Low Molecular Weight Poly(A)$^+$ mRNA Species Encode Factors that Modulate Gating of a non-*Shaker* A-Type K^+ Channel

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ABSTRACT Voltage-dependent K^+ channels control repolarization of action potentials and help establish firing patterns in nerve cells. To determine the nature and role of molecular components that modulate K^+ channel function in vivo, we coinjected *Xenopus* oocytes with cRNA encoding a cloned subthreshold A-type K^+ channel (*mShal*1, also referred to as mKv4.1) and a low molecular weight (LMW) fraction (2–4 kb) of poly(A)$^+$ mRNA (both from rodent brain). Coinjected oocytes exhibited a significant (fourfold) increase in the surface expression of *mShal*1 K^+ channels with no change in the open-channel conductance. Coexpression also modified the gating kinetics of *mShal*1 current in several respects. Macroscopic inactivation of whole oocyte currents was fitted with the sum of two exponential components. Both fast and slow time constants of inactivation were accelerated at all membrane potentials in coinjected oocytes (τ_f = 47.2 ms vs 56.5 ms at 0 mV and τ_s = 157 ms vs 225 ms at 0 mV), and the corresponding ratios of amplitude terms were shifted toward domination by the fast component (A_f/A_s = 2.71 vs 1.17 at 0 mV). Macroscopic activation was characterized in terms of the time-to-peak current, and it was found to be more rapid at all membrane potentials in coinjected oocytes (9.9 ms vs 13.5 ms at 0 mV). Coexpression also leads to more rapid recovery from inactivation (~2.4-fold faster at −100 mV). The coexpressed K^+ currents in oocytes resemble currents expressed in mouse fibroblasts (NIH3T3) transfected only with *mShal*1 cDNA. These results indicate that mammalian regulatory subunits or enzymes encoded by LMW mRNA species, which are apparently missing or expressed at low levels in *Xenopus* oocytes, may modulate gating in some native subthreshold A-type K^+ channels.

INTRODUCTION

A-type or transient K^+ channels that activate and inactivate quickly at subthreshold membrane potentials regulate repetitive firing in nerve cells by controlling the slow subthreshold depolarization during the inter-spike interval (Connor and Stevens, 1971; Rudy, 1988; Hille, 1992). The rates of inactivation and recovery from inactivation influence the speed at which these A-type channels are reprimed and

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thus, the frequency of repetitive firing. These channels also show fast and slow phases of inactivation with macroscopic time constants that are weakly voltage dependent, except near threshold, and they show rapid recovery from inactivation (Solc, Zagotta, and Aldrich, 1987; Solc and Aldrich, 1990). A-type K^+ channels in this group include those encoded by the *Drosophila Shal* gene and its rodent homologue (Wei, Covarrubias, Butler, Baker, Pak, and Salkoff, 1990; Pak, Baker, Covarrubias, Butler, Ratcliffe, and Salkoff, 1991; Baldwin, Tsaur, Lopez, Jan, and Jan, 1991; Roberds and Tamkun, 1991). Although many K^+ channel genes have been cloned that express functional voltage-sensitive K^+ channels in heterologous expression systems (Jan and Jan, 1990; Salkoff, Baker, Butler, Covarrubias, Pak, and Wei, 1992; Pongs, 1992), the possible regulation of these channels in their native environment by factors encoded by other mRNA species has not received much attention. To determine whether there are native regulatory factors that modulate gating in A-type K^+ channels, we coinjected a low molecular weight (LMW) fraction (2–4 kb) of rat brain poly(A)$^+$ mRNA in oocytes along with cRNA encoding *mShal*1 (a mouse brain *Shal* homologue), and we studied the expressed currents using voltage-clamp techniques. Our results suggest that specific functions of K^+ channels in nerve cells may not be completely determined at the transcriptional and translational levels, but are instead fine tuned as the channel undergoes specific post-translational modifications involving structural subunits or regulatory enzymes that are encoded by LMW mRNA species.

Some of this work has appeared in abstract form (Chabala, Bakry, and Covarrubias, 1992; Chabala and Covarrubias, 1993).

MATERIALS AND METHODS

RNA Preparation

*mShal*1 is a cloned K^+ channel isolated from mammalian brain (Pak et al., 1991). In terms of the recent vertebrate gene nomenclature, this clone is referred to as mKv4.1 (Chandy, Douglas, Gutman, Jan, Joho, Kaczmarek, McKinnon, North, Numa, Philipson, Ribera, Rudy, Salkoff, Swanson, Steiner, Tanouye, and Tempel, 1991). Capped runoff transcripts were prepared using 1 μg linearized template *mShal*1 (or *fShal*2) DNA with an mRNA capping kit (Stratagene Corp., La Jolla, CA). The transcription reaction was run in the presence of T3 RNA polymerase for 30 min at 37°C, and it was boosted with a second addition of T3 RNA polymerase for an additional 30 min. To remove unincorporated ribonucleotides and reaction salts, the transcription products (~25 μl) were then dialyzed on a 0.025 μM Millipore VS filter (Millipore Corp., Bedford, MA) against DEPC-treated water in a covered 35-mm tissue culture dish for 1 h in a refrigerator (cf, Silhavy, Berman, and Enquist, 1984). Dialyzed reaction products were then diluted and injected into oocytes. Total poly(A)$^+$ mRNA was isolated from adult rat brain (male, Sprague Dawley) using the RNAzol procedure (Biotecx Laboratories, Inc., Houston, TX), and it was size-fractionated as previously described (Goldin, Snutch, Lübbert, Dowsett, Marshall, Auld, Downey, Fritz, Lester, Dunn, Catterall, and Davidson, 1986). The poly(A)$^+$ mRNA fractions were resuspended at a concentration of 1–1.5 μg/μl.

Transfection of Mouse Fibroblasts

To express *mShal*1 in NIH3T3 mouse fibroblasts, *mShal*1 cDNA was subcloned into a mammalian expression vector (pMAMneo, Clontech Laboratories, Inc., Palo Alto, CA). pMAMneo was first modified by introducing a SacII restriction site between SalI and XhoI in the polylinker region by cassette mutagenesis. *mShal*1 was then excised from pBluescript KS II

with SalI and SacII, and it was directly ligated into pMAMneo. Transient transfections of 3T3 cells were done following standard calcium-phosphate/DNA coprecipitation techniques (Ausubel, Brent, Kingston, Moore, Seidman, Smith, and Struhl, 1991). Selection of cells expressing *mShal*1 K^+ channels was facilitated by cotransfecting 3T3 cells with EBO-pCD-Leu2, where Leu2 encodes a surface antigen (Margolskee, McHendry-Rinde, and Horn, 1992). Expression of *mShal*1 K^+ channels was induced with 1 μM dexamethasone (added 24 h before recording).

