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Functional Characterization of a Minimal K⁺ Channel Expressed from a Synthetic Gene

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ABSTRACT: A gene for a slowly activating, voltage-dependent K⁺-selective ion channel was designed and synthesized on the basis of its known amino acid sequence. The synthetic gene was cloned into a transcription vector, and in vitro transcribed mRNA was injected into *Xenopus* oocytes for electrophysiological assay of the resulting ionic currents. The currents are voltage-dependent and highly selective for K⁺ over Na⁺. The selectivity among monovalent cations follows a familiar K⁺-channel sequence: K⁺ > Rb⁺ > NH₄⁺ > Cs⁺ >> Na⁺, Li⁺. The currents are inhibited by Ba²⁺, Cs⁺, and tetraethylammonium (TEA), common pore blockers of K⁺ channels. Open-channel blockade by Cs⁺ (but not by Ba²⁺ or TEA) depends on applied voltage. The major inhibitory effect of Ba²⁺ is to alter channel gating by favoring the closed state; this effect is specific for Ba²⁺ and is relieved by external K⁺. The results argue that although the polypeptide expressed is very small for a eukaryotic ion channel, 130 amino acid residues in length, the ionic currents observed are indeed mediated by a genuine K⁺-channel protein. This synthetic gene is therefore well suited for a molecular analysis of the basic mechanisms of K⁺-channel function.

Voltage-dependent K⁺ channels are centrally involved in the generation of electric signals in excitable cell membranes (Hille, 1984). In order to fulfill their physiological duties, channels of this class must carry out two essential tasks. First, in response to changes in membrane voltage, the channel protein must alter its conformation to form a water-filled transmembrane pore through which ions can diffuse passively. Second, the open pore thus formed must discriminate strongly among inorganic ions, allowing rapid permeation for K⁺ but preventing Na⁺ from traversing the pore. Very little is known about the molecular mechanisms by which these tasks are accomplished; only in the last few years have cDNA clones for K⁺ channels become available (Papazian et al., 1987; Pongs et al., 1988; Kamb et al., 1988; Frech et al., 1989; Stuhmer et al., 1989; Butler et al., 1990), and work on structure-function relations through site-directed mutagenesis is only now beginning (Hille, 1984; Ruppersberg et al., 1990; MacKinnon & Miller, 1989; Isacoff et al., 1990; MacKinnon & Yellen, 1990; Hoshi et al., 1990). Nearly all K⁺ channels for which genes have been identified belong to a single molecular family consisting of rather large (~70-kDa) polypeptides that span the membrane at least six times. The

functional channel is believed to be formed as a tetramer of these 70-kDa subunits (Catterall, 1988; Jan & Jan, 1989; MacKinnon, 1991).

Recently, several groups employing heterologous expression in *Xenopus* oocytes cloned a gene putatively coding for a voltage-dependent K⁺ channel from mammalian kidney, uterus, and heart (Takumi et al., 1988; Murai et al., 1989; Folander et al., 1990; Pragnell et al., 1990). The molecular characteristics of this cDNA are unprecedented among all other known K⁺ channels. The gene codes for a very small (~15-kDa) polypeptide of only 130 amino acids. Moreover, hydropathy analysis of this polypeptide identifies only a single membrane-spanning α -helix (Takumi et al., 1988). These remarkable properties have prompted workers in the field to name this channel "minK" (minimal K).

There are currently two points of fundamental uncertainty about the nature of this unusual clone. First, it is still unclear whether the K⁺ currents induced in oocytes by expression of this cDNA are in fact mediated by a channel-type K⁺ transporter; since direct observations of single channels underlying this K⁺ current have not yet been achieved, it is still possible that a "carrier-type" mechanism is responsible.

Second, it has not been rigorously shown that this cDNA codes for the K⁺ transporter itself; the cDNA could code for an unspecified "regulator" of a K⁺ transporter endogenous to the oocytes but not normally active. Although either of these alternatives would be highly unusual, the minK cDNA is so unlike any other ion channel that these possibilities should be taken seriously.

In order to approach the molecular nature of the K⁺ currents elicited by the minK polypeptide, we have designed and constructed a synthetic gene coding for minK. Using this gene, we address the first fundamental uncertainty about the minK currents in oocytes: Are these currents mediated by a channel-type mechanism? Our results show that the K⁺ currents display functional properties of voltage-dependent gating, ion selectivity, and specific block similar to those seen in many K⁺ channels but unknown in carrier-type K⁺ transporters.

