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Subunit Composition of MinK Potassium Channels

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Summary

Expression of minK protein in Xenopus oocytes induces a slowly activating, voltage-dependent, potassium-selective current. Point mutations in minK that alter current gating kinetics, ion selectivity, pharmacology, and response to protein kinase C all support the notion that minK is a structural protein for a channel-type transporter. Yet, minK has just 130 amino acids and a single transmembrane domain. Though larger cloned potassium channels form functional channels through tetrameric subunit association, the subunit composition of minK is unknown. Subunit stoichiometry was determined by coexpression of wild-type minK and a dominant lethal point mutant of minK, which reaches the plasma membrane but passes no current. The results support a model for complete minK potassium channels in which just two minK monomers are present, with other, as yet unidentified, non-minK subunits.

Introduction

Shaker-type voltage-activated potassium channels are multimeric protein complexes built through assembly of four identical or similar pore-forming transmembrane α subunits (MacKinnon, 1991; Shen et al., 1994). Cytoplasmic β subunits, which are not required for pore function but profoundly alter channel closing rates, appear to associate with α subunits with $\alpha_4\beta_4$ stoichiometry (Rettig et al., 1994). The multimeric nature of these channels was first demonstrated as hybrid channel behavior upon coexpression in one cell of two pharmacologically or kinetically distinguishable α subunits (Ruppersberg et al., 1990; Isacoff et al., 1990; Christie et al., 1990). That four α subunits aggregate to form functional channels was first demonstrated by determining the fraction of toxin-resistant channels resulting from coexpression of toxin-sensitive and toxin-insensitive Shaker channel α subunits (MacKinnon, 1991) and has been supported extensively (MacKinnon et al., 1993).

First cloned from rat kidney (Takumi et al., 1988), minK has been identified as a single copy gene in rat, mouse, guinea pig, and human (Murai et al., 1989; Iwai et al., 1990; Folander et al., 1990; Lesage et al., 1992; Zhang et al., 1994). The protein is expressed in numerous mammalian tissues, including the kidney, duodenum, T-lymphocytes, uterus, inner ear, and heart (Sugimoto et al., 1990; Sakagami et al., 1991; Honore et al., 1991; Attali et al., 1992). MinK appears to underlie the cardiac delayed rectifier I_{Ks}, which mediates myocardial repolarization (Sanguinetti and Jurkiewicz, 1990; Freeman and Kass, 1993; Varnum et al., 1993; Busch et al., 1994); it is subject to regulation during development (Boyle et al., 1987) and modulation by hormones and second messengers (Busch et al., 1992; Zhang et al., 1994; Blumenthal and Kaczmarek, 1994; for review see Swanson et al., 1993). Yet, minK is structurally distinct with no homology to other voltage-gated potassium channels. This suggests that minK channels will employ unique ion transport mechanisms or functional motifs.

In this paper, we take a quantitative subunit mixing approach to assess the number of functional minK monomers in complete minK potassium channels. Wild-type minK is coexpressed with a lethal minK mutant that inhibits normal channel function. Cell surface expression of wild-type and mutant minK subunits and minK-induced currents are studied. The results demonstrate, first, that the two subunit types express equally well in oocytes; second, that they express in independent fashion; and third, that a single mutant subunit in a channel complex acts dominantly to ablate function. Since the two minK proteins are handled equivalently by oocytes, the array of multimeric channels assembled from the two is expected to follow a simple binomial distribution, and the effect of incorporating mutant subunits can be used to evaluate minK subunit valence by the strategy of MacKinnon (1991).

Results

Expression of wild-type rat minK protein (WT) in Xenopus laevis oocytes leads to a slowly activating, voltage-dependent, potassium-selective current not present in uninjected cells (Figure 1; Takumi et al., 1988; Hausdorff et al., 1991). In contrast, expression of a minK point mutant in which aspartic acid 77 is altered to asparagine (D77N) yields no current across the same voltage range (Figure 1), as shown previously by Takumi and coworkers (1991). Figure 1 shows that when WT and D77N minK cRNAs are coinjected in a 1:1 ratio, oocytes express roughly one-fourth the current seen with WT minK cRNA alone. This result suggests that functional minK channels are formed as multimeric protein complexes whose function is inhibited by incorporation of D77N subunits. For this explanation to be correct, oocyte surface expression of WT and D77N minK proteins must be identical.