Oocyte Preparation and Electrophysiological Recordings

Oocytes were denuded in a Ca^{+2}-free Ringer's solution consisting of (in mM) 82.5 NaCl, 2 KCl, 1 $MgCl_2$, 10 HEPES (pH 7.6) containing 2 mg/ml collagenase (Boehringer type A or B). Defolliculated oocytes were incubated overnight at 18°C in ND96 solution containing (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.6). External tetraethylammonium (TEA, Eastman Kodak Co., Rochester, NY) chloride and 4-aminopyridine (4-AP, Sigma Chemical Co., St. Louis, MO) solutions were made up by equimolar replacements with NaCl using the ND96 protocol. In those pharmacological experiments, at least 3 ml of each solution was perfused into the chamber (~0.4 ml capacity) to ensure complete washout of the previous solution. Mature oocytes were selected and injected with 50–70 nl of the RNA solutions, which contained *mShal*1 cRNA, *mShal*1 cRNA plus LMW poly(A)$^+$ mRNA, or LMW poly(A)$^+$ mRNA. In the coinjection experiments, *mShal*1 cRNA (~4–5 ng/oocyte) was diluted fourfold with the 2–4 kb poly(A)$^+$ mRNA fraction (~40–50 ng/oocyte). Injected oocytes were returned to the incubator, and expressed whole oocyte currents were recorded 2–6 d later in ND96 solution at room temperature (22–24°C) using a Dagan TEV 200 two-electrode voltage clamp (Dagan Corp., Minneapolis, MN).

Oocytes for macropatch recording were prepared as reported (Methfessel, Witzemann, Takahashi, Mishina, Numa, and Sakmann, 1986). We used a three-stage puller (Sutter Instruments, Co., Novato, CA) to obtain pipette tip resistances of ~1 Mohm after fire polishing the tip. The vitelline membrane was removed with fine dissecting forceps after exposing the oocytes to a hypertonic "stripping" solution for ~10 min. Oocytes were then immediately transferred to the recording chamber containing (in mM) 140 potassium aspartate, 10 KCl, 1.8 $CaCl_2$, 10 HEPES (pH 7.2), which clamped the membrane potential near 0 mV. All macropatch recordings were done using the cell-attached configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). Experiments were carried out at room temperature with an Axopatch 200 amplifier (Axon Instruments, Inc., Foster City, CA). The pipette contained ND96 solution.

Whole-cell recording of K^+ currents in 3T3 cells was done in an external solution containing (in mM) 130 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 25 dextrose, 10 HEPES (pH 7.2). The pipette solution consisted of (in mM) 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 HEPES (pH 7.2). Currents were recorded at room temperature using the Axopatch 200 amplifier. Capacitance (electrode and membrane) and series resistance were electronically compensated.

All currents were low-pass filtered at 1 kHz and digitized at 2 kHz. Data acquisition and analysis were done using pClamp (Axon Instruments, Inc.) and curve fitting was carried out using NFIT (Long Island Products, Galveston, TX) or Peakfit (Jandel Scientific, Corta Madera, CA).

RESULTS

Species Encoded by LMW Poly(A)$^+$ mRNA Modify mShal1 Currents

Macroscopic currents expressed in *Xenopus* oocytes injected with *mShal*1 cRNA show pronounced fast and slow phases of inactivation (Fig. 1 *A*). Coexpression of *mShal*1 cRNA and the 2–4 kb fraction of rat brain poly(A)$^+$ mRNA, however, affects both