MATERIALS AND METHODS

Design, Synthesis, and Construction of the MinK Channel Gene. The minK channel gene was designed by use of the constraints for construction of synthetic genes to maximize the number of unique restriction sites in the coding sequence. (Oprian et al., 1987). Seven overlapping oligonucleotide duplexes of approximately 60 base pairs in length were synthesized on an Applied Biosystems 380A DNA synthesizer and used in the construction of the gene. Each oligonucleotide was gel purified, phosphorylated to low specific activity, and analyzed for purity. These were annealed and ligated in a single reaction by T4 DNA ligase to form the full-length gene. The gene was then cloned into the *EcoRI*–*Bam*HI sites of pSP65 to form the plasmid pSF101 and propagated in *Escherichia coli* strain DH1. The sequence of the synthetic gene was confirmed by DNA sequencing of both strands. RNA was prepared by standard in vitro transcription methods with the SP6 polymerase Riboprobe kit (Promega) supplemented with the capping nucleotide 5'GpppG5' (Pharmacia). The template for transcription was pSP101 linearized with *Sal*I.

Oocyte Injection and Electrophysiology. Female *Xenopus laevis* were obtained from *Xenopus* I. Oocytes were harvested, treated with collagenase (Gibco) for removal of the follicle layer, and incubated at 17 °C in a solution of 96 mM NaCl, 5 mM Hepes (pH 7.6), 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 50 µg/mL gentamicin. Defolliculated stage V and VI oocytes were microinjected with 0.4 ng (0.01 µg/µL) of in vitro transcribed RNA. Macroscopic currents were recorded 2 days postinjection by use of a computer-controlled two-electrode voltage clamp (Axoclamp 2A; Axon Instruments, Burlingame, CA). Electrodes with resistances of 0.5–2.5 MΩ were filled with 3 M KCl. The external solution was a low-Ca²⁺ saline composed of 96 mM NaCl, 5 mM Hepes (pH 7.6), 2 mM KCl, 1 mM MgCl₂, and 0.3 mM CaCl₂, which was continuously perfused throughout the experiment. For experiments under bionic conditions, the K⁺ and Na⁺ in the solution were replaced with other monovalent cations. All experiments were done at 21–23 °C.

RESULTS

Construction of the Synthetic Gene. The synthetic gene for the minK channel was designed from the amino acid sequence deduced from the natural cDNA originally cloned from rat kidney (Takumi et al., 1988). We exploited the degeneracy of the genetic code to introduce 40 unique restriction enzyme sites into the sequence, as shown in Figure 1. The 5' untranslated region of the natural gene was deleted and replaced with a Kozak consensus sequence for eukaryotic expression (Kozak, 1989). The gene was flanked with nested restriction

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EcoRI      BglI/HcoI/HaeII      ScaI
GAATTCGCGCCGACC ATG GCG CTG TCG AAC TCG ACA ACA GTA CTG CCT
              Met Ala  Leu Ser Asn  Ser  Thr Thr Val Leu Pro

              NheI              BanII/SmaI
TTT CTG GCT AGC CTG TGG CAG GAA ACA GAT GAG CCC GGG GGC AAT
Phe Leu  Ala Ser  Leu Trp  Gln  Glu Thr  Asp Glu Pro Gly Gly Asn

              PstI              PvuI      PvuII
ATG TCT GCA GAC CTG GCG CGC CGA TCG CAG CTG CGA GAT GAC AGC
Met Ser  Ala Asp  Leu Ala  Arg  Arg  Ser  Gln Leu Arg  Asp Asp Ser

              XhoI/PaeR71/DraII/Eco0109/PaeI      AclI/StyI      MboII
AAG CTC GAG GCC CTG TAT ATC CTC ATG GTC CTT GGT TTC TTC GGC
Lys Leu  Glu  Ala  Leu Tyr  Ile Leu Met  Val Leu Gly  Phe Phe Gly

              HphI              DdeI/MaeIII      BglII/XhoII
TTT TTC ACC CTC GGG ATC ATG CTG AGT TAC ATC AGA TCT AAA AAG
Phe Phe  Thr Leu Gly  Ile Met  Leu Ser Tyr  Ile  Arg Ser Lys Lys

