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Pharmacological Characterization of the Rhythmic Synaptic Drive onto Lumbosacral Motoneurons in the Chick Embryo Spinal Cord

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The isolated spinal cord of the chick embryo generates episodes of rhythmic bursting in which sartorius (hip flexor) and femorotibialis (knee extensor) motoneurons exhibit characteristic patterns of activity. At the beginning of each cycle both sets of motoneurons discharge synchronously. Following this brief synchronous activation sartorius motoneurons stop firing at the time of peak femorotibialis activity, producing a period of alternation between the two sets of motoneurons. Intracellular recording from motoneurons has suggested that the pause is mediated by a synaptically induced shunt conductance. However, the pharmacological basis for this shunt and the nature of the excitatory drive to motoneurons is unknown. To address these questions we have investigated the pharmacology of the rhythmic, synaptic drive to lumbosacral motoneurons using local and bath application of several excitatory and inhibitory antagonists, and documenting their effects on motor output in E10–E12 chick embryos.

Local application of bicuculline or picrotoxin over sartorius motoneurons abolished the pause in firing recorded from the sartorius muscle nerve. As a consequence, the pattern of sartorius and femorotibialis activity was similar and the motoneurons were coactive. The pause in sartorius firing was shortened following local application of the glycine antagonist strychnine the nicotinic, cholinergic antagonists mecamylamine, and dihydro-β-erythroidine and several excitatory amino acid antagonists. Application of the GABA uptake inhibitor nipecotic acid depressed the slow potentials and discharge recorded from the sartorius muscle nerve. These findings suggest that the pause is determined primarily by synaptic inputs acting at motoneuron $GABA_A$ receptors with contributions from glycinergic, cholinergic, and glutamatergic inputs. The actions of locally applied GABA onto spinal neurons are consistent with these findings because the neurotransmitter depolarizes spinal neurons and reduces their input resistance.

Local application of bicuculline, but not strychnine, onto segments containing femorotibialis motoneurons altered the amplitude and duration of femorotibialis discharge and changed the profile of the slow potentials recorded form the muscle nerve. This finding implicates GABAergic inputs in the regulation of femorotibialis discharge.

The pause in sartorius firing was still present and a pause in firing appeared in each cycle of femorotibialis discharge following bath application of bicuculline or strychnine. The pause in both sets of motoneurons could be abolished by local application of the NMDA receptor antagonist AP-5 onto the motoneurons, but not by local application of bicuculline. This action of AP-5 was in contrast to its activity in normal Tyrode's solution where it shortened the pause slightly. These results can be explained by hypothesizing an increase in the number of functionally active somatic glutamate receptors that can shunt the motoneuron membrane and prevent firing.

Local application of NMDA or ampa/kainate receptor antagonists depressed discharge in sartorius and femorotibialis motoneurons but only when used at high concentration, suggesting that motoneuron discharge is regulated by dendritically located excitatory amino acid receptors.

[Key words: locomotion, rhythmic activity, spinal networks, chick]

In lower vertebrates considerable progress has been made in understanding the organization and pharmacology of the spinal networks responsible for rhythmic motor behavior. Glutamate seems to be the major excitatory transmitter in these circuits and is responsible for the excitatory rhythmic drive underlying swimming in the lamprey (for review, see Grillner and Matsushima, 1991) and in the *Xenopus* embryo (Dale and Roberts, 1985; Soffe, 1987). In both species bath application of the glycine receptor antagonist strychnine can synchronize the normally alternating activity of motoneurons on opposite sides of the animal (Cohen and Harris-Warrick, 1984; Alford and Williams, 1989; Hagevik and McClellan, 1994). Activation of $GABA_A$ receptors is not required for left–right alternation during fictive swimming in the lamprey (Grillner and Wallen, 1980; Alford et al., 1991; Tegner et al., 1993; Hagevik and McClellan, 1994) but does appear to be involved, together with $GABA_B$ receptors, in the coordination of locomotor activity (Alford et al., 1991; Tegner et al., 1993). In higher vertebrates, inhibitory processes also govern the alternation of motoneurons on the right and left side of the animal during locomotion. In the neonatal rat cord bath application of the $GABA_A$ receptor antagonist bicuculline or the glycine antagonist strychnine converts the normal left–right alternation into a synchronous bursting pattern (Harder and Schmidt, 1992). Much less is known, however, about the pharmacology regulating the alternation of flexor and extensor motoneurons within a limb, since most of the previous work has focused on left/right alternation. Intracellular recordings from cat

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motoneurons during fictive locomotion have revealed the presence of IPSPs that appear to inhibit motoneurons during the activity of their antagonists (Perret, 1983; Pratt and Jordan, 1987). The pharmacology of these IPSPs has been difficult to address in the intact cat, although there is some evidence that it is glycinergic (Pratt and Jordan, 1987). Surprisingly little work has been devoted to this issue using *in vitro* preparations of the spinal cord, although one recent report in the mouse is compatible with glycinergic mediation of the alternation (Droge and Tao, 1993).

We have been using the isolated lumbosacral spinal cord of the chick embryo to analyze the development of motor activity in a higher vertebrate. This preparation is spontaneously active and produces recurring episodes of rhythmic activity that can be recorded from motoneurons or interneurons (Landmesser and O'Donovan, 1984; O'Donovan, 1989a; O'Donovan and Ritter, 1995). Each episode comprises several cycles of activity in which flexor and extensor motoneurons discharge rhythmically. At the beginning of each cycle both sets of motoneurons discharge synchronously. Following this brief synchronous activation flexor motoneurons stop firing at the time of peak extensor activity and then resume firing for the remainder of the cycle. This pattern of activity produces a period of alternation between these antagonist motoneurons during each cycle. As in other species, bath application of excitatory amino acids is a potent activator of rhythmic activity and can convert the episodic pattern of activation into a continuous one (Barry and O'Donovan, 1987).

Intracellular and whole-cell recordings have identified several types of synaptic input to sartorius and femorotibialis motoneurons, but these inputs have been poorly characterized (O'Donovan, 1989a; Sernagor and O'Donovan, 1991b). Of particular interest is the regulation of the sartorius pause in firing, which is accompanied by a large drop in the impedance of sartorius motoneurons and has been attributed to a depolarizing IPSP of unknown pharmacology (O'Donovan, 1989a).

In general, we know very little about the pharmacology of the rhythmic inputs to motoneurons in the developing spinal cord. Earlier pharmacological studies implicated excitatory amino acids in the excitatory drive, but their interpretation was complicated by the observation that spontaneous activity can persist in the presence of NMDA receptor antagonists (Barry and O'Donovan, 1987). As a consequence, the precise nature of the excitatory drive to lumbosacral motoneurons is unknown. To investigate the pharmacology of the rhythmic inputs to lumbosacral motoneurons we have used local application of antagonists on the ventral surface of the cord or within the motor nucleus in an attempt to restrict their action to the motoneuron synapses. The effect of the drugs was assessed by measuring the discharge patterns of sartorius and femorotibialis motoneurons.

Preliminary results of this study have appeared in abstract form (Sernagor and O'Donovan, 1991a).

Materials and Methods

Isolation of the spinal cord and muscle nerve recording. Both the isolation of the spinal cord and the techniques used to record from the muscle nerves have been well documented previously (Landmesser and O'Donovan, 1984; O'Donovan, 1989a) and, therefore, will be reviewed only briefly. Experiments were performed on isolated spinal cord preparations from white Leghorn chicken embryos aged E10–E12. The embryos were decapitated and eviscerated under continuous superfusion with oxygenated Tyrode's solution (concentration in mM: NaCl 139, KCl 2.9, $NaHCO_3$ 17, glucose 12.2, $CaCl_2$ 3, $MgCl_2$ 1) cooled to 12–15°C. A laminectomy was performed to expose the ventral side of the spinal cord, and the dura was opened to facilitate oxygenation of the tissue. Two muscle nerves (sartorius, a hip flexor and femorotibialis, a knee extensor) were freed from the periphery and, in the majority of experiments, deafferented. The cord, including the segments T4 to LS5, or T4–LS8, was then isolated from the vertebral column, transferred to the experimental chamber, and pinned ventral side up to the Sylgard-lined floor. The dura was cut as close as possible to the lateral edge of the cord to facilitate drug access. The isolated cord was superfused (15–20 ml/min) with Tyrode's solution heated to 27–29°C. Motor activity occurred spontaneously in most cases or could be evoked by a single stimulus (30–40 μA, 0.5 msec) applied to the spinal cord.

Tight-fitting suction electrodes made of polyethylene tubing were used to record from the muscle nerves and to stimulate the spinal cord. The signals were amplified (×10,000), filtered at a high bandwidth (DC to 3–10 kHz), and stored using either a tape recorder (Vetter, model D) or a VCR with a digitizer (Neurodata). Further analysis of the data was performed off line (see section on quantitative analysis).

Pharmacology. We assessed the effects of the drugs by monitoring the changes they induced in the pattern of discharge recorded from motor nerves during episodes of rhythmic activity. We used local application of drugs on the ventral surface of the cord directly over sartorius and femorotibialis motoneurons or within the motoneuron pools to identify the nature of the synaptic rhythmic synaptic drive onto motoneurons without perturbing the rhythm itself. In general, local application over the cord surface and injection within the motor nucleus produced similar results on the motor pattern. However, the effective concentration of the antagonists was lowest when they were injected within the motor nucleus.

We also bath applied the inhibitory antagonists strychnine (20 μM) and bicuculline (5–50 μM) and the GABA uptake inhibitor nipecotic acid (1 mM). We compared the effects of bath application and local application of the inhibitory antagonists for two reasons. First, to test conclusions derived from local application of the drugs. Secondly, to compare the effects of drug application restricted to the motor nucleus with the effects of bath application, which should affect all functionally active inhibitory neurons within the cord.

