A pH-sensitive Yeast Outward Rectifier K⁺ Channel with Two Pore Domains and Novel Gating Properties*

(Received for publication, August 30, 1995, and in revised form, October 10, 1995)

Florian Lesage, Eric Guillemare, Michel Fink, Fabrice Duprat, Michel Lazdunskii, Georges Romy, and Jacques Baranhan
From the Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France

YORK is a newly cloned K⁺ channel from yeast. Unlike all other cloned K⁺ channels, it has two pore domains instead of one. It displays eight transmembrane segments arranged like a covalent assembly of a Shaker-type voltage-dependent K⁺ channel (without S4 transmembrane segments) with an inward rectifier K⁺ channel. When expressed in Xenopus oocytes, YORK does not pass inward currents; it conducts only K⁺-selective outward currents. However, the mechanism responsible for this strict outward rectification is unusual. Like inward rectifiers, its activation potential threshold closely follows the K⁺ equilibrium potential. Unlike inward rectifiers, the rectification is not due to a voltage-dependent Mg²⁺ block. The blocking element is probably intrinsic to the YORK protein itself. YORK activity is decreased at acidic internal pH, with a pKₐ of 6.5.

Pharmacological and regulation properties were analyzed. Ba²⁺ ions and quinine block YORK currents through high and low affinity sites, while tetraethylammonium displays only one affinity for blocking. Activation of protein kinase C indirectly produces an increase of the current, while protein kinase A activation has no effect.

A large number of K⁺ channel subunits have been identified in the last few years (1–6). All of the pore-forming K⁺ channel subunits clonned in vertebrates as well as in plants, paramecia, and bacteria (7–10) share a highly conserved sequence called the pore (or H5) domain (P-domain), which has been shown to be part of the K⁺-selective pore (11–14). The presence of this sequence in a protein is considered the signature of a K⁺ channel structure. In all K⁺ channels so far cloned, there is never more than one P-domain per subunit and a variable number of transmembrane segments (TMS). Voltage-gated K⁺ channels have six TMS, inward rectifier K⁺ channels have two TMS (1–4), and Ca²⁺-dependent K⁺ channels have 10 TMS (15, 16). All six-TMS channels have a positively charged TMS (S4) that is responsible for voltage sensitivity. The two-TMS K⁺ channels do not possess such a domain. Their apparent voltage sensitivity for gating results from a voltage-dependent block by intracellular Mg²⁺ or polyamines (17–22). YORK, the yeast outward rectifying K⁺ channel described in this work, presents both novel structural features and novel functional properties.

EXPERIMENTAL PROCEDURES

Molecular Biology—YORK cDNA was amplified from Saccharomyces cerevisiae DNA by polymerase chain reaction with a low error rate polymerase (Pwo DNA pol, Boehringer Mannheim), doped into the pEXO vector, and sequenced on both strands by automatic sequencing using an Applied Biosystems Model 373A sequencer. Site-directed mutagenesis was carried out using a Transformer system (CLONTECH) according to the manufacturer’s protocol. Wild-type and mutant plasmids were linearized by BamHI digestion, and then capped cRNAs were synthesized using T7 RNA polymerase (Stratagene).

Electrophysiology—Xenopus oocyte preparation, cRNA injection, and electrophysiology have been described elsewhere (23). ND96 solution contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂, and 5 mM HEPES adjusted to pH 7.4 with NaOH.

For inside-out patch recordings, pipettes were filled with a high K⁺ solution (40 mM KCl, 100 mM potassium methanesulfonate, 1.8 mM CaCl₂, 2 mM MgCl₂, 5 mM HEPES adjusted to pH 7.4 with KOH, and 10 μM GdCl₃) to prevent endogenous stretch-activated channel activities. The bath solution contained 140 mM KCl, 3 mM MgCl₂, 5 mM HEPES adjusted to pH 7.2 with KOH. For outside-out recordings, pipettes were filled with a solution identical to that used for the bath solution in the inside-out patch recordings. The external medium contained 140 mM KCl, 0.3 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES adjusted to pH 7.1 with KOH. The external divalent-free solution contained 140 mM KCl, 2 mM EGTA, 2 mM EDTA, and 10 mM HEPES adjusted to pH 7.1 with KOH. Single-channel recording data were filtered at 5 kHz and digitized at 50 kHz.

