Extracellular $K^+$ Induces Locomotor-Like Patterns in the Rat Spinal Cord In Vitro: Comparison With NMDA or 5-HT Induced Activity

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Bracci, E., M. Beato, and A. Nistri. Extracellular $K^+$ induces locomotor-like patterns in the rat spinal cord in vitro: comparison with NMDA or 5-HT induced activity. J. Neurophysiol. 79: 2643-2652, 1998. Bath-application of increasing concentrations of extracellular $K^+$ elicited alternating motor patterns recorded from pairs of various lumbar ventral roots of the neonatal rat (0-2 days old) spinal cord in vitro. The threshold concentration of $K^+$ for this effect was 7.9 ± 0.8 mM (mean ± SD). The suprathreshold concentration range useful to evoke persistent motor patterns (lasting ≥ 10 min) was very narrow (~ 1 mM) as further increments elicited only rhythmic activity lasting from 20 s to a few minutes. On average, the fastest periodic rhythm of patterns was 1.1 ± 0.3 s. Intracellular recording from lumbar motoneurons showed that raised extracellular $K^+$ elicited membrane potential oscillations with superimposed repetitive firing. In the presence of N-methyl-D-aspartate (NMDA) or non-NMDA receptor blockers [R(-)-2-amino-phosphonovaleric acid or 6-cyano-7-nitroquinoxaline-2,3-dione, respectively] extracellular $K^+$ increases could still induce motor patterns although the threshold concentration was raised. Serotonin (5-HT) also induced alternating motor patterns (threshold old 15 ± 7 μM) that were consistently slower than those induced by high $K^+$ or NMDA. Ritanserin (1 μM) prevented the locomotor-like activity of 5-HT but not that of high $K^+$ provided the concentration of the latter was further increased. Subthreshold concentrations of $K^+$ became effective in the presence of subthreshold doses of 5-HT or NMDA, indicating mutual facilitation between these substances. The fastest pattern frequency was observed by raising $K^+$ or by adding NMDA. In the presence of 5-HT, the pattern frequency was never as fast even if NMDA (or high $K^+$) was coapplied. Furthermore, application of 5-HT significantly slowed down the $K^+$- or NMDA-induced rhythm, an effect strongly potentiated in the presence of ritanserin. It is suggested that the operation of the spinal locomotor network was activated by rises in extracellular $K^+$, which presumably led to a broad increase in neuronal excitability. Whenever the efficiency of excitatory synaptic transmission was diminished (for example by glutamate receptor antagonism), a larger concentration of $K^+$ was required to evoke locomotor-like patterns. The complex effect (comprising stimulation and inhibition) of 5-HT on alternating pattern generation appeared to result from a dual action of this substance on the spinal locomotor network.

INTRODUCTION

The neonatal rat spinal cord in vitro provides a useful preparation to study central motor programs (Kudo and Yamada 1987) because it can generate locomotor-like (fictive) patterns of activity. These consist of rhythmic bursts alternating between distinct motoneuronal pools (Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996). Such patterns can be induced by agents such as N-methyl-D-aspartate (NMDA) (Kudo and Yamada 1987), serotonin (5-HT) (Beato et al. 1997; Cazalets et al. 1992), acetylcholine (Cowley and Schmidt 1994), or dopamine (Kiehn and Kjaerulff 1996), although some differences emerge (Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996). NMDA and 5-HT have been used widely alone or in combination to induce fictive locomotion because they reliably elicit long-lasting alternating patterns characterized by relatively high frequencies closer to those observed during locomotion in vivo (Cazalets et al. 1992; Kjaerulff and Kiehn 1996; Squalli-Houssaini et al. 1993). These findings have suggested that serotonergic and glutamatergic fibers from the brain stem locomotor region may be responsible in the intact animal for the activation of the spinal locomotor network, usually referred to as central pattern generator (CPG) (for a recent review, see Rossignol 1996).

Although it has been shown that ionotropic glutamatergic transmission plays a crucial role during locomotor activity (Beato et al. 1997; Cazalets et al. 1992), the mechanisms by which bath application of agents such as NMDA or 5-HT induces alternating patterns in the neonatal rat spinal cord are presently unclear even if the cellular effects of NMDA and 5-HT have been investigated widely. Activation of NMDA receptor gates a cation-permeable channel (Mayer and Westbrook 1987). Furthermore, NMDA endows some neurons with intrinsic oscillatory ability in the rat spinal cord (see Hochman et al. 1994a,b; Kiehn et al. 1996), presumably via the voltage-dependent Mg$^{2+}$ block of NMDA receptors (Mayer and Westbrook 1987). 5-HT has been shown to exert excitatory effects on spinal motoneurons by affecting several membrane conductances (Elliott and Wallis 1992; Takahashi and Berger 1990); such excitatory effects are blocked in the rat spinal cord by the 5-HT$_2$ receptor antagonist ritanserin, which unmask inhibitory effects of 5-HT on motoneuron spontaneous and evoked synaptic transmission (Elliott and Wallis 1992). 5-HT also can induce plateau potentials in spinal motoneurons of cat and turtle (for a review, see Hultborn and Kiehn 1992). Although it seems possible that spinal rhythmogenesis relies on the membrane mechanisms activated by NMDA or 5-HT receptors, it is not clear whether similar mechanisms are required for locomotor activity in the neonatal rat spinal cord.