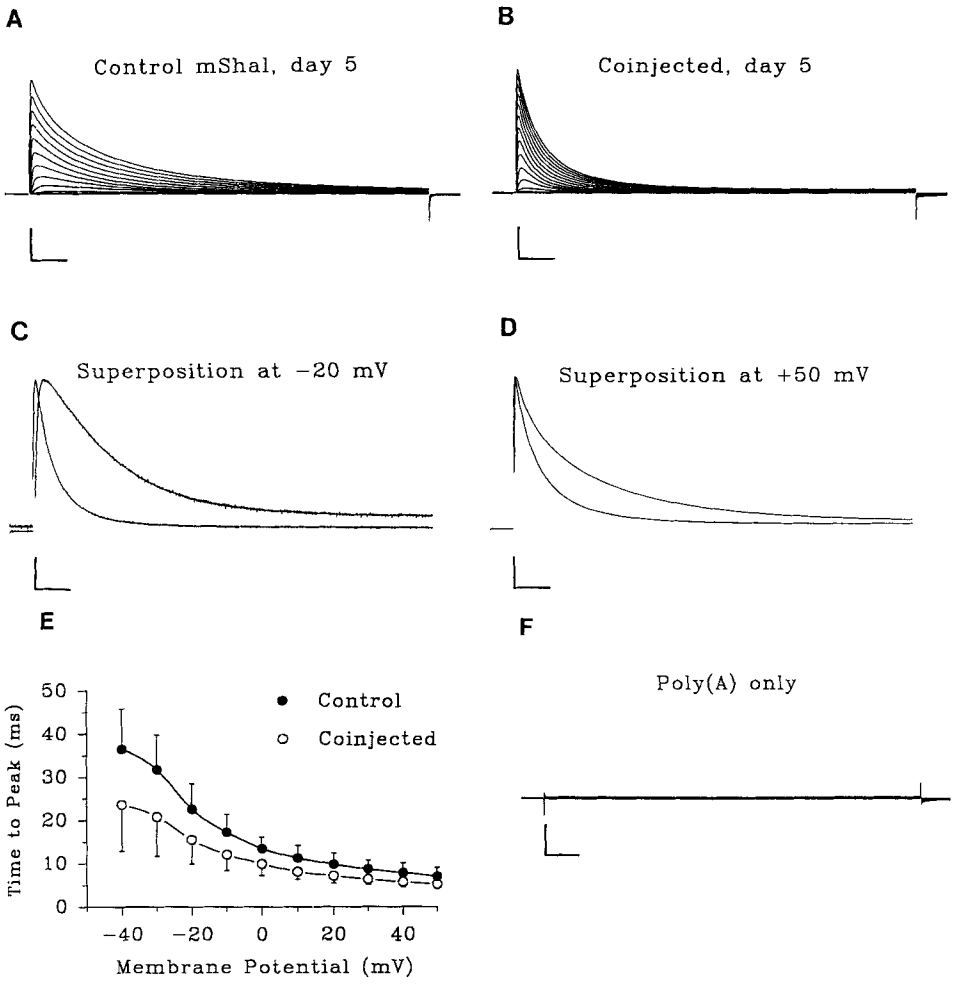


FIGURE 1. Macroscopic currents in control and coinjected oocytes. (*A*) Family of currents from −90 to +50 mV (10-mV steps) from a holding potential (HP) of −100 mV in a control oocyte injected with *mShal*1 cRNA (4–5 ng/oocyte). (*B*) Family of currents from −90 to +50 mV (10 mV steps, HP = −100 mV) in an oocyte injected with *mShal*1 cRNA diluted fourfold with a 2–4 kb fraction (40–50 ng/oocyte) of rat brain poly(A)$^+$ mRNA. Oocytes in *A* and *B* were from the same batch. Injections were done at the same time and experiments were done 5 d later. (*C*) Superposition of (scaled) currents from *A* and *B* at −20 mV. The rapid phase of inactivation was pronounced in both groups near activation threshold, but it was more dominant in coinjected oocytes. (*D*) Superposition of currents from *A* and *B* at +50 mV. (*E*) Time-to-peak current as a function of membrane potential in control (*filled circles*, n = 18, mean ± SD) and coinjected (*open circles*, n = 15) oocytes. Coexpression lead to more rapid rates of *mShal*1 channel activation as measured by the time-to-peak current (t = 4.19, $p < 0.01$, paired comparisons). (*F*) Currents from −90 to +50 mV (10-mV steps, HP = −100 mV) in an oocyte injected only with the 2–4 kb fraction of poly(A)$^+$ mRNA. As previously reported, injection of the LMW fraction by itself did not lead to expression of current (Rudy et al., 1988). Calibration bars: 2 μA and 100 ms (*A*, *B*, and *D*), 0.7 μA and 100 ms (*C*), 1 μA and 100 ms (*F*).

phases of inactivation and results in more complete inactivation of the current during a 1 s test pulse (Fig. 1 *B*). Superposition of currents in control and coinjected oocytes clearly shows the influence of LMW poly(A)$^+$ mRNA on the macroscopic kinetics (Fig. 1, *C* and *D*). Although apparent at all membrane potentials, this effect is most striking at more hyperpolarized membrane potentials. In addition, the rate of activation in terms of the time-to-peak current was faster at all membrane potentials in coinjected oocytes (Fig. 1 *E*). The effects on the kinetics were time dependent, and maximal effects generally occurred 4–6 d after injection of RNA. Injection of the LMW poly(A)$^+$ mRNA fraction alone, however, did not lead to expression of detectable current (Fig. 1 *F*).

Coexpression also resulted in a substantial increase (~fourfold) in surface expression of *mShal*1 K^+ channels without a corresponding change in the open-channel conductance.[1] Both oocytes in Fig. 1, *A* and *B* were injected with RNA at the same time, and they were tested 5 d later, but the amount of *mShal*1 cRNA injected into the oocyte of Fig. 1 *B* was diluted 4:1 with LMW poly(A)$^+$ mRNA. In oocytes that were injected on the same day and tested 4–6 days later, we found that the mean peak current at +50 mV was 8.00 ± 0.83 μA (mean ± SEM) in coinjected oocytes (n = 14) compared to 8.05 ± 0.95 μA in control oocytes (n = 11). In each case, however, the amount of *mShal*1 cRNA was diluted 4:1 in the coinjected oocytes, and all of the *mShal*1 cRNA was prepared from the same transcription reaction. The expression of current was not saturated in the oocytes, because it was necessary to dilute the *mShal*1 cRNA to produce currents in the 3–10-μA range. That is, injection of undiluted *mShal*1 cRNA routinely produced currents in the 30-μA range.

We have also carried out these coexpression experiments using the subthreshold A-type K^+ channel encoded by *Drosophila fShal*2 cDNA, which has more rapid kinetics of inactivation compared to the mammalian A-type K^+ channel encoded by *mShal*1 (Pak et al., 1991). In fact, *fShal*2 currents expressed in oocytes resemble those shown for coinjected oocytes in Fig. 1 *B*. Under the conditions of Fig. 1, coinjection of the LMW poly(A)$^+$ mRNA fraction had no effect on the kinetics of activation or inactivation of *fShal*2 currents, but a similar increase (also ~fourfold) in expressed *fShal*2 current was found. In oocytes that were injected on the same day and tested 4–6 d later, we found that the mean peak current at +50 mV was 1.16 ± 0.14 μA (mean ± SEM) in coinjected oocytes (n = 6) compared to 1.19 ± 0.18 μA in control oocytes (n = 5), but the *fShal*2 cRNA was diluted 4:1 in coinjected oocytes with the LMW poly(A)$^+$ mRNA fraction.

Kinetics of Modification Induced by LMW Poly(A)$^+$ mRNA Species

We found that the falling phases of the macroscopic currents were well described by the sum of two exponential components. The voltage dependence of the fast inactivation time constants (τ_f) from control and coinjected oocytes is shown in Fig.

[1] Increased surface expression is used only in a descriptive sense to indicate that the macroscopic current was on average larger in coinjected oocytes. It is not clear if the increase in surface expression is due to increased protein production, more efficient incorporation of channels into the membrane, or an increase in the fraction of functional channels from the total number of channels already in the membrane. The increase in macroscopic current was not, however, due to an increase in the open-channel conductance, which was found to be 6–8 pS at +50 mV in macropatch recordings.