CO₂-enriched solution was prepared by bubbling 100% CO₂ into the standard ND96 solution for 5 min. For inside-out patch experiments, internal solutions at different pH values were prepared by adding Na₂HPO₄ and NaH₂PO₄ at different ratios. To obtain pH values of 6, 6.4, 6.8, 7.2, 7.6, and 8, the Na₂HPO₄/NaH₂PO₄ ratios were 1.28/76, 2.67/34, 4.85/1, 7.2/28, 8.6/13, and 9.4/0.5 mM, respectively. The curve was the least-square fit of the following equation: \[ I = I_0 \cdot \exp\left(\frac{pH - \text{pK}}{a}\right) \], with pK = 6.5, Iₘₐₓ = 1.4 pA, and a = 0.38.

RESULTS AND DISCUSSION

Structural Properties of YORK—A search in the GenBank/EMBL Data Bank for sequences homologous to the mSlo P-domain (15) led to the identification of a DNA sequence that presented the particularity of displaying two P-like domains in the same open reading frame. This sequence (accession number X77087) was originally obtained from chromosome X of S. cerevisiae in the framework of the European Yeast Genome Sequencing Project (24). The full coding sequence was amplified by polymerase chain reaction from yeast DNA and cloned in pEXO, a vector designed for preparation of efficiently transcribed cRNA in Xenopus oocytes (25).

The YORK gene encodes a 691-amino acid-long protein that does not share significant sequence conservation with previously cloned K⁺ channels, except in the two P-domain se-
Fig. 1. Structural properties of YORK. a, amino acid sequence of YORK. TMSs are boxed, and P-domains are underlined. Consensus sites for glycosylation (□) and for protein kinase C (●), protein kinase A (○), and tyrosine kinase (●) phosphorylation are shown. These sites have been identified by using the profsite server (European Bioinformatics Institute) with the ppssearch software that is derived from the Macpattern program. b, hydropathy profile and deduced topology for YORK. Hydrophobicity values were calculated according to the method of Kyte and Doolittle (37) (window size of 11 amino acids) and are plotted against amino acid position. Shaded hydrophobic peaks correspond to TMSs. PHO, phosphorylating pH; PHI, hydrophilicity, c, alignment of the P-domains of YORK and other K+ channels. Identical and conserved residues are boxed in black and gray, respectively.

quences (Fig. 1, a and c). The hydropathy profile shown in Fig. 1b predicts eight membrane-spanning hydrophobic segments (S1 to S8). The P-domains P1 and P2 are flanked by TMS S5 and S6 and TMS S7 and S8, respectively. Placing the NH2 terminus on the cytoplasmic side, in agreement with the absence of signal peptide, leads to the topology model proposed in Fig. 1b. In this model, the two P-domains are inserted into the membrane from the outside, corresponding to the known ori-

Functional Expression of YORK Channels—The biophysical properties of the YORK channels were characterized after injection of their cRNA into Xenopus oocytes. Large currents of several microamperes were recorded that were strictly outward rectifying. Depolarizations induced a two-step current composed of instantaneous and delayed slow components (Fig. 2a). The delayed current could be fitted by an exponential function, with a time constant varying from 0.7 s at −50 mV to 0.2 s at +50 mV (Fig. 2b, inset). The instantaneous and delayed I-V relationships are similar. No K+ inward currents were recorded (Fig. 2b), not even as a detectable tail current regardless of the repolarization level (see Fig. 7a). The threshold potential for activation followed the change in the K+ equilibrium potential \((E_K)\) as the external K+ concentration decreased (55.4 ± 0.7-mV shift \((n = 5)\) for a 10-fold change in the external K+ concentration) (Fig. 2c). Hence, the YORK activation potential depends on the driving force for K+ \((V - E_K)\) as described for inward rectifier K+ channels (26) rather than on the membrane potential \((V)\) as described for voltage-dependent outward rectifier K+ channels. Moreover, as for inward rectifier K+
Outward, with a unitary conductance of 26.5, progressive increase in the number of open channels (Fig. 2). Inset, voltage dependence of the time constant of activation. c, relationships between the global conductance (G) and the electrochemical potential (V - E K) for different external K+ concentrations. Conductances were calculated from 1-V curves using the following equation: G = 1/((V - E K)). d, outward rectifying currents measured by inside-out patch single-channel recordings in symmetrical K+ solution (140 mM) (holding potential = -20 mV).

