{-4}
(+)-Bicuculline methobromide	1×10^{-4}
Anticonvulsants	
Phenobarbitone	3×10^{-4}
Primidone	3×10^{-5}
Phenylethylmalonic acid diamide	3×10^{-5}
Valproic acid	1×10^{-4}
Ethosuximide	1×10^{-3}
Centrally-acting muscle relaxants	
Chlordiazepoxide hydrochloride	3×10^{-5}
Mephenesin	3×10^{-4}
Others	
Ethanol	1×10^{-1}
Morphone sulphate	1×10^{-5}
Flurazepam dihydrochloride	3×10^{-5}
N,N-dimethylglycine	1×10^{-3}

At least 3 trials were performed with each drug at the concentration indicated.

Discussion

In the experiments described here, intrastriatal stimulation evoked two extracellular field potentials, a short latency N-1 and a longer latency N-2. The following observations suggest that N-1 results from the direct activation of fibre tracts or cell bodies: (1) N-1 was decreased or abolished by procaine but unaffected or increased by low concentrations of calcium; and (2) although uncommon, single-unit extracellular action potentials were recorded that coincided with N-1. The unexpected decrease in N-1 amplitude with stimulus frequencies of 5 Hz and above may be due to the fact that stimulus intensities chosen to produce a maximal N-2 were still sub-maximal for N-1. On the other hand, several observations suggest that N-2 is a synaptically-mediated response: (1) low concentrations of calcium or high concentrations of magnesium decreased or abolished N-2; (2) single-unit extracellular action potentials frequently coincided with N-2; and (3) intracellular recordings showed action potentials with a latency similar to N-2, arising from graded depolarizing potentials (presumed e.p.s.ps).

Misgeld and co-workers (1979, 1980) have previously reported the activation of two extracellular waveforms, N-1 and N-2, by electrical stimulation of neostriatal slices. Pharmacologically, however, the pathway activated by Misgeld *et al.* appears to be different from the pathway studied in experiments described here. In the experiments of Misgeld *et al.* (1979, 1980) N-2 was blocked by the nicotinic antagonists tubocurarine (3 μM) and mecamylamine (10 μM). In our experiments, N-2 was insensitive to these nicotinic antagonists. Although the reason that apparently different pathways were activated in these two investigations is not clear, it presumably results from differences in experimental methodology. The major experimental differences in the two studies are: (1) Misgeld *et al.* used coronal slices, whereas our slices were parasagittal; (2) Misgeld *et al.* recorded from slices positioned at the surface of the bathing solution, in contrast to the use of submerged slices in our experiments; (3) Misgeld *et al.* stimulated the surface of the slice with larger-diameter electrodes, whereas we stimulated the depths of the slice with smaller-diameter electrodes; and (4) Misgeld *et al.* recorded at distances of 500–1000 μM from the stimulating electrode, in contrast to the 150–450 μm recording distances in our study.

While most of the pharmacological studies were obtained at an unphysiologically cool temperature range, we feel the results are representative because the essential features of the pharmacological profile of responsiveness were reproduced at a warmer temperature range, and the graded variation in N-1 and N-2 response latencies with different temperatures

(Figure 2) indicates that the recorded waveforms result from similar physiological processes at the two temperature ranges.

Compounds known to antagonize the postsynaptic responses of excitatory amino acids were found to reduce or block N-2 in the present study. The concentrations at which the antagonists employed exert specific effects have not, in every case, been well-established in the literature. APV, however, has been found to antagonize excitatory amino acid responses mediated by NMDA (N-methyl-D-aspartate)-receptors at concentrations $< 50\ \mu M$ (Davies *et al.*, 1981; Evans *et al.*, 1982; Collins, 1982). Since concentrations of APV greater than 1 mM were required to reduce the N-2 response in the present study, it appears unlikely that NMDA-receptors are involved in this response. On the other hand, GG, PDA, and APB have been found to antagonize selectively excitatory amino acid and synaptic responses in the spinal cord in concentrations similar to those employed in this study (Watkins, 1981). The reduction of N-2 in the present study by these glutamate antagonists thus suggests that the pathway driven to produce the N-2 response in the present experiments may involve a glutamate-like excitatory amino acid transmitter acting on non-NMDA receptors.

The potent blocking effect of baclofen on the N-2 pathway in neostriatum is similar to that found on synapses mediated by an excitatory amino acid elsewhere in the CNS. Baclofen has been found to reduce or block transmission through such pathways in spinal cord (Bein, 1972; Pierau & Zimmerman, 1973; Davidoff & Sears, 1974; Kato *et al.*, 1978), brainstem (Fromm *et al.*, 1980), hippocampus (Lanthorn & Cotman, 1981; Ault & Nadler, 1982), and olfactory cortex (Cain & Simmonds, 1982; Collins *et al.*, 1982).

Biochemical studies in neostriatum (Potashner & Gerard, 1983) and electrophysiological studies in other portions of the CNS (Davidoff & Sears, 1974; Fox *et al.*, 1978; Ault & Evans, 1981; Olpe *et al.*, 1982) suggest that baclofen impairs the release of excitatory amino acids from presynaptic terminals. The present study does not distinguish between a pre- or postsynaptic action but it is of interest to note that, in addition to the potency differences between baclofen and the structural analogues of glutamate in blocking N-2, baclofen exhibits a much broader dose-response curve.

The blocking effect of baclofen does not appear to be mediated by GABA receptors of the classic type ($GABA_A$) since the GABA-antagonists picrotoxin and bicuculline (Robbins, 1959; Eccles *et al.*, 1963; Galindo, 1969; Curtis *et al.*, 1970) did not interfere with its action. It has been hypothesized that baclofen exerts its effect at bicuculline-insensitive GABA receptors ($GABA_B$) (Bowery *et al.*, 1980; Hill & Bowery, 1981). We were unable to test this hypothesis because there are no specific antagonists for these putative GABA receptors.

Fibres from the cerebral cortex are among the most numerous of projections to the neostriatum (Glees, 1944; Webster, 1961; Carman *et al.*, 1963; Kemp & Powell, 1970; 1971). Biochemical investigations employing cortical lesions have suggested that glutamic acid is the natural synaptic transmitter for this afferent pathway (Divac *et al.*, 1977; Kim *et al.*, 1977; McGeer *et al.*, 1977; Reubi & Cuenod, 1979). The cerebral cortex is therefore a possible source for the afferent fibres producing the N-2 response.

The kind gift of drug samples from the following companies is gratefully acknowledged: baclofen, Ciba-Geigy; DS103-282, Sandoz; ethosuximide, Parke-Davis; fluphenazine hydrochloride, Schering; flurazepam dihydrochloride, Hoffmann-LaRoche; primidone, Ayerst; sulpiride, Ravizza. G.C. was a Pharmacology Research Associate of the National Institute of General Medical Sciences.

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(Received December 24, 1985.
Revised April 14, 1986.
Accepted April 30, 1986.)