Equal and Independent Surface Expression of Subunits

To evaluate minK surface expression, a 9 amino acid epitope tag (c-*myc*) was inserted into the coding sequence for both WT and D77N minK proteins between residues 22 and 23, a region shown to be extracellular by Blumenthal and Kaczmarek (1994). The epitope does not appear to alter the expression level, activation, or ion selectivity of WT or D77N minK currents (Figure 1; data not shown).

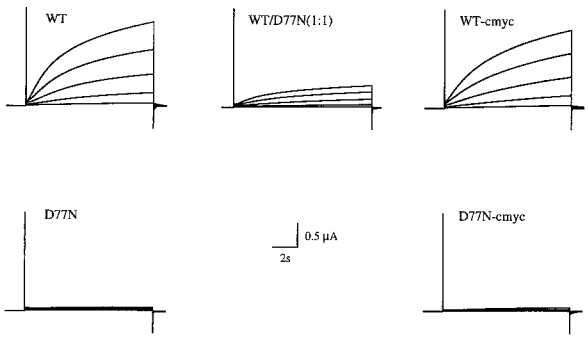

Figure 1. Expression of MinK-Induced Currents in Xenopus Oocytes

Currents were elicited by 10 s depolarizing pulses from −40 to 40 mV in 20 mV steps from a holding potential of −80 mV with a 5 s interpulse interval and are displayed without leak subtraction. Oocytes were injected with 2 ng minK cRNA for wild-type minK (WT), D77N minK (D77N), WT–c-*myc* minK (WT–c*myc*), D77N–c*myc* minK (D77N–c*myc*), or 2 ng WT and 2 ng D77N minK cRNAs (WT:D77N).

Figure 2A compares minK surface expression on groups of oocytes injected with increasing amounts of either D77N–c-*myc* or WT–c-*myc* minK cRNA. Surface levels were evaluated by binding of a monoclonal anti-c-*myc* antibody to individual intact oocytes. In fact, D77N minK protein is transported to and expressed on the oocyte surface in a dose-dependent manner that appears identical to WT–c-*myc* minK protein.

Despite attaining surface levels equal to WT minK, D77N minK protein expression yields no current over the entire dose range tested (Figure 2B). Over the same range, WT minK isochronal currents rise rapidly (Figure 2B). WT minK currents are observed to reach a stable plateau level above 0.1 ng injected cRNA, even though the level of wild-type protein on the surface continues to increase (Figure 2A), as previously described by Blumenthal and Kaczmarek (1994).

To assess whether oocytes exhibit a preference for surface expression of WT or D77N minK protein, surface levels were evaluated for oocytes coinjected with mixtures of WT–c-*myc* and D77N–c-*myc* minK cRNAs (Figure 3). Oocytes received a constant amount of cRNA containing ratios of WT–c-*myc* to D77N–c-*myc* from 8:1 to 1:8; surface levels of c-*myc* epitope remained constant. This shows that each protein has an equal probability of reaching the oocyte surface when the two cRNAs are coinjected and indicates that the mutant does not inhibit channel function by interfering with normal cellular processing of WT minK. Thus, expression of the two subunits is not only equal (see Figure 2), but also independent (Figure 3).

