Volume 242, number 1, 199–206 FEB 06696 December 1988

Potassium channels expressed from rat brain cDNA have delayed rectifier properties

Walter Stühmer, Martin Stocker*, Bert Sakmann, Peter Seeburg+, Arnd Baumann*, Andrew Grupe* and Olaf Pongs*

*Max-Planck-Institut für biophysikalische Chemie, 3400 Göttingen, *Lehrstuhl für Biochemie, Ruhr Universität Bochum, 4630 Bochum* and *+Zentrum für Molekulare Biologie Heidelberg, 6900 Heidelberg, FRG*

Received 25 November 1988

Injection into *Xenopus* oocytes of RNA synthesized in vitro using the rat brain cDNA RCK1 as a template or nuclear injection of the cDNA results in the expression of functional potassium channels. These channels exhibit properties similar to those of the non-inactivating delayed rectifier channel found in mammalian neurons and other excitable cells.

cDNA; cDNA expression; Potassium channel; Delayed rectifier; Single-channel analysis; Mast cell-degranulating peptide; (*Xenopus* oocyte)

1. INTRODUCTION

Excitable membranes display a variety of potassium channels (K^+ channels) with different functional properties. Currents carried by these channels have been separated into voltage-gated currents, such as the delayed rectifier, anomalous rectifier and transient (I_A) currents, and lignd-gated currents, such as Ca^{2+}-activated and ATP-sensitive currents. Recent studies using recombinant DNA techniques indicate that in *Drosophila melanogaster* the *Shaker* gene complex encodes a family of K^+ channels [1–3] with functional properties similar to the channels that mediate the transient, rapidly inactivating I_A current observed in a variety of cell types in different species [4,5]. Using a low stringency hybridization protocol with a *Drosophila Shaker* cDNA probe a homologous cDNA, named RCK1, was isolated from a rat cerebral cortex library [6]. We have now expressed in *Xenopus* oocytes the protein encoded by this cDNA and have characterized the functional and pharmacological properties of the membrane channels formed by this protein. These channels are different from those formed by the *Shaker* gene complex and functionally resemble the delayed rectifier K^+ channel.

Correspondence address: W. Stühmer, Max-Planck-Institut für biophysikalische Chemie, Postfach 2841, 3400 Göttingen, FRG

Abbreviations: cRNA, cDNA derived mRNA; DTX, Dendrotoxin; MCDP, mast-cell degranulating peptide; TEA, tetraethylammonium chloride; 4-AP, 4-aminopyridine

2. MATERIALS AND METHODS

Two alternative strategies were used to translate RCK1 channels. One involved direct injection into the *Xenopus* oocyte nucleus [7,8] of a vector, pCDM8, with a cytomegalo virus (CMV/T7) promoter containing the RCK1 coding region. The other consisted of in vitro transcription of the cDNA into mRNA which was then injected into the cytoplasm of the oocytes. Both injection procedures resulted in expression of whole cell currents with amplitudes in the range of 5–40 μA.

The pCDMs vector, with a length of 4.8 kbp, has a Sup F, CMV/T7 promoter, a polylinker site, a splice and a poly-A coding region and Py, SV40, πVX and M13 origins of replication.

For the preparation of cRNA, RCK1 cDNA was digested with *Eco*RI/*Hin*dIII and overhanging ends were filled in with Klenow-DNA polymerase. The DNA was then cloned into the *Sma*I restriction site of pAS18. This vector was derived from pSPT18 (Pharmacia) by inserting 70 A-residues into the polylinker region between *Sst*I and *Eco*RI sites. The pAS18-RCK1 recombinant was linearized by cutting at the *Eco*RI site. Capped run-off cRNA was synthesized by a standard protocol [9].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

Xenopus oocytes were injected with either the pCDM8 construct or the cRNA and incubated for 2–3 days at 19°C [10]. Whole cell currents were recorded using a standard two microelectrode voltage clamp in normal frog Ringer solution of the following composition (in mM) NaCl 115, KCl 2, $CaCl_2$ 1.8, Hepes 10 (pH 7.2). DTX and MCDP were a generous gift from Drs F. Dreyer and E. Habermann. Single-channel currents were recorded in the cell-attached and in the inside-out patch configuration [11]. Pipettes were filled with extracellular solution unless otherwise stated. The bathing solution in experiments with inside-out patches contained (in mM): KCl 100, EGTA 10, Hepes 10 (pH 7.2). All experiments were carried out at 20–22°C.

