MODIFICATION OF K CHANNEL INACTIVATION BY PAPAIN AND *N*-BROMOACETAMIDE

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ABSTRACT The whole-cell variation of the patch clamp technique was used to study macroscopic K current in voltage clamped GH3 cells. An inactivating, voltage-dependent K current was studied in isolation by inhibiting Ca-activated K currents with internal Ca chelators and external tetraethylammonium ions. Under control conditions, the K current inactivated in two phases with time constants of 25 and 79 ms. After treatment with either a proteolytic enzyme such as papain or the amino acid reagent *N*-bromoacetamide, the K current no longer inactivated rapidly, but decayed very slowly with a time constant of 500 to 750 ms. The action of papain or *N*-bromoacetamide on K channels is comparable to their action on Na channels, suggesting that inactivation in Na and K channels occurs by a similar mechanism.

The primary amino acid sequence of voltage-dependent ionic channels of several types has been resolved, revealing a remarkable degree of structural homology. For example, a dihydropyridine-binding protein from skeletal muscle, thought to be a voltage-dependent Ca channel, has been shown to be ~55% homologous to the Na channel (Tanabe et al., 1987). The Shaker locus of *Drosophila,* thought to be an inactivating, voltage-dependent K channel, has also been sequenced, and the predicted protein has a region of structural homology with the Na channel (Tempel et al., 1987). In addition to the structural similarity, the kinetic behavior of inactivating K channels, although slower, resembles that of Na channels. Na currents and inactivating K currents have similar time courses; the currents activate rapidly to a peak and then inactivate with somewhat slower kinetics (Hodgkin and Huxley, 1952; Connor and Stevens, 1971; Neher, 1971). In fact, based on similarities in the single channel behavior of these two channel types, it was previously suggested that they might be structurally related (Cooper and Shrier, 1985). The comparable structural and functional properties of Na channels and inactivating K channels suggested to us that agents that modify Na channel inactivation might also alter K channel inactivation. Fast inactivation of Na channels is removed by treatment with a variety of proteolytic enzymes (Rojas and Armstrong, 1971; Armstrong et al., 1973; Rojas and Rudy, 1976), or by certain amino acid reagents such as *N*-bromoacetamide (NBA) and *N*-bromosuccinimide (Oxford et al., 1978; Patlak and Horn, 1982). Here we report that the proteolytic enzyme papain or the amino acid reagent NBA selectively remove fast inactivation from voltage-dependent K channels in GH3 cells. The effect of these reagents on inactivating K channels is remarkably similar to their effect on Na channels, suggesting that inactivation in Na and K channels may occur by a similar mechanism.

We have used the whole-cell variation of the patch clamp technique (Hamill et al., 1981) to voltage clamp GH3 cells using techniques which have been described previously (Matteson and Armstrong, 1984 and 1986). Sodium current was blocked by adding 200 nM tetrodotoxin (TTX) to the standard external solution, which contained 130 mM NaCl, 5 mM KCl, 10 mM $CaCl_2$ and 10 mM Hepes (pH 7.4). Ca channel blockers were not used because many of them were found to block or alter K channel properties. Ca currents are a very minor contaminant in these experiments, because their maximum value is about an order of magnitude smaller than the voltage-dependent K currents. The internal solution contained 80 mM K glutamate, 30 mM KCl, 25 mM KF, 2 mM $MgCl_2$, 10 mM EGTA (KOH), and 10 mM Hepes (pH 7.2). All experiments were performed at room temperature (19–20°C). The holding potential used throughout was −80 mV. Linear leakage and capacitive currents were subtracted with a P/2 procedure using control pulses from −120 mV (Armstrong and Bezanilla, 1974).