Local application of drugs. We used the inhibitory antagonists bicuculline methiodide, picrotoxin, and strychnine hydrochloride and the GABA uptake inhibitor nipecotic acid. Excitatory antagonists included 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 1±/-2-amino-5-phosphonopentanoic acid (AP-5), kynurenic acid, and 2,4-dihydroxyphenylacetic acid (2,4 DHPAA). We also used the nicotinic cholinergic antagonists curare, mecamylamine hydrochloride, and dihydro-β-erythroidine hydrobromide (DHβE). The drugs were dissolved in Tyrode's solution at concentrations that depended on their mode of application as described below. Drugs were obtained from Sigma, Tocris Neuramin, or Research Biochemicals International.

Drugs were pressure ejected either on the ventral surface of the spinal cord or within the motor nucleus from glass micropipettes using a pneumatic pico pump (PV 800, WPI) at pressures varying between 4 and 35 p.s.i. The pipettes were pulled to a diameter of about 100 μm when the agents were applied onto the ventral surface of the cord (1 or 10 mM in Tyrode's solution) and to 10–20 μm when they were injected within the motor nucleus (50 μM, 100 μM, 1 or 10 mM). The long axis of the spinal cord was positioned in the direction of the perfusate and the drugs were ejected in parallel with the flow. The pressure used in each case depended on the pipette diameter. Generally, the pressure was chosen so that a drop of solution formed around the tip of the pipette within 5 sec after the ejection started. The type of ejection (onto the ventral surface of the cord or inside the motor nucleus), its duration, and the concentration of the drug were also determined for each case, since some drugs were more effective than others, depending on their nature, their possible site of action, and the dimensions of the spinal cord. As a consequence, these parameters were usually adjusted to achieve a maximal effect on the response recorded from the motor nerve without perturbing the rhythm.

Since the largest fraction of the sartorius pool is located in the segment LS1 and that of the femorotibialis pool in LS3 (Landmesser, 1978; Hollyday, 1980) the ventral roots of T7 and LS2 (which also contribute axons, but to a lesser extent, to the sartorius and the femorotibialis nerves) were cut in some of the experiments, so that the response recorded from the sartorius nerve originated exclusively from the LS1 root and the response from the femorotibialis from the LS3 root. Drug ejections were usually made onto or within either of these segments to

maximize our ability to detect drug-induced changes in the pattern of nerve activity. In other experiments the ventral roots were left intact.

The pipette was first appropriately positioned onto or inside the cord and then the pressure ejection was started. In some experiments we visualized the spread of the drug by including fast green in the drug solution. We found that injections within the motor nucleus were restricted to the injected segment and to approximately half the diameter of the hemicord. This indicates that the action of the antagonists is probably not restricted to synapses on motoneurons.

Drugs was ejected for 5–60 sec and motor activity was evoked by electrical stimulation of the cord surface toward the end of the ejection. On some occasions the pressure pulse itself stimulated the cord mechanically. This was indicated by the initiation of motor activity, which happened mainly when the drug was delivered inside the motor nucleus. This problem could be avoided by starting the pressure while the electrode was advanced toward and inside the tissue. In each experiment every drug was generally applied more than once and repeated only when the pattern of recorded activity had returned to that recorded before drug application. When the drugs were applied onto the surface of the cord recovery was achieved within 10–15 min, after which time the next stimulus or drug would be applied. With ejections inside the tissue, recovery was slightly slower and took 20–25 min at most.

Since pressure ejection within the nucleus could result in mechanical effects due to local swelling, we performed controls in which Tyrode's solution without drug was injected into the cord. However, such injections were without significant effects on the nerve activity (see Fig. 3*D*).

Quantitative analysis of the effects of the drugs on the pattern of motor activity. Quantification of the effects of the different drugs was accomplished by comparing the integral and peak of the averaged rectified neurogram for an averaged cycle (see below and O'Donovan, 1989a) and the duration of the pause in the sartorius neurogram (see Fig. 1 and legend for definitions). Several cycles were averaged after filtering the recorded neurograms at a bandwidth of 50 Hz to 10 kHz to isolate the spike activity. The spike activity was then rectified and integrated (time constant, 50 msec), and digitized at 1 kHz using a commercially available A-D converter (RC electronics) in an AT class personal computer. In each episode all the cycles that could be clearly discerned were included in the analysis. Sartorius cycles were generally aligned according to the pause onset (Fig. 1*A*). Femorotibialis cycles (and sartorius cycles in which the inhibition was pharmacologically blocked) were aligned according to the initial discharge. A pretrigger time of 400 msec was included for the averaging. As discussed in an earlier report (O'Donovan, 1989a) cycles could not be normalized because certain parameters of the cycle (e.g., the sartorius pause) stay constant, whereas others vary throughout the episode (O'Donovan and Landmesser, 1987; Ho and O'Donovan, 1993). Therefore, we averaged cycles and allowed the cycle length to vary in accordance with the duration of activity in that cycle. The averaged responses were then imported into a spreadsheet (Quatropro) in order to measure the parameters illustrated in Figure 1. These included: the total amount (or the integral) of activity, the amplitude of the peak discharge (for femorotibialis neurograms) and the duration of the pause (for sartorius neurograms). All measures were made before and after drug application and during the wash period. Measures are expressed as the mean ± SD.

Local application of drugs did not affect the expression of rhythmic activity nor did it substantially alter the temporal structure of the episode (Fig. 1*C*). This point was important to establish because some of the cycle parameters can vary during the episode. If the episode structure was altered by the drugs, this would affect the selection of cycles used for averaging and could bias the average artifactually.

Whole-cell recording of the action of pressure-ejected GABA onto spinal neurons. We examined the effects of locally applied GABA on the membrane potential and conductance of spinal neurons in the ventral part of the cord. In addition, we determined the ability of bicuculline and strychnine to antagonize the GABA effects. GABA was pressure ejected at a concentration of 100 μM or 1 mM either onto the ventral surface of the cord or into the motor nucleus while recording from a ventral root in the same segment or from single neurons in spinal cord slices. The antagonists bicuculline or strychnine were bath applied at a concentration of 100 μM. To test the specificity of these antagonists at the single cell level, whole-cell patch clamp recordings were made from ventrally located neurons in transverse slices of E11–E13 spinal cord. The slices were prepared by dissecting the cord out as described above, cleaning the dura between the roots, and slicing the cord transversely

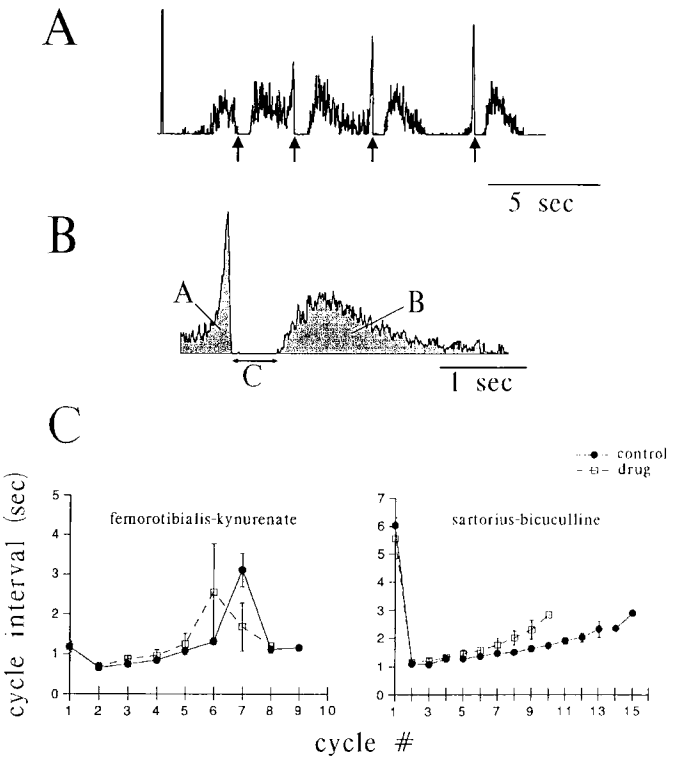

Figure 1. Quantification of the effects of drug application. *A* illustrates how consecutive cycles of activity (within a single episode) were aligned for averaging. The *arrows* indicate the point from which cycles were aligned. The example displayed in this figure is a rectified integrated episode of activity from a sartorius neurogram, and the alignment was performed from the abrupt onset of the pause in activity. *B* shows the average response from the four cycles in *A* and the different parameters calculated from this average response. The *shaded area* represents the total amount of activity measured for the average. The duration of the pause in sartorius firing (*C*) is also indicated. *C* shows that the episode structure exhibits only minor changes following local application of drugs. Both panels illustrate the intercycle interval as a function of cycle number within an episode of activity in control conditions (*filled symbols*) and in the presence of a drug (*open symbols*). The *left* panel illustrates the effects of a kynurenate injection (10 mM, inside LS3) on femorotibialis discharge, and the *right* panel, the effects of bicuculline (100 μM, on the surface of the cord) on sartorius discharge. Although profound changes in discharge parameters within a single cycle were observed in both cases, the temporal pattern of the entire episode was not affected significantly.

with a vibrating blade between the roots at segmental levels LS2–LS6. Patch-clamp recordings were made as described previously (Sernagor and O'Donovan, 1991b) using patch solution that contained in mM: potassium methylsulfonate 140, EGTA 5, Hepes 10, $MgCl_2$ 1, $CaCl_2$ 0.5, diNa ATP 1. Liquid junctions potentials were measured and corrected as described by Neher (1992). GABA, at 100 μM, was pressure ejected from a pipette located adjacent to the recording electrode, and antagonists were bath applied at 100 μM. In both sets of experiments, recordings were done either in the presence of 1 μM TTX or in low Ca^{2+}, high Mg^{2+} solution to eliminate responses due to synaptic activation. In the whole-cell patch-clamp experiments, the solution was not changed to low Ca^{2+} or TTX until whole-cell recording had been achieved.