              AccI              HinfI/FokI
CTG GAA CAC TCG CAC GAC CCT TTC AAC GTC TAC ATC GAG TCG GAT
Leu Glu  His  Ser  His Asp  Pro  Phe Asn Val Tyr  Ile  Glu Ser Asp

              NsiI              HindIII      MluI      XbaI
GCA TGG CAG GAG AAA GGC AAA GCT TTG TTC CAG GCA GCG GTT CTA
Ala Trp  Gln Glu  Lys  Gly Lys  Ala Leu Phe  Gln Ala  Arg Val Leu

              SphI      SnaBI              SacII
GAG AGC TTC AGA GCA TGC TAC GTA ATT GAA AAC CAG GCC GCG GTA
Glu Ser  Phe Arg  Ala  Cys Tyr Val  Ile  Glu Asn Gln Ala Ala Val

              BspHI              BspHII      SacI/HglAI
GAA CAA CCT GCC ACC CAC CTT CCG GAG CTC AAG CCA CTG TCA TGA
Glu Gln Pro Ala Thr  His Leu Pro  Glu Leu Lys  Pro Leu Ser End

NotI XmaIII/BamHI
GCGGCCGCGATCC

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FIGURE 1: Sequence of the synthetic minK gene. The 5' untranslated region includes the Kozak consensus sequence for eukaryotic expression, GCGGCCACC. The putative membrane-spanning region is printed in bold and underlined. Unique restriction sites are printed above their recognition sequences.

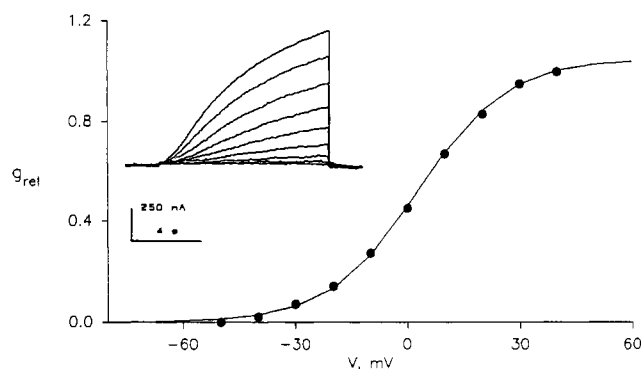


FIGURE 2: Expression of the synthetic gene in *Xenopus* oocytes. Currents were elicited in oocytes injected with 0.4 ng of cRNA by 10-s depolarizing pulses from a holding voltage of -80 mV; pulses were from -40 to +40 mV in 10-mV steps (inset). The voltage activation curve was derived by normalizing minK-mediated conductance to its value at 40 mV, after leak subtraction and measurement of reversal potential for each oocyte. The solid curve is drawn according to $g_{rel} = g_{max}[1 + \exp(z'F(V - V_0)/RT)]^{-1}$, with $g_{max} = 1.05$, $z' = 2.1$, and $V_0 = 3$ mV (Labarca et al., 1980).

sites for ease of insertion into different expression vectors. For in vitro transcription, we constructed a plasmid, pSF101, in which the minK gene is placed behind the promoter for SP6 RNA polymerase.

Expression of the Synthetic Gene in *Xenopus* Oocytes. Two days after injection of mRNA, *Xenopus* oocytes were assayed for voltage-dependent K⁺ currents via standard two-electrode voltage clamp methods. Oocytes were held at -80 mV and depolarized for 5 or 10 s to voltages from -40 to +40 mV. We observed a slowly activating voltage-dependent outward current (Figure 2, inset) not present in uninjected cells. This current appears identical with that seen in oocytes injected with the native uterine mRNA or cRNA (Boyle et al., 1987; Takumi et al., 1988; Folander et al., 1990; Pragnell et al., 1990).

The normalized voltage activation curve is shown Figure 2. Here, we present the conductance 10 s after switching to

Table I: Ionic Selectivity of MinK Currents^a

	K	Rb	NH ₄	Cs	Na	Li
V_{rev} (mV)	-1 ± 1	-11 ± 1	-44 ± 2	-70 ± 1	<-151 ± 9	<-155 ± 8
P_K/P_X	1.1 ± 0.1	1.5 ± 0.1	5.9 ± 0.4	16.3 ± 0.5	>400	>400

^a Reversal potentials were measured under nearly biionic conditions, with 98 mM of the indicated cation in the external solution. Values represent the mean ± SEM of four to eight oocytes. Permeability ratios are defined as $P_K/P_X = \exp(-FV_{rev}/RT)$.