A related issue is whether the CPG operation requires selective activation of a distinct subgroup of spinal neurons or whether rhythmicity results from a widespread increase in neuronal excitability. The answer to this question is complicated by the fact that the CPG has not been precisely
identified in anatomic terms. Recent experiments have indicated a ventral localization of the spinal rhythmogenic networks (Bracci et al. 1996b; Cowley and Schmidt 1997; Ho and O'Donovan 1993; Kjaerulff and Kiehn 1996). Furthermore, it recently has been proposed that 5-HT and NMDA activate distinct rhythmogenic circuits (Cowley and Schmidt 1997) rather than the same CPG.

To investigate these latter issues, we increased extracellular K\textsuperscript{+} to elicit neuronal depolarization because this manipulation provides a tool to investigate those properties of the network that depend on the level of excitability of spinal neurons. We report the novel finding that high K\textsuperscript{+} solution induced well-coordinated alternating activity in the neonatal rat spinal cord. The sensitivity of this activity to glutamate or 5-HT antagonists has been studied to establish whether spinal rhythmogenesis requires activation of any particular receptor class. The interaction among 5-HT, NMDA, and high K\textsuperscript{+} solution also has been investigated to clarify whether these agents induce locomotion through independent mechanisms.

**METHODS**

Standard procedures were used to obtain (and record from) spinal cord preparations of neonatal Wistar rats (0–2 days old) (Ballerini et al. 1995; Bracci et al. 1996a). The isolated spinal cord (from mid-thoracic region to the conus medullaris) was fixed to the bottom of a recording chamber and continuously superfused (7.5 ml/min) with Krebs solution [containing (in mM) 113 NaCl, 4.5 KCl, 1 MgCl\textsubscript{2} \textcdot 7H\textsubscript{2}O, 2 CaCl\textsubscript{2}, 1 NaH\textsubscript{2}PO\textsubscript{4}, 25 NaHCO\textsubscript{3}, and 11 glucose, gassed with 95\% O\textsubscript{2}-5\% CO\textsubscript{2}, pH 7.4] at room temperature. High K\textsuperscript{+} solution (\textlessthan;20 mM) was obtained by diluting the required amount of 1 M KCl in control solution. All agents were bath-applied via the superfusing solution at the concentrations mentioned in the text. Any application (3–20 min) of K\textsuperscript{+} was always followed by a prolonged (>15 min) wash-out in control solution.

DC-coupled ventral root (VR) recordings were performed from L\textsubscript{2} and L\textsubscript{5} VRs (that mainly contain flexor and extensor motor axons, respectively) (Kiehn and Kjaerulff 1996) with tight-fitting suction pipettes containing an Ag/AgCl pellet. Usually two roots were monitored bilaterally (at the L\textsubscript{2} or L\textsubscript{5} segmental level) or ipsilaterally (at the L\textsubscript{2} and the L\textsubscript{5} levels). In certain experiments, four VRs (left and right L\textsubscript{2} and L\textsubscript{5}) were monitored simultaneously. L\textsubscript{5} motoneurons (MN) were identified functionally by ventral root stimulation (Fulton and Walton 1986) and recorded from with 3 M KCl-filled microelectrodes (25–60 M\Omega) under current-clamp conditions. During intracellular experiments, the activity of left and right L\textsubscript{2} VRs was recorded simultaneously. DC-coupled VR and MN responses were amplified, displayed on-line on a chart recorder (Gould RS3400), and digitally stored on DAT tape (acquisition rate 11 kHz). No filtering was applied to these signals, unless explicitly mentioned in the figure legend.

Data were quantified as means ± SD; statistical significance was assessed with Student’s t-test for paired data (or analysis of variance test for normalized data). The minimum cycle period of transient motor patterns (<10 min duration) induced by a certain treatment was measured by averaging the duration of the last (and fastest) 10 cycles that were clearly discernible before VR activity eventually turned into tonic discharge. When persistent patterns (>10 min) were observed, analogous measurements over 20 consecutive cycles were performed after 10–15 min of stable rhythmic activity. For each application, the minimum period therefore is expressed as mean ± SD.

