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Differentiation of I_{K_A} in Amphibian Spinal Neurons

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We have examined the development of an inactivating outward current in embryonic amphibian neurons differentiating in culture. On the basis of ionic selectivity, voltage dependence of activation and inactivation and pharmacological sensitivity, it is similar to A currents described in other neurons. In these embryonic neurons the A current appears later than other voltage-dependent currents studied previously. Furthermore, its maturation extends to times later than those required for the differentiation of another potassium current, the voltage-dependent delayed rectifier. These changes in A current are consistent with parallel changes in the action potential and excitability of the developing neurons.

The initial expression and later maturation of electrical excitability is an essential feature of neuronal differentiation. It involves the appearance of ionic currents, the balance of which determines the form of excitability characteristic of different developmental stages of neurons and different classes of mature cells. Understanding the relationship between the appearance of different ionic currents and the form and frequency of impulse activity is presently a goal of developmental neurobiology.

Embryonic spinal neurons dissociated from the neural plate of *Xenopus* embryos exhibit voltage-dependent Ca, Na, K, and Ca-dependent K currents at the time of primary neurite extension early in culture (O'Dowd et al., 1988). During the first day *in vitro,* these neurons undergo marked changes in the ionic dependence and duration of the action potential. Increases in density of Na, delayed rectifier and Ca-dependent K currents and accelerated kinetics of K currents during the same period contribute to this maturation. The delayed rectifier current plays the most prominent role, as it increases 3-fold in density and activates twice as rapidly during the first day in culture (O'Dowd et al., 1988). It shows no further increase in its density or rate of activation during the second day (Ribera and Spitzer, 1989).

These neurons express a third K current—an A current (I_{K_A})—during the second day *in vitro*; this current is absent from most neurons during the first day. Thus, the initial appearance of the A current follows that of the delayed rectifier current, and its maturation extends to times later than those involved in the development of the delayed rectifier, as observed for several other systems (Bader et al., 1985; Nerbonne et al., 1986; Nerbonne and Gurney, 1989). We have characterized the properties of this current in developing spinal neurons. Furthermore, we have investigated how it may contribute to the changes in impulse duration and firing properties of developing spinal neurons. A preliminary account of some of these findings has appeared (Ribera and Spitzer, 1988).

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Materials and Methods

Cell cultures. Dissociated cell cultures were prepared from neural plate stage *Xenopus* embryos according to methods previously described (Spitzer and Lamborghini, 1976; Blair, 1983; Ribera and Spitzer, 1989); cells from a single embryo were plated onto 35 mm Falcon culture dishes and grown for 2 d in 2 ml of a fully defined medium (in mM: NaCl, 116; KCl, 3; $CaCl_2$, 9.4; $Ca(NO_3)_2$, 0.6; $MgSO_4$, 0.6; Tris-base, 4.6, titrated to pH 7.8 with NaOH) that was not changed during the culture period.

Biophysical techniques. Whole-cell currents were recorded using patch-clamp methods (Hamill et al., 1981; O'Dowd et al., 1988; Dagan 8900 amplifier). Pipets were filled with 100 mM KCl, 10 mM EGTA, 10 mM HEPES, pH 7.4, and had resistances of 2–4 MΩ. The composition of the bath saline was (in mM): NaCl, 80; KCl, 3; $MgCl_2$, 5; $CoCl_2$, 10; HEPES, 5. In the cases indicated, NaCl was reduced to 40 mM and 40 mM TEA was added (high external TEA solution). Na currents were blocked by addition of 10^{-7} M TTX (Sigma); Ca currents were avoided by omission of $CaCl_2$ from the saline and addition of 10 mM $CoCl_2$. Solutions containing the blockers 4-aminopyridine and 3,4-diaminopyridine were prepared just prior to use; the pH was readjusted to 7.4 after dissolution of the aminopyridine.