Under our experimental conditions, the macroscopic currents evoked by depolarizing voltage clamp steps are mainly K currents. GH3 cells contain two populations of potassium channels: Ca-activated K channels and inactivating, voltage-dependent K channels (May and Oxford, 1986; Ritchie, 1987). EGTA and F^- were included in our internal solution to help minimize current through Ca-activated K channels. Isolation of the voltage-dependent K current was further aided pharmacologically, using tetraethylammonium (TEA) to block the Ca-activated currents (Fig. 1). The cell illustrated in Fig. 1 *A* had a relatively large, noninactivating K current at the end of a 370-ms

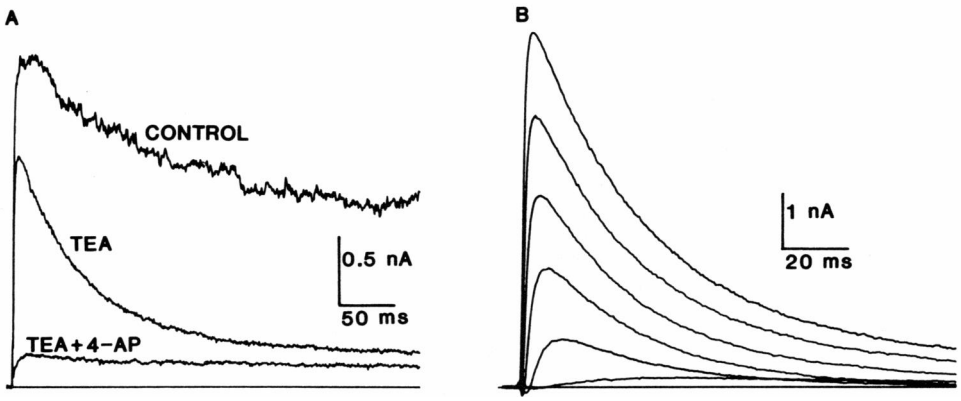

FIGURE 1. Isolation of voltage-dependent K currents. (*A*) The illustrated currents were recorded during 370 ms steps to +70 mV from a holding potential of −80 mV. The trace labeled TEA was recorded after switching the external solution from control to one containing 10 mM TEA (TEA substituted for 10 mM NaCl in the control external solution). The smallest current (TEA+4-AP) was then recorded after adding 5 mM 4-AP to the TEA containing external solution. (*B*) The superimposed currents were generated by voltage-clamp steps to −30, −10, 10, 30, 50, and 70 mV. These currents are from a different cell than the one illustrated in *A*. Experiment AU247A.

depolarization to +70 mV. Most of this steady-state current was blocked by 10 mM TEA, which also dramatically reduced the current fluctuations. These effects of TEA are due to block of Ca-activated K channels, which are more sensitive to block by external TEA than are the inactivating K channels (May and Oxford, 1986; Ritchie, 1987; Thompson, 1977). The current remaining after addition of TEA is an inactivating, voltage-dependent outward current, which can be blocked by 4-aminopyridine (Fig. 1 *A;* May and Oxford, 1986; Ritchie, 1987). This transient outward current reverses near the potassium equilibrium potential indicating that K selective channels are involved.

The rest of the data shown in this paper was obtained in the presence of 10 mM TEA. Fig 1 *B* illustrates a family of inactivating K currents recorded at various voltages. Threshold for activation of this voltage-dependent current is −40 to −50 mV. The current activates rapidly to a peak in 4 to 5 ms at +70 mV and then decays toward a sustained outward level. By measuring the instantaneous conductance at various times during the decay phase, it can be shown that conductance is decreasing (data not shown). Thus, the current decay results from K channel inactivation. The time course of inactivation at +70 mV is best fit by the sum of two exponentials, with time constants of 25 ± 3.8 ms and 79 ± 11 ms (mean ± SD, n = 17).

The inactivating K channels in GH3 cells are similar in many respects to the so-called A-current channels which were first described in molluscan neurons (Connor and Stevens, 1971). Inactivating K currents have now been found in a variety of excitable cells (Rogawski, 1985). In *Drosophila* flight muscle, A-current has the following properties. It is selectively blocked by external aminopyridines, the activation threshold is near −40 mV, the current rises to a peak in a few milliseconds and then inactivates with a time constant of ~80 ms at +15 mV (Salkoff and Wyman, 1981*a* and *b*). Thus, in GH3 cells we have recorded the activity of K channels which are similar, although not identical, to the A-current channels in *Drosophila.*