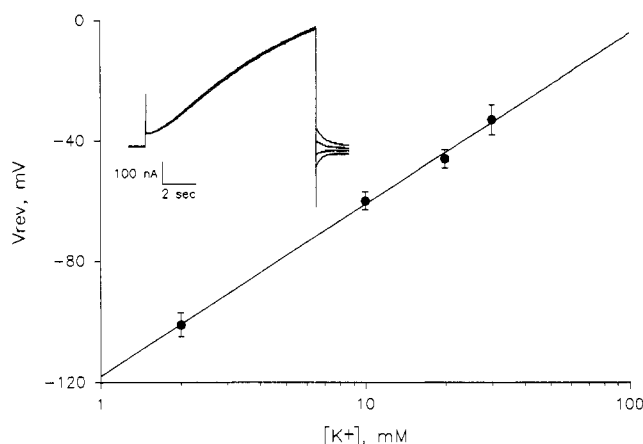


FIGURE 3: Reversal potentials of minK tail currents. Cell were depolarized to +30 mV for 5 s, and tail currents were observed by repolarizing to voltages near the reversal potential (inset). External solutions contained the indicated K⁺ concentrations (adjusted by replacement for Na⁺). The solid line is drawn with a slope of 58 mV/decade.

a test voltage, normalized to its value at 40 mV. We emphasize that because of the complicated kinetics of channel activation the conductance at 10 s is only a crude approximation to the steady-state value, so this activation curve is not an equilibrium measurement. Nevertheless, channel activation is clearly voltage-dependent. As the membrane is depolarized, the conductance increases with an *e*-fold enhancement per 12-mV depolarization and approaches saturation by 40 mV. In 14 such experiments, the voltage at which the conductance is 50% activated is -5 ± 2 mV. The first report of this current (Boyle et al., 1987) highlighted its complicated activation kinetics, demonstrating a strong "memory effect" of the holding potential. We have also observed this phenomenon and have accordingly employed a 10-s interpulse "resting" period and a single holding potential in all experiments.

Ionic Selectivity. Previous studies showed qualitatively that this channel is selective for K⁺ over Na⁺ (Boyle et al., 1987; Takumi et al., 1988; Folander et al., 1990; Pragnell et al., 1990). We carried out several experiments to determine in finer detail the selectivity of the channel for K⁺ over other ions. Oocytes were held at -80 mV and pulsed to a positive potential to open the channels. The oocytes were then repolarized to various test voltages, and the initial slopes of the "tail currents" were examined to determine the reversal potential, the voltage at which the currents reversed from outward to inward (Figure 3, inset). As external K⁺ is varied (Figure 3), the reversal potential exhibits precise Nernstian behavior, varying 58 mV per 10-fold change in K⁺ concentration; the channel is therefore highly selective for K⁺ over both Na⁺ and Cl⁻.

To assess the channel's selectivity among monovalent cations, we measured the tail-current reversal potential under biionic conditions, i.e., with a single species of monovalent cation in the external solution and K⁺ as the major internal cation. The measured reversal potentials and calculated "permeability ratios" for the different monovalent cations are shown in Table I. These potentials demonstrate a selectivity sequence conventionally observed for virtually all known K⁺