Voltage-clamp protocols were executed with a PDP 11/23 computer (Indec Systems). The membrane potential was usually held at −80 mV and stepped to depolarized voltages ranging between −20 and 25 mV in increments of 5 mV for 30, 150, or 300 msec. Neurons with short processes (<50 μm; O'Dowd et al., 1988) were selected to assure adequate control of membrane voltage. Cell capacitance was determined from the capacitative current transient to evaluate the membrane surface area (Marty and Neher, 1983). Series resistance was assessed by measuring the time constant of decay of the capacitative current when sampled at 33 μsec intervals and filtered at 16 kHz (Frequency Devices 8-pole low-pass Bessel filter). This resistance was partially compensated electronically (20–90%); the residual uncompensated series resistance was 1–5 MΩ. Currents were digitized at 100 or 1000 μsec and filtered at 5 kHz. Subtraction of currents and evaluation of current densities, times to half-maximal current, and time constants of inactivation were achieved by computer programs.

Action potentials were elicited and recorded using current-clamp methods (Axoclamp-2A amplifier). Microelectrodes were filled with 3 M KCl and had resistances ranging from 60 to 100 MΩ. The resting potentials recorded from neurons ranged from −40 to −70 mV regardless of age; injection of a small steady current was used to hold the membrane potential near −80 mV. A single action potential was generated with a 2.5 msec depolarizing current pulse using a bridge circuit. The amplitude of minimum current required to elicit an action potential (2.5 msec rheobase current) was then used for longer current injections, up to 1–2 sec for young neurons and 250 msec for cells at 1–2 d. The rheobase current varied between 0.25 and 0.5 nA in 1 and 2 d neurons and between 0.25 and 0.75 nA in young neurons. Cells were continuously superfused with a saline of the following composition (in mM): NaCl, 125; KCl, 3; $CaCl_2$, 10; HEPES, 5. Data were recorded with a chart recorder (Gould Brush 220); the frequency of action potential firing was measured directly from the recordings. Data are presented as

