1992

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 33, Issue of November 25, pp. 23742-23745, 1992
Printed in U.S.A.

Cooperative Interactions among Subunits of a Voltage-dependent Potassium Channel

EVIDENCE FROM EXPRESSION OF CONCATENATED cDNAs*

(Received for publication, July 31, 1992)

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Four copies of the coding sequence for a voltage-dependent potassium channel (RBK1, rat Kv1.1) were ligated contiguously and transcribed *in vitro*. The resulting RNA encodes four covalently linked subunit domains ([4]RBK1). Injection of this RNA into *Xenopus* oocytes resulted in the expression of voltage-dependent potassium currents. A single amino acid substitution, Tyr → Val, located within the outer mouth of the pore, introduced into the equivalent position of any of the four domains, reduced affinity for external tetraethylammonium by approximately the same amount. In constructs containing 0, 1, 2, 3, or 4 Tyr residues the free energy of binding tetraethylammonium was linearly related to the number of Tyr residues. A different amino acid substitution, Leu → Ile, located in the S4 region, was made in the equivalent position of one, two, three, or four domains. The depolarization required for channel activation increased approximately linearly with the number of Ile residues, whereas models of independent gating of each domain predict marked nonlinearity. Expression of this concatenated channel provides direct evidence that voltage-dependent potassium channels have four subunits positioned symmetrically around a central permeation pathway and that these subunits interact cooperatively during channel activation.

Subunits of voltage-dependent potassium channels are thought to have six α-helical membrane-spanning domains (S1–S6) and two shorter β-sheeted segments that contribute to the ionic pore (termed P, or SS1/SS2; Refs. 1 and 2). There is evidence that the channel is made from four such subunits, analogous to voltage-dependent sodium and calcium channels (3–5). Diversity can be generated by heteropolymerization of different subunits expressed in the same cell (6, 7; for review, see Ref. 8), and heteropolymeric channels have been identified after injecting RNAs encoding two subunits into *Xenopus* oocytes (9–11). Heteropolymerization occurs among channel subunits within the *Shaker*, *Shab*, *Shaw*, and *Shal* families of channels, although not between subunits belonging to different families (8, 12). The injection of two RNAs in various proportions has been used to express *mixtures* of channels in proportions expected from binomial theory (5, 13), but that approach relies on untested assumptions of equal translation and random association of subunits. More importantly, such co-injection experiments do not allow one to vary systematically the quaternary structure of a *single* homogeneous channel species.

Tetrameric channels of known subunit composition are necessary to test models of subunit interaction. For example, the Hodgkin and Huxley (14) model of the action potential implied that four subunits (or gating particles) operate independently during activation of the potassium current by depolarization. However, gating currents of the *Shaker* H4 channel measured using the cut-open oocyte technique suggest otherwise (15). The time course of on-gating currents indicates that during activation, transitions that occur later (*i.e.* closer to the open state) are faster than those which occur earlier. This suggests that the conformational change that a subunit undergoes when it is activated becomes progressively easier as surrounding subunits are activated (see also 16).

The main purpose of the present experiments was the expression of potassium channels of known subunit composition so as to test the effects of mutations in individual subunits on channel activation. Rather than using separate subunits, the potassium channel was expressed as a contiguous polypeptide from four concatenated cDNAs. A rat homolog of *Shaker* was used (RBK1,[1] Ref. 17; also called Kv1.1, Ref. 18), and the concatenated cDNA and channel are termed [4]RBK1. The first part of the paper describes experiments which verify that the resultant channel was indeed formed as a continuous polypeptide with four domains. To do this, we studied the effects of a single amino acid mutation in one or more subunits on the action of extracellular TEA, which is known to block the channel by entering the conducting pore (19–21).

The second part of the paper describes experiments to determine whether there is cooperative interaction among subunits during channel activation. The voltage dependence of activation is markedly affected by a single-point mutation of a hydrophobic residue within the S4 region of a *Shaker* subunit (22, 23); in that case, the expressed channels would

* This work was supported by Department of Health and Human Services Grants DA03160 and NS28504 and a fellowship (to J. Y.) from the Ford Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: RBK1, potassium channel subunit clone from rat brain or protein that it encodes; RBK2, potassium channel subunit clone from rat brain or the protein that it encodes; [4]RBK1, a cDNA clone with four concatenated copies of RBK1 coding sequences, or the protein that this encodes; TEA, tetraethylammonium; G/G_{max}, potassium conductance as a fraction of the maximal value; $V_{0.5}$, potential at which potassium conductance is half its maximal value; V_L and V_I, potential at which a domain of [4] RBK1 containing Leu or Ile, respectively, has a 50% probability of being in the open conformation; K, apparent equilibrium constant; p_o, probability that a channel is open.

have four subunits each bearing the mutation. Therefore, a similar mutation was introduced into the S4 region of one, two, three, or four domains of the concatenated channel, and the voltage dependence of their activation was compared with that expected for independent noninteracting domains.