3. RESULTS

3.1. *Whole cell currents in* Xenopus *oocytes expressing RCK1*

Depolarizing voltage steps in *Xenopus* oocytes injected with RCK1 cRNA elicited outward currents (fig.1A). This current was voltage-dependent and started to activate at potentials positive to −30 mV (fig.1B). Control (uninjected) oocytes never showed currents of any type with amplitudes greater than 100 nA. In contrast, more than 90% of injected oocytes, taken from 8 frogs, exhibited outward currents greater than 2 μA. Inactivation of these currents was very slow, with current magnitude decreasing less than 20% during 10 s voltage steps to 0 mV membrane potential. Activation was fast, being in the range of milliseconds. From similar experiments with sodium channels [12], which have activation kinetics in the submillisecond range, we estimate that the change in potential is complete in much less than a millisecond. Thus the activation of these currents is not disturbed by the limited clamp speed.

Ion substitution experiments indicated that the reversal potential of the outward currents depends on the external potassium concentration, $[K]_o$, as expected for a K^+-selective channel. Tail currents recorded at various membrane potentials from an oocyte expressing RCK1 cRNA are shown in fig.2A. In this particular example, recorded in 50 mM $[K]_o$, the tail current reverses direction at about −30 mV. The value of the reversal potential varied with the extracellular potassium concentration. The dependence of reversal potential on $[K]_o$ is plotted on semi-logarithmic coordinates for $[K]_o$ ranging from 2.5 (normal frog Ringers) to 100 mM in fig.2B. The data were well fitted by the Nernst equation, giving a slope of 55.1 mV for a 10-fold change in $[K]_o$. This indicates that the channels have a higher selectivity for K^+ over Na^+ and Cl^-. The datapoint at 2.5 mM $[K]_o$ was not considered reliable due to the fact that at this low potassium concentration it was difficult to measure reversal potentials because inward tail currents were very small.

We next examined the sensitivity of the K channel to various pharmacological agents. The RCK1 K^+ channel was blocked by external application of TEA, 4-AP, DTX and MCDP. Concentration-response curves for these blockers were obtained by measuring the amplitude of outward current at the end of a 120 ms pulse to +20 mV (fig.3). The concentrations of channel blocker or toxin which inhibited the ionic currents by 50% (IC_{50}) were: 12 nM for DTX, 45 nM for MCDP, 0.6 mM for TEA and 1.0 mM for 4-AP. The concentration-response curves did not depend on the value of the test potential chosen and did not show any use- or time-dependent block. Although the effects of TEA, DTX and MCDP on current amplitude were completely reversible, MCDP-treated channels showed slower activation kinetics after washout of the peptide (not shown). The 4-AP block was only partially reversible, recovering to only 20% of control values. The currents were insensitive to apamin (1 μM), which inhibits a TEA-insensitive Ca^{2+}-activated K^+ channel of small conductance [13,14], or β-bungarotoxin (200 nM), which inhibits a DTX-sensitive delayed rectifier in dorsal root ganglion neurons [15].

3.2. *Single-channel currents in* Xenopus *oocytes expressing RCK1*

Currents through single-channels were recorded from inside-out patches where the extracellular side was exposed to normal frog Ringer's solution and the cytoplasmic side to a solution containing 100 mM K^+. The four upper traces in fig.4A represent responses to a depolarizing voltage step to 0 mV from a holding potential of −60 mV. After a variable latency, voltage steps activated elementary outward currents. This record probably represents activation of a single channel as no superimposition of elementary currents to higher levels was seen. This was not the case in other patches isolated from oocytes with a higher channel density (as judged from the larger whole-cell current response of the oocytes). Elementary currents were superim-