Papain has recently been shown to remove fast Na inactivation in neuroblastoma cells, and also to cause minimal injury to the cell in whole-cell patch clamp experiments (Gonoi and Hille, 1987). Fig. 2 illustrates the effect of internal papain on K channel inactivation in GH3 cells. The inactivating current in Fig. 2 *A* was recorded 15 s after breaking into the cell with internal solution containing 0.15 mg/ml papain. At this point papain has not yet acted, and inactivation is comparable to that seen in control cells; the time constants of the fast and slow inactivation components in this trace are 26 and 66 ms. 6 min later fast inactivation is removed, leaving a large, very slowly inactivating current. In control experiments, we found that inactivation was not modified in this way during dialysis of GH3 cells with control internal solution. In all of the 11 cells we have studied using 0.1–0.7 mg/ml papain, fast inactivation of K channels was removed in the manner shown in Fig. 2. Fig. 2 *A* also shows that 4-AP blocks 90% of the current after papain action, indicating that the slowly inactivating current flows through the 4-AP-sensitive K channels. An additional effect of papain was to increase the magnitude of the K current. In 10 of the 11 cells studied papain increased the current at +70 mV by an average of 42%, and in the 11th cell the current decreased by 5%. Papain-containing internal solution, which had been boiled to heat-inactivate the enzyme, had no effect on K channel inactivation. Another proteolytic enzyme mixture, pronase, removed inactivation in a manner similar to papain.

Examination of K currents during very long depolarizations (3 s) revealed the time course of the very slow inactivation process which remained after papain action. Inactivation was less complete but not completely abolished by papain. In the cell illustrated in Fig. 2 *B,* control

