Cytoplasmic and Extracellular IsK Peptides Activate Endogenous K\(^+\) and Cl\(^-\) Channels in Xenopus Oocytes

EVIDENCE FOR REGULATORY FUNCTION*

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IsK is a 14.5-kDa type III membrane glycoprotein which induces slowly activating K\(^+\) and Cl\(^-\) currents when expressed in Xenopus oocytes and HEK 293 cells. Recently, mutagenesis experiments identified amino- and carboxyl-terminal domains of IsK as critical for induction of Cl\(^-\) and K\(^+\) currents, respectively. This hypothesis was tested by examining effects of synthetic IsK hydrophilic peptides on untreated Xenopus oocytes. In agreement with IsK membrane topology, we show here that peptides derived from carboxyl and amino termini are sufficient to activate slow K\(^+\) and Cl\(^-\) channel. Alkaline biophysical and pharmacological characteristics are similar to those exhibited by the native IsK protein. That data provide further evidence that IsK is a regulatory subunit of pre-existing silent channel complexes rather than a channel per se.

The last few years have been an exciting time for our understanding of the molecular structure and diversity of voltage-gated K\(^+\) channels belonging to the Shaker-like superfamily (for review, see Refs. 1–5). Contrastingly, since its original cloning by expression in Xenopus oocytes, the nature of the IsK protein (or minK) remained a mystery (for review, see Refs. 6 and 7). From a structural point of view, IsK is an exception to the family of K\(^+\) channels. IsK is a 14.5-kDa type III glycoprotein with one transmembrane segment which has no sequence homology with other cloned functional channels (8). It is a member of a family of small bitopic membrane proteins which induce upon expression in Xenopus oocytes slowly activating voltage-dependent currents (6, 7, 9). This family includes phospholemman (10), influenza virus M2 protein (11), CHIF (12), Xenopus oocytes, and pholemman (10), influenza virus M2 protein (11), CHIF (12), and Mat-8 (13). When expressed in Xenopus oocytes or in HEK 293 cells, the IsK protein evokes a unique slowly activating, voltage-dependent K \(^+\) -selective current that closely resembles slow K\(^+\) channel in a variety of host cells and to an endogenous oocyte factor or with pre-existing silent channels (18–20). Furthermore, IsK mutagenesis suggested that the amino-terminal domain is critical for the induction of Cl\(^-\) currents while the carboxyl-terminal domain is critical for the activation of K\(^+\) channel activity (18). These findings hinted at the possibility that IsK is a regulatory subunit of heteromultimeric channel complexes rather than a channel per se.

To test this hypothesis, synthetic IsK hydrophilic peptides were applied to untreated Xenopus oocytes. In agreement with IsK membrane topology, we show here that internal or external application of IsK peptides derived from the carboxyl- and amino-terminal domains are sufficient to activate slow K\(^+\) or Cl\(^-\) currents, respectively. The peptide-activated channels displayed characteristics similar to those exhibited by the native IsK protein, namely voltage dependence, activation kinetics, ion selectivity, and pharmacology. Our data provide clear evidence for the nature of IsK as a prototypic member of a family of short membrane transport proteins with regulatory function.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Peptides were synthesized by a solid-phase method on pyridine-2-aldoximine methyl-amino acid resin (0.15 meq) (21), as described previously (22). The peptides were subjected to amino acid analysis to confirm their composition.

Electrophysiology—Xenopus laevis were purchased from C.R.B.M. (Montpellier, France). Ovarian lobes were surgically removed as described (23) in a Ca\(^+\)\(^-\)-free OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 5 mM HEPES (pH 7.5 with NaOH)). Oocytes were kept for 2–9 days in OR2 standard solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 5 mM HEPES (pH 7.5 with NaOH)) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin.