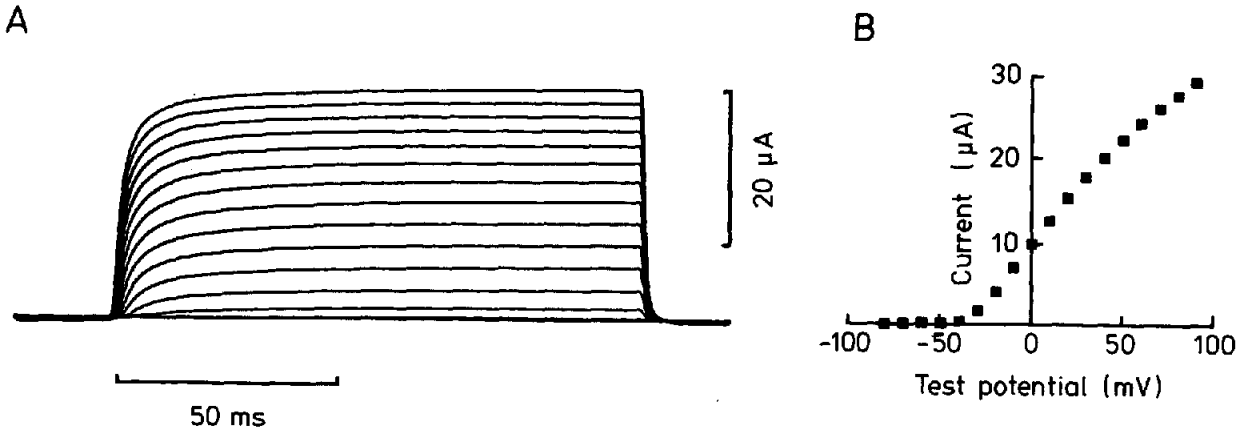


Fig.1. Delayed, non-inactivating outward currents in *Xenopus* oocytes injected with mRNA derived from RCK1 cDNA. (A) Outward currents in response to depolarizing voltage steps. The membrane potential was held at −80 mV and stepped at 2 s intervals to test potentials ranging from −80 to +90 mV in 10 mV increments. (B) Steady-state currents from the experiment shown in A are plotted as a function of membrane potential after leak subtraction.

posed on an average current (lowermost trace) which had an activation time course (3.7 ms half-activation rise time) comparable to that of the whole cell current, elicited by depolarization to 0 mV. Elementary currents were not inactivated within seconds (fig.5A). However, during frequent and repetitive stimulation at 1 Hz, 'blank' responses occurred, where the channel did not open (not shown). Blank responses always clustered into groups, suggesting the existence of a very slow inactivation process (see below).

Amplitude histograms collected at 0 mV membrane potential from current records such as those shown in figs 4A and 5A indicate that elementary currents have a most frequently occurring 'main' amplitude of 0.78 ± 0.06 pA (mean ± SD, $n = 5$). In addition, two less frequently occurring sublevels were observed in the seven patches examined