A Dominant Lethal Phenotype

When WT and D77N minK cRNAs are coinjected in a 1:1 ratio, the current that results is only a small fraction of that seen with WT minK cRNA alone (see Figure 1). This suggests that D77N mutant subunits have a dominant effect, such that incorporation of a single D77N subunit into a channel complex makes it nonfunctional. If this is the case, only fully WT minK channels will conduct, and currents that result from coinjection of WT and D77N minK

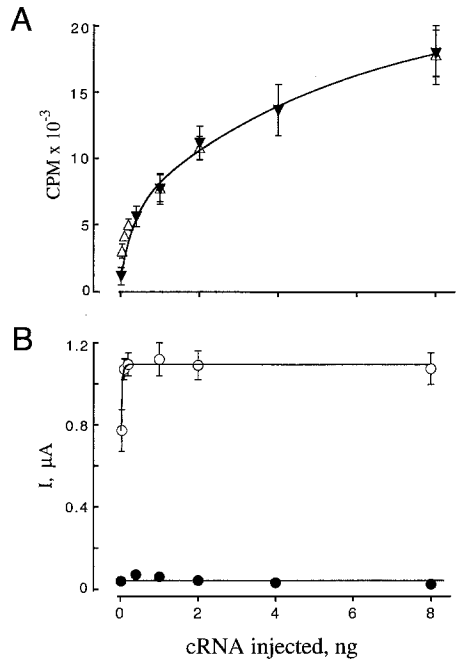

Figure 2. WT–c-*myc* MinK or D77N-c-*myc* MinK Surface Expression and Induced Currents

(A) CPM specific binding per oocyte for WT–c-*myc* (open triangles) or D77N–c-*myc* (closed triangles).

(B) Current per oocyte for WT–c-*myc* (open circles) or D77N-c-*myc* (closed circles). Surface minK expression was measured as described in Experimental Procedures. Isochronal currents were measured at the end of a 10 s test pulse to 20 mV from a holding voltage of −80 mV. To control for the sensitivity of rat minK-induced currents to temperature and repetitive stimulation, all oocytes were moved from 17°C to 21°C 1 day prior to study, placed into voltage clamp at −80 mV until holding current was less than 5 nA with constant perfusion, and stimulated once to the command potential. The three lowest doses of WT–c-*myc* minK cRNA injected were 0.05, 0.10, and 0.20 ng/oocyte. Points are mean ± SEM for groups of eight oocytes.

cRNAs would be expected to behave like wild-type channels in their pharmacology, ion selectivity, and gating kinetics. Table 1 shows this to be the case. Oocytes injected with WT minK cRNA or a WT:D77N minK cRNA mixture that results in ~60% of wild-type current level were studied. Both groups exhibit nearly identical sensitivity to block by barium, tetraethylammonium, cesium, and azimilide. Whereas barium and tetraethylammonium block minK near the membrane surface, cesium blockade occurs at a binding site inside the pore (Goldstein and Miller, 1991); the site of block by the class III anti-arrhythmic is unknown. All four channel blocking sites appear unaltered. This suggests that the local environment at these four positions is identical in the two groups. That the voltage dependence of cesium block is the same argues that this blocker binds in an equivalent position in the ion conduction pathways of both groups (Table 1).

Ion selectivity of currents expressed by oocytes injected with WT minK cRNA or the WT:D77N minK cRNA mixture also appeared to be identical. Tail current reversal potentials in control solutions were the same in the two groups and changed identically with external potassium concen-

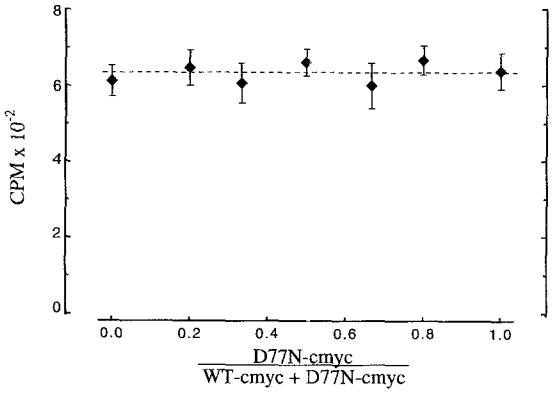

Figure 3. Surface Expression of WT–c-*myc* MinK and D77N–c-*myc* MinK Are Equal and Independent

Oocytes were injected with a constant amount of cRNA (2 ng) encoding WT–c-*myc* minK, or D77N-c-*myc* minK, or ratios of the two as indicated and specific binding evaluated as in Figure 2. Points are mean ± SEM for groups of eight oocytes.

tration in a strictly Nernstian fashion (Table 1). As expected, both groups were ideally selective for potassium over sodium and chloride (Goldstein and Miller, 1991).