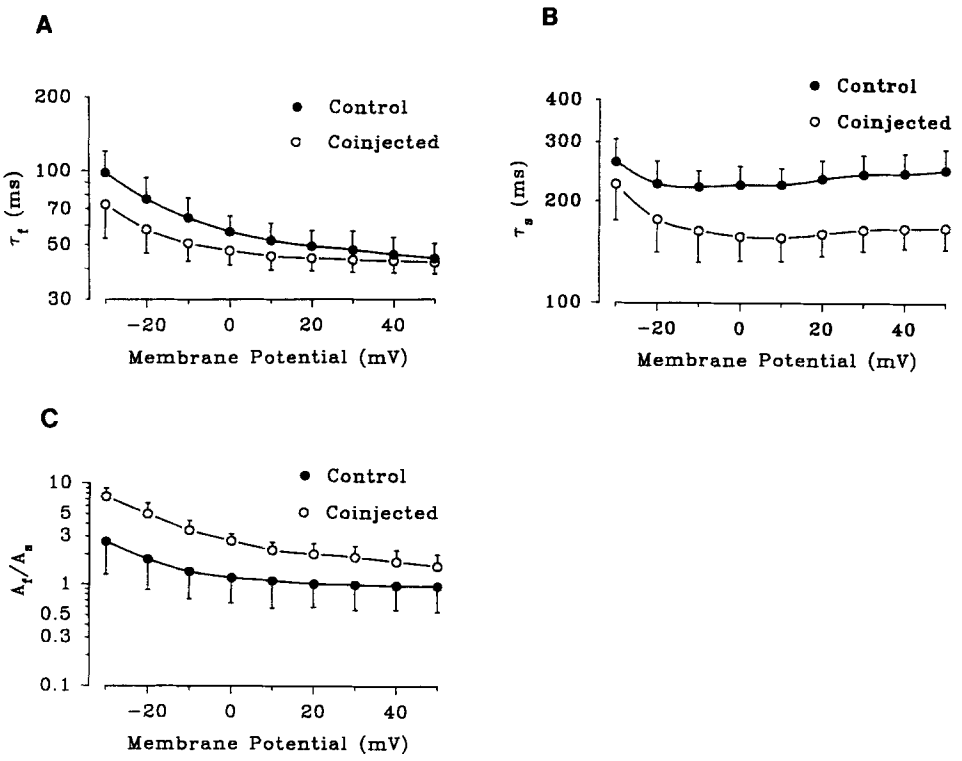


FIGURE 2. Voltage dependence of inactivation. The decay phase of the macroscopic currents was fitted with the sum of two exponential components, $A_f\exp(-t/\tau_f) + A_s\exp(-t/\tau_s)$, where A and τ are the amplitude terms and time constants of inactivation for the fast (f) and slow (s) components, respectively. (A) τ_f in control oocytes (*filled circles*, $n = 18$, mean ± SD) and coinjected (*open circles*, $n = 15$) oocytes. τ_f in coinjected oocytes was systematically reduced ($t = 3.65$, $p < 0.01$, paired comparisons), and the corresponding slope between −20 and +50 mV was reduced from −135 to −260 mV/e-fold change ($t = 2.75$, $p < 0.02$). (B) τ_s in the same control (*filled circles*) and coinjected (*open circles*) oocytes. τ_s was significantly faster in coinjected oocytes ($t = 13.33$, $p < 0.01$, paired comparisons). In control oocytes, τ_s shows only a slight positive slope between −20 and +50 mV (592 mV/e-fold change). In coinjected oocytes, however, τ_s was reduced about 30% over most of the voltage range, and the voltage dependence was somewhat flatter between −20 and +50 mV (7,200 mV/e-fold change, $t = 2.50$, $p < 0.05$). (C) Voltage dependence of the amplitude ratio A_f/A_s for control (*filled circles*) and coinjected (*open circles*) oocytes. A_f/A_s was systematically larger in coinjected oocytes ($t = 3.80$, $p < 0.01$, paired comparisons).

2 *A*, whereas the voltage dependence of the corresponding slow inactivation time constants (τ_s) is shown in Fig. 2 *B*. Both time constants were faster and less voltage dependent in coinjected oocytes, and the ratio of the amplitude terms associated with the time constants (i.e., A_f/A_s) was systematically modified throughout the voltage range (Fig. 2 *C*). The A_f/A_s ratio at +50 mV was near unity in control *mShal*1 oocytes but became dominated by the fast component as the membrane potential became more hyperpolarized. A similar trend was found in coinjected oocytes, but the A_f/A_s

ratio was uniformly larger across the range of membrane potentials studied. When the ratio of *mShal*1 cRNA to poly(A)⁺ mRNA was reduced (i.e., 1:1 rather than 4:1), the results with respect to the inactivation time constants, amplitude terms, and time-to-peak current were intermediate between those shown in Figs. 1 and 2 for control and coinjected oocytes.

Macroscopic recovery from inactivation is steeply voltage dependent in *mShal*1 with more rapid recovery at more hyperpolarized membrane potentials (Pak et al., 1991). We found that coexpression of the LMW poly(A)⁺ mRNA fraction speeded up recovery from inactivation in *mShal*1 on average about 2.4-fold at −100 mV (Fig. 3).

Peak current-voltage relations for control and coinjected oocytes were identical and showed channel activation near −50 mV. Prepulse inactivation curves were fitted with

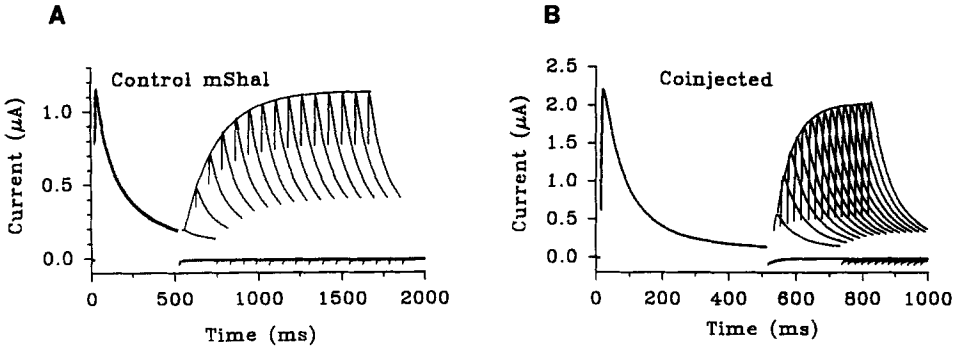


FIGURE 3. Recovery from inactivation. (*A*) Macroscopic recovery protocol at −100 mV in a control *mShal*1 oocyte. A double pulse paradigm was used with a first pulse (500 ms in duration) to +50 mV. The inter-pulse recovery interval (at −100 mV) was incremented in 80 ms steps. Envelopes of peak currents during recovery were fitted to a single exponential function to estimate the recovery time constant (τ_r). In this example, τ_r = 199 ms. (*B*) Recovery protocol at −100 mV in a coinjected oocyte. First pulse as in *A* but with inter-pulse recovery interval that was incremented in 20-ms steps. In this oocyte, τ_r = 51 ms. The mean recovery time at −100 mV was 165.8 ± 8.8 ms (n = 12, mean ± SEM) for control *mShal*1 oocytes and 68.9 ± 4.7 ms (n = 15) for coinjected oocytes. Coexpression accelerated recovery from inactivation in *mShal*1 about 2.4-fold at −100 mV (t = 10.66, $p < 0.01$).