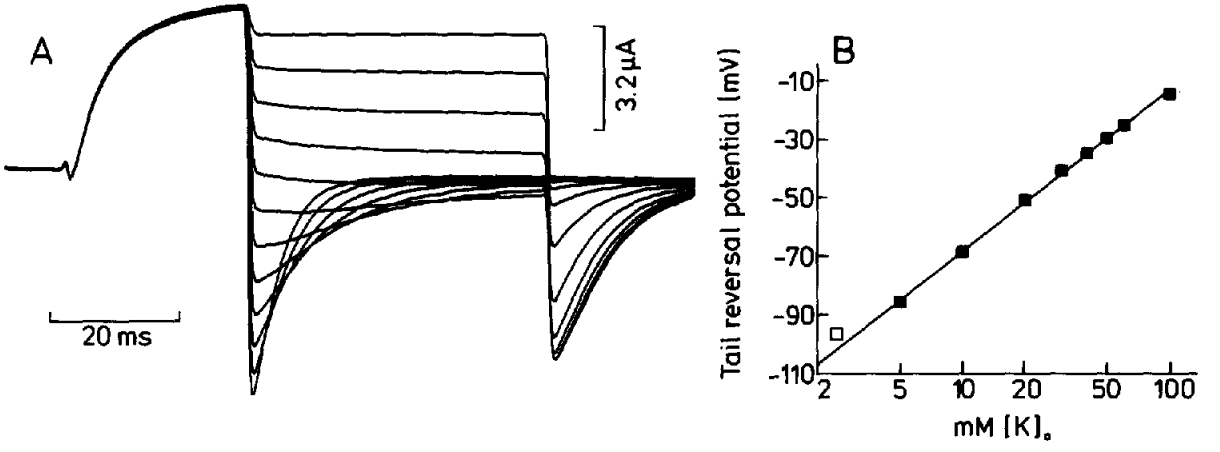


Fig.2. Potassium concentration dependence of the reversal potential of outward currents in *Xenopus* oocytes injected with RCK1 cRNA. (A) Tail currents recorded in an RCK1 expressing oocyte bathed in a solution containing 50 mM $[K]_o$. Following a depolarizing prepulse to +20 mV, the membrane potential was stepped to tail potentials ranging from −100 to +10 mV. The initial amplitude of the tail current was determined by extrapolating the current to the beginning of the pulse. The reversal potential was obtained as the potential, at which the tail currents reversed direction. (B) Reversal potentials obtained from tail current analysis. Current reversal potential plotted as a function of $[K]_o$. The K^+ concentration was raised by replacing with isoosmolar amounts of Na^+.