EXPERIMENTAL PROCEDURES

Concatenated cDNA Construction and Site-directed Mutagenesis—RBK1 coding sequences were concatenated by making four unique cDNAs of RBK1 that could be ligated together in predetermined orientation (Fig. 1*A*). The amino-terminal subunit (domain I) was constructed by the introduction of 36 bases of DNA, encoding 10 glutamine residues, at the 3′ end of an RBK1 cDNA. The glutamine linker was engineered to generate the correct reading frame such that the first Gln codon replaced the natural RBK1 stop codon. The Gln linker region contained a *Bam*HI restriction site immediately 3′ to the 10th Gln codon and added amino acids (Gly and Ser). The second subunit (domain II) was constructed by introducing a *Bam*HI restriction site immediately 5′ to the usual RBK1 translation initiation codon. In addition, a linker region of 10 glutamines followed by a *Sal*I restriction site (encoding Val and Asp) was introduced at the 3′ end, replacing the usual stop codon in RBK1. The third subunit (domain III) was constructed in an analogous manner with a *Sal*I restriction site 5′ to the initiator ATG and a linker region of 10 Gln followed by a *Pst*I restriction site (encoding Leu and Gln) at the 3′ end. The fourth subunit (domain IV) was generated by inserting a *Pst*I site 5′ to the initiating ATG. Domain I was ligated to domain II across the *Bam*HI site, and domain III was ligated to domain IV across the *Pst*I site to generate two unique RBK1 doublets. Ligation products were transformed into *Escherichia coli* host strain JM109, and individual clones were analyzed by restriction analysis and DNA sequencing. Appropriate restriction fragments were isolated and ligated across the *Sal*I restriction site to generate the [4]RBK1 construct. This ligation reaction was transformed into *E. coli* host strain Sure (Stratagene, La Jolla, CA). The resulting plasmid was verified to contain four RBK1 coding sequences joined in-frame with the Gln-encoding linker regions by DNA sequencing and restriction analysis.

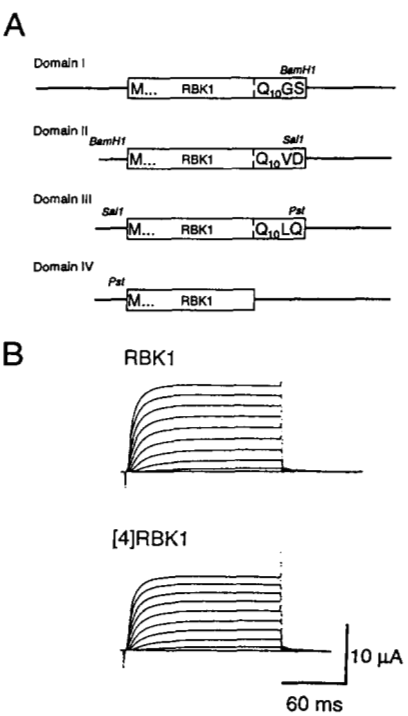

FIG. 1. *A*, construction of [4]RBK1. Each subunit used to construct [4]RBK1 was generated by oligonucleotide-directed mutagenesis. The first set of ligations produced two unique dimers (I-II and III-IV). The dimers were then ligated across the *Sal*I site to make a quadruplet RBK1. *B*, currents in oocytes expressing RBK1 subunits and [4]RBK1 constructs. In each case the oocyte was held at −80 mV, and voltage-clamp depolarizations were applied to −60, −50, −40, −30, −20, −10, 0, 10, and 20 mV.

RBK1 subunits containing the mutations Y379V or L305I were generated by site-directed mutagenesis (24), using the individual subunit coding sequences which already contained the Gln linker regions and the restriction sites. The DNA constructs which encoded the mutated subunits were ligated to other RBK1 wild-type or mutated coding sequences as described above to generate the various four domain channels. All plasmid constructs were performed in the pS⁻ vector, a derivative of pSelect (Promega, Madison, WI) in which the f1 origin of replication had been reversed to permit rescue of the coding strand by helper phage-mediated superinfection.