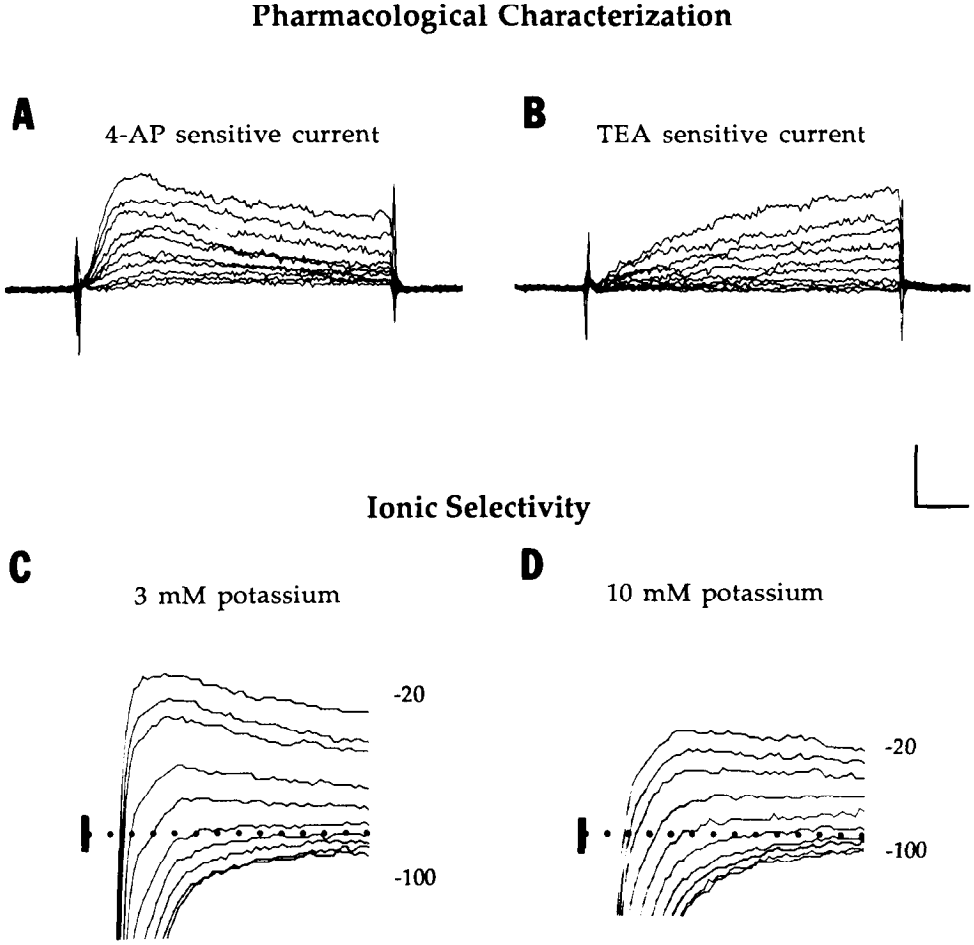

Figure 1. Properties of the inactivating current in neurons at 2 d in culture. *A* and *B*, Pharmacological characterization. Records from a single 2-d-old neuron held at −80 mV and stepped to depolarized potentials (−20 to 25 mV in 5 mV increments). The voltage-clamp protocol was applied during the absence and then during the presence of 4-aminopyridine or TEA; records obtained in the presence of the drug were digitally subtracted from those obtained in its absence to reveal the drug-sensitive current. The effects of the drugs on the outward currents were readily reversible in this neuron. *A*, Current sensitive to 4-aminopyridine current (1 mM). *B*, Current sensitive to TEA (2.5 mM). *C* and *D*, Ionic selectivity. Recordings were obtained in the presence of 40 mM TEA and either 3 mM (*C*) or 10 mM external potassium (*D*). The potassium equilibrium potentials predicted by the Nernst equation under these 2 conditions are −86 and −56 mV, respectively. The voltage-clamp protocol stepped the neuron to 25 mV and then returned to −100, −90, −80, −70, −60, −50, −40, −30, −25, or −20. In the example shown, the reversal potential shifted ~30 mV upon changing the external K concentration (~−65 mV in 3 mM K and ~−35 mV in 10 mM K). Scale bar: top, 250 pA and 5 msec; bottom, 250 pA and 2.5 msec.

means ± SEM for the number of cells indicated in parentheses; levels of significance were evaluated by the Student's *t*-test.

Results

Characterization of inactivating current

An inactivating outward current appears superimposed on a larger sustained delayed rectifier current in recordings in the absence of external calcium and presence of TTX. The pharmacological sensitivity of this inactivating outward current was evaluated to define the means for its isolation and further characterization. The current was studied first in neurons at 2 d in culture, when it is expressed in all cells examined. Comparisons of the effects of 1–2 mM 4-aminopyridine and 2.5 mM TEA on the same neuron suggest that these agents are relatively specific in their effects on different currents (n = 5; Fig. 1*A–B*). The delayed rectifier K current is not completely blocked by 20 mM external TEA during recording of whole cell outward currents in the absence of external Ca (O'Dowd et al., 1988). Nonetheless, in the presence of 40 mM TEA, 80% of the inactivating current is present, whereas only 20% of the sustained current remains (n = 5; see also Hernandez-Cruz, 1987), indicating that the inactivating current is relatively insensitive to TEA. However, 1–2 mM 4-aminopyridine not only blocks 60% of the inactivating current but reduces the delayed rectifier current by 25% as well, and this effect is usually not rapidly and fully reversible. Higher concentrations of this agent proved toxic to the neurons. Subtraction of records before and after its application thus reveals difference currents comprised of the irreversibly blocked delayed rectifier in addition to A current. For this reason, 4-aminopyridine was not used to isolate A current by subtraction. TEA proved the most reliable pharmacological tool with which to study the inactivating outward current in isolation.