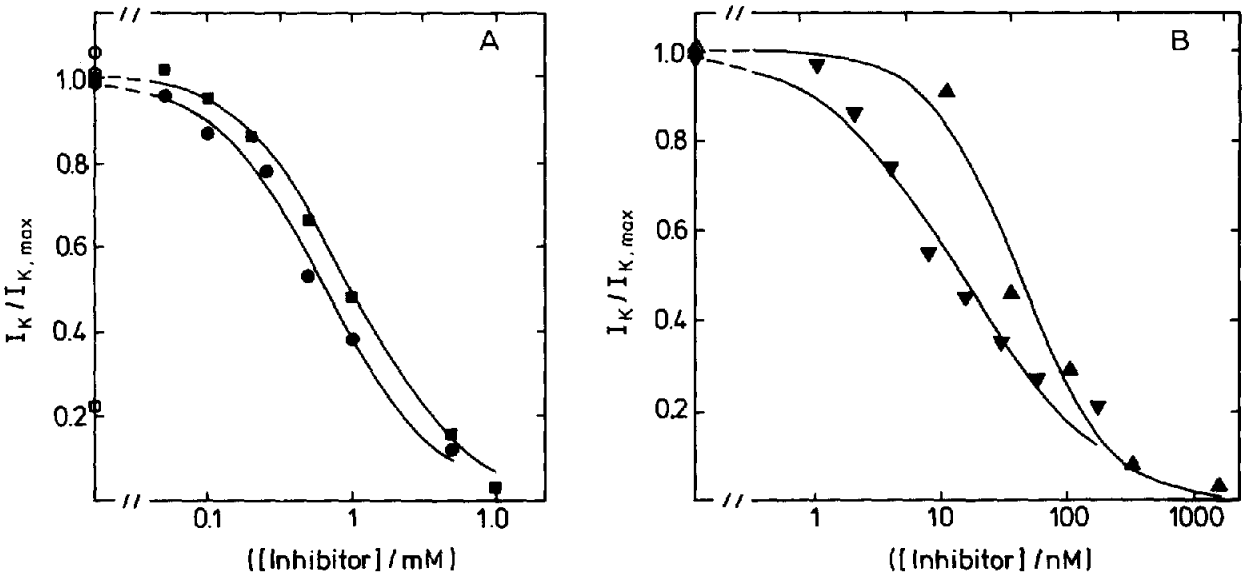


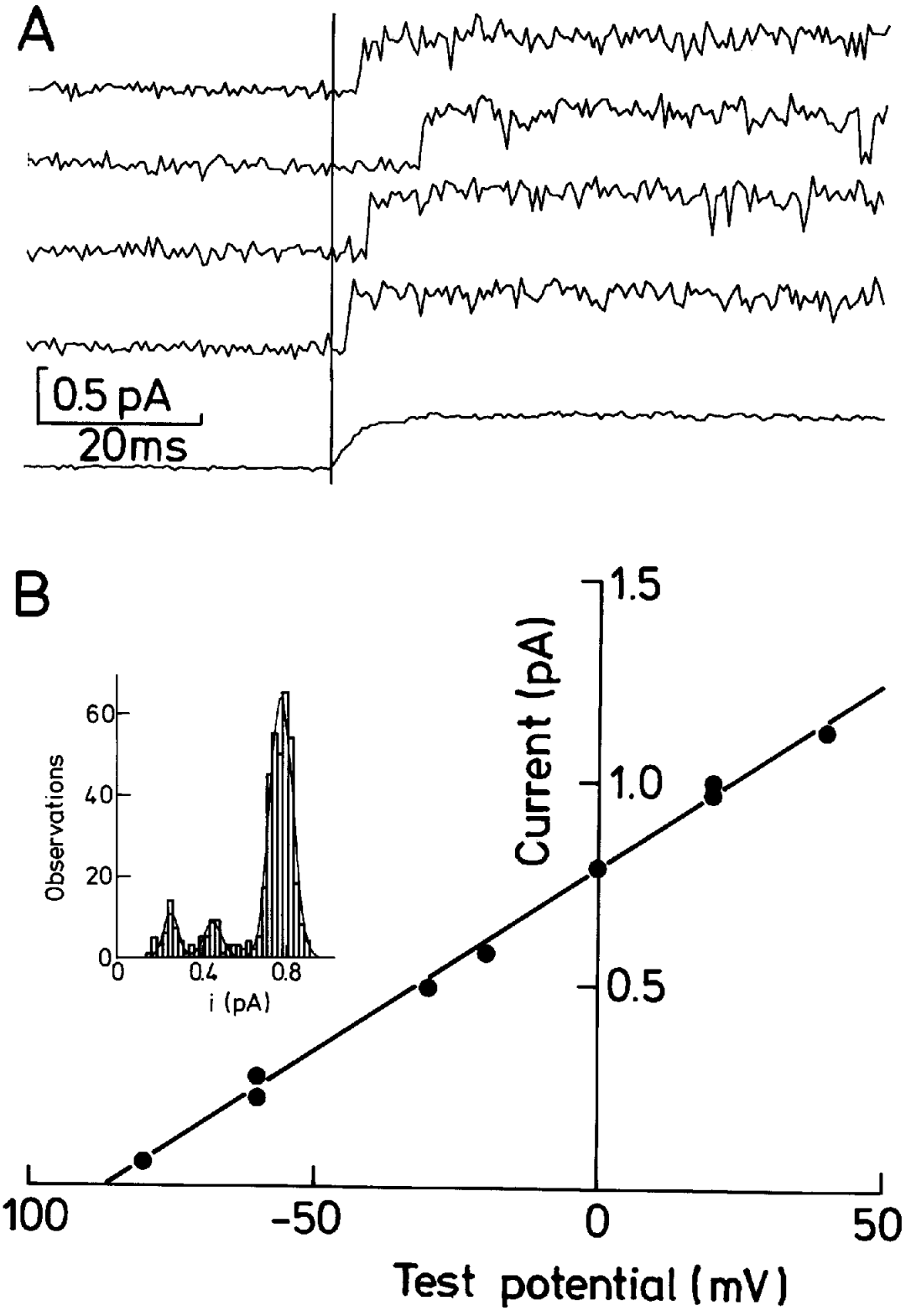
Fig.3. Sensitivity of outward currents elicited after injection of RCK1 cRNA into oocytes towards K^+-channel blockers added to the bathing solution (normal frog Ringer's). (A) Concentration dependence of TEA (●) and of 4-AP block (■). (B) Concentration dependence of DTX (▼) and of MCDP block (▲). Open symbols indicate the recovery of outward current when the bathing solution with the highest blocker concentration was replaced by normal frog Ringer's. Peak currents at +20 mV test potentials were 15.5 μA (TEA), 15.7 μA (4-AP), 5.1 μA (DTX) and 2.2 μA (MCDP), respectively.

(fig.4B, inset). The slope conductances of the RCK1 channel in its main open state, as determined in two patches over a potential range from −60 mV to 40 mV, was 8.9 and 9.3 pS (fig.4B). The current reversal potential, obtained by linear back-extrapolation, was −82 mV. This value is close to the value of −89 mV predicted from the Nernst equation for a K^+-selective channel. The single-channel conductance increased to 22 pS (for the main state) when the K^+ concentration on the extracellular side of the membrane was increased to 100 mM K^+ (not shown).