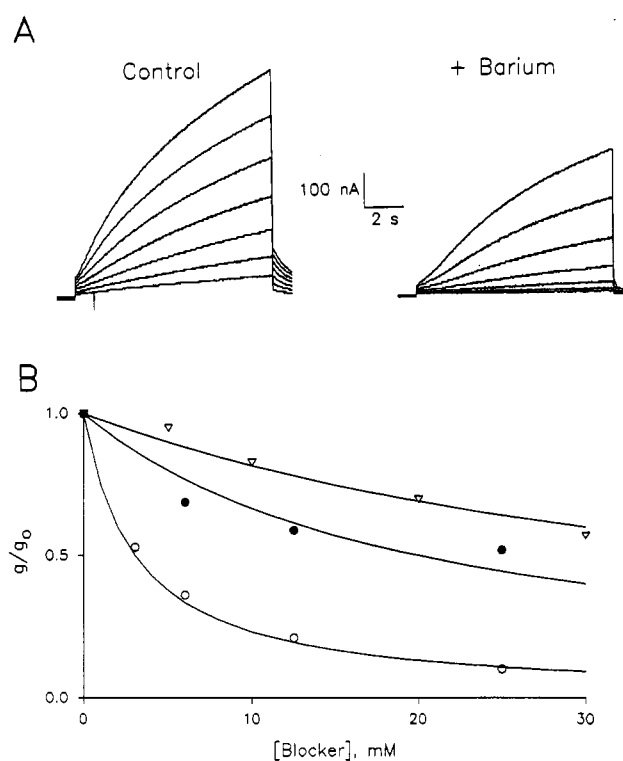


FIGURE 4: Steady-state block of minK currents by Ba²⁺, Cs⁺, and TEA. Panel A shows raw records in the absence or the presence of 5 mM Ba²⁺. Currents were elicited by 10-s pulses to voltages of -40 to +20 mV in 10-mV steps. Panel B shows relative inhibition curves for the blockers TEA (Δ), Cs⁺ (●), and Ba²⁺ (○), measured at zero voltage. Each data set is from a single oocyte but is representative of the entire body of data (Table II).

channels: K⁺ > Rb⁺ > NH₄⁺ > Cs⁺ >> Na⁺, Li⁺ (Latorre & Miller, 1983; Yellen, 1987; Adams, 1989). By reversal potential measurement, the channel is at least 400-fold more permeable to K⁺ than to Na⁺.

Block by Ba²⁺, Cs⁺, and TEA. We examined the effect on the minK channel of Ba²⁺, Cs⁺, and tetraethylammonium (TEA), common pore blockers of many K⁺ channels (Latorre & Miller, 1983; Yellen, 1987). To distinguish open-channel block from effects on channel gating, we used two different protocols to study the inhibitory effects of these agents. With the "steady-state" method, oocytes were held at -80 mV and depolarized to various test potentials in the absence or the presence of blocker. The currents measured after a 5-s pulse reflect the blocker's action on both open-channel current and channel gating. Both Ba²⁺ and Cs⁺ reduce steady-state currents effectively (Figure 4), with zero-voltage inhibition constants of 2.6 and 21 mM, respectively; external TEA inhibits weakly ($K_i > 45$ mM).

If these blockers act by binding reversibly within the conduction pore, their effects should depend on applied voltage (Woodhull, 1973; Coronado & Miller, 1979)

$$K_i(V) = K_i(0) \exp(z\delta FV/RT) \quad (1)$$

where $K_i(0)$ is the zero-voltage inhibition constant, *z* is the valence of the blocking ion, and δ is the fraction of the applied voltage drop experienced at the blocker's binding site. Table

Table II: Blocking Parameters for Three K⁺-Channel Blockers^a

	steady state		instantaneous	
	K _i (0) (mM)	δ	K _i (0) (mM)	δ
Ba ²⁺	2.4 ± 0.3	0.72 ± 0.03	3.6 ± 0.1	0.08 ± 0.01
Cs ⁺	21 ± 3	0.38 ± 0.04	40 ± 2	0.39 ± 0.01
TEA	>45	<0.15	nd	nd

^aInhibition of minK currents was measured at various concentrations of Ba²⁺, Cs²⁺, or TEA in the external solution. Following eq 1, the inhibition constant at zero voltage, K_i(0), and electrical distance, δ , are reported for each blocker. For steady-state blockade, current was measured 5 or 10 s after depolarization to potentials in the range -40 to +40 mV. In the instantaneous protocol, channels were opened for 5 s at 20 mV and then currents were measured 25 ms after repolarizing to test potentials in the range -50 to +20 mV. In both cases, block was calculated by normalization to conductance in the absence of blocker, after correction for leak currents; minK reversal potentials were measured in each experiment. Each value represents the mean ± SEM of three to six oocytes. Equation 1 does not strictly apply to block by Cs⁺, since this ion is slightly permeant. However, it may be used as a good approximation, as has been documented in a different K⁺ channel (Cukierman et al., 1985). Instantaneous blockade by TEA was not done (nd).