Results

To identify the nature of the rhythmic synaptic drive to motoneurons we applied several classes of antagonist locally over motoneurons or within the motor nucleus and monitored the effects on the pattern of discharge recorded from motor nerves.

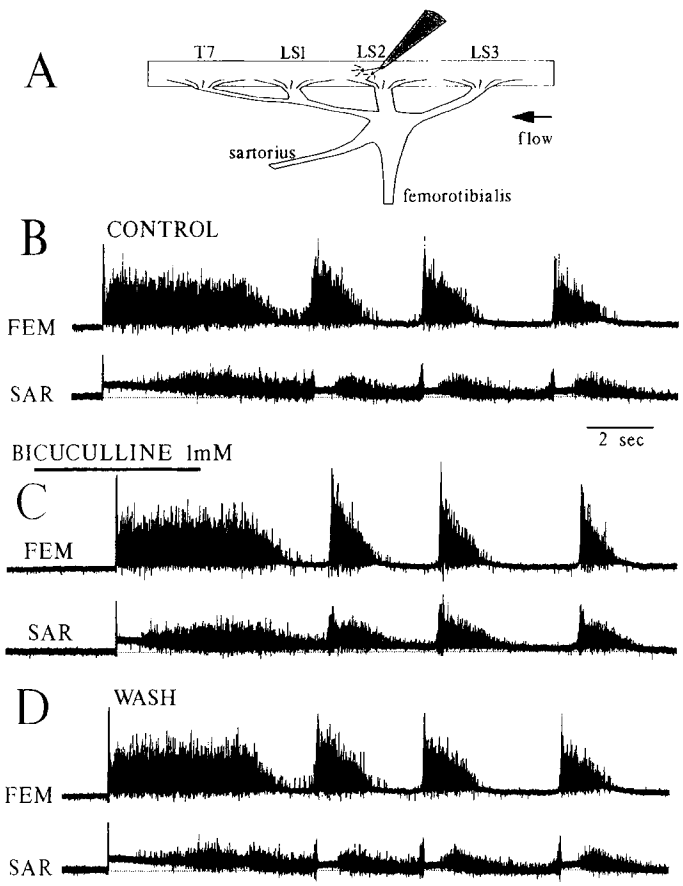

Figure 2. Bicuculline, a $GABA_A$ receptor antagonist, abolishes the pause in sartorius discharge. *A*, A diagram showing how the drugs were ejected from an electrode (*shaded*) onto the ventral surface of the spinal cord. The *arrow* on the right side shows the direction of the perfusion flow. The flow from the electrode was aligned with the flow in the chamber. *B*, *C*, and *D* illustrate one episode of activity in a femorotibialis (*upper recording*) and sartorius (*lower recording*) nerves from an E10 spinal cord in control conditions (*B*), in the presence of bicuculline (*C*, 1 mM ejected onto the surface of the cord), and after bicuculline was washed out (*D*). The duration of bicuculline ejection in *B* is indicated by the *horizontal bar* above the femorotibialis recording.

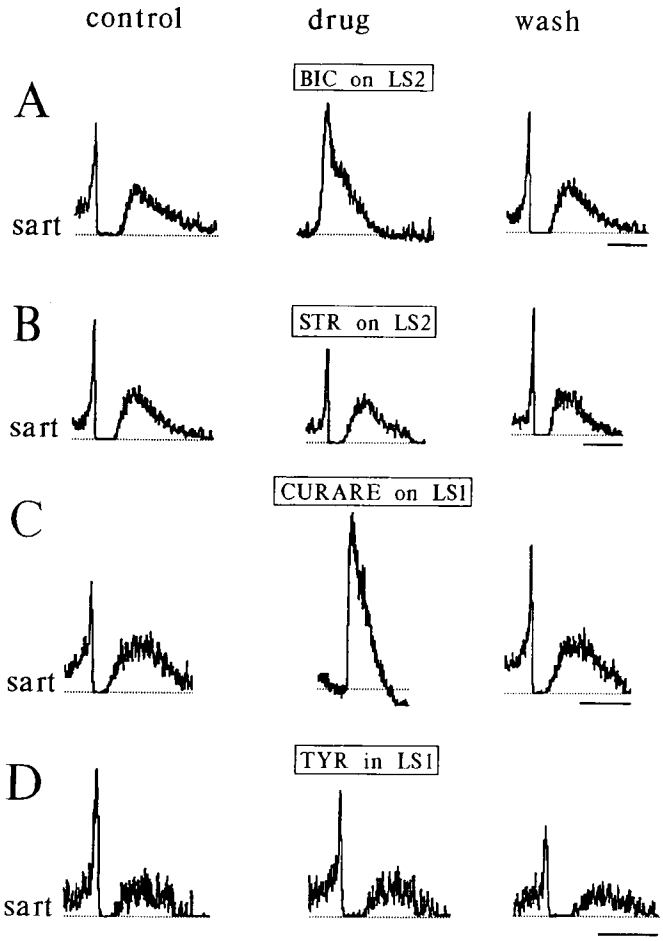

Figure 3. The inhibition in sartorius discharge is affected not only by blockade of GABAergic receptors but also by blockade of glycinergic and cholinergic receptors. These recordings are rectified integrated averaged responses. *A* summarizes the effect of bicuculline on the sartorius discharge (average of six cycles, same experiment as illustrated in Fig. 2). *B*, The effect of strychnine (1 mM, ejected onto the cord), a glycinergic antagonist, on sartorius discharge (average of eight cycles, same experiment as in *A*). *C*, The effect of curare (1 mM, onto a E10 spinal cord), a cholinergic nicotinic blocker, on sartorius discharge (average of seven cycles). *D*, The effect of an injection of Tyrode's solution inside LS1 on the sartorius discharge (average from seven cycles, E11 spinal cord).

The goal was to block or substantially reduce specific synaptic inputs to motoneurons without disturbing the rhythm.

The effects of locally applied drugs on the pause in sartorius discharge

We found that the pause in sartorius discharge could be abolished by application of bicuculline or picrotoxin over sartorius motoneurons. The pause was shortened by strychnine, by nicotinic, cholinergic antagonists, and by excitatory amino acid antagonists.

Bicuculline and picrotoxin. Figures 2 and 3*A* illustrate the effect of bicuculline, a competitive $GABA_A$ antagonist, on the inhibition expressed in sartorius motoneurons during motor activity. Figure 2 shows the neurograms obtained, respectively, from the femorotibialis and the sartorius nerves (upper and lower traces in each pair) before (*B*), during (*C*), and after (*D*) the application of the drug on an E10 spinal cord. Bicuculline (1 mM) was ejected for 5 sec onto the ventral surface of the cord, at the rostral end of LS2, as shown in the upper diagram (*A*). In this particular case, the ventral roots of T7 and LS2 were not transected (see Materials and Methods).

The pause in the sartorius neurogram was completely abolished in the presence of bicuculline, and the pattern of activity recorded in the sartorius nerve looked similar to that of femorotibialis motoneurons. Very little change was seen in femorotibialis motoneurons, presumably because the LS3 segment, containing the majority of femorotibialis motoneurons, was not in contact with the drug, which was ejected in the direction of the perfusion stream over LS2. In five E10 cords and one E11 cord (total of 52 cycles), bicuculline (1 mM or 100 μM), ejected either on the ventral surface of the cord or inside the sartorius nucleus, completely abolished the pause in sartorius activity. In three out of three experiments, intranuclear injection of 100 μM picrotoxin also abolished the pause in sartorius activity.

Strychnine. In the next set of experiments we investigated the role of glycinergic inhibition in regulating the pause in sartorius activity during each cycle. Pressure ejection of the glycinergic antagonist strychnine (100 μM or 1 mM) reduced the duration of the pause by 63 ± 22% in six E10–11 spinal cords (total of

82 cycles). However, complete blockade was never observed. When the concentration of strychnine was increased to 10 mM, rhythmic activity was abolished in an irreversible manner and the spikes disappeared. Figure 3*B* summarizes the effect of strychnine (1 mM) on sartorius motoneurons (average of eight cycles) in the same experiment as in Figure 2. In this experiment, strychnine depressed the duration of the sartorius inhibition by 32% and recovery was incomplete.

Nicotinic, cholinergic antagonists. We also investigated the effects of local ejections of the nicotinic antagonists curare, mecamylamine, and DHβE on the pause in sartorius firing during motor activity. Curare (100 μM inside the motor nucleus or 1 mM on the ventral surface of the spinal cord) completely blocked the pause (measured in 23 cycles) in three E10 preparations and shortened it by 80% in one E11 embryo. Figure 3*C* illustrates an example of the effect of curare ejected on LS1 in one of those spinal cords (1 mM for 5 sec).

Since curare can antagonize GABA agonists in spinal motoneurons (Perrins and Roberts, 1994) it is possible that its effect on the pause might be due, in part, to blockade of $GABA_A$ receptors. For this reason we used two other cholinergic antagonists, mecamylamine and DHβE, to establish if there is a cholinergic component to the pause. In developing *Xenopus* motoneurons mecamylamine does not antagonize the effects of the $GABA_A$ agonist muscimol, whereas curare does (Perrins and Roberts, 1994). In the present work, we found that both drugs (mecamylamine and DHβE) depressed the pause in sartorius discharge, although it was not abolished (Fig. 4). Mecamylamine reduced the duration of the pause by 62 ± 9% (34 cycles in four embryos). DHβE decreased the pause duration by 74 ± 13% (48 cycles in four embryos; see Fig. 4). For most trials, both drugs were used at a concentration of 100 μM and injected into LS1, although one application of both drugs was made onto the ventral surface of LS1 at a concentration of 1 mM.