5-HT was purchased from Sigma; NMDA, R(-)-2-amino-phosphonoovaleric acid (APV), and 6-cyano-7-nitroquinazoline-2,3-di-

**RESULTS**

Spontaneous activity in high K\textsuperscript{+} solution

In control solution (4.5 mM K\textsuperscript{+}), spontaneous events of variable amplitude occurred irregularly in lumbar VRs; these events often were detected synchronously in left and right VRs at the same segmental level or in ipsilateral L\textsubscript{2} and L\textsubscript{5} VRs. In the majority of preparations, the average occurrence of such events was <3/min so that long quiescent periods were observed, as illustrated in the example of Fig. 1A. Moderate increases in K\textsuperscript{+} concentration (Fig. 1A, 7.5 mM K\textsuperscript{+}) were invariably followed by an increase in the frequency of irregular spontaneous events. A concentration of 8.5 mM K\textsuperscript{+} elicited rhythmic alternating patterns of activity in L\textsubscript{5} VRs (Fig. 1A). A similar phenomenon was observed by recording from L\textsubscript{2} or L\textsubscript{5} VRs of 71 of 81 preparations.
The minimum K\(^+\) concentration sufficient to induce this activity was investigated in 30 preparations (Fig. 1B; average value 7.9 ± 0.8 mM). The duration of rhythmic activity could vary from a minimum of 20 s (transient pattern) to >10 min (persistent pattern). In the attempt to elicit persistent patterns via small changes in K\(^+\) concentrations, it became evident that there was a narrow window of K\(^+\) doses (applied sequentially in 0.5 mM increments with intermediate washout) capable of generating a sustained alternating rhythm. In fact, stable frequency persisted (for = 10 min) in 12 of 19 preparations over a narrow range of above-threshold K\(^+\) concentrations (0.5–1 mM). Figure 1C shows that in the presence of various K\(^+\) concentrations (17 tests in 12 preparations), the cycle period of persistent patterns ranged between 1 and 2 s. In 7 of 19 experiments, alternating activity was observed only transiently after application of any above-threshold dose of K\(^+\).

When doses of K\(^+\) >2 mM above threshold were applied, rhythmic alternating activity was observed only transiently in all preparations as cycle period decreased until rhythmic activity (3–6 min after the application onset) turned into tonic firing (an example of this phenomenon is shown in Fig. 7A) and eventually into block. The last locomotor cycles preceding the onset of tonic activity were therefore characterized by the fastest period the duration of which (on average 1.1 ± 0.3 s) was independent of the K\(^+\) concentration applied (≤20 mM) although the larger K\(^+\) doses converted rhythmic activity into tonic firing earlier.

The alternating patterns observed in high K\(^+\) solutions were similar to the standard fictive locomotor rhythm typically induced by 5-HT and NMDA (Kjaerulf and Kiehn 1996) because rhythmic bursts appeared alternately in left and right L2 (predominantly flexor motor outputs; n = 14) or L3 VRs (predominantly extensor motor outputs; n = 44) and in ipsilateral L2 and L3 VRs (n = 10). Furthermore, simultaneous records from L2 VRs and L3 VRs of the same preparation displayed a typical pattern of alternated activity, as shown in Fig. 2. In this example, the persistent, fictive locomotor pattern was induced by 9.5 mM K\(^+\) (8.5 mM threshold) with 1.9 ± 0.2 s periodicity and phase alternation between left and right roots as well as between homolateral L2 and L3 roots. Similar phase relations were observed in L2 and L3 VRs with a mixture of 5-HT (30 μM) and NMDA (6 μM) although the fictive locomotor rhythm was slower (2.7 ± 0.3 s period). Analogous results were obtained in three preparations.

**Intracellular recording**

Simultaneous recordings were obtained from single L5 motoneurons and L2 VRs in seven preparations. In control solution, under current-clamp conditions, the resting potential was −71 ± 9 mV (n = 9). Application of high K\(^+\) solution (9–12 mM) elicited large-amplitude depolarizations (33 ± 8 mV) peaking after 237 ± 71 s from the onset of application. Washout to control solution was followed by complete repolarization. In 8 of 9 motoneurones, rhythmic membrane potential oscillations were observed during the rising phase of the depolarization induced by high K\(^+\) solution. An example is shown in Fig. 3A: in this case, the resting membrane potential of a left L5 motoneuron was −77 mV in control solution and reached a plateau of −45 mV after 290 s application of 10 mM K\(^+\). Spontaneous action potentials, induced by ongoing synaptic activity, were infrequent in control solution but increased in frequency during high K\(^+\) application when the membrane potential was between −65 and −50 mV. At more positive potentials, a decrease in spike amplitude and frequency was observed. Rhythmic oscillations were observed transiently during the depolarizing phase, when membrane potential was between −63 and −55 mV. This activity was approximately in phase with the one recorded from right L2 VR and out of phase with that recorded from left L2 VR (Fig. 3B, fast time scale trace of motoneurone response is shown alongside traces from L2 VRs of the same preparation). In this case, action potentials were generated preferentially (but not exclusively) during the positive phase of each oscillation (Fig. 3B). Another example, recorded from a different preparation is shown in Fig. 3C: in this case, on a left L5 motoneuron, rhythmic activity was observed in the range between −56 and −49 mV when a cluster of 7–10 action potentials was generated exclusively during the depolarized phase of each oscillation. This firing activity was out of phase with that recorded from left L2 VR. Rhythmic activity with a similar phase relation with respect to L2 VRs was observed in all L5 motoneurons in which oscillations appeared, during high K\(^+\) application, at membrane potentials between −63 and −40 mV. These observations indicate that, in the presence of high K\(^+\) solution, the intracellularly recorded L5 motoneurons had temporally distinct outputs from those present in the L2 segment of the same side, consistent with a locomotor-like pattern.