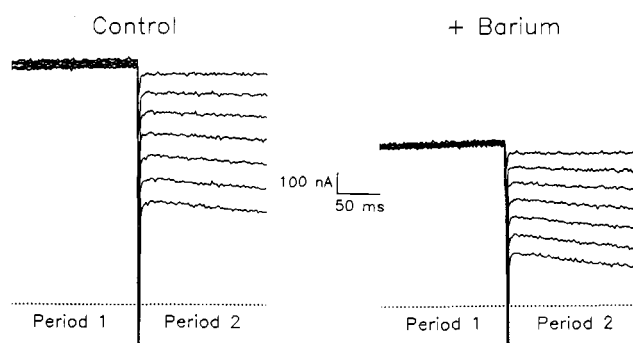


FIGURE 5: Instantaneous protocol for block of minK currents. Raw records in the absence or the presence of 5 mM Ba²⁺ are shown. Oocytes were held at -80 mV, depolarized to 20 mV for 5 s (period 1, final 150 ms displayed), repolarized to test potentials (-50 to +10 mV in 10-mV steps) for 300 ms (period 2, final 150 ms displayed), and then returned to -80 mV. For simplicity, currents are displayed only for periods 1 and 2. The dashed line represents the zero-current level.

II summarizes the effects of voltage on the blockers. While TEA inhibition is only weakly voltage-dependent ($\delta < 0.15$), both Cs⁺ and Ba²⁺ display strong voltage-dependent block ($\delta = 0.38$ and 0.72 , respectively). The simplest interpretation of these results is that TEA binds near the externally facing pore entrance, that Cs⁺ blocks part of the way through the pore, and that Ba²⁺ reaches a blocking site deeply within the pore.

We used a second protocol to test this interpretation; the effect of blockers on the "instantaneous" current-voltage relation confirms the voltage-dependent block of Cs⁺ but shows that Ba²⁺ inhibition operates mainly by a different mechanism (Table II; Figure 5). Here, channels were opened for 5 s at 20 mV and then currents were examined 25 ms after voltage was shifted to various test potentials, a time too short for channel gating to occur. Ba²⁺ inhibition of this 25-ms current (Figure 5) is nearly independent of voltage, in striking contrast to the steady-state current, which is 9-fold more sensitive to voltage (Table II). The inhibition constant for Ba²⁺ is similar with both methods because Ba²⁺ effects on gating are not removed by this analysis but simply held constant during the 25-ms analysis pulse.

We are forced to conclude, then, that Ba²⁺ inhibits minK currents through an effect on channel gating, i.e., by favoring the channel's closed state. The tail currents in Figure 4 clearly show that Ba²⁺ accelerates the rate of channel closing upon

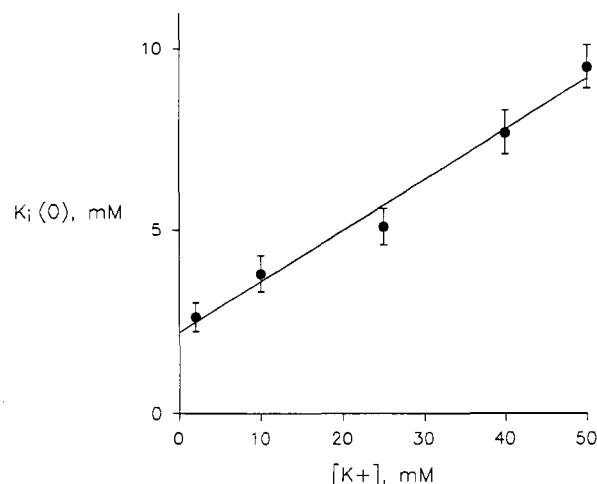


FIGURE 6: Competition for a site in minK by Ba²⁺ and K⁺. The inhibition constant for Ba²⁺ at 0 mV was measured at 2, 10, 25, 40, and 50 mM external K⁺; data are from three to six oocytes.

repolarization to -80 mV, as expected if the blocking ion "shifts" the voltage-activation curve toward more positive potentials. This is a specific effect of Ba²⁺ and not a result of surface charge screening (Frankenhauser & Hodgkin, 1956), since neither Mg²⁺ nor Ca²⁺ affects minK currents (data not shown). We do not know the site of Ba²⁺ action. However, we favor the idea that it binds in a pore-associated region of the channel, since its effects are relieved competitively by external K⁺, as shown in Figure 6.