a single Boltzmann function to estimate the midpoint potential and the slope factor (cf, Zagotta and Aldrich, 1990). These curves, which were generated using 10-s long prepulses and 500 ms duration test pulses to +40 mV (cf, Pak et al., 1991), were similar for both groups, although coinjected oocytes tended to show a modest 2–3 mV hyperpolarization in the midpoint potential (70.5 ± 3.8 mV vs 68.1 ± 3.2 mV, mean ± SEM), a 10% more shallow slope (5.6 ± 0.6 mV/e-fold change vs 5.1 ± 0.4 mV/e-fold change), and more complete inactivation (cf, Fig. 1).

Pharmacological Sensitivity

We also measured the pharmacological sensitivity to TEA ions and to 4-AP in control and coinjected oocytes. As previous reported *mShal*1 is comparatively insensitive to

external TEA (Pak et al., 1991). We found that control and coinjected oocytes showed similar insensitivities to external TEA with an IC_{50} of ~180–200 mM at +50 mV in both groups (n = 3 in each group). Coinjected oocytes (Fig. 4), however, were more sensitive to external 4-AP. On average the IC_{50} blocking affinity was reduced ~2.4-fold at +50 mV in coinjected oocytes. As suggested in Fig. 4, however, 4-AP has a much larger effect in blocking the fast component of inactivation. Because the fast component of inactivation is controlled by a cytoplasmic domain (cf, Discussion),

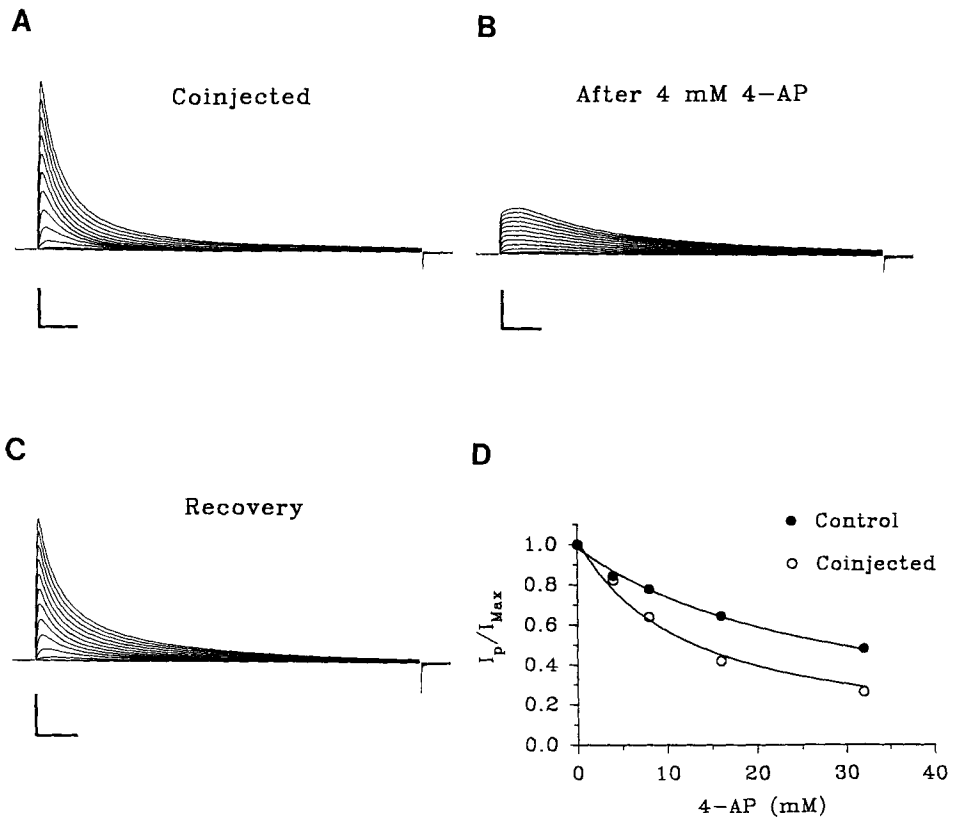


FIGURE 4. Sensitivity to 4-aminopyridine. (*A*) Family of control currents in a coinjected oocyte from −90 to +50 mV in 10 mV steps. (*B*) Same family of currents after exposure to 32 mM 4-AP. (*C*) Family of recovery currents in the same oocyte after perfusing ~18 ml of ND96 solution into the chamber (~0.4 ml capacity). 4-AP produced a preferential block of the fast component of inactivation. The time constant of the residual slow component of inactivation was also reduced ~30–40% at 32 mM 4-AP. A similar preferential block of the fast component of inactivation occurred in oocytes injected only with *mShal1* cRNA. (*D*) Graph of peak currents fit with a single binding isotherm in the coinjected oocyte and in a control oocyte injected only with *mShal1* cRNA. The IC_{50} values were 29.0 and 12.5 mM at +50 mV for the control and coinjected oocytes, respectively. On average, we found IC_{50} = 28.4 ± 1.6 mM (n = 3, mean ± SEM) at +50 mV in control *mShal1* oocytes compared to IC_{50} = 12.1 ± 1.8 mM (n = 3) in coinjected oocytes. Binding of 4-AP was voltage dependent with less tight binding at more hyperpolarized membrane potentials. Bars, 2 μA and 100 ms (*A–C*).

4-AP may have an internal site of action (cf, Kirsch, Verner, Drewe, and Brown, 1993) in our experiments. In support of the latter idea, we note that substantial washout time is necessary to show recovery from 4-AP inhibition while TEA inhibition reverses readily.