**Sensitivity of K\(^+\)-induced activity to glutamate antagonists**

Because 5-HT–induced rhythmic activity was sensitive to NMDA or non-NMDA glutamate receptor antagonists (Beato et al. 1997), tests were made to determine if high K\(^+\)-induced patterns showed similar sensitivity.

An example of the effects of NMDA receptor antagonism on K\(^+\)–induced activity is shown in Fig. 4A. Application of 8.5 mM K\(^+\) elicited an alternating pattern in left and right L3 VRs (Fig. 4A). In the presence of 20 μM APV (a concentration previously tested in our laboratory for its ability to suppress NMDA receptor mediated responses) (Baranauskas and Nistri 1998; Beato et al. 1997), the same K\(^+\) concentration merely increased the frequency of irregular spontaneous events. In the presence of APV, rhythmic alternating activity was observed when a larger dose (12 mM) of K\(^+\) was applied. Similar results were obtained in eight preparations. On average, K\(^+\) threshold concentration was increased by 59 ± 12% in the presence of the NMDA receptor antagonist APV (20 μM) (see histogram of Fig. 4C).

The effects observed after non-NMDA receptor block are illustrated in Fig. 4B. Application of 9 mM K\(^+\) elicited an alternating pattern suppressed in the presence of the non-NMDA antagonist CNQX (10 μM, a concentration previously tested in our laboratory for its ability to suppress non-NMDA glutamate receptor mediated responses) (Baranauskas and Nistri 1998; Beato et al. 1997). A larger dose (11.5 mM) of K\(^+\) induced alternating activity even in the presence of the non-NMDA antagonist. Similar results were
Sensitivity of $K^+$-induced activity to ritanserin

The excitatory effects of 5-HT on neonatal rat spinal motoneurons are blocked fully by the 5-HT$_2$ antagonist ritanserin (Elliott and Wallis 1992). We found that ritanserin also inhibited 5-HT–induced locomotor activity. In fact, in 4 of 4 preparations in which 5-HT induced locomotor activity (with a threshold concentration of 15 ± 7 μM), prolonged (>1 h) (see Elliott and Wallis 1992) application of ritanserin (1 μM) prevented any locomotor effect of 5-HT (≤200 μM; see example with 30 μM 5-HT in Fig. 4C). In this case, 5-HT depressed spontaneous background activity as reported by Elliott and Wallis (1992). In the presence of ritanserin, 9.5 mM $K^+$ still elicited alternating patterns (Fig. 5D) that maintained a cycle period similar to that observed in control solution (1.1 ± 0.2 and 1.2 ± 0.2 s, respectively, despite a slight change in pattern waveforms). In 2 of 8 preparations, ritanserin failed to produce detectable effects on $K^+$ activity in terms of threshold concentration or cycle period. In the remaining six preparations, ritanserin produced an increase of 14 ± 4% in $K^+$ threshold concentration; in these cases, the cycle period measured with the same concentration of $K^+$ was significantly ($P < 0.05$) larger in the presence of ritanserin (by 21 ± 15%).

Since the rhythm evoked by high extracellular $K^+$ persisted during block of excitatory 5-HT receptors, we tested whether it depended on activation of NMDA receptors by endogenous glutamate. Hence on the same preparation, the NMDA antagonist APV (20 μM) was applied in the presence of ritanserin. Under these conditions 9.5 mM $K^+$ (sufficient to elicit an alternating pattern in control solution and in the presence of ritanserin) failed to induce any pattern (Fig. 5E). A larger dose of $K^+$ (11.5 mM), however, evoked an alternating pattern in left and right 15 VRs (Fig. 5F). Similar results were observed in 3 of 4 preparations, in which, in the presence of ritanserin and APV, $K^+$ still could induce alternating patterns, although with a threshold concentration increased by 62 ± 26% with respect to control solution.

Synergy between NMDA, 5-HT, and high $K^+$

To gain insight into the relation between locomotor patterns induced by $K^+$ and those induced by 5-HT and NMDA, we investigated whether mutual facilitation between these agents could take place. Figure 6 illustrates a typical example of the interaction between high $K^+$ and NMDA. In this preparation, $K^+$ threshold concentration for alternating activity was 7 mM (Fig. 6A). A pattern remarkably similar to that induced by 7 mM $K^+$ was elicited by 4 μM NMDA (Fig. 6B). When subthreshold doses of $K^+$ and NMDA were applied simultaneously (6 mM and 3 μM, respectively), a regular rhythmic pattern was observed (Fig. 6C). Figure 6D illustrates the relation of cycle period to NMDA concentration for 4.5 or 6 mM $K^+$ in this preparation, indicating that the higher $K^+$ concentration facilitated the effect of NMDA. A similar facilitation between subthreshold doses of $K^+$ and NMDA was observed in 4 of 4 preparations (in which $K^+$ threshold was 8.2 ± 1.1 mM and NMDA threshold was 3.8 ± 1.2 μM).