DISCUSSION

In recent years, the union of channel biophysics and molecular genetics has initiated a determined effort to understand basic ion-channel mechanisms in molecular detail (Miller, 1989; Jan & Jan, 1989). This undertaking is hampered by the large size and complexity of most channel proteins, which are built from polypeptides in the range of 50–400 kDa. If minK is in fact a structural protein for a K⁺ channel, this channel will be far simpler at the molecular level than any other known ion channel. Such a "minimal" voltage-dependent K⁺ channel would provide an advantageous focus for structure-function studies on the fundamental mechanisms of K⁺ channels.

Our present results strongly suggest that the K⁺ currents induced in frog oocytes by the expression of minK mRNA are specifically mediated by a channel-type transport protein. We have characterized these currents in detail to compare them to the properties of other known K⁺ channels. The fundamental similarities are striking in three separate areas: gating, ion selectivity, and blocker pharmacology. First, the activation of current by depolarizing voltages is commonly observed with numerous K⁺ channels. The voltage dependence of minK currents, *e*-fold activation per 12-mV depolarization, is in the range found for several different types of K⁺ channels, such as classical delayed rectifiers (Hodgkin & Huxley, 1952), *Shaker* A-type channels (Zagotta & Aldrich, 1990), *Shaker* homologues *shal* and *shaw* (Butler et al., 1990) high-conductance Ca²⁺-activated K⁺ channels (Barrett et al., 1982; Moczydlowski & Latorre, 1983; Magleby & Pallotta, 1983), and sarcoplasmic reticulum K⁺-selective channels (Labarca et al., 1980), which give *e*-fold activation for 4, 4, 7, 15, 11, and 23 mV of depolarization, respectively. The kinetics of activation displayed by the minK currents are much slower than for neuronal K⁺ channels but are conventional in their voltage dependence. This type of time- and voltage-dependent

activation of K⁺ currents has been observed only for channel-type transporters; it has not been described for any carrier-type transport system, such as an energy-requiring "pump" or a coupled cotransporter.

The ion selectivity sequence of the minK currents, K⁺ > Rb⁺ > NH₄⁺ > Cs⁺ >> Na⁺, Li⁺, is identical with that found in virtually all known K⁺ channels. As a class, K⁺ channels are defined by a strong discrimination for K⁺ over Na⁺, as is seen here. Such channels also allow passage of the close K⁺ analogues Rb⁺ and NH₄⁺, though with lower permeabilities (Latorre & Miller, 1983). Furthermore, K⁺ channels only grudgingly permit Cs⁺ permeation, even though this cation is just slightly larger in crystal radius than K⁺. While this selectivity profile is familiar for K⁺ channels, it is not standard for other types of K⁺-selective transport proteins, some of which utilize Rb⁺ and NH₄⁺ even more efficiently than K⁺ (Shaffer et al., 1977; Gache et al., 1979). Finally, minK-induced K⁺ currents are reversibly inhibited by three ions, Ba²⁺, Cs⁺, and TEA, known to act in the millimolar range as blockers of many K⁺ channels (Latorre & Miller, 1983). Inhibition by Cs⁺ is voltage-dependent, as expected if the blocking site is located within the K⁺ conduction pathway and as seen in all K⁺ channels (Yellen, 1987). The specific inhibition by Ba²⁺, however, operates on the voltage-dependent gating process. Though this kind of effect is less common than simple pore blocking, Ba²⁺ has been observed to alter gating of other K⁺ channels by binding in the pore (Armstrong & Taylor, 1980; Miller et al., 1987). While Ba²⁺ does inhibit K⁺ transport in carriers such as the Na/K/Cl coupled cotransporter and the Na/K ATPase, this phenomenon is not specific to Ba²⁺, but can be seen with other divalent cations (Kim et al., 1988; Gache et al., 1979).

In the absence of direct single-channel observations, the case presented by these three lines of evidence is only circumstantial; nevertheless, we consider it strong. Accordingly, we conclude that the K⁺-selective currents induced by minK mRNA are brought about by a voltage-dependent K⁺-channel protein. It is still not known whether the minK protein forms the channel itself or only regulates an endogenous "silent" K⁺ channel in oocytes. However, preliminary results (S. Goldstein and C. Miller, unpublished results) showing that point mutations in the minK gene alter specific functional properties of the currents, including voltage-dependent gating and ion selectivity, imply that the expressed protein forms an integral part of the channel itself. We are currently pursuing the purification and functional reconstitution of the expressed minK protein to test this proposal directly. We anticipate that the use of the synthetic gene described here, with its many unique restriction sites, will facilitate future structure-function investigations of this minimal K⁺ channel.