The activation kinetics of minK currents are complex; minK currents do not saturate; they exhibit sensitivity to cRNA dose and time after injection and can be described only by stipulating both pulse duration and the specific experimental condition under study (Hice et al., 1994; Cui et al., 1994; Blumenthal and Kaczmarek, 1994). Activation kinetics, evaluated here only qualitatively using a simple ratio of time-dependent current amplitudes, were found to be the same in oocytes injected with WT minK cRNA or the WT:D77N minK cRNA mixture (Table 1). Deactivation kinetics are insensitive to expression level (Blumenthal and Kaczmarek, 1994) but sensitive to amino acid differences present between minK isolates (Hice et al., 1994) and are well described as a biexponential decay. Oocytes injected with WT minK cRNA or the WT:D77N minK cRNA mixture show identical deactivation kinetics (Table 1). That all four external and pore blocking sites were unaltered by subunit mixing, and that the potassium selectivity determining regions and activation and deactivation determining domains behaved identically in the two groups argues strongly that only fully WT channels were under study in both groups of oocytes, and thus that channels with one or more D77N subunits were silent.

Binomial Analysis

Given that WT and D77N minK proteins are handled identically and independently by oocytes and that only fully WT channels conduct, how should increasing amounts of D77N minK cRNA be expected to influence the current seen if a constant amount of WT minK cRNA is coinjected? The array of multimeric channels assembled are expected to follow a binomial distribution (MacKinnon, 1991), and the measured current (I) to be given by the sum of the currents contributed by each species of channel i:

Table 1. Blocking Parameters, Potassium Selectivity, and Gating Kinetics of Currents Induced in Oocytes by WT MinK or WT:D77N MinK Coexpression

	WT	WT:D77N
Ba^{2+} (K_i, mM, 0 mV)	1.6 ± 0.1	1.5 ± 0.1
TEA (K_i, mM, 0 mV)	97 ± 5	82 ± 11
Azimilide (K_i, μM, 0 mV)	2.8 ± 0.5	3.0 ± 0.4
Cs^+ (K_i, mM, −40 mV)	18 ± 3	16 ± 4
Cs^+ (δ)	0.34 ± 0.01	0.33 ± 0.01
V_{rev}, mV (2 mM K_{ext})	88 ± 2	88 ± 3
ΔV_{rev}, mV (2–20 mM K_{ext})	59 ± 1	58 ± 3
Activation		
$I_{10\,s}/I_{2\,s}$	3.0 ± 0.1	3.2 ± 0.1
Deactivation		
τ_1 (ms)	760 ± 40	790 ± 60
τ_2 (ms)	2940 ± 110	2920 ± 70
$A_1/(A_1 + A_2)$	0.12 ± 0.02	0.10 ± 0.01