Electrophysiology—Oocytes (stages V-VI) were injected with approximately 1 ng of *in vitro* transcribed RNA. Membrane currents were recorded 24–96 h later by a two-electrode voltage clamp (17). The voltage dependence of activation was measured by clamping the oocyte membrane at −80 mV and depolarizing to a series of test potentials. Leak-subtracted peak currents were divided by the driving force (assuming a reversal potential of −90 mV) to obtain the conductance, and this was expressed as a fraction of its maximal value (G/G_{max}). Inhibition by TEA was measured by clamping oocytes at −80 mV and recording currents during 250-ms pulses to 0 mV, repeated every 30 s. TEA was applied by superfusion, and the concentration was increased cumulatively. Current amplitude was measured when it reached its new steady state in the presence of TEA.

Data Analysis—Inhibition by TEA (I, in percent) was fitted by sum of squares minimization to $I = 100 \cdot [TEA]^n/([TEA]^n + K^n)$ to provide an estimate of K; when mixtures of two channel species were expressed (*e.g.* [4]RBK1 and RBK2), the dose-response curves were fitted to $I = (100 \cdot r \cdot [TEA]/([TEA] + K_1)) + (100 \cdot (1 - r) \cdot [TEA]/([TEA] + K_2))$ or to $I = (100 \cdot r_1 \cdot [TEA]/([TEA] + K_1)) + (100 \cdot r_2 \cdot [TEA]/([TEA] + K_2)) + (100 \cdot (1 - r_1 - r_2) \cdot [TEA]/([TEA] + K_3))$, where K_1 and K_2 are the apparent TEA equilibrium constants for the channels when expressed singly, and K_3 is the apparent dissociation constant for all heteropolymeric channels. The mean inhibition (n = three to eight oocytes) was used for this fitting. For estimation of the affinity of TEA for individual channel species, the results from single oocytes (four to eight TEA concentrations) were fitted to logistic functions; the K estimate was converted to the change in free energy that occurs on binding TEA (ΔG) from $\Delta G = -RT\ln K$.

The normalized conductance (G/G_{max}) was fit to $G/G_{max} = 1/(1 + \exp((V_{0.5} - V)/k))$ where $V_{0.5}$ is the potential at which $G/G_{max} = 0.5$, and k is the number of mV required to cause e-fold shift in conductance. For the constructs containing Leu → Ile mutations, the activation curves predicted for independently activating domains (subunits) were obtained from $G/G_{max} = (1/(1 + \exp((V_L - V)/k_L)))^n \cdot (1/(1 + \exp((V_I - V)/k_I)))^{(4-n)}$. n is the number of domains containing L; $(4 - n)$ domains contain I. V_L, V_I, k_L, and k_I are the values of $V_{0.5}$ and k for the individual domains containing Leu or Ile; these were estimated by least squares fitting to the data for $n = 4$ and $n = 0$, respectively.

RESULTS

Expression of [4]RBK1—Currents in oocytes injected with the [4]RBK1 RNA activated within a few milliseconds (τ at 0 mV was 3.6 ± 0.6 mV, $n = 5$) (Fig. 1*B*). The voltage dependence of activation was well fit by a Boltzmann function, where $V_{0.5}$ was −13.5 ± 6.4 mV and k was 13.0 ± 0.9 mV ($n = 5$). (The values for RBK1 in parallel experiments were $\tau = 5.4 \pm 0.7$ ms, $V_{0.5}$ −16.9 ± 2.1 mV, $k = 11.1 \pm 1.1$ mV, $n = 5$.) The reversal potential of the current changed with the potassium concentration as expected from the Nernst equation.

The concentration of TEA that gave half-maximal current inhibition (apparent equilibrium constant, K) was similar to that for RBK1 (Fig. 2), and the inhibition was well described by $I(\%) = 100 \cdot [TEA]^n/([TEA]^n + K^n)$; the averaged inhibition data were fitted by $n = 0.95$. The inhibition of [4]RBK1 current by α-dendrotoxin ($K = 0.5 \pm 0.2$ nM, $n = 3$) was also similar to that observed with RBK1 ($K = 0.3 \pm 0.1$ nM, $n = 3$).