The ionic selectivity of the inactivating component was investigated by examination of tail currents in the presence of 40 mM TEA. In 3 mM external K (E_K = −86 mV), neurons were voltage-clamped at a holding potential of −80 mV and then stepped for 15 msec to +20 mV and returned to potentials between −100 and −20 mV. Under these conditions, the inactivating current is strongly activated, and most of the current consists of this component. Neurons were then perfused with recording solution containing 10 mM external K (E_K = −56 mV), and the same voltage-clamp protocol was repeated. The tail currents reverse at a potential of −59 ± 4 mV in 3 mM K, and at −37 ± 3 mV in 10 mM K (n = 7; Fig. 1*C–D*). The shift in reversal potential predicted by the Nernst equation is 30 mV; a 32 mV shift was observed. This agreement between theoretical and observed values indicates that the A-like current is carried largely if not completely by K ions. The discrepancy between E_{rev} and E_K suggests charge screening at the external face of the channel or local K accumulation in this region (Hille, 1984). The pharmacological susceptibility of this current further supports its identity as a K current.

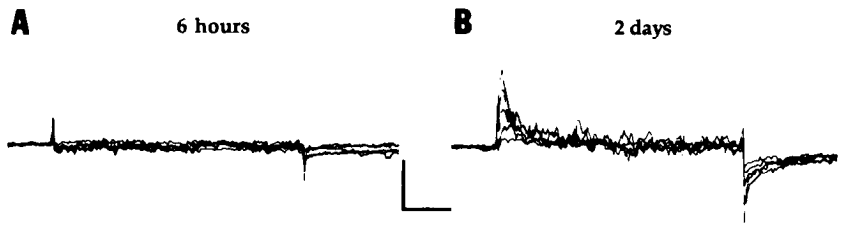

Figure 2. Developmental appearance of A current, isolated by the voltage protocol. Currents were elicited by depolarized voltage steps (−20, −10, 0, 10, and 20 mV shown) from a holding potential of −80 and then of −40 mV; the 2 sets of records were subtracted from each other to yield a difference current. Difference currents obtained from (*A*) 6 hr or (*B*) 2 d neurons. Scale bar: 250 pA, 25 msec. The "inward" tail currents that appear after termination of the voltage pulse are the outward tail currents recorded at a holding potential of −40 mV that have been inverted by the subtraction procedure.

An additional method for separation of the inactivating current was achieved by isolating the current inactivated at a depolarized holding potential of −40 mV (Ribera and Spitzer, 1989). An inactivating current component was isolated by subtracting the outward currents recorded in response to depolarizing voltage steps from a holding potential of −40 mV from those recorded to steps from a holding potential of −80 mV. In contrast to the delayed rectifier, this current was activated at potentials in the more hyperpolarized range of −35 to −30 mV (n = 10). By criteria of voltage dependence of activation and inactivation, ionic selectivity and pharmacology, this inactivating current is an A current.

Developmental appearance of A current

The differentiation of A current in *Xenopus* embryonic spinal neurons was studied during the first 2 d of their development in culture. Recordings from 2-d-old neurons reveal that an inactivating component of the total outward current can be isolated by the voltage protocol in 100% of cells (n = 23). Pharmacological isolation was avoided initially because of the possibility of developmental changes in sensitivities to various blockers (Harris and Marshall, 1973; Matsuda et al., 1978; Veselovsky et al., 1979; Strichartz et al., 1983; Kano et al., 1989). The same voltage protocol applied to 1-d-old neurons indicates that 50% of the neurons (n = 18) possess a detectable A current (Fig. 2). At early times (6–9 hr in culture), only 10% of the neurons exhibit an A current (n = 10).