Oocytes were injected with 2 ng WT minK cRNA (WT) or with 2 ng WT and 0.5 ng D77N minK cRNA (WT:D77N); coinjection yields 0.63 ± 0.04 of WT minK-induced current. Zero-voltage inhibition constants (K_i, 0 mV) were determined by repetitive pulses to 0 mV from a holding voltage of −80 mV and measurement of currents at the end of each 10 s pulse in the absence and presence of blockers; block is calculated after leak subtraction (Goldstein and Miller, 1991). Tail current reversal potentials and voltage-dependent blockade were studied by opening channels with a 10 s pulse to 20 mV and measuring current 25 ms after shifting to test potentials of from −110 to −10 mV (V_{rev}) or −50 to 10 mV (block), as described previously (Goldstein and Miller, 1991). Electrical distance (δ) was calculated from $K_i(V) = K_i(0)\exp(z\delta FV/RT)$, 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 (Woodhull, 1973; Coronado and Miller, 1979). Activation kinetics were evaluated by opening channels with a 10 s test pulse to 20 mV from −80 mV and taking the ratio of macroscopic currents at 10 and 2 s; deactivation kinetics were studied by the same protocol in the presence of 20 mM external KCl. Deactivation was fit according to $I(t) = A_0 + A_1 \exp(t/\tau_1) + A_2\exp(t/\tau_2)$ where A_0 represents the current at steady state, and A_1 and A_2 the amplitudes of the components represented by time constants τ_1 and τ_2, respectively. All controls were performed in ND-96 with 2mM KCl as described in Experimental Procedures; inhibition constants were determined after isotonic substitution of NaCl by 5 mM barium chloride (Ba^{2+}), or 75 mM tetraethylammonium chloride (TEA), or 50 mM cesium chloride (Ca^+), or upon addition of 5 μM azimilide (Busch et al., 1994); external potassium was altered by isotonic substitution of NaCl with 18 mM KCl. All values are the mean ± SEM for 3–6 oocytes.

$$I = \sum_{i=0}^{n} F_i Z_i I_{WT} \quad (1)$$

where F_i is the fraction of channels that are i type, Z_i is the fraction of wild-type current passed by channels that are i type, and I_{WT} is the macroscopic current measured when all channels in the oocyte are wild type. F_i is given by the binomial distribution

$$F_i = \binom{n}{i} f_{WT}^{i} f_{D77N}^{(n-i)} \quad (2)$$

where f_{WT} and f_{D77N} are the fractions of wild-type and D77N-type subunits, and n is the subunit stoichiometry. This describes the fraction of channels of each mixing type independent of the total number of subunits. Expanding equation 2 and substituting into equation 1 gives

Table 2. MinK Subunit Stoichiometry Evaluated at Various Mixing Ratios of WT and D77N MinK cRNA

F_{WT}	cRNAs	Coinjected	I/I_{WT}	$\ln (I/I_{WT})/\ln(f_{WT})$
0.89	WT–c-*myc*	D77N–c-*myc*	0.81	1.8 ± 0.3 (5)
	WT–c-*myc*	D77N	0.83	1.6 ± 0.3 (9)
	WT	D77N	0.87	1.2 ± 0.1 (8)
0.80	WT–c-*myc*	D77N–c-*myc*	0.64	2.0 ± 0.1 (13)
	WT–c-*myc*	D77N	0.64	2.0 ± 0.1 (10)
	WT	D77N	0.61	2.2 ± 0.2 (7)
0.67	WT–c-*myc*	D77N–c-*myc*	0.45	2.0 ± 0.1 (12)
	WT–c-*myc*	D77N	0.43	2.1 ± 0.1 (11)
	WT	D77N	0.47	1.9 ± 0.2 (6)
0.50	WT–c-*myc*	D77N–c-*myc*	0.25	2.0 ± 0.1 (4)
	WT–c-*myc*	D77N	0.25	2.0 ± 0.1 (14)
	WT	D77N	0.31	1.7 ± 0.1 (7)
0.33	WT–c-*myc*	D77N–c-*myc*	0.09	2.2 ± 0.1 (4)
	WT–c-*myc*	D77N	0.14	1.8 ± 0.1 (11)
	WT	D77N	0.15	1.7 ± 0.1 (8)
0.20	WT–c-*myc*	D77N–c-*myc*	0.01	2.9 ± 0.2 (4)
	WT–c-*myc*	D77N	0.04	2.0 ± 0.1 (10)
	WT	D77N	0.08	1.6 ± 0.1 (8)
0.11	WT–c-*myc*	D77N–c-*myc*	0.01	2.2 ± 0.1 (4)
	WT–c-*myc*	D77N	0.01	2.0 ± 0.1 (8)
	WT	D77N	0.04	1.5 ± 0.2 (7)