Command pulse protocols, data acquisition, and analyses were performed using pClamp software (Axon Instruments, Foster City, CA) as described (23). Oocytes were perfused in OR2 standard solution, and all experiments were carried out with the oocyte membrane held at –80 mV or –60 mV. Oocytes were injected with full-length mouse IsK cDNA or full-length dog phospholemman (10 ng/oocyte), and the resulting currents were recorded 2 days following DNA injection. For intracellular peptide injection, a volumetrically calibrated micropipette was used and peptide content in 20 nl was ejected by brief pressure pulses. The peptides were dissolved in 20 mM HEPES (pH 7.4), 120 mM KCl and were briefly sonicated before microinjection. To record depolarization-activated currents, 20-ns depolarizing pulses were applied from a holding potential of –60 mV at 45-s intervals and 1 min after microinjecting or superfusing the peptides. For C-27-activated currents, the reversal potential determination was performed by changing sequentially the perfusate to a series of OR2 solutions in which Na\(^+\) was replaced by K\(^+\). For extracellular peptide perfusion, the peptides were dissolved in OR2 superfusing solutions. To record hyperpolarization-activated currents, 5-ns hyperpolarizing pulses were evoked from a holding potential of –10
of the slow K⁺ current of IsK is essential for the induction of C-27-activated currents, since it did not reach steady-state even after several minutes (at +40 mV, t_half = 6–12 s, by fitting the 20-s pulse). Unlike deactivation kinetics, the activation rates varied considerably among oocytes. Tail current deactivation was faster than activation kinetics, requiring seconds for full relaxation (Fig. 2B), and was fit as a biexponential decay (at −80 mV repolarization, t_half = 0.27 ± 0.08 s and t_half = 1.80 ± 0.25 s; n = 7; 3 batches). The current was selective for K⁺ ions. The slope of the tail current reversal potentials at various [K⁺]o (substituted for Na⁺) was 52.6 mV per decade, consistent with a K⁺ selectivity (Fig. 2D; n = 4, in 2 batches). In Xenopus oocytes, voltage steps to potentials above −20 mV usually give rise to transient Ca²⁺-activated outward Cl⁻ currents in OR2 standard recording solutions. To examine a possible interference of outward Cl⁻ conductance, we studied the C-27-activated outward Cl⁻ currents under Cl⁻-free recording solutions ([Cl⁻]o substituted with gluconate). Under these conditions, Cl⁻ influx was prevented and no Ca²⁺-activated Cl⁻ currents could be recorded in the presence of the Ca²⁺ ionophore A23187 (1 µM) at voltage steps from −20 mV up to 60 mV (not shown). In gluconate recording solutions, intracellularly applied C-27 (100 µM) was able to evoke slowly activating K⁺-selective currents upon step depolarization to 40 mV (Fig. 4A, control trace). Although the activation was slightly faster than in Cl⁻-containing solutions, the pharmacology was nearly identical with that found with the expression of the native IsK protein. Fig. 4A shows that barium (5 mM) and dophilium (100 µM) blocked the C-27-induced K⁺ current by 70 ± 9% and 60 ± 10%, respectively (n = 5; 3 different batches). Tetraethylammonium (30 mM) caused a 50 ± 11% blockade of

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**RESULTS**

Mutagenesis experiments have shown that an intracellular carboxyl-terminal domain of IsK is essential for the induction of the slow K⁺ current in Xenopus oocytes (18). A 27-mer hydrophilic peptide (C-27), spanning a carboxyl-terminal region of the rat IsK sequence (positions 68–94) (22) and located immediately downstream from the transmembrane segment, was synthesized and microinjected into Xenopus oocytes (Fig. 1). Within 30 s after C-27 peptide injection, membrane depolarization above a threshold of −50 mV evoked a slowly activating voltage-dependent outward current (Fig. 2, A and C). This current was very similar to the slow K⁺ current produced by the native IsK. The depolarization-activated current could last for more than 20 min after injection and then declined to 0 after 30 min. Current amplitude was partly dependent on peptide concentration, being activated above 20 µM C-27, but then rapidly saturating above 100 µM C-27 (not shown). No currents could be evoked, when internally applied C-27 was tested upon hyperpolarization or when it was perfused in the external bath solution (n = 4, 3 batches). A 34-mer hydrophilic peptide (N-34), spanning an amino-terminal region of the rat IsK sequence (position 10–43) (22), did not evoke any current when injected into the oocyte (at 200 µM; n = 5, 3 batches). Other non-IsK peptides such as P1, P2, and P3 (Fig. 1) were ineffective at 200 µM (n = 5, 3 batches).