The current density and time to half-maximum current were determined for 1- and 2-d-old neurons; current was isolated both by the voltage protocol and by high external TEA. The mean current density of A current recorded in the presence of high TEA was 20% greater than that resolved by the voltage protocol, as expected, reflecting contamination by residual delayed rectifier current with the former procedure in addition to steady-state inactivation of A current at −40 mV (see below) with the latter procedure. However, there was no difference in the voltage dependence of activation of A current and the time to half-maximum. The mean current density, voltage of activation, and the time to half-maximum current do not change between 1 and 2 d in culture (Fig. 3). Although the cultures contain sensory, motor, and interneurons (Spitzer and Lamborghini, 1976; Bixby and Spitzer, 1984; Lamborghini and Iles, 1985), the neurons all have their birthdates at an early stage of development (Spitzer and Lamborghini, 1976) and constitute a relatively homogeneous population with respect to age. Accordingly, the properties of currents of functionally different neurons appear very similar during this period. These findings also suggest that the relative sensitivities of K and A current to TEA do not change during this developmental interval.

Table 1. I_{K_A} inactivation time constant (τ, msec)

Time in culture	Membrane potential +20	+10	0	−10
Current isolation by voltage				
1 d (4)	19 ± 7	25 ± 11	40 ± 25	
2 d (5)	12 ± 3	11 ± 2	12 ± 1	
Current isolation by TEA				
1 d (5)	20 ± 5	21 ± 5	23 ± 8	16 ± 2
2 d (8)	23 ± 5	20 ± 3	16 ± 3	25 ± 7

Levels of statistically significant difference were evaluated with the Student's t-test. Values were compared within each current isolation procedure as well as between them. The resulting p values were all >0.2 except for comparisons between the 2 d entries of both sections for +20 mV ($p < 0.12$) and +10 mV ($p < 0.06$).

Inactivation properties of A current

The current inactivates during a depolarizing voltage pulse. A time constant of inactivation, τ, was evaluated by regression fit of the data with

$$I = I_{max} e^{-t/\tau} + I_{ss}$$

where I_{max} is the maximum current and I_{ss} is the steady-state current. Single exponentials provided satisfactory fits. This parameter was evaluated at both 1 and 2 d, and for data obtained either in the presence of 40 mM TEA or A current isolated by voltage (Table 1). This analysis indicates that τ is neither developmentally regulated nor voltage sensitive, and its determination is unaffected by the method of isolation of A current. The time constant is on the order of 20 msec.

The steady-state inactivation of A current was measured in the presence of high external TEA, since the voltage protocol does not allow isolation of currents elicited from strongly depolarized holding potentials. Current was activated by stepping to +25 mV from holding potentials ranging between −100 and −20 mV. Inactivation was determined by measuring the amplitude of the current elicited from each holding potential and dividing the values by the current amplitude recorded from a holding potential of −100 mV. The shape of the curve does not change between 1 and 2 d in culture; for neurons at either age, half-inactivation is obtained at ~−40 mV (Fig. 4). A satisfactory fit was achieved with the Boltzmann relation $\{I_x/I_0 = 1/(1 + \exp[(39 + V)/9])\}$ that specifies the ratio of activated to inactivated channels at equilibrium in terms of the energy difference between the 2 states. The equivalent gating charge for this curve is approximately 4.