Nipecotic acid: A GABA uptake inhibitor. To provide additional evidence for the participation of $GABA_A$ receptor involvement in the sartorius pause we examined the effects of nipecotic acid, a GABA uptake inhibitor on the pattern of sartorius discharge. Figure 5 illustrates the effect of nipecotic acid injected inside LS1 in an E11 spinal cord (10 mM for 30 sec). Figure 5*A* shows the neurograms of sartorius (S) and femorotibialis (F) before, during, and after the drug injection. The upper three traces in Figure 5*B* represent 17 averaged, rectified, and integrated cycles of sartorius activity from the same experiment illustrated in part *A* of Figure 5 and show that blockade of GABA uptake depresses sartorius discharge. Bath application of nipecotic acid (n = 4, 1 mM) produced similar effects to the intranuclear injection, and resulted in the abolition of sartorius discharge and a depression of the slow potentials recorded from the muscle nerve.

Because nipecotic acid increases the amount of GABA near GABA release sites, it is reasonable to assume that bicuculline, which competes with the endogenous neurotransmitter for receptor occupancy, will be less effective in the presence of nipecotic acid. We found that this was true, as is illustrated in Figure 5*B*, middle and lower traces. Bicuculline, which at a concentration of 100 μM reduced the inhibition by 64% (in five cycles) when injected inside the motor nucleus in this preparation, had no effect when injected together with nipecotic acid 10 mM (Fig. 5*B*, middle traces, seven cycles). Under these conditions, the sartorius discharge was almost abolished. However, when the concentration of the antagonist was increased 10-fold,

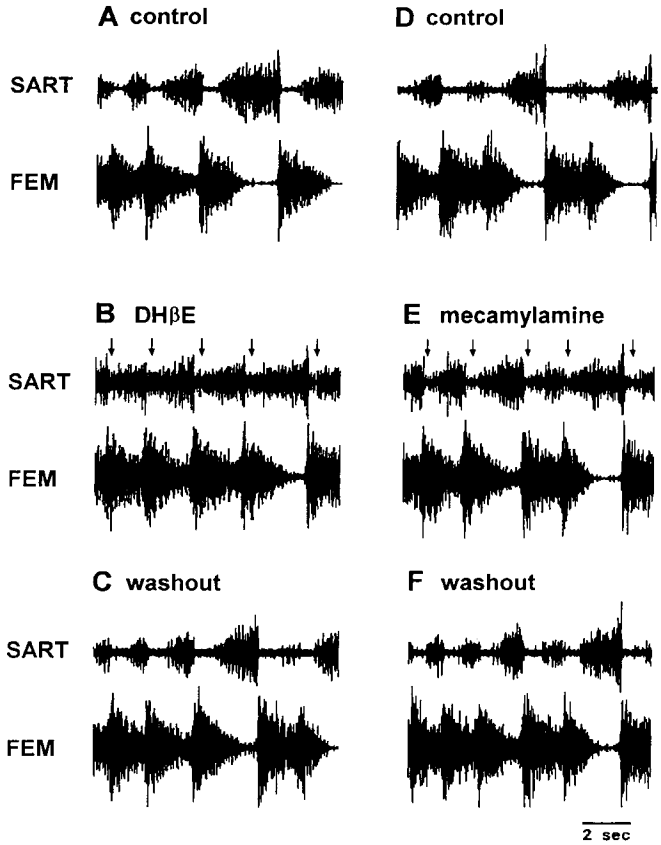

Figure 4. The effects of locally applied mecamylamine and DHβE on the pause in sartorius firing. Each trace is a neurogram recorded from the sartorius (*SART*) or femorotibialis (*FEM*) muscle nerves before (*A* and *D, Control*), during (*B* and *E, Drugs*), and after (*C* and *F, Washout*) local application of cholinergic antagonists. In the drug recordings, the location of the pause is indicated by the *arrows*. Both drugs were injected into LS1 20 sec before rhythmic activity was initiated by a stimulus to the cord. The recordings were made in a spinal cord from an E10 embryo.

the inhibition was completely abolished both in the presence of the antagonist alone (in eight cycles) and in the presence of the antagonist together with nipecotic acid (Fig. 5*B*, lower traces, four cycles) in a similar manner to the experiment illustrated in Figure 2.

The fact that bicuculline can antagonize the actions of nipecotic acid suggests that the actions of nipecotic acid on the pattern of motor discharge are due to a prolonged action of GABA on $GABA_A$ receptors rather than on other GABA receptor subtypes.

As illustrated in Figure 5, nipecotic acid injected into LS1 appeared also to influence the pattern of femorotibialis discharge. This effect could be due to spread of the drug onto femorotibialis motoneurons because femorotibialis discharge was affected in a similar manner when the drug was bath applied. Alternatively, the drug may have spread to affect presynaptic interneurons common to both sets of motoneurons.

Taken together, the results of puffing antagonists onto sartorius motoneurons favor the hypothesis that the pause in sartorius discharge is due primarily to the action of synaptically released GABA on $GABA_A$ receptors. All of the other antagonists we studied were not as effective as bicuculline or picrotoxin at depressing the pause in sartorius discharge. The observation that several antagonists including strychnine, cholinergic antagonists,

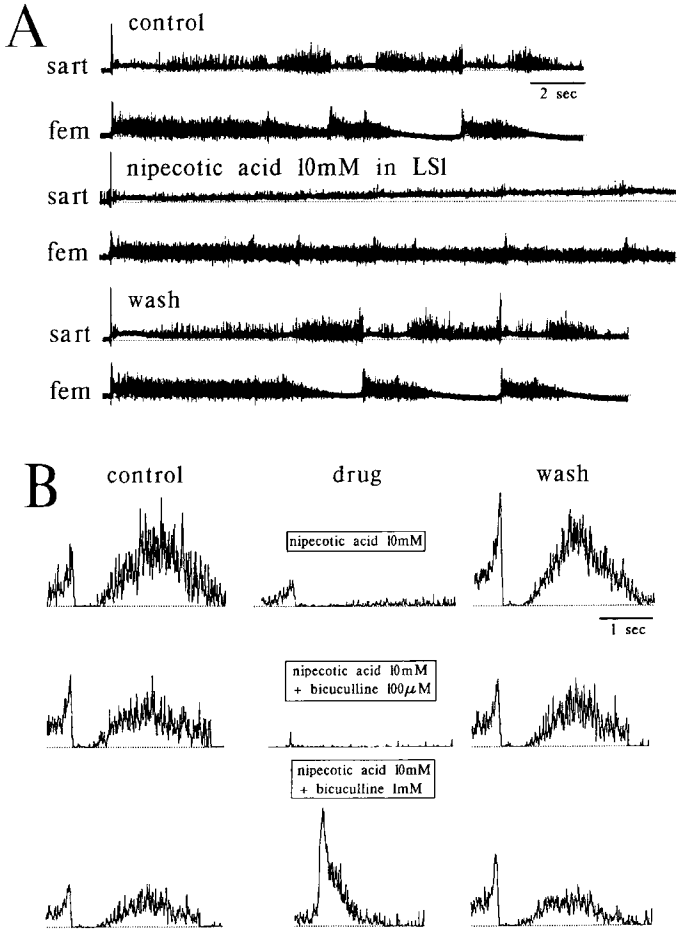

Figure 5. Inhibition of GABA uptake in the presence of nipecotic acid depresses discharge recorded from the sartorius nerve. *A* illustrates the effect of nipecotic acid (10 mM, injected inside LS1 for 30 sec, E11 spinal cord) on the nerve (*sart*, sartorius; *fem*, femorotibialis) discharges. The *two upper recordings* are the control, the *two middle recordings* were obtained in the presence of nipecotic acid, and the *two lower recordings* show the recovery from this GABA uptake inhibitor. These recordings represent entire episodes of activity. *B* shows how nipecotic acid, which enhances the effects of GABA, competes with bicuculline, which blocks the effects of GABA. These traces are rectified, integrated averaged cycles obtained from the same preparation as in *A*. The *middle upper trace* (*drug*) shows the enhancing effect of nipecotic acid (10 mM) on the sartorius inhibition (average from seven cycles). The *middle trace on the second row* shows how bicuculline (100 mM) lacks any effect on sartorius discharge when applied together with nipecotic acid (10 mM; average from seven cycles). However, the effect of bicuculline was recovered when the concentration of the drug was increased 10-fold without changing the nipecotic acid concentration, as illustrated in the *middle panel of the third row* (average from four cycles).

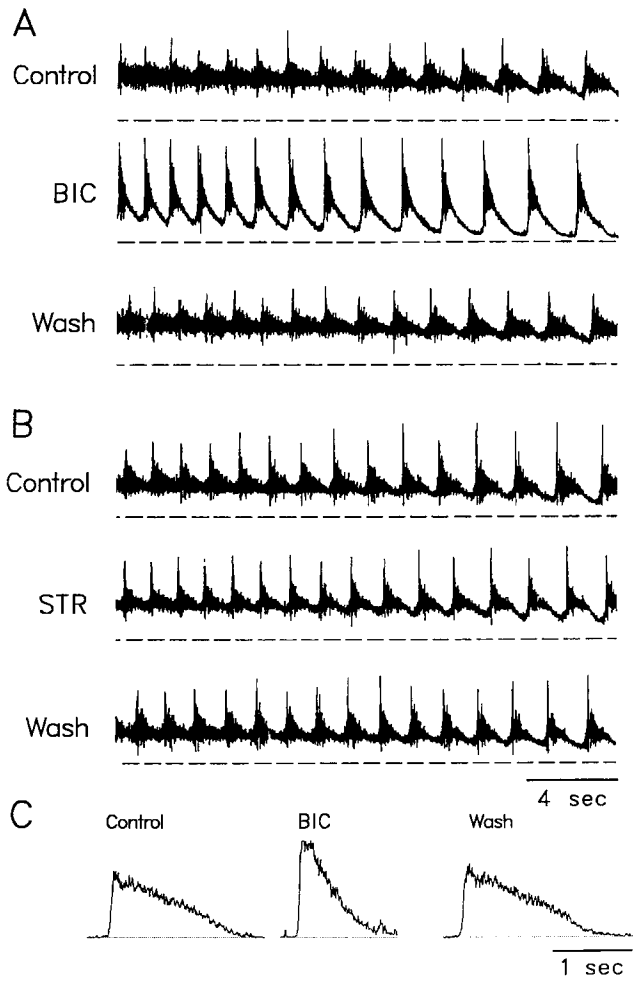

Figure 6. The effects of local application of inhibitory antagonists on the firing and slow potentials recorded from the femorotibialis muscle nerve. *A*, The effects of bicuculline (BIC, 100 μM) applied inside LS2–3 20 sec before the onset of evoked rhythmic motor activity. The *dashed line* in each record shows the baseline for the slow potentials. *B*, Local application of strychnine (*STR*, 100 μM) inside LS2–3 has less effect than bicuculline on the discharge and slow potential activity. Recordings in *A* and *B* were made from the same E11 embryo. *C*, Recordings from a different embryo of the rectified averaged femorotibialis neurogram in control Tyrode's, following local application of 1 mM bicuculline onto LS3 (*BIC*) and after washout of the drug.

and excitatory amino acid antagonists (see below) reduced the pause in sartorius discharge suggests that other input systems are involved in addition to GABAergic inputs (see Discussion).