It was difficult to describe accurately the activation kinetics of C-27-activated currents, since it did not reach steady-state even after several minutes (at +40 mV, t_half = 6–12 s, by fitting the 20-s pulse). Unlike deactivation kinetics, the activation rates varied considerably among oocytes. Tail current deactivation was faster than activation kinetics, requiring seconds for full relaxation (Fig. 2B), and was fit as a biexponential decay (at −80 mV repolarization, t_half = 0.27 ± 0.08 s and t_half = 1.80 ± 0.25 s; n = 7; 3 batches). The current was selective for K⁺ ions. The slope of the tail current reversal potentials at various [K⁺]o (substituted for Na⁺) was 52.6 mV per decade, consistent with a K⁺ selectivity (Fig. 2D; n = 4, in 2 batches). In Xenopus oocytes, voltage steps to potentials above −20 mV usually give rise to transient Ca²⁺-activated outward Cl⁻ currents in OR2 standard recording solutions. To examine a possible interference of outward Cl⁻ conductance, we studied the C-27-activated outward currents under Cl⁻-free recording solutions ([Cl⁻]o substituted with gluconate). Under these conditions, Cl⁻ influx was prevented and no Ca²⁺-activated Cl⁻ currents could be recorded in the presence of the Ca²⁺ ionophore A23187 (1 µM) at voltage steps from −20 mV up to 60 mV (not shown). In gluconate recording solutions, intracellularly applied C-27 (100 µM) was able to evoke slowly activating K⁺-selective currents upon step depolarization to 40 mV (Fig. 4A, control trace). Although the activation was slightly faster than in Cl⁻-containing solutions, the pharmacology was nearly identical with that found with the expression of the native IsK protein. Fig. 4A shows that barium (5 mM) and dophilium (100 µM) blocked the C-27-induced K⁺ current by 70 ± 9% and 60 ± 10%, respectively (n = 5; 3 different batches). Tetraethylammonium (30 mM) caused a 50 ± 11% blockade of...
pulses from 0 mV to current amplitudes were measured at the end of a 5-s hyperpolarizing slow Cl solutions (not shown). were obtained with aspartate or methanesulfonate recording are necessary to obtain an effective inhibition. Similar results a previous report (24) and suggests that other domains of IsK 3 batches). The lack of lanthanum blockade is in contrast with

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15 s and could be readily reversed upon washing out of the peptide, suggesting that the peptide only weakly associates with the endogenous oocyte channel. Like C-27 and above a threshold of 30 μM, current amplitude was not strictly depend-

slowly developing inward current was evoked above a thresh-

old of 90 mV (Fig. 3). This current was very similar to that induced by phospholemman or by IsK (at high cRNA concentration) (10, 18). Since some batches of untreated oocytes did express a similar slow inward current, we always tested the effects of the peptides in oocytes that did not exhibit this endogenous inward current. The N-34 action occurred within 15 s and could be readily reversed upon washing out of the peptide, suggesting that the peptide only weakly associates with the endogenous oocyte channel. Like C-27 and above a threshold of 30 μM, current amplitude was not strictly depend-

ential currents measured by the tail current reversal potentials (open circles). The current amplitudes were measured at the end of a 5-s hyperpolarizing pulses from 0 mV to 200 mV. D, Cl selectivity of N-34-induced currents measured by the tail current reversal potentials (open circles). The straight line is the Nernst relationship for a perfectly selective Cl current with a [Cl]o = 65 mM. Points shown are the means ± S.E. of 3 independent experiments.