Macropatch Data Confirm Results with Whole Oocytes

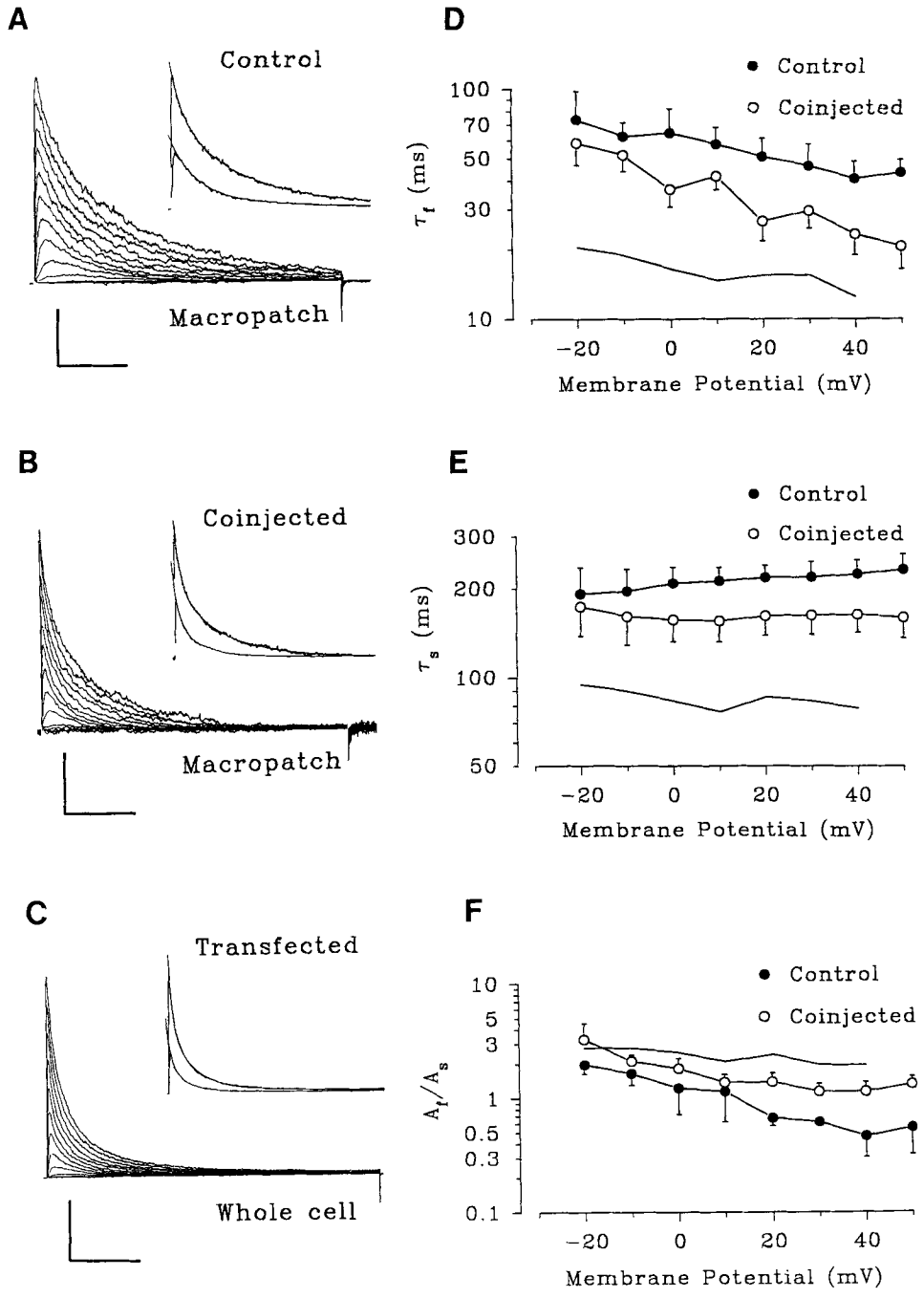
To rule out possible errors in interpretation of the two-electrode voltage clamp results (cf, Stühmer, 1992), we also carried out cell-attached macropatch recordings in control and coinjected oocytes (Fig. 5). Those results agreed with the two-electrode voltage clamp results, which indicates that all experiments were done under proper voltage control. We also checked for proper voltage control by doing two-electrode voltage clamp experiments with and without series resistance compensation. Although peak currents were slightly larger and time constants of inactivation were slightly faster with 660 ohms of series resistance compensation, the basic results were identical. Indeed, we did not observe current size-dependent kinetics for oocytes with two-electrode peak currents under 15 μA in amplitude. We occasionally recorded from membrane patches in some coinjected oocytes (as in Fig. 5) that evidently contained a higher density of channels with even faster kinetics of inactivation. We suspect that in the steady state, coinjected oocytes may contain both modified and normal *mShal*1 channels distributed in a nonrandom manner in the membrane, and the total current is represented by the sum of those two populations. Certain heterogeneous membrane patches might then be expected to show even more rapid kinetics compared to macroscopic recordings.

mShal1 Currents in a Mammalian Expression System

To compare our results in oocytes with another expression system that might be capable of inducing different posttranslational modifications, we also transfected a mouse cell line (NIH3T3) with *mShal*1 cDNA. The NIH3T3 cell line was chosen because it does not appear to have endogenous depolarization-activated K^+ channels. Whole-cell currents in these cells (Fig. 5 *C*) were qualitatively similar (although with faster kinetics) compared to those expressed in coinjected oocytes, indicating that a mammalian expression system produces factors, possibly encoded by LMW mRNA species, that can modify *mShal*1 K^+ channels in vivo. We also isolated total poly(A)$^+$ mRNA from the NIH3T3 cells and coinjected it into oocytes along with *mShal*1 cRNA. The results were qualitatively similar to those shown in Fig. 1, which reinforces the idea that mammalian poly(A)$^+$ mRNA encodes factors that modify gating in *mShal*1 K^+ channels.