For the outwardly rectifying YORK channel might be blockbypenetration of an impermeant ion in the pore. Mg2+ with striking similarities to the biophysical properties of inward rectifiers. The absence of an S4 voltage sensor structure, associated with an instantaneous activity followed by a progressive increase in the number of open channels (Fig. 2d). Single-channel recordings showed that the current was strictly outward, with a unitary conductance of 26 ± 2 picoSiemens (n = 5), and had a high tendency to flicker, with a mean open time of 0.19 ± 0.01 ms (six inside-out patches) (Fig. 3, a and b).

The absence of an S4 voltage sensor structure, associated with striking similarities to the biophysical properties of inward rectifier K+ channels, suggested that the gating mechanism for the outwardly rectifying YORK channel might be analogous to that of inward rectifiers, i.e., a voltage-dependent block by penetration of an impermeant ion in the pore. Mg2+ or polyamine cations play this role for inward rectifier channels (17-22). Surprisingly, Mg2+ and Ca2+ removal from the external solution has no effect on the outward rectifying properties of YORK whole cell currents (data not shown). This lack of effect of divalent cations on YORK rectification was fully confirmed on excised patches. In outside-out configuration, the complete chelation of divalent cations by EGTA and EDTA in the external bathing solution did not allow inward currents to be recorded (Fig. 3c). This finding has been validated on seven independent patches and differs from the results of Ketchum et al. (27) that appeared while this paper was submitted for publication. Therefore, it cannot be concluded, as these authors did, that the mechanism leading to YORK outward rectification is strictly the same as in inward rectifiers, but in the opposite direction. The blocking element responsible for outward rectification probably belongs to the channel protein itself and obstructs the permeation pathway when the membrane potential is below E K.

Pharmacological Properties—The pharmacology of YORK channels was studied under two-microelectrode conditions.

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Fig. 2. Biophysical properties of YORK currents. a, currents recorded under two-electrode voltage clamp in ND96 solution from oocytes injected with 40 ng of cRNA. Superimposed current traces were induced by voltage steps from a holding potential of -80 mV. b, mean I-V curves (n = 5) measured at the onset of voltage steps (C) and at the end of stimulation (D). Inset, voltage dependence of the time constant of activation. c, relationships between the global conductance (G) and the electrochemical potential (V - E K) for different external K+ concentrations. Conductances were calculated from 1-V curves using the following equation: G = 1/((V - E K)). d, outward rectifying currents measured by inside-out patch single-channel recordings in symmetrical K+ solution (140 mM) (holding potential = -20 mV).

Channels (26), the maximum chord conductance of YORK was approximately proportional to the square root of the external K+ concentration (Fig. 2c). Chord conductance was independent of the presence of Na+ in the bath, demonstrating a high K+ versus Na+ selectivity (data not shown).

In inside-out patches containing several YORK channels, responses to depolarizing voltage steps, from a holding potential of -20 mV, were consistent with those obtained in whole cell measurements, i.e., an instantaneous activity followed by a progressive increase in the number of open channels (Fig. 2d). Single-channel recordings showed that the current was strictly outward, with a unitary conductance of 26 ± 2 picoSiemens (n = 5), and had a high tendency to flicker, with a mean open time of 0.19 ± 0.01 ms (six inside-out patches) (Fig. 3, a and b).

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Outward rectification of YORK currents in excised membrane patches. a, steady-state single-channel activities at the indicated potentials; inside-out (I/O) patch mode in symmetrical K+ solution. b, I-V plot of the mean single-channel currents recorded as in a. c, absence of inward current in divalent-free external solution (n = 7). YORK currents were recorded in outside-out (O/O) patch mode at -60 or +60 mV in symmetrical K+ solution. The same patch was bathed in solution containing either 1 mM Mg2+ (left traces) or 2 mM EDTA and no added divalent cations (right traces).