[4]RBK1 Forms Channels as a Single Intact Polypeptide—It is possible that [4]RBK1 formed channels by polymerization of individual subunits that form as a result of RNA degradation in the oocyte, translation initiation at internal

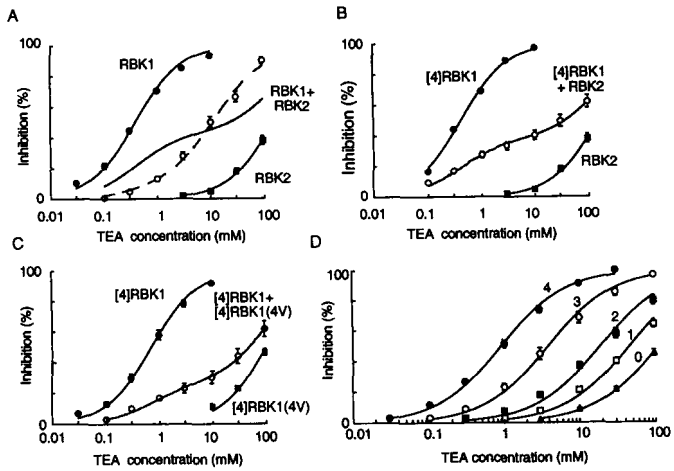

FIG. 2. **Dose-response curves for TEA indicate that [4] RBK1 does not heteropolymerize.** *A*, RBK1 and RBK2 heteropolymerize. *B* and *C*, [4]RBK1 does not heteropolymerize with RBK2 or with [4]RBK1(4V) ([4]RBK1 with Tyr → Val substitution in each domain). *Filled circles* are the values observed when the subunits or constructs were injected alone. *Lines* through these *points* are fitted by residual squares minimization to $I = 100 \cdot [TEA]/([TEA] + K_1)$ for the *leftmost* data (*filled circles*) and $I = 100 \cdot [TEA]/([TEA] + K_2)$ for the *rightmost* data (*filled squares*). *Open circles* are the points determined by experiment from oocytes co-injected with approximately equal amounts of RNA encoding RBK1 and RBK2 (*A*), [4] RBK1 and RBK2 (*B*), and [4]RBK1 and [4]RBK1(4V)(*C*). The *solid lines* (*A*, *B*, and *C*) show the dose-response curve expected if *no heteropolymerization* occurs: they are the best fits to $I = (100 \cdot r \cdot [TEA]/([TEA] + K_1)) + (100 \cdot (1 - r) \cdot [TEA]/([TEA] + K_2))$ when r was allowed to vary. The broken line (*A*) shows the dose-response curves expected if *heteropolymerization* occurs: it is the best fit to $I = (100 \cdot r_1 \cdot [TEA]/([TEA] + K_1)) + (100 \cdot r_2 \cdot [TEA]/([TEA] + K_2)) + (100 \cdot (1 - r_1 - r_2) \cdot [TEA]/([TEA] + K_3))$ where r_1, r_2, and K_3 were allowed to vary. *D*, Inhibition by TEA of current through concatenated [4]RBK1 channels with differing numbers of Tyr residues in the channel mouth. The inhibition is shown as the mean ± S.E. for each group of experiments; the number of Tyr residues at the channel mouth in each construct is shown beside the *trace*. Individual estimates of K for all variants tested are shown in Table I, which also gives the number of oocytes tested in each group.

methionines, or proteolysis. They could also form by polymerization of intact [4]RBK1 molecules. In any of these cases, polymerization should be possible with other distinct potassium channel subunits if these are co-expressed in the oocyte. RBK2 is a potassium channel subunit that is homologous to RBK1 but is much less sensitive to TEA; it readily heteropolymerizes with RBK1 (9). We therefore co-expressed [4] RBK1 with RBK2. The concentration-response curves for inhibition of current by TEA were well fit by the sum of two logistic functions, demonstrating that current passes only through two distinct channel populations, [4]RBK1 and homomeric RBK2 (Fig. 2*B*). This result is in contrast to experiments with co-expression of RBK1 and RBK2. As reported previously (9, 13), the TEA concentration-response curve clearly indicated the formation of channel species having intermediate sensitivity to TEA (Fig. 2*A*). Finally, we have demonstrated previously that Tyr → Val substitution at position 379 in RBK1 results in the expression of a current that is approximately 30-fold less sensitive to TEA; co-expression of [4]RBK1 with [4]RBK1 containing the Tyr → Val substitution in each domain also provided no evidence for heteropolymerization (Fig. 2*C*).