Effects of the Rat Brain Na^+ Channel β_1 Subunit

Coexpression of the rat brain Na^+ channel β_1 subunit in oocytes along with the main rat brain Na^+ channel α subunit produces effects that resemble our coexpression results presented in Fig. 1 (cf, Isom, De Jongh, Patton, Reber, Offord, Charbonneau, Walsh, Goldin, and Catterall, 1992). Because of this similarity and because of the increasing evidence that there may be LMW β subunits associated with some voltage-dependent K^+ channels (cf, below), we carried out experiments to determine


A
Control
Macropatch
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C
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D
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100
70
50
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10
τ_f (ms)
−20
0
20
40
Membrane Potential (mV)
E
Control
Coinjected
300
200
100
50
τ_s (ms)
−20
0
20
40
Membrane Potential (mV)
F
Control
Coinjected
10
5
3
1
0.5
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A_f/A_s
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Membrane Potential (mV)

FIGURE 5

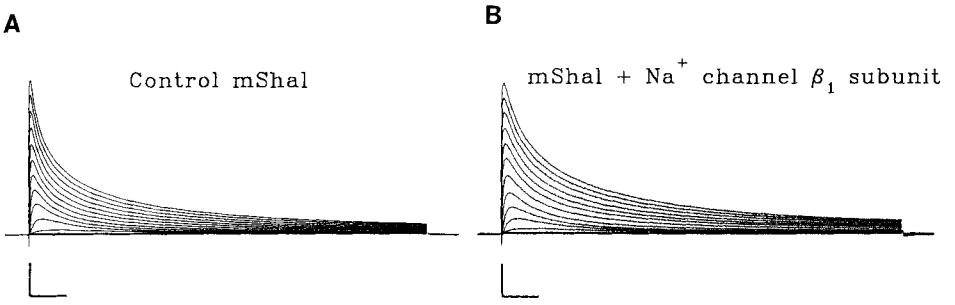

FIGURE 6. Effects of the β_1 subunit of the rat brain Na^+ channel. (*A*) Family of control currents from −90 to +50 mV (10 mV steps, HP = −100 mV) in an oocyte injected with 4–5 ng of *mShal*1 cRNA. (*B*) Similar family of currents in an oocyte (same batch as in *A*) injected with *mShal*1 cRNA diluted 4:1 with cRNA coding for the rat brain Na^+ channel β_1 subunit (5–7 ng). The oocytes in *A* and *B* were injected at the same time, and they were tested 5 d later with the two-electrode voltage clamp. Coinjected oocytes had ~4–5 times as much of the β_1 cRNA as the *mShal*1 cRNA. Under these conditions, there was no acceleration in the kinetics of inactivation or in the time-to-peak current. In fact, the kinetics of inactivation were slightly slower in coinjected oocytes. Peak currents in coinjected oocytes were reduced in proportion to the amount of *mShal*1 cRNA injected. Similar results were obtained when *mShal*1 cRNA was diluted an additional two- or fivefold so that the ratio of β_1 cRNA to *mShal*1 cRNA in coinjected oocytes was 8–10:1 or 20–25:1. Bars: (*A*) 2 μA and 100 ms; (*B*) 0.8 μA and 100 ms.

if the rat brain Na^+ channel β_1 subunit could modify the kinetics or expression of *mShal*1 currents. As shown in Fig. 6, coexpression of cRNA coding for the rat brain Na^+ channel β_1 subunit along with *mShal*1 cRNA did not accelerate the kinetics of activation or inactivation, and it did not lead to upregulation of the expressed current. The kinetics of inactivation were in fact slightly slower in coinjected oocytes, but because this small effect is in the opposite direction to account for our observations we have not pursued the matter further. In oocytes that were injected at the same time and tested 4–6 d later, we found that the peak current at +50 mV was

FIGURE 5 (*opposite*). Macroscopic *mShal*1 K^+ currents in oocyte macropatches and whole-cell NIH3T3 fibroblasts. (*A*) Cell-attached macropatch currents from an oocyte injected with *mShal*1 cRNA (4 d post-injection). Currents were elicited by 10-mV steps from −80 to +50 mV (HP = −100 mV). (*B*) Macropatch currents from an oocyte injected with *mShal*1 cRNA and LMW poly(A)⁺ mRNA from rat brain (4 d post-injection). Macropatch currents are the average of 5 or 10 traces at each membrane potential. (*C*) Whole-cell currents from 3T3 fibroblasts transfected with *mShal*1 cDNA. Currents were elicited by 10-mV steps from −80 to +40 mV (HP = −90 mV). Currents in the insets were fitted with the sum of two exponential components (0 and +50 mV in *A* and *B*, −10 and +40 mV in *C*). (*D*) Voltage dependence of τ_f from a two exponential fit as in Fig. 2. (*E*) Voltage dependence of τ_s. (*F*) Voltage dependence of A_f/A_s. Closed circles are for control macropatches expressing *mShal*1 (n = 4, mean ± SD), whereas open circles are for macropatches from oocytes coexpressing *mShal*1 and LMW poly(A)⁺ mRNA (n = 4), and the solid line is for NIH3T3 fibroblasts expressing *mShal*1 (n = 1). Because macropatch currents were nosier than whole-oocyte currents, we constrained τ_s in the fit to the mean values obtained from the fits of whole oocyte currents. Bars: (*A*) 40 pA and 200 ms; (*B*) 20 pA and 200 ms; (*C*) 300 pA and 200 ms.

reduced by 70.8 ± 4.7% (mean ± SEM) in coinjected oocytes (n = 4 control and coinjected oocytes), which is roughly equivalent to the dilution of *mShal*1 cRNA by the Na^+ channel β_1 subunit cRNA.

DISCUSSION

We have shown that LMW mammalian poly(A)$^+$ mRNA encodes factors that modify several macroscopic gating properties of the *mShal*1 K^+ channel. The observed decrease in time-to-peak current and the decrease in τ_f and τ_s coupled to an increase in the A_f/A_s ratio may be linked to a modification of one or more microscopic rate constants affecting channel activation and inactivation in a homogeneous population of channels with complex gating (cf, Solc and Aldrich, 1990).[2] Site-directed mutagenesis has shown that fast and slow inactivation in *Shaker* K^+ channels are controlled by separate intracellular domains in the NH_2- and COOH-terminal regions (Hoshi, Zagotta, and Aldrich, 1990, 1991), respectively, and similar features are shared by K^+ channels encoded by the *Shal* gene (Pak et al., 1991; Baldwin et al., 1991). Coexpression of LMW poly(A)$^+$ mRNA along with *mShal*1 cRNA speeds up both phases of inactivation in *mShal*1 currents. Thus, there may be direct or induced posttranslational modifications at NH_2- and COOH-terminal sites that control inactivation. Because channel activation and inactivation are probably coupled (Aldrich and Stevens, 1987; Zagotta and Aldrich, 1990), another possibility is that the activation process is modified at a single site that speeds up the time-to-peak current and indirectly alters macroscopic inactivation. We note, however, that the time constants of macroscopic inactivation are slow compared to the rise time of the currents (Figs. 1 and 2); thus, an acceleration of *both* activation and inactivation rate constants in a coupled sequential model (cf, Solc and Aldrich, 1990) may be needed to account for our observations. An additional possibility is that the underlying mechanism consists of a shift in gating modes (Hess, Lansman, and Tsien, 1984) as has been proposed for similar results (cf, below) obtained in cloned Na^+ channels (Zhou, Potts, Trimmer, Agnew, and Sigworth, 1991). In the latter case, however, only the amplitude terms associated with fast and slow time constants of inactivation were changed.