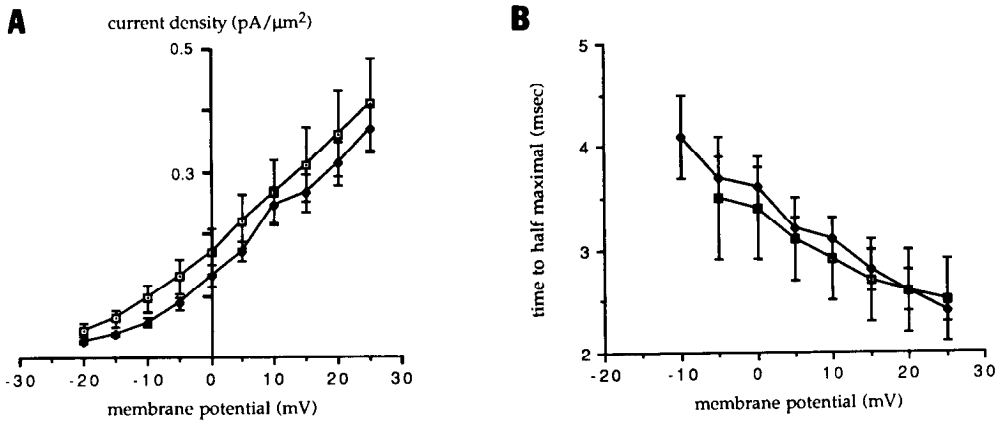

Figure 3. Amplitude and activation of A current at 1 and 2 d in culture, isolated by application of 40 mM TEA. *A*, Current density as a function of voltage (*open symbols*, 1 d neurons, $n = 8$; *filled symbols*, 2 d neurons, $n = 9$). *B*, Time to half-maximum current as a function of voltage (*open symbols*, 1 d neurons, $n = 8$; *filled symbols*, 2 d neurons, $n = 9$). These properties of A current were also evaluated with the voltage protocol which revealed currents that were 20% smaller; the difference is probably due to the half-inactivation point for A current at −40 mV and the small reduction of A current by 40 mM TEA. Nonetheless, similar voltage dependencies were obtained by both methods.

Rate of action potential firing

The frequency of action potentials elicited by constant current pulses of various durations was measured at 6–9 hr, 1 d, and 2 d in culture. In young cells, the duration of the action potential ranges between 5 and 250 msec in response to brief (1–2 msec) current pulses (Ribera and Spitzer, 1989). Brief rheobase current pulses were used to reduce the impact of variation in input resistance from cell to cell which ranged between 100–200 MΩ. The typical response for rheobase current injection of 500 msec or less was the firing of a single action potential that was sustained for the duration of the pulse. At 1 and 2 d, the durations of action potentials elicited by brief pulses ranged between 1–2 msec (Ribera and Spitzer, 1989). In response to rheobase current injection on the order of 100–200 msec, 96% of 1-d-old neurons fired repetitively ($n = 25/26$; Fig. 5*A*). In neurons in which several impulses were generated, repetitive firing was generally observed only at the onset of the pulse ($n = 9/11$; 82%), and the impulse frequency was 88 ± 9 Hz (mean ± SEM). At 2 d, only 71% of neurons fired repetitively ($n = 20/28$; Fig. 5*B*), although they were depolarized beyond threshold for the remainder of the current pulse. In these cells, 60% fired more than 2 impulses at a rate of 88 ± 9 Hz ($n = 9/12$; 250 msec current injection; Table 2).

Developmental changes in the time to reach threshold were also examined. In these experiments the amplitude of injected current was reduced below the 2.5 msec rheobase value to the point at which a single action potential was elicited during a 200 msec pulse. A larger percentage of rheobase was required to bring 2 d neurons to threshold (Table 2). In summary, these assays reveal that young cells typically fire only single impulses, neurons at 1 d fire repetitively, while older neurons fire repetitively less often and are less excitable.

The effects of blockers of A current on firing frequency were evaluated. The spike overshoot typically increased shortly after application of 4-aminopyridine or 3,4-diaminopyridine, and there was an increase in impulse duration and decrease in the number of action potentials elicited by long current pulses ($n = 3/4$). These effects could be due to the action of the aminopyridines on both the A current and the delayed rectifier current. However, after 2–3 min, cells were no longer excitable, even when the amplitude of the injected current was increased ($n = 4$). These toxic effects of the aminopyridines were not reversible, and prevented comparison of the properties of excitability before and after their application.

Discussion

This current is activated rapidly, inactivates during a voltage step and at depolarized holding potentials, is sensitive to millimolar concentrations of 4-aminopyridine and thus resembles "A" currents of other neurons (Connor and Stevens, 1971a, b; Neher, 1971; Rogawski, 1988; see Rogawski, 1985, for review). TEA as well as 4-AP reduce both the delayed rectifier and the A current; however, 4-AP has a greater effect on A current, whereas TEA is more selective for the delayed rectifier as observed by others (Thompson, 1977; Bader et al., 1985; Cull-Candy et al., 1989). Its relatively depolarized half-inactivation point of −40 mV is similar to that reported for the *Drosophila* A current that is absent in *Shaker* mutants (Salkoff, 1983), the current induced in oocytes by *Shaker* cRNA (Iverson et al., 1988; Timpe et al., 1988a, b) and the inactivating K current recently described in GH_3 pituitary cells (Oxford and Wagoner, 1989). The inactivation kinetics of this current vary considerably from neuron to neuron as reported for A current in other neurons