When K^+ on the cytoplasmic face was replaced by Na^+, elementary currents were no longer detectable. Partial replacement of K^+ by 50 mM Cs^+ blocked elementary currents with an apparent half blocking concentration of 15–18 mM Cs^+. Similarly, addition of 2 mM TEA to the bath solution facing the cytoplasmic side of the patch, reduced the size of single-channel currents to less than half the control value (one experiment for each of these three conditions).

The gating behaviour of single RCK1 channels was not homogeneous. In five of the seven patches examined the channel predominantly occupied one of its open, conducting states after being activated, closing only briefly at irregular intervals. The average open and closed times measured in the patch illustrated in fig.5A (apparently containing only one channel) were 4.6 ms and 1.5 ms, respectively. The average open probability at 0 mV was 0.76. Comparable values were found in a second

Fig.4. Currents through single channels in inside-out patches from an oocyte injected with RCK1 cRNA. Pipette contained normal frog Ringer's, bathing solution contained 100 mM K^+ solution facing the cytoplasmic side of the membrane. (A) Successive sweeps of current responses to voltage steps from −60 mV to 0 mV. The lowermost trace represents the averaged response from 70 successive depolarizations. Leakage and capacitive currents were subtracted digitally. Filter: 1 kHz (−3 dB). (B) The inset shows an amplitude histogram of outward currents at 0 mV test potential. Amplitudes were measured as the distance between the baseline and current levels lasting at least 20 ms. Fit of three gaussians with means of 0.78, 0.49 and 0.24 pA, respectively. The graph shows the single channel current-voltage relation (*i-V*). Each point represents the mean amplitude of at least 20 determinations of the largest and most frequently occurring unitary current level. Linear regression yields a channel conductance of 9.3 pS for the main open state. Current amplitudes at −60 and −80 mV were obtained from openings observed briefly after repolarization (tail currents).


A
0.5 pA
20ms
B
Observations
60
40
20
0
0
0.4
0.8
i (pA)
Current (pA)
1.5
1.0
0.5
-100
-50
0
50
Test potential (mV)