The expression of two contiguous potassium channel subunits has been used previously to provide evidence for heteropolymerization (10). When only one of the domains contained the Tyr → Val substitution, the resulting currents had a sensitivity to TEA that was intermediate between that found when both domains contained Tyr and when both domains contained Val (13, 21). In other experiments (13), mixtures of single subunits were expressed which contained either Tyr or Val, and the contribution of the Tyr residue from each subunit was estimated by fitting of the TEA inhibition curves. These results indicated that 4 Tyr residues, 1 from each subunit, take part in the binding of a single TEA ion (13, 21).

Therefore, concatenated channels were made in which the Tyr of the first, second, third and fourth domains was replaced by Val, either singly or in combination. These mutated concatenates are denoted I*-II-III-IV, I-II*-III-IV etc., where * indicates the domains in which Tyr was replaced by Val. The inhibition by TEA of the currents in oocytes expressing these channels is shown in Fig. 2*D*. The successive introduction of Tyr residues into the concatenate progressively decreased the sensitivity to TEA, and in each case the inhibition was fit by a logistic function with unit Hill coefficient. Substitution of the same number of Tyr residues by Val had approximately the same effect independent of the domains into which they were substituted (Table I).

Cooperative Interaction between Subunits during Activation—The RBK1 homolog in which Leu^{305} was mutated to Ile was expressed; the currents activated only at very positive membrane potentials. Therefore, RBK1(L305I) was used as the basis to construct and express the concatenated channels in which this Leu residue in S4 was changed to Ile in one, two, three, or four domains (I*-II-III-IV, I*-II*-III-IV, I*-II*-III-IV*, and I*-II*-III*-IV*). Fig. 3 shows that the voltage dependence of activation was shifted progressively to depolarized potentials as Ile residues were introduced, 1 into each domain; the shifts were approximately equal for each substitution. Fig. 3 also shows (*broken lines*) the expected curves if each of the four domains activated independently (see "Experimental Procedures"). Clearly, much less depolarization is required to activate channels having one, two, or three Ile-containing domains than expected if each activated independently. This implies positive cooperativity among the domains

TABLE I

Changes in free energy on dissociation of TEA from [4]RBK1

I-II-III-IV is [4]RBK1, the concatenate of four RBK1 subunits. * indicates a domain with Tyr → Val substitution. The Tyr was in the equivalent position in each domain (residue 379 in I, 886 in II, 1393 in III, 1900 in IV). Mean values are independent of the domain that contained the Tyr → Val substitution.

Construct	ΔG	S.D.	*n*
I-II-III-IV	17.3	0.8	9
I*-II-III-IV	12.7	0.4	15
I-II*-III-IV	14.0	0.8	7
I-II-III*-IV	13.2	0.8	9
I-II-III-IV*	14.6	1.1	10
Mean	13.5	1.1	41
I*-II*-III-IV	10.2	0.7	16
I*-II-III*-IV	8.8	0.8	18
I-II*-III*-IV	9.3	0.7	15
I-II*-III-IV*	10.4	0.7	14
Mean	9.7	0.9	63
I*-II*-III*-IV	7.0	1.6	20
I*-II*-III-IV*	8.2	1.2	14
I*-II-III*-IV*	7.3	1.3	13
I-II*-III*-IV*	6.9	1.4	13
Mean	7.4	1.4	60
I*-II*-III*-IV*	5.2	1.0	17