Currents are blocked by Ba2+ (Fig. 4a). The instantaneous component of the current is 150 times more sensitive than the delayed component. The complete dose-response curve is bimodal, with IC50 values of 20 μM and 3 mM for the instantaneous and delayed components, respectively (Fig. 4b). Quinidine is also a good blocker of YORK current. Like Ba2+, this compound is more potent on the instantaneous component, with an IC50 of 90 μM (Fig. 4d). The sensitivity of the delayed component to quinidine is by far lower and was not accurately determined. Both current components are equally sensitive to tetraethylammonium (IC50 = 5.5 mV) (Fig. 4c). Known blockers of other K+ channels such as Cs+ (10 mM), 4-aminopyridine (1 mM), dextrotoxin (100 mM), charrybotoxin (3 nM), apamin (300 nM), tidesamil (30 μM), and glibenclamide (10 μM) and K+ channel openers such as pinacidil and P1060 (both at 100 μM) had no effect on YORK currents.

Regulation of YORK Channels by Internal pH and Phosphorylation—YORK channel activity was inhibited at acidic pH (Fig. 5, a and b). Inhibition by protons was conserved in inside-out excised patches and shown to result from a decrease in the number of active channels rather than from changes in their unitary conductance or kinetic parameters (Fig. 5c). The pH dependence of the open probability (PO) had a pKα of 6.5, which suggests participation of one of the YORK histidine residues in the blockade (Fig. 5d). Since external pH (from 4.3 to 8) had no effect on YORK activity, it is likely that the pH-sensitive groups are located intracellularly.

Activation of protein kinase C by PMA (30 nM) increased the YORK current (Fig. 6, a and b). The time course of activation (Fig. 6c) and the range of PMA concentrations needed to observe the effect (between 1 and 60 nM) (Fig. 6e) are those expected for the protein kinase C activation in Xenopus oocytes (28, 29). Moreover, the inactive phorbol ester 4α-phorbol 12,13-didecanoate was without effect (Fig. 6c), as was PMA after preincubation with the protein kinase C inhibitor staurosporine (30 μM) (Fig. 6e). Activation of protein kinase C can also be achieved in a more physiological way by activating a receptor coupled to phospholipase C through G proteins. When the cloned 5-HT2 serotonin receptor (30) was coexpressed with YORK, serotonin, similarly to PMA, induced an increase of the K+ current (Fig. 6d). All these results taken together clearly indicate that protein kinase C is involved in YORK regulation. To our knowledge, this is the first description of activation of a cloned K+ channel by protein kinase C. However, when protein
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kinase C was directly applied on excised patches, it did not increase channel activity (data not shown). It is therefore likely that the protein kinase C regulation is indirect and requires the involvement of soluble cytoplasmic components. Elevation of [Ca²⁺], by application of A23187 (1 μM) or microinjection of inositol triphosphate (1 μM) did not affect YORK activity. Therefore, YORK seems insensitive to variations of internal Ca²⁺. Protein kinase A activation by application of 8-chloro-cAMP (300 μM) or forskolin (10 μM) had no effect on channel function.

Gating Kinetics and Proposed Model for YORK Rectification—Tail currents were absent in the voltage range of −120 to +30 mV following voltage pulses to +30 mV, at which the K⁺ channels are fully activated (Fig. 7a). In a simplified kinetic model of the YORK channel comprising only one open state and one closed state, the lack of any detectable time course of the tail currents suggests a very fast transition from the open to the closed state. The ratio between the slow and the instantaneous components of the YORK currents during step depolarizations is dependent on the holding potential. Membrane hyperpolarization clearly increases this ratio, as shown in Fig. 7b, where the same outside-out patch was stimulated repeatedly, first in a polarized condition and then in a depolarized condition. Voltage-dependent changes in the distribution of the two components of the YORK channel currents are also shown in Fig. 7 (c and d). A brief (40 ms) membrane potential change during the onset of YORK current following a step depolarization is enough to modify the ratio between the slow and the instantaneous components of the K⁺ current. The majority of the initial slow component reappeared after a brief hyperpolarization to −200 mV. The time dependence for recovery of the slow component from a voltage pulse to +30 mV is analyzed in Fig. 7 (e and f) by changing the time interval between pulses. The time for 50% recovery of the slow component was −0.5 s. The absence of tail current kinetics and the voltage and time dependences for the recovery of the slow component suggest a conversion of the slow component into an instantaneous component.