The effects of locally applied inhibitory antagonists on femorotibialis discharge

Local application of bicuculline (100 μM inside LS3 or 1 mM over the ventral surface, $n = 5$ experiments) decreased the duration of the femorotibialis discharge, reduced the integrated activity, and increased in the peak discharge (Fig. 6*A*,*C*). Bicuculline (1 mM, ejected on the ventral side of the cord) increased the peak femorotibialis discharge by 40 ± 14% while reducing the integrated discharge by 20 ± 9% (13 cycles measured in two preparations). The profile of the slow potentials recorded from the femorotibialis muscle nerve was also altered following application of bicuculline (Fig. 6*A*, BIC). The antagonist increased the peak amplitude of these potentials in each cycle and reduced the level of the depolarization between cycles.

Locally applied strychnine (100 μM inside LS3) had little effect on either the pattern of discharge or the slow potentials recorded from the muscle nerve (Fig. 6*B*).

The action of GABA on spinal neurons and the specificity of inhibitory antagonists

Because our experiments suggested a primary role for GABA in the pause of sartorius discharge, we investigated the effects of GABA application on the membrane potential and conductance of spinal neurons. Earlier work using intracellular or whole-cell recording from identified sartorius motoneurons had

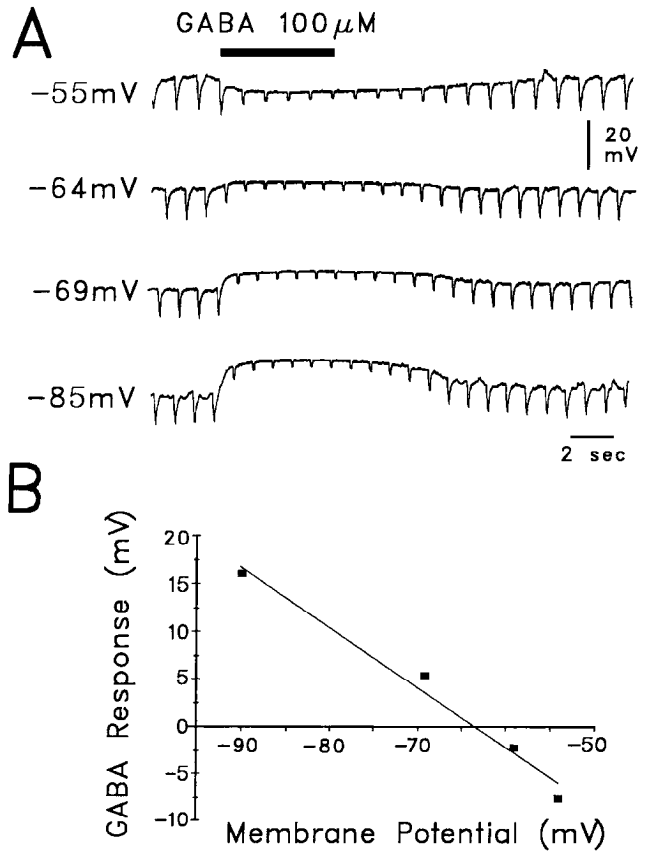

Figure 7. A. Whole-cell patch recording from a ventrally located neuron in transverse slice of an E11 spinal cord. Recordings were done in Tyrode's solution in which no calcium had been added. DC current was injected to hold the membrane potential of the cell at different levels, and then GABA (100 μM) was pressure ejected adjacent to the recording site. Short, hyperpolarizing pulses were administered continuously. Notice the decrease in conductance that accompanies the GABA-induced depolarization. *B,* Plot of the amplitude of the GABA-induced membrane potential change (*GABA response*) against the holding potential (*membrane potential*) in a different cell from that shown in *A* during application of 100 μM GABA.

revealed the pause was accompanied by a depolarizing synaptic potential and a large increase in membrane conductance (O'Donovan, 1989a; Sernagor and O'Donovan, 1991a; O'Donovan et al., 1992). Although GABA is known to produce depolarizing responses in developing rat motoneurons when recorded with sharp electrodes (Wu et al., 1992), its effects on motoneurons in the isolated chick spinal cord have not been documented. Our experiments allowed us to establish the specificity of the inhibitory antagonists bicuculline and strychnine on the membrane potential changes produced by GABA and to examine the conductance changes accompanying GABA application.

Whole-cell patch recordings were made from ventrally located neurons in transverse slices of spinal cord one to two segments thick. Recordings were made in the presence of 1 μM TTX or in zero Ca^{2+} Tyrode's solution to block synaptic transmission. GABA (100 μM) pressure ejected close to the slice surface resulted in a depolarization in 21 out of 23 recorded cells (Fig. 7*A*). The amplitude of this depolarization at rest ranged between 5 and 15 mV. Injection of short (20 msec) hyperpolarizing pulses during the GABA-induced depolarization revealed a decrease in membrane resistance. In the cell illustrated in Figure 7, the membrane resistance decreased by 75% during the application of GABA. Both the depolarization and the impedance change were blocked by bicuculline (n = 11) but not by strychnine (n = 4). The reversal potential for this GABA-induced response was determined under current clamp by injecting depolarizing DC current and puffing on GABA at different holding potentials. The reversal potential averaged −45 ± 17 mV (n = 8 cells, see Fig. 7*B*), which is substantially more positive than the expected reversal potential for chloride (see Discussion), although there was considerable variation between neurons (range −16 to −64 mV). The variability may have been due to a number of factors, including distance from the recording to the site of the GABA application, fidelity of the clamp, or incomplete dialysis of the intracellular contents. Despite this variability, it is clear that at the developmental stages examined here, a large proportion of ventrally located neurons depolarize when exposed to GABA, and that this response displays a pharmacology characteristic of $GABA_A$ receptors.

These findings were confirmed by experiments in which the GABA-induced depolarization was recorded in the ventral roots of the isolated cord instead of intracellularly from slices. The ventral root potential was blocked reversibly by bicuculline (n = 6) or by picrotoxin (n = 3), but not by strychnine (n = 4; each antagonist was used at a bath concentration of 100 μM).

These findings are consistent for a role for GABA in regulating the pause in sartorius discharge. Furthermore, they indicate that the reduction of the pause caused by strychnine is unlikely to be due to a lack of specificity for this inhibitory antagonist.

The effects of bath-applied inhibitory antagonists on the pattern of flexor and extensor activity

To obtain further evidence for the role of inhibitory amino acids in the patterning of flexor and extensor activity we bath applied glycine and $GABA_A$ antagonists and determined their effects on rhythmic motor activity, in particular, on the patterning of sartorius and femorotibialis discharge. We hypothesized that if $GABA_A$ or glycine receptors were the major determinants of the sartorius pause, then it should be abolished in the presence of the bath-applied antagonists. However, this was not the case. In the presence of bicuculline (50 μM, n = 6/6) we found that the pause in sartorius was still present and, in addition, a pause was induced in the firing of femorotibialis motoneurons. The induction of a pause in femorotibialis motoneurons was age dependent and was not observed in embryos younger than E10. In E11 and E12 embryos, bath application of 5–10 μM bicuculline resulted in the appearance of a small pause in the femorotibialis discharge (Fig. 8*B*, arrows). At this concentration, the pause was mainly induced in those cycles at the end of an episode. As the bath concentration of bicuculline was increased, the duration of the pause in femorotibialis discharge increased in a dose-dependent manner (Fig. 8*B–E*) and the profile of the femorotibialis discharge was altered. In control recordings, the femorotibialis burst declines throughout the cycle in contrast to the sartorius discharge, which augments after the pause. At 20, 30, and 50 μM, the femorotibialis discharge exhibited a similar profile to that of sartorius (Fig. 8*C,D*).

Strychnine had the same effect as bicuculline, provided that the extracellular concentration of K^+ was elevated from 3 mM to 6 mM (Fig. 9*B*). Elevation of the extracellular concentration of K^+ was necessary because strychnine depressed the number of cycles in an episode in normal Tyrode's, and this effect could be corrected by increasing the concentration of extracellular K^+.