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Characterization of a Putative Clone for the 67-Kilodalton Elastin/Laminin Receptor Suggests That It Encodes a Cytoplasmic Protein Rather than a Cell Surface Receptor[†]

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ABSTRACT: The 67-kDa elastin binding protein shares many immunological and structural properties with the high-affinity 67-kDa tumor cell laminin receptor. Taking advantage of these similarities, we have screened a bovine cDNA library with a partial cDNA probe for the laminin receptor and have isolated and characterized a cDNA clone of 1038 bp that hybridizes to a single-size mRNA of 1.3 kb. The clone encodes a protein with a predicted molecular weight of 33K that lacks an N-terminal leader sequence, shows no posttranslational processing when translated in vitro in the presence of microsomes, and does not bind to elastin affinity columns. Although the bovine clone is nearly identical with clones encoding human and mouse proteins reported to be 67-kDa laminin receptor, physical and functional characteristics of the encoded protein suggest that it is a cytoplasmic protein that does not bind elastin. This finding calls into question the earlier conclusion that the clone encodes the 67-kDa receptor.

It is becoming increasingly evident that most extracellular matrix (ECM) macromolecules interact with specific binding proteins on the surface of cells. The best characterized receptors are members of the integrin family (Hynes, 1987), although other matrix binding proteins have been identified. One component of the extracellular matrix that lacks Arg-Gly-Asp (RGD) sequences and does not interact with integrin receptors is elastin, an abundant ECM component of elastic tissues (Mecham et al., 1989a). Instead, elastin associates with one or more cell surface proteins that exhibit properties of typical receptors as assessed by high-affinity binding that is both saturable and reversible (Blood et al., 1988; Hornebeck et al., 1986; Mecham et al., 1989a). One of these elastin receptors is a 67-kDa protein that is structurally and functionally similar to, if not identical with a high-affinity 67-kDa laminin receptor (Graf et al., 1987; Lesot et al., 1983; Rao et al., 1983; Terranova et al., 1983).

cDNA clones thought to encode the 67-kDa protein have been identified and characterized. Using a monoclonal antibody generated against the 67-kDa laminin binding protein, Wewer et al. (1986) isolated a partial clone (clone pLR4-4) from a human umbilical vein endothelial expression cDNA

library. This partial sequence was then used to select full-length clones from a transformed murine fibroblast cDNA library [clone pMLR21 (Rao et al., 1989)]. Yow et al. (1988) characterized a human cDNA clone, J9, selected because of its increased expression in colon carcinomas. J9 was found to have extensive homology with the mouse cDNA, suggesting that receptor sequences are highly conserved between species.

Because the 67-kDa elastin binding protein exhibits immunological and functional similarity to the laminin receptor, we used a partial cDNA clone to the laminin receptor to screen a cDNA library made from mRNA isolated from bovine ligamentum nuchae. We felt that there was a high likelihood that selected hybridizable clones would be the elastin receptor because the predominant cell type in the ligament is a fibroblast that produces elastin but not laminin. In this report, we describe the isolation, characterization, and DNA sequence of a bovine clone that hybridizes with the putative laminin receptor probe. One clone is highly homologous to the laminin receptor clones, but functional and physical characteristics suggest it encodes a cytoplasmic protein that lacks a transmembrane domain. This protein neither binds elastin nor undergoes posttranslational processing that might account for the difference in size between the encoded protein and the isolated elastin/laminin receptor.

MATERIALS AND METHODS

Materials. T₄ DNA ligase, T₄ DNA polymerase, exonuclease III, SP6 RNA polymerase, pGEM-4Z, rabbit reticulocyte lysate, canine pancreatic microsomes, and mRNAs encoding the *Escherichia coli* β -lactamase and *Saccharomyces cerevisiae* α -factor were obtained from Promega (Madison, WI). EcoRI, BamHI, Klenow DNA polymerase, and DNA polymerase I were from New England Biolabs (Beverly, MA).

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