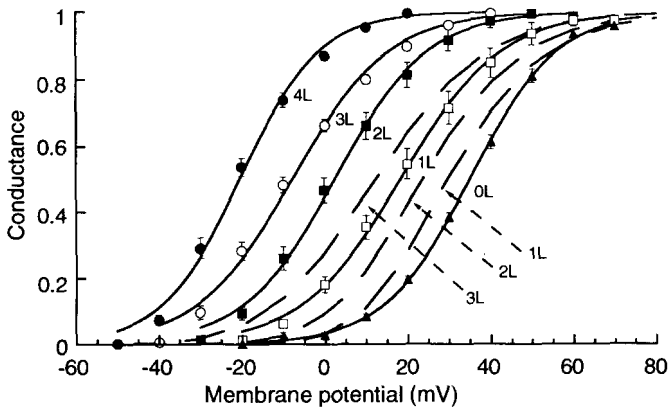

FIG. 3. **Activation curves for [4]RBK1 and its homologs with one, two, three, or four Leu → Ile substitutions at the equivalent position in each domain (within S4).** *4L* indicates [4]RBK1, *3L* indicates [4]RBK1(L305I)(I*-II-III-IV), *2L* indicates [4]RBK1(L305I, L811I) (I*-II*-III-IV), *1L* indicates [4]RBK1(L305I, L811I, L1823I) (I*-II*-III-IV*), and *0L* indicates [4]RBK1(L305I, L811I, L1317I, L1823I)(I*-II*-III*-IV*). Data are shown as mean ± S.E.; the *solid lines* through the *points* are fitted to simple Boltzmann functions. The fits to individual oocytes (see "Experimental Procedures") gave for *4L*: $V_{0.5} = -20.5 \pm 1.2$ and $k = 9.3 \pm 0.2$ ($n = 5$); for *3L*: $V_{0.5} = -7.9 \pm 1.2$ and $k = 11.5 \pm 0.4$ ($n = 5$); for *2L*: $V_{0.5} = 2.6 \pm 2.1$ and $k = 10.9 \pm 0.8$ ($n = 5$); for *1L*: $V_{0.5} = 18.6 \pm 2.5$ and $k = 11.8 \pm 0.7$ ($n = 5$); and for *0L*: $V_{0.5} = 34.9 \pm 0.9$ and $k = 10.3 \pm 0.3$ ($n = 5$). The *broken lines* represent the expected activation curves for four independently activating domains, assuming that the channel conducts only when all four are in the open conformation (see "Experimental Procedures").

of [4]RBK1 and, presumably, the subunits of native potassium channels.

DISCUSSION

The expression of [4]RBK1 resulted in functional potassium channels with properties that did not differ markedly from those of RBK1. The external vestibule of the channel appears to form normally, as evidenced by the finding that TEA and dendrotoxin blocked [4]RBK1 channels at the same concentrations as those required to block the RBK1 channels. The results depicted in Fig. 2 allow us to conclude that within the resolution of our measurements, [4]RBK1 channels form exclusively as intact polypeptides. [4]RBK1 did not polymerize with RBK2 (Fig. 2*B*), and [4]RBK1 did not heteropolymerize with [4]RBK1(4Y → 4V) (Fig. 2*C*). In contrast, monomeric RBK1 subunits readily heteropolymerize both with RBK2 subunits (Fig. 2*A*; also Ref. 9) and with RBK1(Y379V) subunits. Simulations of the dose-response curves for the mixture of [4]RBK1 with RBK2 (Fig. 2*B*) indicated that any heteropolymeric channel of intermediate sensitivity contributes less than 5% of the total current.

Previous experiments using mixtures of wild-type and mutant channel subunits, and tandem constructs (I-II, I*-II, I-II*, I*-II*), have indicated that the Tyr^{379} residue on each of the four subunits of the RBK1 channel contributes to the binding of TEA (13, 21). The present results (Table I) show that [4]RBK1 forms channels with essentially the same symmetry as RBK1 itself, and they provide the most direct evidence to date that each of the 4 Tyr residues contributes approximately equally to the formation of the TEA binding site.

The depolarization required to activate concantenated channels with a Leu → Ile substitution in one or more S4 segments was considerably less than expected if the domains operated independently during activation. For example, for the I*-II*-III-IV construct, the model of independent activation indicates that $V_{0.5}$ is 23 mV (*broken line arrowed* as *2L* in Fig. 3). In other words, at 23 mV the expected probability of a channel being open (p_o) is 0.5, and the energies of the open and closed states are equal. The experimentally observed value for p_o is 0.87 at this potential (from *solid line* marked as *2L* in Fig. 3). This increased p_o corresponds to an energy difference of $RT\ln((1 - p_o)/p_o)$ or 4.6 kJ/mol; presumably this is supplied from the cooperative interactions among the domains.

It seems likely that concatenated channels may provide a useful model system for the kind of quantitative studies of subunit interaction that have been attempted for other tetrameric proteins (*e.g.* 25, 26). The overall similarities between the properties of this concantenated channel and that formed from individual subunits provides confidence that similar interactions would occur among the subunits of multimeric native channels.

Acknowledgment—We thank Yan-Na Wu for injecting oocytes.

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