Oocytes were injected with WT minK cRNA or WT:D77N minK cRNA mixtures as described in Experimental Procedures. To address uncertainty in the mixing ratio (f_{WT}), each of the 4 cRNA species was synthesized on two occasions. At each mixing ratio, at least 6 separate groups of oocytes were studied on different days with different cRNA pairs. Currents (I and I_{WT}) were measured as in Figure 2 and the value for n as determined by equation 5 is reported as the mean ± SEM for the number of oocytes indicated in parentheses.

$$I = f_{WT}^{n} Z_0 I_{WT} + n f_{WT}^{(n-1)} f_{D77N} Z_1 I_{WT} + \ldots + f_{D77N}^{n} Z_n I_{WT} \quad (3)$$

If all channels with one or more D77N subunits are silent, Z_0 is 1, all other Z values are 0, and equation 1 simplifies to

$$I = f_{WT}^{n} I_{WT} \quad (4)$$

and, thus, taking the logarithm yields

$$n = \frac{1}{\ln (f_{WT})} \ln \left(\frac{I}{I_{WT}}\right) \quad (5)$$

Since I and I_{WT} are measurable and f_{WT} is known, n, the subunit stoichiometry, can be determined.

Subunit Stoichiometry

Table 2 shows the results of mixing experiments in which increasing amounts of D77N minK cRNA were coinjected with a constant amount of WT minK cRNA. Evaluation of 170 oocytes gave a value for n of 1.9 ± 0.1, suggesting that two functional minK monomers are present in each minK potassium channel. The same value for n is obtained at all seven mixing ratios; this is expected, since the value of n determined from equation 5 should not show dependence on the mixing ratio. Intermediate mixing ratios (f_{WT} = 0.80, 0.67, 0.50, and 0.33) were the most informative, inasmuch as the extremes yielded either small currents or limited effects. Experiments were performed in which a c-*myc* epitope was present in both WT to D77N minK, in which it was present only in WT minK, and with no epitope present. In each case, the value of n was near 2 (Table 2).

As expected, the value determined for n was also independent of test voltage (Table 3). At two mixing ratios and command voltages from 50 to -10 mV the value of n was close to 2. If subunit aggregation is a voltage-dependent process, as suggested by Varnum et al. (1995), it is unaltered by coexpression of the two subunit types.

Table 3. MinK Subunit Stoichiometry Evaluated at Various Test Voltages and Mixing Ratios

f_{WT}	Command Voltage (mV)	$\ln (I/I_{WT})/\ln(f_{WT})$
0.80	50	1.8 ± 0.1 (5)
	40	1.8 ± 0.1 (5)
	30	1.7 ± 0.1 (6)
	20	1.9 ± 0.1 (6)
	10	1.8 ± 0.1 (6)
	0	2.1 ± 0.2 (6)
	-10	2.0 ± 0.2 (6)
0.67	40	2.2 ± 0.1 (3)
	30	2.0 ± 0.1 (3)
	20	2.0 ± 0.1 (3)
	10	1.8 ± 0.1 (3)
	0	1.7 ± 0.1 (3)
	-10	1.7 ± 0.1 (3)

Oocytes were injected with WT minK cRNA or WT:D77N minK cRNA mixtures as described in Experimental Procedures and studied at the indicated command voltages using a 10 s test pulse from -80 mV and an interpulse interval of 2 min. The value for n as determined by equation 5 is reported as the mean ± SEM for the number of oocytes indicated in parentheses.