Similar facilitation was found between 5-HT and $K^+$ application. In the four preparations tested simultaneous application of 5-HT (7.5–15 μM) and high $K^+$ (6–7.5 mM), which per se failed to elicit any rhythmic activity, produced an alternating pattern (cycle period 1.9 ± 0.7 s).
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dependent periods at concentrations ≤7 μM (cycle period was 1.5 ± 0.1 s at 4 μM; Fig. 7A, middle). When an 8 μM dose of NMDA was used, only transient patterns (1.2 ± 0.2 s minimum period) that evolved into tonic firing after ~380 s were seen. The minimum cycle period attained with high K⁺ and with NMDA was not significantly different. Furthermore, the minimum period observed with several combinations of K⁺ and NMDA was also not significantly different from the values observed with K⁺ or NMDA alone (not shown).

In the same preparation, a stable pattern (3.4 ± 0.4 s period) was elicited by 30 μM 5-HT (Fig. 7A, bottom), whereas larger 5-HT concentrations induced transient patterns without decreasing cycle period (see Beato et al. 1997). Nevertheless, it was possible to accelerate 5-HT-evoked activity by increasing external K⁺ (12.5 mM in the example of Fig. 7A, bottom) with cycle period decreasing...

FIG. 3. Concomitant intra- and extracellular recording of activity induced by high K⁺ solutions. A: intracellular trace of single left L5 motoneuron (IL5 MN; response during application of 10 mM K⁺ (top horizontal bar). Initial resting potential: −77 mV. Note increased spike activity accompanying membrane depolarization (downward deflections were electrotonic potentials elicited by 0.1-nA, 100-ms pulses; ongoing synaptic activity usually prevented measurements of cell input resistance) until spike amplitude was severely depressed. B: simultaneous records of the same motoneuron membrane potential (IL5 MN; taken during the time indicated by small horizontal bar in A) and right and left L2 VRs. Note oscillations in membrane potential at single cell level (with spike activity) in phase with L2 patterns and in antiphase with IL2 activity. C: simultaneous record of single motoneuron potential (IL5 MN) and rhythmic pattern of IL2 VR in a different preparation from A and B. Note motoneuronal firing bursts (around −52 mV membrane potential) in antiphase with L2 ventral root activity. Initial resting potential: −72 mV.

Fastest cycle period of K⁺, NMDA, and 5-HT induced patterns

It has been shown that rhythmic activity induced by coapplication of 5-HT and NMDA is accelerated by increases in K⁺ concentration and that NMDA-induced patterns are slowed down by 5-HT application (Sqalli-Houssaini et al. 1993). We investigated the fastest cycle period attainable with different combinations of K⁺, NMDA, and 5-HT. As previously described, large doses of K⁺ resulted in cycle acceleration eventually followed by tonic firing activity as illustrated by the example of Fig. 7A, top. In this case, with 7 mM K⁺ the cycle period (1.6 ± 0.2 s) was stable, whereas with 12.5 mM K⁺, after 5 min the cycle period reached its minimum value (1.2 ± 0.1 s) and the rhythmic pattern turned into tonic activity (see trace recorded 50 s later). In the same preparation, NMDA induced stable patterns with dose-
seemed possible that although the excitatory action facilitated locomotor activity, the inhibitory action might oppose it. Although the 5-HT–mediated excitation is selectively antagonized by ritanserin (Elliott and Wallis 1992), the inhibitory one consisting of depression of excitatory postsynaptic potentials (EPSPs) on motoneurons (Elliott and Wallis 1992) has so far been resistant to selective pharmacological antagonists. Experiments thus were undertaken to explore if the inhibitory action of 5-HT might have been responsible for the fact that the alternating rhythm induced by this agent was always consistently slower than the one evoked by NMDA or high K⁺. To test this possibility, we studied the effects of 5-HT on alternating patterns induced by K⁺ or NMDA in the presence of ritanserin. Figure 8A (middle) shows that 20 μM 5-HT (plus ritanserin) prevented the generation of the alternating pattern by 12.5 mM K⁺ (Fig. 8A, left). Subsequent application of 15.5 mM K⁺ reinstated the rhythmic activity although a slower rate (Fig. 8A, right). In four preparations bathed in ritanserin solution, 5-HT (10–50 μM) produced an increase of 15±5% in K⁺ threshold concentration. Comparable block, rhythm slowdown, or agonist threshold rise were induced by 5-HT in the case of NMDA generated patterns. This is exemplified in Fig. 8B in which 5 μM NMDA elicited a stable alternating pattern (left) slowed down by 50 μM 5-HT (57±11% period increase) even in the absence of ritanserin (right). Subse-

FIG. 5. Ritanserin blocks the excitatory effect of 5-HT but not of high K⁺ solution. Top: alternating rhythms recorded from left and right L5 VRs during application of 30 μM 5-HT (A) or 9.5 mM K⁺ (B). Middle: ritanserin (1 μM) blocks response to 5-HT (C) with only slight decrease in the one to high K⁺ (D). Bottom: subsequent application of 20 μM APV prevented the action of 9.5 mM K⁺ (E) although 11.5 mM K⁺ elicited an alternating rhythm (F).