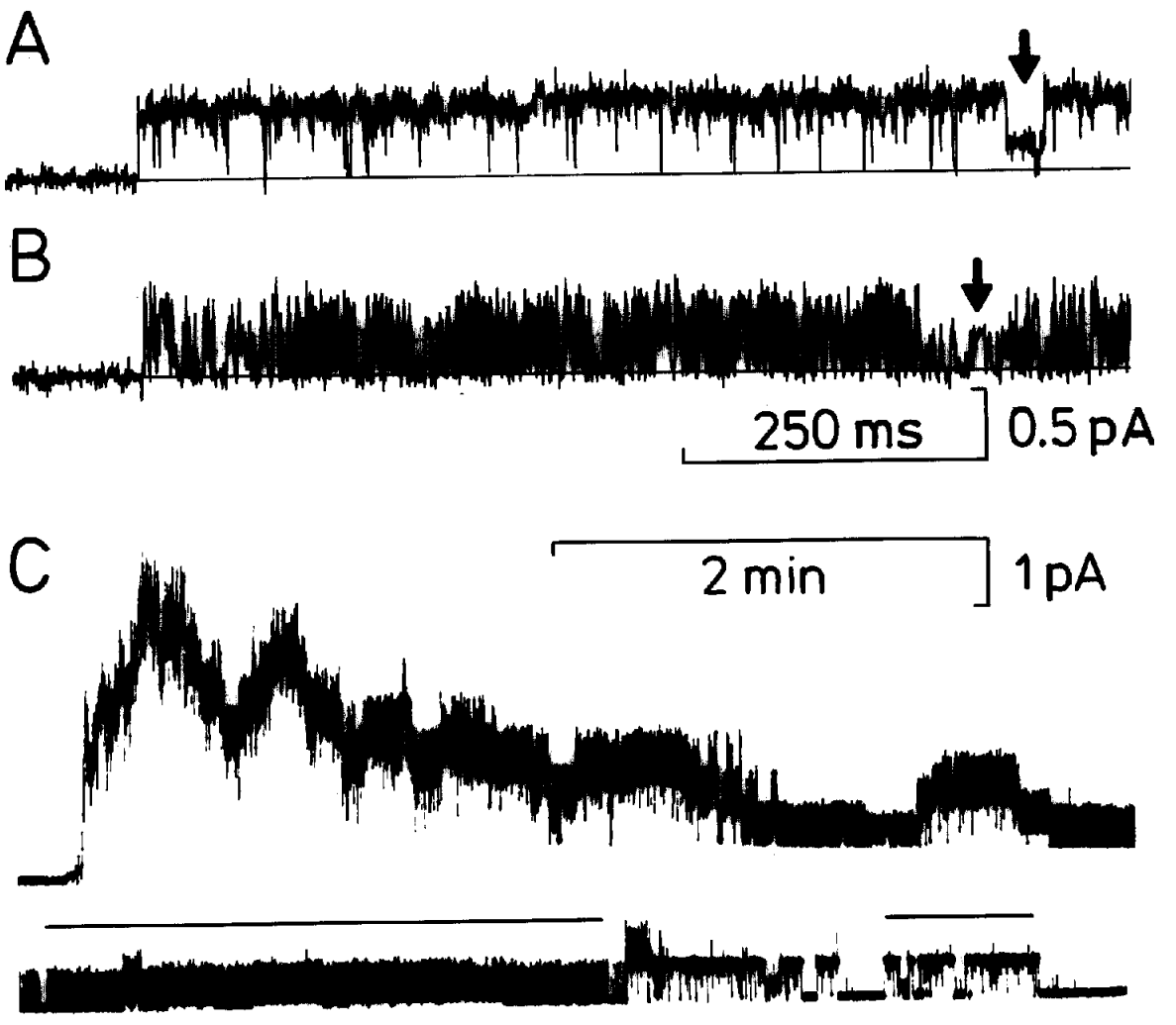


Fig.5. Gating behaviour of single RCK1 channels in inside-out patches. (A,B) Different gating modes. Both traces are the initial part of a current response to a voltage step from −60 to 0 mV (1 kHz filtering, −3 dB). Upper trace shows the predominant type of gating with high open probability (average P_o = 0.76). Same patch as in fig.4A. Lower trace is a recording under same conditions from another patch which shows the less frequently occurring type of gating characteristically with lower open probability (P_o = 0.44) of the channel. P_o values were measured during 3–4 trials and epochs of 0.5–3 s duration were analysed in each trial. Threshold criterion for transitions between open and closed states was 0.4 pA and minimum duration of an open or closed state was 0.5 ms. Average open and closed times were determined by dividing the total time the channel spent in either of the two states by the number of openings and closings. Arrows mark substates. (C) Inactivation of RCK1 channels in the time range of tens of seconds. Pen-writer recording, 0.4 kHz filter. Up to six channels are open simultaneously after changing the membrane potential manually from −60 to 0 mV, which gradually close. The lower trace is a continuation of the recording shown in the upper trace and shows that different gating behaviour occurs with channels in the same patch.

patch containing only a single channel. RCK1 channels can also adopt a different gating behaviour which consists predominantly of a rapid succession of brief openings and closures, both with average durations of milliseconds (fig.5B). This type of gating was observed in three of seven pathces. One of the three patches with channels showing this behaviour contained apparently only one channel. The average open and closed times were 2.1 ms and 2.8 ms, respectively, for this

patch. The average open probability at 0 mV was 0.44. In three multichannel patches both gating modes were observed. In single-channel patches the gating mode remained unchanged for long periods (minutes) and transitions between gating modes were rare, suggesting that individual channels preferentially adopt a single mode of gating. It cannot be determined at present whether the two gating modes derive from a single-channel type or from different molecular forms of the RCK1 channel (e.g. homooligomers with different numbers of subunits). The fact that the current amplitude distributions in different gating modes are similar suggests that the structural differences responsible for these different forms of gating may be small.