Coinjection of LMW brain poly(A)$^+$ mRNA also results in significant increase (~fourfold) in the surface expression of *mShal*1 K^+ channels. This effect with LMW poly(A)$^+$ mRNA has also been noticed in cloned Na^+ channels (Auld, Goldin, Krafte, Marshall, Dunn, Catterall, Lester, Davidson, and Dunn, 1988). The increase in surface expression of *mShal*1 currents, however, may involve different mRNA species compared to those that modify the kinetics of *mShal*1 currents. In support of the latter idea, we found a similar increase in *Drosophila fShal*2 current with no change in kinetics of *fShal*2 currents. In fact, we note that coexpression of *mShal*1 and LMW poly(A)$^+$ mRNA in oocytes and transfection of 3T3 fibroblasts with *mShal*1 produces

[2] As indicated in Results there may well be two homogeneous populations of channels with complex gating, normal *mShal*1 channels and modified *mShal*1 channels. It seems likely that under our coexpression conditions (Fig. 1) not all channels are modified, and we note that in the mammalian system (Fig. 4), where a larger fraction of channels may be modified by more efficiently expressed regulatory factors encoded by poly(A)$^+$ mRNA, the kinetics are even more rapid.

macroscopic currents that closely resemble *fShal2* currents expressed in oocytes (Pak et al., 1991), and they also resemble the non-*Shaker* A_2 currents found in larval *Drosophila* neurons (Solc et al., 1987; Solc and Aldrich, 1990), which may well be coded for by the *Shal* gene.

Although no functional LMW β subunits for K^+ channels have yet been identified, 38–42 kD peptides do copurify with dendrotoxin (DTX) receptors from rodent brain (Rehm and Tempel, 1991; Parcej, Scott, and Dolly, 1992) and a similar peptide copurifies with the rat brain *drk*1 polypeptide (Trimmer, 1991). It is clear, however, that some properties of expressed K^+ channels can be modulated by posttranslational processes. Increased or decreased surface expression of voltage-gated K^+ channels can occur without altering the kinetics through phosphorylation by protein kinases (Moran, Dascal, and Lotan, 1991; Hoger, Walter, Vance, Yu, Lester, and Davidson, 1991; Busch, Varnum, North, and Adelman, 1992; Covarrubias, Pak, and Sorensen, 1992). In contrast, oxidation of cysteine residues in the NH_2-terminal region of the mammalian transient K^+ channel clones *RCK*4 and *Raw*3 leads to loss of fast inactivation (Ruppersberg, Stocker, Pongs, Heinemann, Frank, and Koenen, 1991). In other work, nearly identical kinetics have been found for mammalian *Shaker*-type K^+ channels expressed in oocytes or mammalian cells (Ruppersberg, Schröter, Sakmann, Stocker, Sewing, and Pongs, 1990; Koren, Liman, Logothetis, Nadal-Ginard, and Hess, 1990; Garcia-Guzman, Calvo, Cena, and Criado, 1992), although wide differences in toxin pharmacology have been reported. The *Shaker* K^+ channel expressed in oocytes is blocked by low concentrations of charybdotoxin (CTX), whereas the native *Shaker* K^+ channel in *Drosophila* muscle is insensitive to CTX (Zagotta, Germeraad, Garber, Hoshi, and Aldrich, 1989). Native *Shaker* K^+ channels in *Drosophila* larval muscle, however, are blocked by low concentration of DTX (Wu, Tsai, Chen, Zhong, Singh, and Lee, 1989), whereas *Shaker* K^+ channels expressed in oocytes are insensitive to DTX (Stocker, Stühmer, Wittka, Wang, Müller, Ferrus, and Pongs, 1990). The latter observations on differential toxin sensitivity may indicate a deficiency in posttranslational processing by oocytes compared to native tissue (cf, Soreq, 1985).

In a previous study, it was reported that A-type K^+ currents encoded by a 5–7-kb rat brain poly(A)$^+$ mRNA fraction decay more rapidly than A-type K^+ currents from total brain poly(A)$^+$ mRNA, and the slower inactivation is recovered by coexpression of a 2–4 kb poly(A)$^+$ mRNA fraction along with the 5–7-kb fraction (Rudy et al., 1988). Our results indicate an apparent opposite effect, although we note that our results are for a specific cloned A-type K^+ channel, whereas the observations of Rudy et al. (1988) apply to a group of heterogeneous native K^+ channels encoded by the 5–7-kb poly(A)$^+$ mRNA fraction. In work that is more analogous to ours in terms of results, it has been reported that macroscopic inactivation exhibited by the α subunit of the tetrodotoxin-sensitive Na^+ channel from rat brain or skeletal muscle is abnormally slow when the channels are expressed in oocytes (Auld et al., 1988; Zhou et al., 1991; Ukomadu, Zhou, Sigworth, and Agnew, 1992; Krafte, Goldin, Auld, Dunn, Davidson, and Lester, 1990; Isom et al., 1992; Chen, Chahine, Kallen, Barchi, and Horn, 1992). Coexpression of various mRNA species (Auld et al., 1988; Zhou et al., 1991; Ukomadu et al., 1992; Krafte et al., 1990) or the rat brain Na^+ channel β_1 subunit cRNA (Isom et al., 1992), accelerates the kinetics of inactivation and results

in an increase in the surface expression of the Na^+ current (Auld et al., 1988; Zhou et al., 1991; Isom et al., 1992). The latter observations indicate that LMW subunits or regulatory components encoded by poly(A)$^+$ mRNA species may modulate function in some Na^+ channels. Similar results have been reported after coexpression of the α_1 subunit of cardiac Ca^{2+} channel along with its LMW subunits (Singer, Biel, Lotan, Flockerzi, Hofmann, and Dascal, 1991).

In conclusion, our results indicate that one or more mammalian regulatory factors modify the expression and kinetics of certain subthreshold A-type K^+ channels and that these factors are encoded by 2–4 kb poly(A)$^+$ mRNA species. It is not clear, however, whether these factors are structural subunits or regulatory enzymes or both.

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