Effects of 5-HT on alternating activity in the presence of ritanserin

Because 5-HT is known to exert a mixed excitatory-inhibitory action on spinal neurons (Elliott and Wallis 1992), it to 1.8±0.1 s before patterned activity turned into tonic firing. This minimum cycle period was significantly longer than those obtained with K⁺, NMDA, or their combinations (P < 0.001). Similarly, application of NMDA (8 μM) in the presence of 30 μM 5-HT induced analogous pattern acceleration with a minimum period of 2.0±0.3 s. The difference in periodicity due to high K⁺, 5-HT, or NMDA is quantified in Fig. 7B (pooled data from 3 preparations) where the fastest pattern response observed in high K⁺ solution (8–16 mM) is taken as 100% (see horizontal dotted line). The maximal response evoked by NMDA (4–10 μM) was nearly the same as the one induced by K⁺, whereas in the presence of 30–50 μM 5-HT (despite addition of 8–16 mM K⁺), the rhythmic pattern was consistently slower (P < 0.001) as it resulted into nearly doubling of the period elicited by K⁺ alone. These results imply that in the presence of 5-HT the locomotor network was intrinsically unable to produce patterns as fast as those recorded with K⁺ or NMDA alone.

FIG. 6. High K⁺ and NMDA show synergistic effects on motor patterns. A: traces of left and right L5 VRs in 6 mM (left) or 7 mM (right) K⁺ solution. Only the latter concentration induced alternating motor patterns. B: in the same preparation, 3 μM NMDA was ineffective (left), whereas 4 μM (right) induced motor patterns similar to the one caused by 7 mM K⁺. C: coapplication of 6 mM K⁺ and 3 μM NMDA evoked alternating motor patterns. D: graph shows leftward shift of NMDA dose/cycle period by 6 mM K⁺ solution (filled symbols). Each symbol refers to the mean ±SD of periods measured over 20 cycles in the same preparation.
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result of network depolarization. An additional result was the complex action of 5-HT on locomotor activity comprising facilitatory effects (mediated by ritanserin-sensitive receptors) and inhibitory effects (mediated by ritanserin-insensitive receptors).

Characteristics of high K⁺-induced rhythmic activity

High K⁺ solution was used to induce depolarization of spinal neurons. Such a manipulation per se was sufficient to activate the locomotor network in the neonatal rat spinal cord because 7–10.5 mM K⁺ induced alternating VR potential oscillations in the large majority of the preparations tested. Such oscillations appeared out of phase in left and right L₂ (or L₃) VRs and in ipsilateral L₂ and L₃ VRs, thus sharing the same characteristics of those induced by NMDA and 5-HT (Kiehn and Kjaerulff 1996; Kjaerulff and Kiehn 1996; Kudo et al. 1991; present study) and regarded as typical of...

DISCUSSION

The present study has provided a novel observation, namely that in the neonatal rat spinal cord high external K⁺ induces alternating motor patterns (even in the presence of NMDA and 5-HT₂ receptor antagonists) apparently as a...

![Diagram](http://jn.physiology.org/)

FIG. 7. Maximum speed attained with high K⁺ solutions, NMDA, or 5-HT by motor patterns of left and right L₅ VRs. A: top: 7 mM K⁺ solution induced a persistent, alternating motor pattern; 12.5 mM K⁺ elicited a transient pattern that accelerated during the first 300 s of application, and disappeared 50 s later being replaced by sustained firing activity. Middle: similar effects were observed with 4 and 8 μM NMDA, respectively. Bottom: the action of 30 μM 5-HT (maximum dose capable of supporting persistent motor patterns) was accelerated by 12.5 mM K⁺ (last and fastest cycle at 210 s) until tonic firing developed (40 s later). Note that coapplication of 12.5 mM K⁺ solution plus 30 μM 5-HT evoked a motor pattern slower than the one due to 12.5 mM K⁺ alone. B: bar chart of minimum period values observed in 8–16 mM K⁺ solution after addition of NMDA (4–10 μM) or 5-HT (30–50 μM). Values were normalized for each preparation to the minimum period observed with high K⁺ alone. n = 4 preparations. Note that although NMDA evoked responses as fast as those induced by high K⁺, 5-HT elicited consistently slower rhythms despite the presence of high K⁺ solution.