The role of the amino-terminal domain of IsK in activating slow Cl currents was examined by applying extracellularly N-34 (Fig. 1) to untreated oocytes. Upon hyperpolarization, a slowly developing inward current was evoked above a threshold of 90 mV (Fig. 3). This current was very similar to that induced by phospholemman or by IsK (at high cRNA concentration) (10, 18). Since some batches of untreated oocytes did express a similar slow inward current, we always tested the effects of the peptides in oocytes that did not exhibit this endogenous inward current. The N-34 action occurred within 15 s and could be readily reversed upon washing out of the peptide, suggesting that the peptide only weakly associates with the endogenous oocyte channel. Like C-27 and above a threshold of 30 μM, current amplitude was not strictly depend-

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In this report we have shown that intracellular or extracellular application of specific hydrophilic IsK peptides was sufficient to mimic the native IsK induction of slow K⁺ and Cl⁻ currents (6–8, 18). It is clear that these peptides could not form by themselves ion-conducting pores, since they do not interact with the membrane and their action is not irreversible. The peptide-induced channel activity is specific since non-IsK peptides were ineffective. The specificity of the peptide interaction is further evidenced by the ability of intracellular carboxyl terminus and extracellular amino terminus peptides to exclusively evoke K⁺ or Cl⁻ currents, respectively. This is in agreement with the IsK membrane topology. Our data strongly support the notion that IsK must associate with some endogenous oocyte component to form a functional channel complex (18–20). However, mutations in the transmembrane domain of IsK were found to alter channel selectivity, open channel block, and gating kinetics (15, 16). To explain this apparent controversy, we suggest that IsK, acting as a regulatory subunit, has some role in the modulation of channel gating. This is actually the case with the β1 subunit of voltage-dependent Na⁺ channels (same membrane topology as IsK) and voltage-dependent Ca²⁺ channels which alter the voltage dependence of channel gating (26).

It has been suggested recently that IsK might become functional by interacting with either a rare lipid, a cytoskeletal protein, or a channel protein subunit (19). Regarding our data, we favor the latter proposal for two main reasons. First, it is energetically unfavorable for a hydrophilic peptide to associate with a lipid to form a functional channel. Second, it seems unlikely that an extracellularly applied peptide such as N-34 will interact with an intracellular cytoskeletal component to produce a current. Naturally, it does not exclude that the whole channel complex could be linked to the cytoskeleton. Thus, the reasonable explanation is that the IsK peptides interact with endogenous oocyte channels to activate them. In such a model, IsK may act at least in one of the two ways. 1) It could function as an activator or a regulatory subunit which activates pre-existing silent channels by direct protein-protein interactions. 2) Alternatively, IsK could act by recruiting inactive channels and functions as a chaperone that facilitates assembly of multimeric channel complexes. However, the relatively fast peptide action together with the ability of externally applied N-34 or N-13 to evoke slow Cl⁻ currents are not compatible with this view.

The oligomeric nature of the IsK channel complex remains unknown; however, recent studies suggested that it could be made of just two IsK monomers associated with as yet unidentified non-IsK subunits (20). The very slow gating kinetics of IsK suggest that it could activate by a unique mechanism. A model in which IsK channels activate by voltage-dependent subunit aggregation has been proposed (27). Cross-linking or Hg²⁺-induced chelation of IsK subunits were found to hold the channel complex in an open conducting state (27, 28). This mechanism of subunit aggregation also accommodates our view of a heteromultimeric subunit oligomerization process.

It is clear that the slow K⁺ and Cl⁻ currents are not specifically and exclusively induced by the IsK protein in Xenopus oocytes. Other structurally similar small bitopic membrane proteins like CHIF, Mat-8, or phospholemman are also able to activate these slowly activating currents (10, 12, 13). For example, IsK and CHIF evoke slow K⁺ currents which fail to reach steady state within tens of seconds and are sensitive to block by Ba²⁺ and clofilium. Since these proteins share no sequence similarity, it suggests that the specificity requirements for binding to the endogenous channel complex are relatively low. The conformation that the peptides adopt upon binding is unknown; however, the various peptidic motifs must share some yet undefined structural or conformational similarities. The transient character of the peptide action suggests that the transmembrane domain and may be other regions such as the extreme amino terminus may help in stabilizing the physical interaction between IsK and other subunits of the channel complex, independently of the gating process.

In conclusion, our findings provide further evidence for the nature of IsK as a member of a family of short bitopic membrane proteins which are capable of activating endogenous and otherwise silent ion channels. It is now crucial to investigate which physiological roles subserve IsK-channel protein interactions in epithelia, T lymphocytes, or cardiac cells and what are the molecular structures of these interacting channel proteins.

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