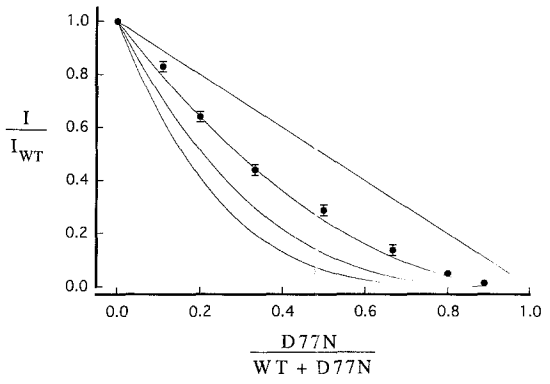

Figure 4. Coexpression of Mixtures of WT and D77N MinK Subunits Predicts a Subunit Stoichiometry of 2

Data are combined by mixing ratio for 170 oocytes studied as described in Table 2. Solid curves are drawn according to equation 4 with n = 1–4. Experimental data lie near the theoretical curve for n = 2. χ^2 analysis indicates that a subunit number of 2 is ~100 times more likely than is n = 1 and ~20 times more likely than is n = 3.

Figure 4 displays the fraction of unblocked current (I/I_{WT}) as the fraction of D77N minK cRNA is increased, combining the results by the mixing ratio for all experiments in Table 2. Theoretical curves based on equation 4 for values of n from 1 to 4 are drawn. The collected data most closely approximate a value for n of 2.

Discussion

This study provides evidence that complete minK channels contain two functional minK monomers. This conclusion is based on three assumptions. The first assumption is that WT and D77N minK subunits are expressed by oocytes in an equal and independent fashion. Experiments measuring surface expression of the two minK proteins support this idea (see Figure 2 and Figure 3). The second assumption is that WT and D77N minK subunits do not preferentially self- or transassociate. We have no evidence for aggregation bias; indeed, the observation that activation and deactivation kinetics are wild type in oocytes coexpressing WT and D77N minK (Table 1) and that the value determined for n is independent of voltage (Table 3) both argue strongly against it. The third assumption is that D77N minK has a dominantly lethal effect such that only fully WT channels are functional in mixing experiments. This is supported by the finding that oocytes expressing WT:D77N minK cRNA mixtures exhibit functional behavior virtually indistinguishable from WT minK (Table 1).

Because they appear to be so small, it is not currently possible to monitor minK single-channel events. Thus, we cannot show directly that D77N minK completely suppresses channel function. Even fluctuation analysis appears too insensitive to rule out the existence of mixed WT:D77N channel species with subconductances (Yang and Sigworth, 1995). Therefore, we consider two cases of incomplete suppression explicitly. First, suppose that mixed channels with one D77N subunit conduct like fully WT channels (and thus that Z_1 in equation 3 has a value of 1). In this case, the collected data are poorly described; the results for $f_{WT} = 0.80$ are consistent with a 6 subunit channel, whereas those for $f_{WT} = 0.67$ and 0.50 are consistent with a 5 and 4 subunit channel, respectively. Alternatively, suppose that mixed channels display subconductance levels. This can allow for larger values for n; an n of 3 approximates the collected data well if WT_2D77N_1 channels are assigned a Z_1 subconductance of 0.35, whereas an n of 4 fits the data if WT_3D77N_1 channels have a Z_1 of 0.5 and WT_2D77N_2 channels a Z_2 of 0.25. Indeed, any value of $n \geq 2$ can be fit to the data by altering the subconductances assigned to each hybrid channel species. A minK monomer stoichiometry of 2 is, however, the simplest model that satisfies all the data.

We have argued previously that minK is a structural component of a potassium-selective pore (Goldstein and Miller, 1991). In support of this idea, minK expression in oocytes and in the kidney-derived cell line HEK293 induces a current with characteristic potassium selectivity, pharmacology, and gating kinetics (Freeman and Kass, 1993; Hausdorff et al., 1991). Further, point mutations in minK alter the ion selectivity, open-channel blockade, gating kinetics, and regulation of this induced current (Goldstein and Miller, 1991; Takumi et al., 1991; Busch et al., 1992; Freeman and Kass, 1993). However, other cell types that express the protein do not appear to show this current (Lesage et al., 1993). This suggests that oocytes provide a necessary component for minK channel function, such as another channel subunit. Observations that isochronal minK currents saturate despite increasing levels of minK protein on the oocyte membrane surface (see Figure 2) support the idea that this required oocyte subunit is present only in limiting amounts, as suggested by Blumenthal and Kaczmarek (1994). Our results endorse a model in which complete minK channels contain just two minK monomers with other, as yet unidentified, non-minK subunits.