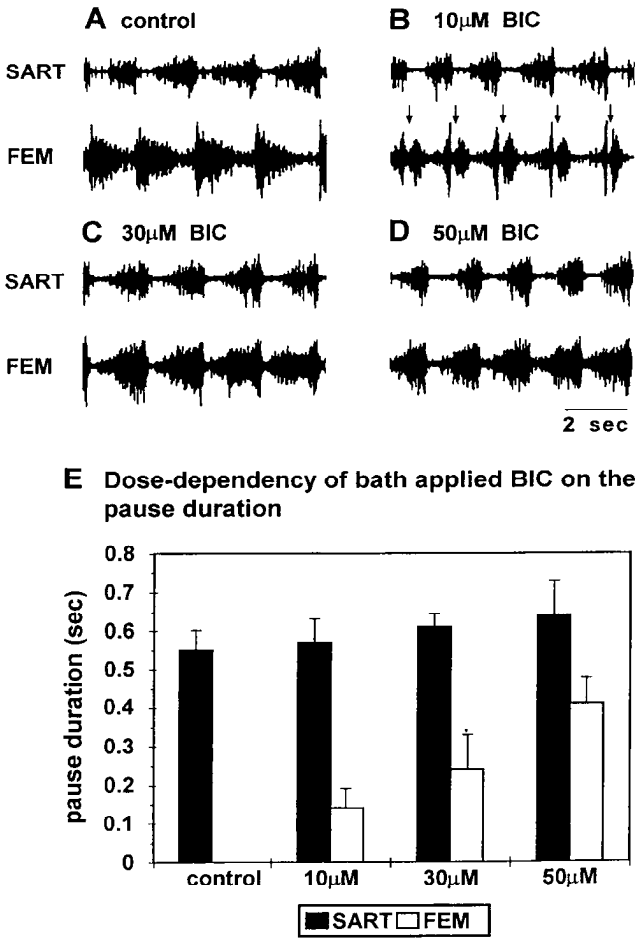

Figure 8. The effects of bath-applied bicuculline on the timing of activity in sartorius (*SART*) and femorotibialis (*FEM*) motoneurons. Several cycles of control activity are shown in *A*. Following bath application of 10 μM bicuculline (*BIC*), a pause is induced in each cycle of femorotibialis activity (*arrows in B*). Higher concentrations (30 μM, *C* and 50 μM, *D*) of bicuculline increased the duration of the pause in femorotibialis and altered the profile of the femorotibialis discharge from a decreasing profile to a recruiting profile characteristic of sartorius discharge. *E*, Dose dependency of the average pause duration (+ SD) in sartorius and femorotibialis motoneurons measured in an E11 embryo. The neurograms were filtered between 100 Hz and 3 kHz.

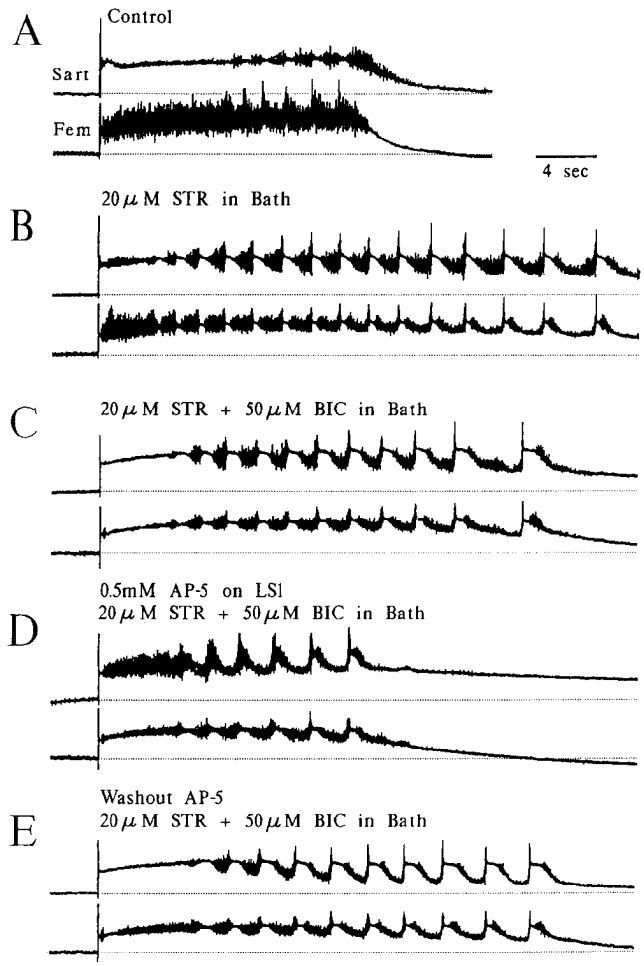

Figure 9. The effects of blockade of $GABA_A$ and glycinergic inhibition on the pattern of discharge in sartorius (*sart*) and femorotibialis (*fem*) motoneurons in an E11 embryo. *A*, Control recording in normal Tyrode's solution. *B*, Addition of 20 μM strychnine to the bath induces a pause in the discharge of femorotibialis motoneurons and synchronizes their activity with sartorius motoneurons. Recordings were made in 6 mM KCl. *C*, The pattern of activity is similar when 50 μM bicuculline is added to the strychnine. *D*, Local application of AP-5 onto LS1 depresses the inhibition of sartorius firing. *E*, Recovery of the synchronized pattern after washout of the locally applied AP-5.

In the presence of both inhibitory antagonists the pause in the discharge of both sartorius and femorotibialis motoneurons was preserved (Fig. 9*C*).

As would be predicted, local application of bicuculline had no effect on the pause in sartorius discharge in the presence of bath-applied bicuculline and strychnine, in contrast to its action in normal Tyrode's solution. However, local application of the NMDA-receptor antagonist AP-5 over sartorius motoneurons (0.3–0.5 mM, 20 sec, $n = 7$) or femorotibialis motoneurons (0.3–0.5 mM, 20 sec, $n = 4$) reversibly abolished the pause in firing recorded from either nerve (Fig. 9*D*). In control recordings made in the normal Tyrode's solution, local application of AP-5 (0.5 mM) reduced the sartorius pause only slightly (by 24 ± 13%, $n = 4$ embryos, 54 cycles, see below).

These findings suggested that glutamatergic inputs might be capable of suppressing sartorius and femorotibialis discharge in the disinhibited cord.

The effects of excitatory amino acid antagonists on the pattern of motoneuron discharge

Glutamate plays a major role in the excitatory locomotor synaptic drive in several to species. In the chick cord, preliminary experiments have estimated the reversal potential for the rhythmic synaptic drive to femorotibialis motoneurons to be close to 0 mV (O'Donovan et al., 1992; Sernagor and O'Donovan, 1990, 1991b) consistent with the action of glutamatergic synapses. The excitatory drive to sartorius motoneurons has not been well characterized. To investigate the pharmacology of the excitatory synaptic drive to flexor and extensor motoneurons we applied antagonists of excitatory amino acids in a similar manner to the inhibitory antagonists.

Femorotibialis. NMDA, non-NMDA, and glutamate receptor antagonists all reduced the amount of activity expressed in femorotibialis motoneurons. Figure 10*A* shows the effect of intranuclear injection of kynurenate (10 mM, middle record), a "broad spectrum" competitive glutamate antagonist and AP-5 (10 mM, Fig. 10*B*, middle record), a specific competitive NMDA

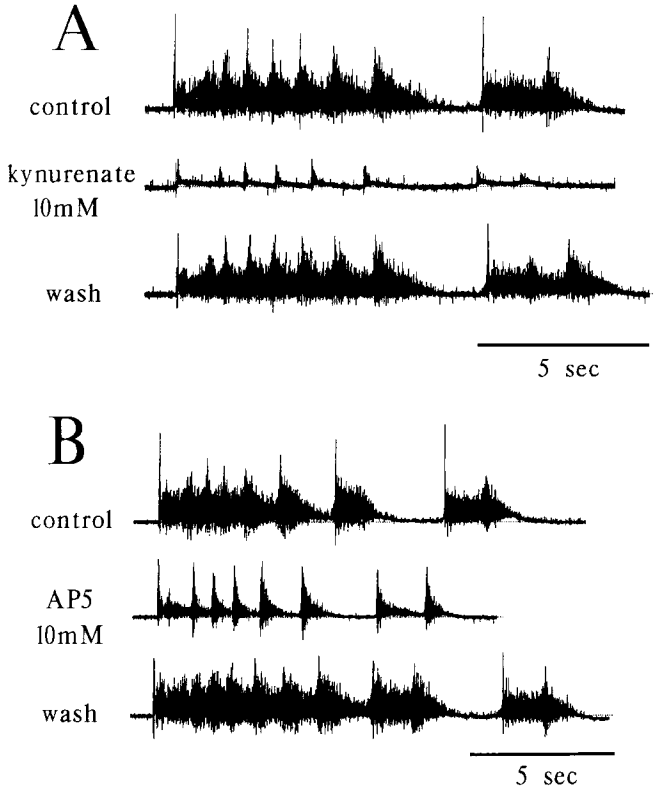

Figure 10. The effects of excitatory amino acid antagonists on femorotibialis discharge. The *three upper panels* show how kynurenate, a broad spectrum glutamate antagonist (10 mM, injected inside LS3 for 30 sec, E10 spinal cord) affects the femorotibialis discharge. Both the amplitude and the duration of the discharge are reduced. The *three lower panels* show the effect of AP5, a NMDA receptor antagonist (10 mM, inside LS3) on femorotibialis discharge in the same embryo as illustrated in *A*.