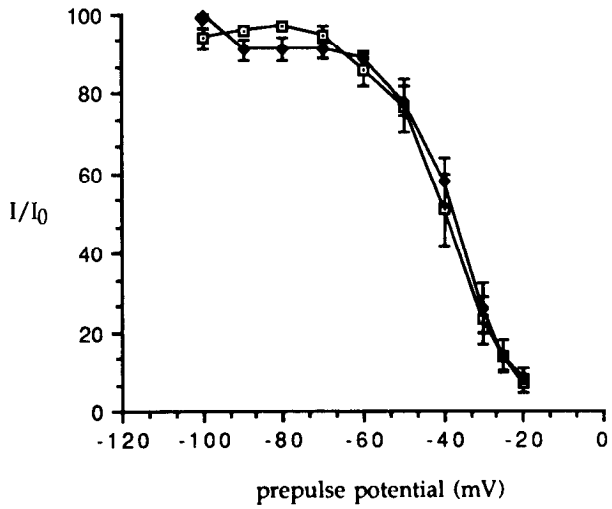

Figure 4. Steady-state inactivation of A current does not change during development. Recordings obtained in the presence of 40 mM TEA; neurons were stepped to 25 mV after holding them for 100 msec at test potentials ranging between −100 and −20 mV (*open symbols*, 1 d neurons, $n = 10$; *filled symbols*, 2 d neurons, $n = 8$). The amplitude of the current elicited from each test potential was normalized to that elicited from −100 mV, I/I_0.

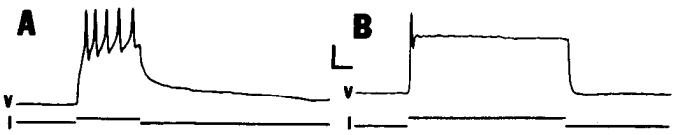

Figure 5. The extent of repetitive firing decreases as neurons acquire A current. Intracellular voltage recordings (V) in response to injection of current (I). *A*, At 1 d, a neuron fires repetitively in response to depolarizing current injection. The membrane potential was set at rest to −88 mV by a small hyperpolarizing d.c. current. *B*, At 2 d, a neuron fires a single action potential in response to a larger current pulse. The membrane potential was set at rest to −80 mV by a small hyperpolarizing d.c. current. Scale bar, 25 mV or 0.5 nA and 30 msec.

(Aguayo, 1989; Cull-Candy et al., 1989). Furthermore, no obvious voltage dependence for the time constant of inactivation was noted. In a variety of different neurons examined at the whole-cell level, this parameter has been found in other studies to be voltage independent (Connor and Stevens, 1971a, b), weakly voltage dependent (Thompson, 1977; Numann et al., 1987; Cull-Candy et al., 1989), or voltage dependent (Neher, 1971). Single exponentials describe the current decay at both 1 and 2 d *in vitro*. Double exponentials are required to fit currents induced in oocytes by several splice variants of the *Drosophila Shaker* gene product (Iverson et al., 1988; Timpe et al., 1988a, b), as well as the A current recorded *in vitro* from *Drosophila* myotubes (Solc et al., 1987).