The approach applied here to determine subunit stoichiometry, combined assay of surface expression and protein function, may prove applicable to other membrane complexes in which a dominant lethal mutant is available. The ongoing controversy over identification of minK as a channel- or carrier-type transporter (Goldstein and Miller, 1991) or structural transport protein with regulatory function (Attali et al., 1993) does not alter the assumptions or conclusions reached here about the number of functional minK monomers in each surface complex. Direct biochemical assessment of minK subunit composition is now being attempted to evaluate this model.

Experimental Procedures

Molecular Biology

Cassette mutagenesis was performed by standard techniques using a synthetic rat minK gene (Hausdorff et al., 1991; Goldstein and Miller, 1991). The point mutation D77N was produced by polymerase chain reaction using a mismatched oligonucleotide and, after digestion with PstI and AccI, was unidirectionally ligated into pSDminK. To add the c-*myc* epitope (QKLISEEDL) between minK amino acids 22 and 23, a pair of synthetic oligonucleotides were phosphorylated, annealed, and ligated into pSDminK or pSD-D77NminK previously digested with NheI and PstI. pSDminK is a modified pGEM-9Zf$^-$ (Promega) vector

carrying the synthetic rat minK gene between a 5′UTR from Shaker H4, which yields high expression of channel proteins in oocytes (Marom et al., 1993), and a 3′UTR from Kv1.3 containing a poly(A) tail (Blumenthal and Kaczmarek, 1994). DNA sequences were confirmed by sequencing of both strands. cRNA was prepared by linearization with NotI and transcription by T7 polymerase as described (Goldstein et al., 1994). Transcript concentration was estimated spectrophotometrically, and aliquots were stored at −80°C.

Measurement of Oocyte Surface minK Levels

Groups of 8 oocytes were studied 72 hr after injection of cRNA. All steps were performed at 4°C in phosphate-buffered saline buffer containing 137 mM NaCl, 9 mM Na_2HPO_4, 1.4 NaH_2PO_4 (pH 7.4), and 2% bovine serum albumin. Oocytes were washed in buffer, incubated for 2 hr in 0.5 ml of 1:1000 ascites fluid containing mouse anti-c-*myc* monoclonal antibody 9E10 (Evan et al., 1985), washed, incubated in 0.4 ml of 0.3 μg/ml biotinylated goat anti-mouse antibody (Oncogene Science, MA) for 45 min, washed, incubated in 0.3 ml with 0.3 μCi [^{125}I]streptavidin (Amersham, MA) for 40 min, washed, and assessed directly by gamma counter.

Electrophysiology

Xenopus oocytes were isolated and injected with 46 nl of cRNA as described previously (Goldstein et al., 1994). Whole oocyte currents were recorded 2 or 3 days after injection using a two electrode voltage clamp (GeneClamp 500, Axon Instruments) with constant perfusion. Unless otherwise indicated, bath solution was ND-96, which contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, and 5 mM HEPES (pH 7.6). All experiments were performed at 21°C. Reproducible minK isochronal current measurements were achieved using the protocol described in the legend to Figure 2.

Subunit Coexpression Protocol

Mixtures of cRNA were prepared immediately prior to injection with a calibrated pipette. Measures to address the uncertainty in cRNA mixing ratios are detailed in the legend to Table 2. In mixing experiments, oocytes were injected with WT minK cRNA (2 ng) or WT minK cRNA (2 ng) and D77N minK cRNA (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, or 16.0 ng). The fraction of WT minK cRNA is defined as f_{WT} = (WT minK cRNA)/(WT minK cRNA + D77N minK cRNA). When WT-c-*myc* minK surface expression was evaluated on oocytes coexpressing WT-c-*myc* minK and D77N minK, surface expression remained at levels that would yield maximal minK currents in the absence of D77N subunits at all mixing ratios (data not shown).

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