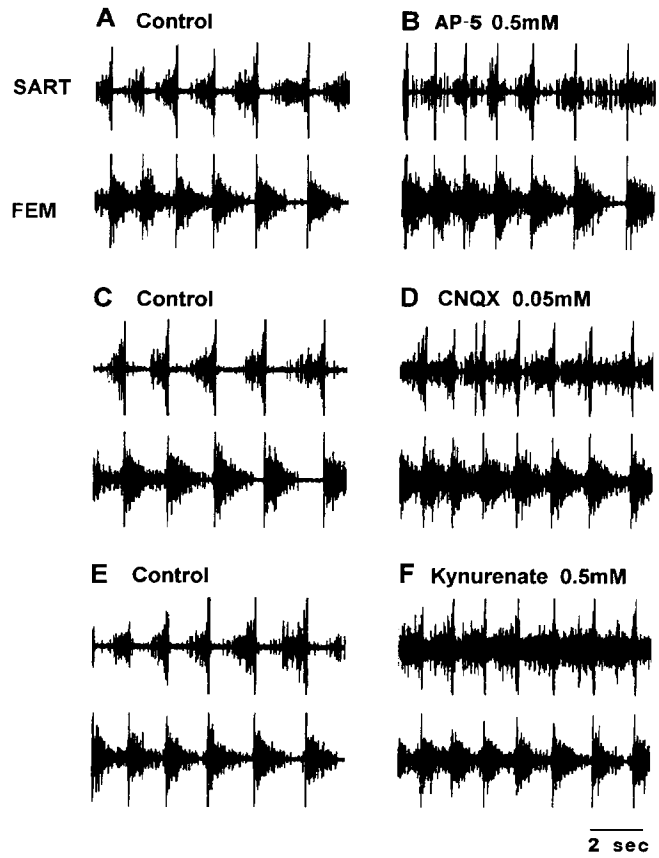

Figure 11. The effects of local application of excitatory amino acid antagonists on the pattern of discharge in sartorius muscle nerve. The *left* set of panels (*A*, *C*, and *E*) show control recordings of the sartorius (*SART*) and femorotibialis (*FEM*) muscle nerve activity. The *right panels* show the responses to drugs locally applied inside LS1 at the concentrations indicated. For brevity, washouts are not illustrated. All recordings were obtained from the same E11 embryo.

receptor antagonist on femorotibialis nerve activity in an E10 spinal cord. Both kynurenate (5–10 mM, $n = 5$ experiments) and AP-5 (1–10 mM, $n = 6$ experiments) depressed the integrated femorotibialis nerve activity, respectively, by 84 ± 2% (measured in 31 cycles from two experiments with intranuclear injection) and 45 ± 20% (47 cycles measured in three experiments; two with ejections on the ventral surface and one with injection inside the nucleus). The femorotibialis discharge was also depressed by 2,4-dihydroxyphenylacetic acid (2,4DHPAA), a specific competitive glutamate antagonist (1–10 mM, $n = 3$), and CNQX, an ampa/kainate antagonist (0.05 mM, $n = 3$). All these antagonists had to be used at a high concentration in order to produce depression of discharge and were most effective when injected inside the nucleus. Although the concentration of these antagonists was high, it is important to emphasize that the effective tissue concentration of the drugs will be lower as they diffuse from the injection site.

Sartorius. Analysis of the excitatory drive to sartorius motoneurons was complicated by the fact that excitatory amino acids antagonists delivered to the sartorius nucleus affect the inhibitory component of the response. As a result, it was difficult to isolate and quantify specific actions on the excitation. Figure 11 illustrates the actions of several excitatory amino acid antagonists applied within the motor nucleus on the discharge of sartorius motoneurons. The NMDA receptor antagonist AP-5 (0.5 mM injected inside LS1) had the least effect on the sartorius discharge pattern. Following application of this drug, the pause in firing was reduced by 24 ± 13% (four embryos, 54 cycles). CNQX, an antagonist at ampa/kainate receptors, shortened and could abolish the pause in sartorius firing (average decrease in duration 61 ± 11%, three embryos, 88 cycles). Kynurenate (0.5 mM, $n = 3$ embryos) was the most effective antagonist and reduced the sartorius pause by 93 ± 6%, three embryos, 117 cycles). At 500 μM, kynurenate did not depress sartorius discharge significantly (Fig. 11*F*), but in three out of four experiments, firing was depressed using kynurenate at a higher concentration (10 mM).

Discussion

This work has investigated the pharmacology of the synaptic drive onto flexor and extensor motoneurons during rhythmic motor activity in the developing spinal cord of the chick embryo. The results demonstrate that the timing of flexor and extensor activity is regulated predominantly by a GABAergic inhibitory mechanism although nicotinic, cholinergic, glycinergic, and even glutamatergic inputs can contribute to the pause in sartorius discharge. Under conditions of disinhibition produced by blockade with inhibitory amino acids, glutamatergic inputs appear to be the dominant regulator of the pause in sartorius discharge (see below).

The nature of the pause in sartorius discharge

The pause in sartorius discharge was blocked by locally applied bicuculline or picrotoxin, implicating a primary role for GABA on $GABA_A$ receptors. Although curare mimicked the actions of bicuculline, its effects may be complicated by an action on GABA receptors. Curare has been reported to antagonize the effects of GABA or its agonists at $GABA_A$ receptors on developing neurons in spinal cord (Bixby and Spitzer, 1982; Perrins and Roberts, 1994) and hippocampus (Siebler et al., 1988). Additional evidence in favor of GABAergic mechanisms comes from the demonstration that the GABA uptake inhibitor nipecotic acid could block the sartorius discharge and depress the slow potentials recorded from the muscle nerve. This action is compatible with a prolonged action of endogenously released GABA at active terminals.

The conclusion that synaptically released GABA acting on $GABA_A$ receptors is a major determinant of the pause is consistent with the actions of GABA puffed onto spinal neurons. Exogenously applied GABA depolarizes spinal neurons and produces a significant increase in membrane conductance. Both of these properties have been shown to characterize the synaptic potential in sartorius motoneurons that accompanies their pause in firing (O'Donovan, 1989a; Sernagor and O'Donovan, 1991; O'Donovan et al., 1992). However, because we used whole-cell patch recording to monitor the GABA-induced effects, the intracellular chloride concentration should be dictated by the concentration of chloride in the electrode—assuming complete dialysis of the intracellular contents. Under this assumption the chloride reversal potential would be determined by the chloride concentration of the electrode contents and the Tyrode's solution, and would be greater than −100 mV for the solutions used in the present work. Nevertheless, we found that the reversal potential for the GABA-induced depolarizations averaged −45 mV, significantly more positive than the calculated chloride reversal potential. At present, we cannot account for this discrepancy. Possible explanations are that the intracellular chloride concentration is not determined exclusively by the electrode contents or, alternatively, that ions other than chloride contribute to the GABA-induced depolarizations. Resolution of this issue will require analysis of the ionic dependence of the GABA-induced responses.

Our findings can be used to explain the altered pattern of firing in sartorius motoneurons when the pause is abolished by bicuculline or picrotoxin. In normal Tyrode's solution the depolarizing GABAergic drive summates with the excitatory drive to sartorius motoneurons, producing a shunt at the peak depolarization (Fig. 12*A*). As the drive and shunt decay, the sartorius firing resumes. When $GABA_A$ receptors are blocked, the depolarization and the shunt are reduced, which shortens the duration of the discharge and advances it in the cycle. In this way, the discharge of sartorius now closely resembles that of femorotibialis motoneurons (Fig. 12*B*).

Another possibility to account for the effects of bicuculline on the sartorius pause is that the antagonist blocks presynaptic inhibition (Stuart and Redman, 1992). However, we consider this an unlikely explanation because the pause persisted in the presence of bath-applied bicuculline. Furthermore, the pause was reduced by excitatory and cholinergic antagonists, which cannot be explained by an action on classical GABAergic presynaptic inhibitory mechanisms. It is also possible that synaptically released GABA might act also on $GABA_B$ receptors, although such an effect should not be antagonized by bicuculline. Further experiments will be required to establish if $GABA_B$ receptors play any role in the sartorius inhibition.

Although $GABA_A$ receptor antagonists produced the most complete blockage of the pause, several other antagonists reduced its duration. In particular, strychnine and the nicotinic, cholinergic antagonists mecamylamine and DHβE both depressed the pause in firing. It is unlikely that the effects of strychnine are due to an action on $GABA_A$ receptors because 100 μM strychnine did not antagonize the effects of GABA applied to spinal neurons. In addition, previous studies have reported that strychnine and bicuculline specifically antagonize glycine and GABA, respectively, in neonatal rat spinal neurons (Wu et al., 1992) and in cultured chick spinal neurons (Obata et al., 1978). We did not test the ability of mecamylamine and DHβE to antagonize locally applied GABA in the chick spinal cord. However, mecamylamine does not antagonize the actions of muscimol, a $GABA_A$ agonist, on spinal motoneurons in *Xenopus* embryos, even though curare does (Perrins and Roberts, 1994).

The effects of the cholinergic antagonists mecamylamine and DHβE could be explained by postulating a cholinergic pathway parallel to, or in series with, the inhibitory pathways. One possible pathway with the latter pharmacology is the recurrent inhibitory circuit, which is depressed by cholinergic and inhibitory antagonists (Velumian, 1984; O'Donovan 1989b). The participation of recurrent inhibition is plausible because the onset of each cycle of activity in sartorius can be associated with a prominent discharge, which is synchronized in several motoneuron pools (Landmesser and O'Donovan, 1984; O'Donovan, 1989a). Furthermore, the cholinergic motoneuron axon collaterals are likely to be located close to the lateral motor column where they should be susceptible to the action of locally applied antagonists. It is also possible that the cholinergic antagonists might act on receptors located on motoneurons rather than on interneurons. Finally, the nicotinic antagonists could act on nicotinic, presynaptic receptors on the inhibitory GABAergic terminals projecting to motoneurons. In several systems, including the embryonic chick brain (McMahon et al., 1994) presynaptic nicotinic receptors have been shown to facilitate GABA release (Wonnacott et al., 1989), so that their blockade might reduce release.

The pause in sartorius discharge was also depressed by the excitatory amino acid antagonist kynurenate and the ampa/kainate receptor antagonist CNQX, and to a lesser degree by the NMDA-receptor antagonist AP-5. The effects of the excitatory amino acid antagonists could be explained by postulating blockade of inhibitory interneurons or alternatively by blockade of receptors on the motoneuron membrane. An action on interneurons is plausible because the drug injections inside the nucleus are probably not limited to motoneurons and a region critical for the pause has been shown to be located just dorsomedial to the lateral motor column (Ho and O'Donovan, 1993). However, an action on inhibitory interneurons cannot account for the ability of the NMDA-receptor antagonist AP-5 to block the pause in sartorius and femorotibialis discharge in cords in which inhibition was blocked by bicuculline and strychnine.