![Diagram](http://jn.physiology.org/)

FIG. 8. 5-HT effects on motor activity in the presence of ritanserin (1 μM). A: rhythmic motor pattern (left and right L₅ VRs) induced by 12.5 mM K⁺ solution (left) was prevented by 20 μM 5-HT (middle), and returned with 15.5 mM K⁺ application (right). All traces were in the presence of ritanserin. B: motor pattern induced by 5 μM NMDA was slowed down by 50 μM 5-HT in control solution (top). Subsequent addition of ritanserin (bottom) slightly reduced the speed of the NMDA-induced pattern and amplified the decelerating action of the same dose of 5-HT on the NMDA-induced rhythm. C: bar chart of 5-HT–induced increase in period of NMDA pattern in control solution or in the presence of ritanserin. n = 4 preparations. Note that in the latter solution the reduction in speed by 5-HT was approximately doubled.

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fictive locomotor patterns. Stable patterns were detected only within a very narrow range of K⁺ concentrations, a feature that might explain why locomotor activity induced by K⁺ has not been reported in previous studies. It is presently difficult to account for such a limited window of effective K⁺ concentrations. One might envisage that generation of alternating patterns depended critically on the combination of factors such as membrane depolarization, nonlinear relation between release of excitatory transmitters and depolarization, interplay between voltage-dependent membrane conductances, and differential sensitivity of network elements to a given K⁺ level. In future experiments, it would be desirable to explore more closely the relative roles of membrane depolarization versus release process, an approach that may be based on the use of various combinations of extracellular low Ca²⁺ and high Mg²⁺ to induce discrete depression of synaptic transmission.

It would have been particularly interesting to observe the effects of high K⁺ solutions directly on CPG interneurons. Because their identity remains elusive, as a first approximation intracellular recordings were performed on motoneurons. During high K⁺ applications, such cells were depolarized and displayed rhythmic oscillations typically observed at membrane potential values ranging between −60 and −50 mV and generated full-amplitude action potentials. Whenever larger depolarizations were attained, oscillations disappeared, and spike amplitude decreased, presumably due to inactivation of the Na⁺ spike-generating mechanism. The observed depolarization of motoneurons by K⁺ was larger than the one predicted by the Nernst equation for a membrane solely permeable to this cation. This discrepancy is not surprising in view of the intense spike activity that reinforced the depolarization via local release of neurotransmitters and the activation of intrinsic voltage-sensitive conductances. We presume that the action of high K⁺ solution on CPG interneurons was qualitatively (though not strictly comparable in quantitative terms) similar to the one observed directly on motoneurons and that the ensuing membrane depolarization triggered alternating patterns.

**Mechanisms for high K⁺-induced rhythmic activity**

How was locomotor-like activity generated during high K⁺ application? It seems likely that rhythmogenesis resulted from increased release of excitatory transmitters from spinal neurons depolarized by high K⁺ application. In particular, activation of transmitter receptors especially by endogenous glutamate with subsequent changes in membrane potential and thus presumably in voltage-activated conductances appeared necessary for locomotor activity. In the neonatal rat spinal cord, the CPG can be activated by agents such as NMDA and 5-HT to generate a motor program (Cazalets et al. 1992; Kjaerulff and Kiehn 1996; Kremer and Lev-Tov 1997; Sqalli-Houssaini et al. 1993) that is expressed within the complex locomotor network via excitatory signaling crucially dependent on glutamatergic transmission (Beato et al. 1997; Cazalets et al. 1996; Kremer and Lev-Tov 1997). In the presence of NMDA receptor antagonism, it was still possible to observe locomotor-like activity (albeit at a slower rate), provided that the K⁺ dose was further increased presumably to enhance release of endogenous glutamate onto non-NMDA receptors, thus compensating at least partly a deficit in glutamatergic transmission. Comparable results were obtained when the non-NMDA receptors were inhibited by CNQX, indicating that either receptor class could support locomotor-like activity (see also Beato et al. 1997). It is currently unclear whether this phenomenon involved recruitment of spare receptors on the same cells or of silent network neurons. The second possibility is compatible with the suggestion of redundant oscillators distributed within the lumbar region (Cowley and Schmidt 1997). Another possibility is that by adding a higher concentration of K⁺, the resulting larger neuronal depolarization compensated for the reduced size of glutamatergic transmission by lowering firing threshold and thus facilitating signal propagation within the same network.