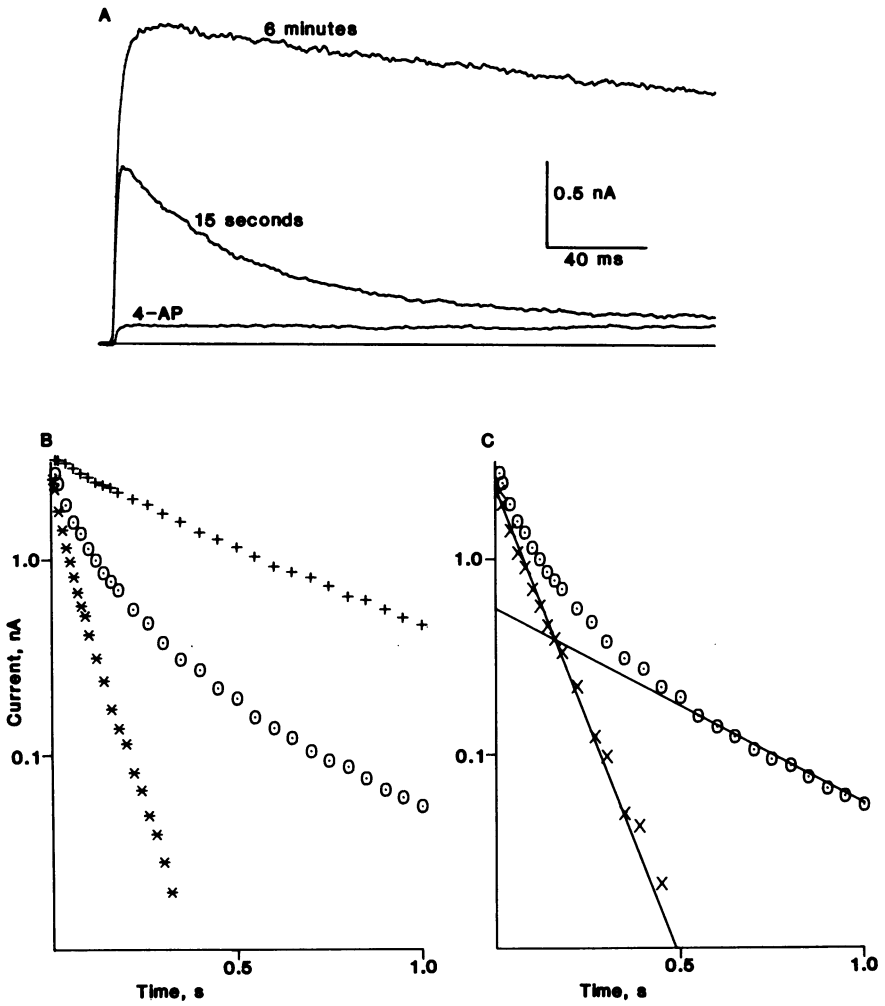

FIGURE 2 Papain removes fast inactivation from K channels. (*A*) The illustrated currents were recorded during 370 ms steps to +70 mV. The inactivating current (trace labeled 15 s) was recorded 15 s after breaking into a cell with an internal solution containing 0.15 mg/ml papain. The larger, slowly inactivating current was recorded 6 min later. The slowly inactivating current was blocked by adding 5 mM 4-AP externally (trace labeled 4-AP). Experiment AU067B. (*B*) Semilog plots of K current vs. time. K currents during 3-s depolarizations to +70 mV were recorded 15 s (*), 2 min (0), and 5 min (+) after breaking into the cell with internal solution containing 0.7 mg/ml papain. The noninactivating (steady-state) component of the current was subtracted before the data were plotted. The magnitude of the steady-state current was 0.18 nA shortly after breaking into the cell and 0.63 nA 5 min after papain action. Experiment AU127A. (*C*) Separation of partially modified current into two exponential components. The later time points were first fit with an exponential having a time constant of 435 ms. This fitted slow exponential was subtracted out and an exponential was fitted to the remaining current (tau = 90 ms). The straight lines are the fitted exponentials.

inactivation proceeded with fast and slow time constants of 23.6 and 75.5 ms at +70 mV (*, recorded 15 s after breaking into the cell). 5 min after obtaining the whole-cell configuration with 0.7 mg/ml papain in the pipette, the inactivation time constant was 509 ms (+, Fig. 2 *B*). At an intermediate time (2 min after obtaining the whole cell configuration), inactivation is partially modified. The decay of the K current at this time occurs in two phases, as shown in Fig. 2 *C*. The fast and slow components of the decay had time constants of 90 and 435 ms. This partial modification of inactivation may reflect two populations of K channels. One set of channels is inactivating normally and the other set has had fast inactivation removed by papain. It has previously been shown that in addition to fast inactivation, K channels undergo a very slow inactivation process (Kasai et al., 1986). The fact that K channels still inactivate slowly after treatment with proteolytic enzymes is similar to the situation in Na channels. Na channels undergo a very slow inactivation process upon depolarization (Adelman and Palti, 1969; Chandler and Meves, 1970; Schauf et al., 1976), and although fast Na inactivation is removed by proteolysis, slow sodium inactivation is unaffected (Rudy, 1978).

The amino acid reagent NBA also dramatically slowed K channel inactivation in GH3 cells. Because NBA is membrane permeant, it was applied externally, so that stable control records could be obtained before application. Removal of fast inactivation is illustrated by the K currents shown in Fig. 3 *A*. This effect was observed in each of 11 cells, using from 3 to 100 μM NBA. After addition of NBA, the magnitude of the K current at +70 mV was increased by an average of 17%. 4-AP reversibly blocked nearly all of the K current after NBA treatment (data not shown). The NBA trace in Fig. 3 *A* shows that a very slow inactivation process remains after NBA treatment. Na channels also continue to inactivate slowly after removal of fast inactivation with NBA (Oxford et al., 1978).

In contrast to its dramatic effect on K channel inactivation, NBA had little or no effect on activation kinetics, as shown in Fig. 3, *B–E*. At each of four different voltages, the superimposed currents in Fig. 3 show that after NBA treatment the potassium current initially rises at the same rate as the control current. Similar results were obtained with papain.