An alternative explanation is that the antagonists are blocking receptors on the motoneuron membrane. We hypothesize that in the disinhibited cord there is an increase in the number of functionally active glutamate receptors on the soma and dendrites of both sartorius and femorotibialis motoneurons (see Fig. 12*C*). Activation of receptors on or near the soma could shunt the

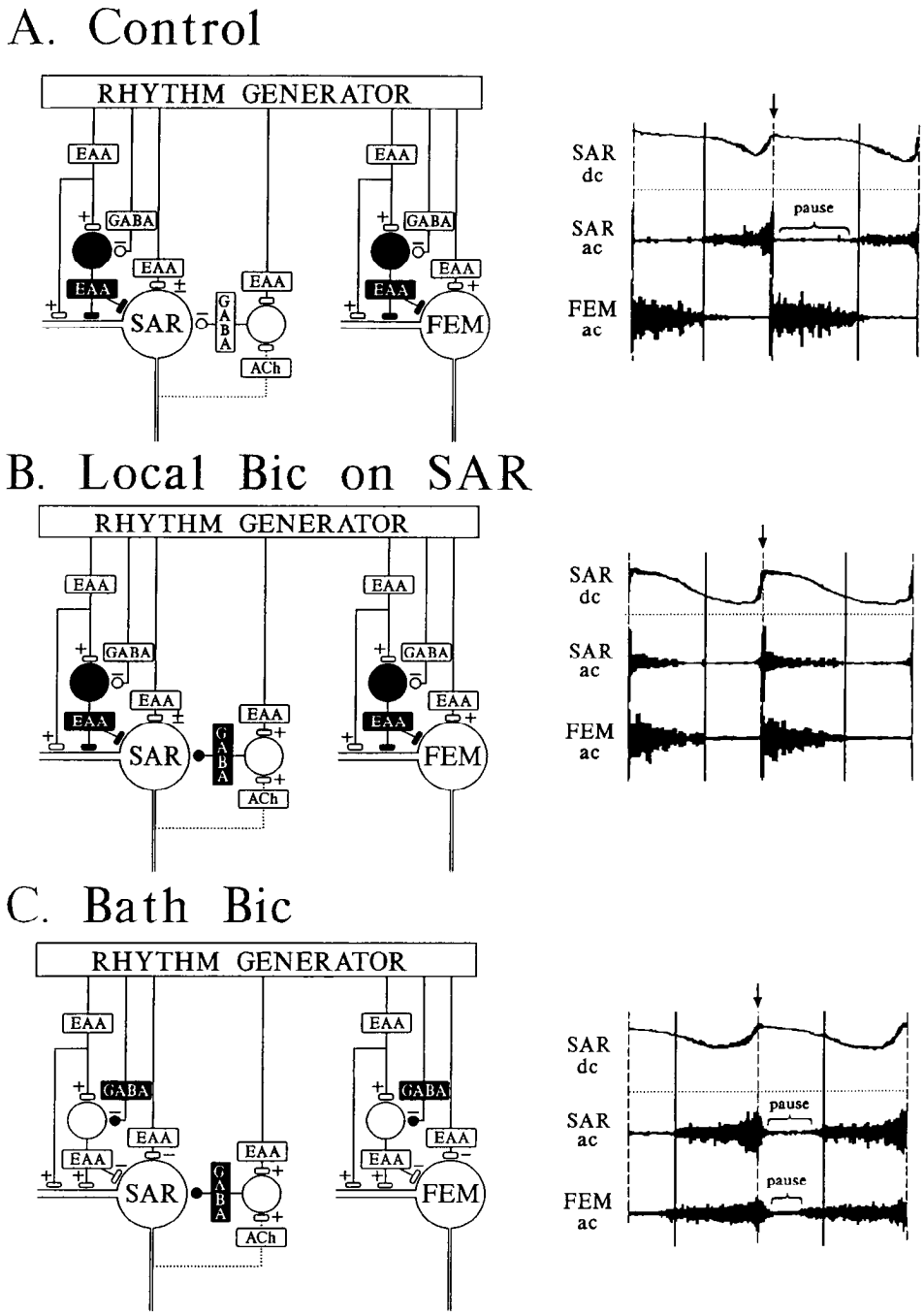

Figure 12. Summary of the synaptic connections of sartorius and femorotibialis motoneurons and the patterns of nerve activity under various conditions. In each figure an *inactive* connection is indicated in *black*. The transmitter is indicated in the box on each connection. *EAA*: Excitatory amino acid. *GABA*: GABA acting at $GABA_A$ receptors. *ACh*: Acetylcholine acting at nicotinic ACh receptors. If the connection is functionally excitatory, this is indicated by (+). If the connection is functionally inhibitory, this is indicated by (−). In the *right panels* are the activity patterns under the different conditions. The upper trace in each record is the slow potential recorded from the sartorius muscle nerve, which acts as a reference for each cycle. The *lower two traces* in each of the *right panels* show the filtered discharge of the sartorius and femorotibialis muscle nerves. The *arrows* mark the peak of the slow depolarization in each panel, which coincides with the peak depolarization in the both sets of motoneurons. *A*, Synaptic connections and activity patterns in normal Tyrode's solution. In this and the other figures we have presented the cholinergic connections as part of the recurrent inhibitory pathway. However, the cholinergic connections could be directly onto the motoneurons. For simplicity, we have not illustrated glycinergic inputs to the motoneurons or the GABAergic input to femorotibialis motoneurons (see text for details). *B*, Connections and activity during local application of bicuculline over sartorius motoneurons, which abolishes the pause in the discharge of sartorius motoneurons. *C*, Connections and activity during bath application of bicuculline, which induces a pause in femorotibialis activity.

membrane in a similar manner to the action of GABA and result in a lengthening of the sartorius pause and the appearance of a pause in femorotibialis (Fig. 12*C*). The idea that synaptically released glutamate might be functionally inhibitory will require careful confirmation in future experiments by examining the effects of direct application of glutamate or its agonists onto discharging motoneurons.

Excitatory drive to sartorius and femorotibialis motoneurons

The discharge of sartorius and femorotibialis motoneurons was depressed by several classes of excitatory amino acid antagonists. The drugs had to be used at higher concentration than the antagonists affecting the pause and never resulted in a complete suppression of firing. This presumably occurred because the excitatory inputs are located on dendrites rather than on the soma. Because the drugs had to be injected at high concentration into the nucleus, it is likely that they will have spread to affect interneurons. Thus, the results of excitatory blockade may be a combination of direct effects on motoneuron excitatory amino acid receptors and excitatory inputs to interneurons. A major role of excitatory amino acid involvement in the excitatory drive to motoneurons is consistent with whole-cell recordings of the rhythmic excitatory synaptic drive in femorotibialis motoneurons, which has an equilibrium potential near 0 mV (Sernagor and O'Donovan, 1990, 1991b; O'Donovan et al., 1992).

Inhibitory drive to femorotibialis motoneurons

Local application of bicuculline resulted in an increase in the peak discharge of femorotibialis motoneurons and a reduction

in the duration of firing during the cycle. In addition, the profile of the slow potentials recorded from the muscle nerve was altered so that the modulation of each cycle was more pronounced with a higher peak amplitude and a lower trough between cycles. This finding suggests that femorotibialis motoneurons may receive a tonic GABAergic input in addition to their documented excitatory drive (Sernagor and O'Donovan, 1991; O'Donovan et al., 1992).

Comparison with other species

Two different types of inhibitory control of motoneuron activity have been studied during rhythmic motor activity in other species. The first is the inhibitory mechanism controlling the alternation of motoneurons of the left and right sides of the spinal cord during swimming. This has been most extensively studied in the lamprey and *Xenopus* spinal cords. In both species the alternation of the contralateral motoneurons appears to be predominantly glycinergic, and candidate glycinergic interneurons that mediate this inhibition have been identified (Buchanan, 1982; Soffe et al., 1984).

The other class of inhibitory control is the regulation of the flexor and extensor alternation that is the object of the present study. Much less is known about this type of inhibition, and the interneurons responsible for its mediation are unknown. In the cat, rat, and the mouse, glycinergic mechanisms have been implicated (Pratt and Jordan, 1987; Droge and Tao, 1993; Noga et al., 1993). By contrast, in the developing chick cord, the alternation appears to be predominantly controlled by GABAergic synapses. At present we do not know how the circuitry controlling embryonic movements becomes transformed into the adult networks controlling locomotion. However, it seems reasonable to speculate that the GABAergic control of motoneuron activity might be a developmental property of the networks that becomes less important with maturity. Consistent with this idea are the results of previous work in the chick and the rat showing that ventral neurons transiently express GABA-like immunoreactivity during development. Early in development most of the GABAergic neurons are found in the ventral part of the cord, whereas in the adult animal they are dorsally located (Ma et al., 1992; Antal et al., 1994). At the age our recordings were made there are still a significant number of neurons with GABA-like immunoreactivity in the ventral spinal cord (Antal et al., 1994) where the inhibitory control seems to be located (Ho and O'Donovan, 1993). The mechanisms responsible for the disappearance of ventrally located neurons with GABA-like immunoreactivity are unknown, but one possibility is that they experience a change in transmitter because interneuron cell death has not been described for spinal interneurons of the chick embryo (Mackay and Oppenheim, 1988). This raises the interesting possibility that the inhibitory interneurons responsible for antagonist alternation might change their transmitter from GABA to glycine (see Berki et al., 1995). This question can only be answered by studies of the changes that occur in the networks controlling motor activity throughout development.

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