The model presented in Fig. 7g takes into account all the properties of YORK channels described in this study. (i) The currents are outwardly rectifying. (ii) As for inward rectifiers, the activation potential is coupled to Eₚ. (iii) The rectification persists in the absence of external divalent cations. (iv) Two kinetically distinct current components are observed.

In this model, an endogenous blocking particle such as an external peptide domain of the channel enters the permeation pathway upon hyperpolarization (Fig. 7g). The presence of two binding sites is hypothesized to account for the instantaneous and the delayed slow currents. A “deep” binding site for the blocking particle is favored in hyperpolarization against a “shallow” binding site. Under polarized conditions, YORK channels are either in the deep blocked state (C₁) or in the shallow blocked state (C₂). Upon depolarization, the channels undergo either a “delayed” conformational change from C₂ to C₁ or an “instantaneous” transition from C₁ to the open state (O). The transition C₁ —> O will give the instantaneous component of the current, while the transitions C₁ —> C₂ —> O will impose the delayed component. Upon repolarization, the only “observable” transition is O —> C₁, which is instantaneous. The model involves two binding sites for the putative blocking element. This view is supported by the fact that both Ba²⁺ and quinine display two affinities for YORK blockade (Fig. 4).
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rents.

Inset b, voltage clamp protocol. c, 1 μA voltage pulse illustrated in the inset of d (1-min interval between each pulse). d, plot of the percentage of recovery for the slow component as a function of membrane potential; same experiment as in Fig. 7e. e, superimposed current traces obtained with the pulse protocol illustrated in the inset of d (1-min interval between each pulse). f, plot of the percentage of recovery for the slow component as a function of the time between two successive test pulses on the recovery of the slow component. g, superimposed current traces at increasing membrane potential.

Conclusions—Outward rectifier K⁺ channels have been described in yeast (31, 32). Unlike YORK, they are Ca²⁺-sensitive, but like YORK, they are tetraethylammonium-sensitive, and their relevant gating parameter appears to be V – E_K instead of V (32). This peculiar property suggests that these channels function in balancing charge movements during nutrient transport in Saccharomyces (33, 34). The gating properties of YORK, its slow activation kinetics, and its inhibition by intracellular acidosis are similar to the properties of K⁺ channels described in stomatal guard cells (35, 36). It is thus likely that the YORK channel has at least a functional counterpart in plant cells and perhaps in other higher organisms as well. A search in the GenBank/EMBL Data Bank with the YORK sequence led to the identification of a Caenorhabditis elegans gene characterized in the framework of the Nematode Sequencing Project (accession number Z35719). This gene encodes a putative K⁺ channel containing two P-domains with GYG and GYG sequences. It seems likely that the presence of two P-domains with the motifs GYG and GLG is a hallmark shared by members of a novel class of K⁺ channels. Whether these structural relationships are associated with functional similarities remains to be determined after cloning and expression of the C. elegans gene.

Acknowledgments—We thank M. M. Larroque, M. Jodar, and N. Leroudier for expert technical assistance and A. Douy for secretarial assistance.

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Fig. 7. Voltage dependence of gating kinetics of YORK currents, a, absence of tail current following a voltage pulse to +30 mV. Inset, voltage clamp protocol. b, same outside-out patch in symmetrical K⁺ solution (140 mV). Average currents were obtained from 30 successive voltage pulses to +60 mV from holding potentials (HP) of -60 mV (left trace) and 0 mV (right trace) (n = 10). c and d, voltage dependence of the recovery of the slow component of the YORK current obtained after a short interval (40 ms) of the test pulse to potentials from -200 to +30 mV. c, superimposed current traces obtained with the pulse protocol illustrated in the inset of d (1-min interval between each pulse). d, plot of the percentage of recovery for the slow component as a function of membrane potential; same experiment as in c. e and f, effect of the time interval between two successive test pulses on the recovery of the slow component. g, superimposed current traces at increasing membrane potential.
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J. Biol. Chem. 1996, 271:4183-4187.
doi: 10.1074/jbc.271.8.4183

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