We have shown that two different types of protein modifiers, a proteolytic enzyme or the amino acid reagent

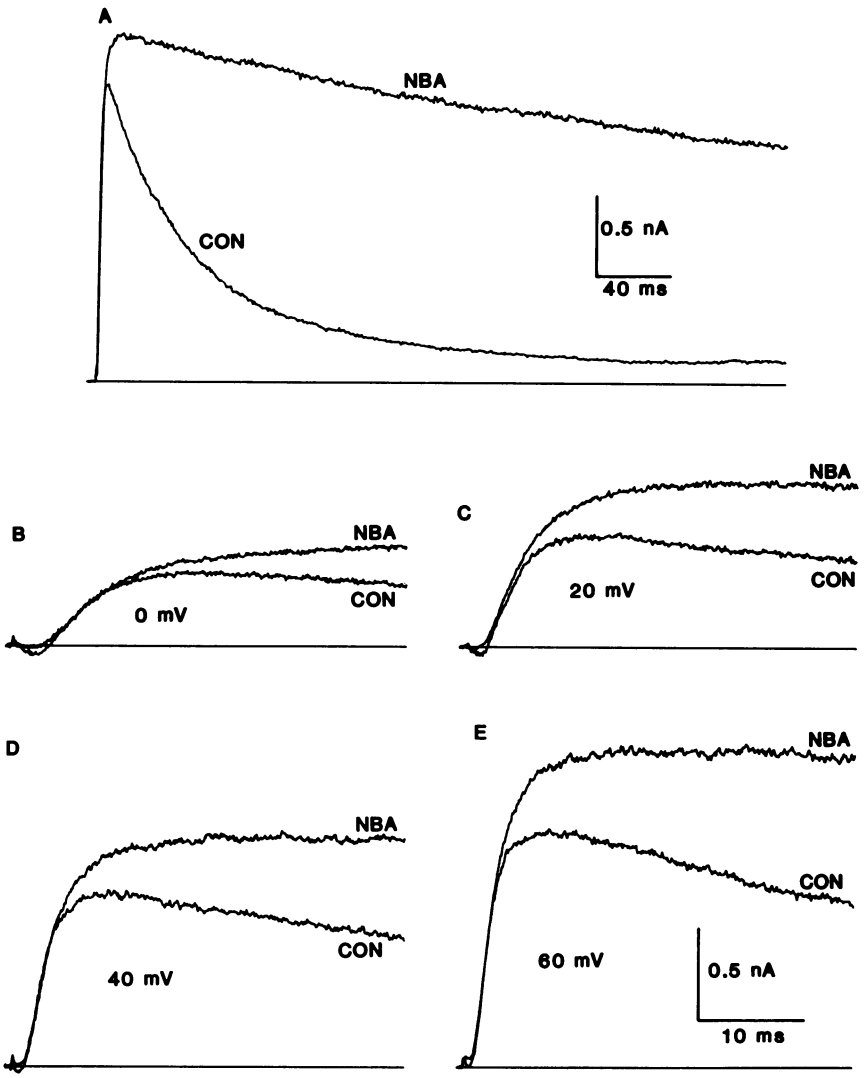

FIGURE 3 NBA removes fast inactivation without significant effects on channel activation. (*A*) Currents recorded during 370 ms steps to +70 mV, before *CON* and after *NBA* adding 30 μM NBA externally. Experiment AU077A. (*B–E*) Each pair of records compares a control K current with an NBA-treated current. The K currents were generated by 37 ms voltage-clamp steps to the indicated voltages. Experiment AU067A.

NBA, dramatically slow K channel inactivation gating with little or no effect on activation kinetics. The selective removal of fast, but not slow, K channel inactivation by these modifiers is remarkably similar to their effect on Na channel inactivation gating. The fact that the amino acid sequences of the Na and inactivating K channels have a homologous region, coupled with our results with inactivation modifiers, strongly suggests that the structures of the inactivation gates in the two channel types are alike. The relative accessibility of the Na channel inactivation gate to internal proteases led to a model in which the gate was assumed to be loosely coupled to the inner mouth of the channel (Armstrong and Bezanilla, 1977). Based in part on this idea, a three-dimensional structural model of the Na channel included a positively charged alpha helical hairpin region at the cytoplasmic side of the membrane which was postulated to be the inactivation gate (Guy, 1987). It will be interesting to see if structural models of the inactivating K channel contain a similar region.

Note added in proof. While this paper was in press we learned that another group has obtained results very similar to ours concerning the action of NBA on inactivating channels (cf. Wagoner, P. K., and G. S. Oxford. 1987. *Biophys. J.* 51:365a).

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