Its functional appearance is developmentally regulated in developing *Xenopus* spinal neurons, and the developmental time course of appearance of A current is later than that of other K currents reported in these neurons (O'Dowd et al., 1988; see also Barish, 1986). Both the delayed rectifier current and the Ca-activated K current are already present at early times in culture, and the delayed rectifier is mature by 1 d *in vitro* (Ribera and Spitzer, 1989). In contrast, the differentiation of A current continues throughout the second day in culture. Similar developmental time courses for delayed rectifier and A currents have been observed in other neuronal systems (Bader et al., 1985; Nerbonne et al., 1986; Nerbonne and Gurney, 1989). The maturation of A current in chick spinal motoneurons also follows that of the delayed rectifier (Best et al., 1988). In rat sciatic nerve, mature axons are less sensitive to 4-aminopyridine than are young nerves; this may reflect the development of myelination and lack of access of 4-aminopyridine to sensitive channels rather than their loss (Eng et al., 1988). The functional expression of A current is dependent upon transcription, as is the maturation of delayed rectifier current (Ribera and Spitzer, 1989).

What are the possible functional roles of A current in these neurons? The A current has been shown to be involved in action potential repolarization in a variety of neurons (Dubois, 1983; Belluzzi et al., 1985; Adams and Galvan, 1986; Storm, 1987). The action potential duration of amphibian spinal neurons continues to decrease between 1 and 2 d in culture (20% decrease from 1.6 to 1.3 msec), even though no changes are detected in the delayed rectifier current during this period (Ribera and Spitzer, 1989). However, during this time significant changes are noted in A current, notably its frequency of appearance, and thus it is likely that it is involved in this late maturation of the impulse.

In early reports of A current, it was shown to be important in controlling the pattern of repetitive firing in molluscan neurons (see Connor and Stevens, 1971b). A similar purpose may be served in amphibian spinal neurons. Repetitive firing becomes prominent in neurons at 1 d in culture (Xie et al., 1989), consistent with maturation of the delayed rectifier serving to repolarize cells so that they can be depolarized to threshold again. It is of particular significance that a larger percentage of the neurons fire only once (not repetitively) at 2 d. In addition, more current is required to bring neurons to threshold at 2 d. These changes may also reflect alteration in the density of Na currents in neuronal cell bodies during this period. Kidokoro and Sand (1989) have examined the Na current during this later period and found that its density decreases in the cell body in parallel with an increase in varicosities of neurites. Finally, A current could serve to reduce the effect of delayed depolarization following the impulse that can lead to repetitive firing (Spitzer, 1984). All these findings indicate a decrease in excitability, during a period in which A current is acquired by all neurons.

Selective elimination of A current will provide definitive evidence concerning its functional role. Pharmacological blockade is complicated by overlapping effects on the delayed rectifier

Table 2. Neuronal impulse frequency

	Changes in electrical excitability			
Time in culture	6–9 hr	1 d	2 d	
Frequency of single impulses				
% of neurons firing only once	78 (7/9)	4 (1/26)	29 (8/28)	
Properties of repetitive firing[a]				
Number of APs fired	3.2 ± 1.5 (2)	10.7 ± 2.3 (11)	9.7 ± 2.1 (12)	
Frequency		88 ± 9 (11)	88 ± 9 (12)	
Threshold properties[b]				
Time to action potential		46 ± 6 (14)	33 ± 7 (13)	$p < 0.1684$
% rheobase		18 ± 3 (14)	42 ± 10 (13)	$p < 0.02$

[a] A single action potential was elicited with a 2.5 msec depolarizing current pulse. The amplitude of minimum current required (2.5 msec rheobase current) was then used for longer pulses.

[b] A single action potential was elicited with a minimal current pulse 2.5 msec in duration (rheobase current). The duration of current injection was extended to 100 msec, and current amplitude was reduced to the point at which a single action potential was produced. The time to elicit the action potential and the amplitude of the current as a fraction of the rheobase value were determined.

current. A promising approach emerges from recent advances in the molecular biology of voltage-dependent K channels (for review, see Jan and Jan, 1989). Cloning of the *Shaker* gene that encodes an A current in *Drosophila* (Iverson et al., 1988; Timpe et al., 1988a, b) has permitted identification of *Xenopus Shaker* homologue (Ribera et al., 1988). Introduction of antisense RNA encoding A current may prevent translation from endogenous transcripts and thus block functional expression.

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