One also might hypothesize that the enhanced release of glutamate by the increased concentration of extracellular K⁺ could have displaced the pharmacological antagonist from the receptors, thus overcoming the block and restoring the rhythm. It is difficult to estimate the concentration of this transmitter in the synaptic cleft in an intact preparation during intense neuronal activity. Nevertheless in our laboratory, large increases in the strength and duration of dorsal root stimuli able to recruit even C fibers and thus probably representing maximal release of endogenous glutamate via trans-synaptic activation failed to restore excitatory synaptic transmission blocked by APV and CNQX at the same concentrations employed in the present study (Baranauskas and Nistri 1998). Furthermore, in the presence of both APV and CNQX, K⁺ could not induce any rhythmic activity. Because the doses of K⁺ used were relatively small, it seems unlikely that they were largely superior to very strong electrical stimulation in liberating glutamate. These observations suggest that even robust rises in the release of endogenous glutamate could not revert the pharmacological antagonism. In the presence of the 5-HT₂ antagonist ritanserin, which blocks excitatory actions of 5-HT on spinal motoneurons (Elliott and Wallis 1992) and 5-HT-induced locomotor patterns, a small increment in K⁺ concentration was sufficient to evoke alternating patterns with only a limited difference in cycle period. This finding suggests that the excitatory action of endogenous 5-HT was not a crucial determinant of the normal operation of the locomotor network as ritanserin produced only a 21% decrease in the rhythm periodicity evoked by K⁺. Furthermore, K⁺ induced alternating activity also could be observed in the simultaneous presence of APV and ritanserin. Thus it seems likely that activation of NMDA or 5-HT receptors was not an absolute requirement for the alternating activity induced by high K⁺ that presumably relied on activation of non-NMDA receptors (see also Beato et al. 1997) when APV and ritanserin were applied.

The identity of the CPG interneurons remains elusive even if they are thought to be present throughout the lumbar ventral spinal cord (Kjaerulff and Kiehn 1996) with a predominance in the more rostral segments (Cazalets et al. 1996; Cowley and Schmidt 1997). In addition to the report of oscillatory interneurons surrounding the central canal of the spinal cord (Hochman et al. 1994a), recent studies have shown the existence of sparse interneurons in the intermediate gray matter with similar intrinsic properties (Kiehn et al. 1996): perhaps at least some of these cells were activated...
by superfusion with high $K^+$ and contributed to the alternating rhythm detected in the present study. Because these cells are activated by NMDA alone (Hochman et al. 1994a; Kiehn et al. 1996), or in combination with 5-HT and cholinergic agents (Kiehn et al. 1996), and the pattern persists in the presence of full NMDA receptor block (Beato et al. 1997), it seems that their participation to the rhythm generation is not essential. Another possibility is that generation of the alternating patterns depended on how the interneurons within the CPG network were wired together rather than on the oscillatory properties of individual neurons.

High $K^+$, NMDA, and 5-HT exhibited mutual facilitation of locomotor-like activity. In fact, coapplication of two of these agents in subthreshold doses was effective in activating the alternating patterns. Because the expression of the rhythm induced by high $K^+$, NMDA (the present study), or 5-HT (Beato et al. 1997) required mainly glutamatergic transmission, it may be suggested that bath-applied 5-HT, NMDA, or high $K^+$ solution could directly act as triggers for the operation of the same CPG interneurons by increasing their excitability. One alternative possibility is that high $K^+$ solution (or NMDA) activated, in part, a distinct network complementary to the one stimulated by 5-HT. Nevertheless, as a clear synergy between 5-HT and high $K^+$ (and NMDA) was present, one should notice that, even if there were separate networks, their activity at spinal level was functionally indistinguishable under the present conditions.

**Influence of ritanserin on 5-HT effects on locomotor activity**

The minimum cycle period observed with NMDA was not significantly different from the one observed with $K^+$ (typically $\sim 1$ s). On the other hand, even though it was possible to accelerate 5-HT–induced patterns by elevating $K^+$ concentrations or by adding NMDA, in this case, the minimum period was significantly longer (typically $\sim 2$ s) than the one obtained with $K^+$ and/or NMDA in the absence of 5-HT. These results suggest that in the presence of 5-HT the locomotor network, regardless of the presence of $K^+$ or NMDA, was constrained into a slow mode of operation.

The present findings suggest that the comparatively slower rhythmic activity induced by 5-HT was mainly due to its dual action on distinct receptor classes, each one of them responsible for pattern induction or inhibition, respectively. Ritanserin has been shown to unmask an inhibitory action of 5-HT on spinal synaptic transmission (Elliott and Wallis 1992): this consists of a reversible depression of spontaneously and electrically evoked EPSPs probably via a presynaptic mechanism that involves an apparently novel 5-HT receptor resistant to conventional 5-HT antagonists (Manuel et al. 1995). It is difficult to detect this effect in control solution because of the concomitant, large depolarization induced by 5-HT (through ritanserin-sensitive receptors) that may have facilitated a hyperpolarization-activated slow inward rectifier (Takahashi and Berger 1990) and reduced an outward $K^+$ current (Elliott and Wallis 1992). As far as locomotor activity is concerned, we found that in the presence of ritanserin, the action of 5-HT changed from facilitatory to inhibitory during high $K^+$ application. Furthermore, in the presence of ritanserin, 5-HT slowed down NMDA-induced patterns significantly more than in control solution. A similar dual action of 5-HT has been reported in other central neurons (reviewed by Anwyl 1990).

In conclusion, the present results suggest that whenever the efficiency of glutamatergic synaptic transmission was impaired either by application of its selective receptor blockers or by activation of ritanserin insensitive 5-HT receptors, the spinal network, despite the increased depolarizing signal needed to activate it, could not support rhythmogenesis as efficiently as in standard conditions.

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**REFERENCES**