Although RCK1 channels do not seem to be inactivated in the time range of seconds, a very slow inactivation, in the range of tens of seconds, was observed. Fig.5C shows a recording from a patch containing several RCK1 channels. Upon depolarization of the membrane/potential from −60 mV to 0 mV the patch current fluctuated between several levels, which were multiples of the unitary level and which reflected the simultaneous opening of 5–6 channels. The number of open channels then decreased slowly until the current fluctuated between the baseline and the first open level and eventually settled at the baseline. At irregular intervals after this, channel activity reappeared for periods of several seconds. During this time the occurrence of two gating modes for RCK1 channels was obvious (indicated by bars in fig.5C, lower trace). Repolarization of the patch to −80 mV removed the slow inactivation process and currents through single channels could again be elicited by depolarizing steps.

4. DISCUSSION

The results reported here indicate that the RCK1 cDNA isolated from rat cortex encodes a protein which, when inserted into the surface membrane of *Xenopus* oocytes, forms voltage-gated channels. These channels are highly selective for K^+ and the macroscopic currents mediated by these channels have time courses resembling those of the classical, delayed outward K^+ currents described for nerve and muscle [16,17]. The properties of single RCK1 channels are not completely homogeneous; they can adopt multiple-conductance states and switch between different gating modes. This may reflect intrinsic properties of a single type of RCK1 channel formed by several identical subunits or it may indicate heterogeneity of RCK1 channels, perhaps due to RCK1 channels assembled with a different number of subunits.

The currents mediated by RCK1 channels in *Xenopus* oocytes share several properties with those mediated by delayed rectifier channels in nerve membranes. Both channel types are highly selective for K^+, activate at a membrane potential of −35 mV to −30 mV and do not inactivate in the time range of hundreds of milliseconds. They are sensitive to K^+ channel blocking agents, such as TEA and 4-AP, and are particularly sensitive to the toxins DTX and MCDP [18–20]. Analysis of single-channel currents in other systems has shown that whole-cell delayed K^+ currents may be mediated by several different subclasses of K^+ channels [21,22]. It is possible that the channels we describe here contribute to the delayed K^+ current seen in neurons. Alternatively, the channels we have studied may represent an 'incomplete' channel version lacking additional subunits or may be composed of different numbers of subunits than the channels found in neurons. Single-channel current recordings from pyramidal cells in slices of rat hippocampus show that non-inactivating K^+ channels of small conductance (10 pS) are present in the soma membrane of hippocampal neurons (Edwards, F. and B.S., unpublished). Further, a similar small conductance K^+ channel has been described recently in frog spinal neurons in tissue culture [21]. These observations suggest that RCK1 channels are expressed in CNS neurons and constitute a 'low conductance subclass' of K^+ channels that contribute to delayed outward currents. The similar conductance of the RCK1 channels that we have observed could indicate that the small conductance K^+ channels seen in rat brain are homooligomers of the RCK1 polypeptide.

The major difference between the properties of the RCK1 channels described here and those of channels expressed in *Xenopus* oocytes following injection of the *Drosophila Shaker* complex derived cRNAs (ShC channels) [5,23] is their inactivation behaviour. The various ShC channels described so far are inactivated in the time range of tens of milliseconds [5,23,24], while the RCK1 channels are inactivated over tens of seconds.

These channels are very similar, however, in their single-channel conductances, voltage dependence of activation and ion selectivity [23]. It has been suggested that inactivation is dependent on amino acids close to the C- or N-terminal end of the channel peptide [23,24]. This suggestion is consistent with our results, because the major differences between the RCK1 protein and the ShC proteins are found in the C-terminal region neighbouring the core domain [6]. Small differences in amino acid sequence between highly homologous channels could lead to large differences in gating properties of channels which have similar or even identical ion transport and voltage sensing properties. The highly conserved core region suggests a common ancestor for ShC and RCK1 channels. It could be that during evolution different K^+ channel subtypes were generated by modifying the 3'- and 5'-ends of the peptide giving rise to I_A-type and delayed rectifier currents.

Acknowledgements: We thank Drs F. Dreyer and E. Habermann for the generous gift of DTX and MCDP, X. Wang for help with the oocyte injections and Dr G. Augustine for comments on the manuscript. This work was supported by a grant of the Deutsche Forschungsgemeinschaft to O.P.

REFERENCES

[1] Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. and Jan, L.Y. (1988) Nature 331, 137–142.
[2] Pongs, O., Kecskemethy, N., Müller, R., Krah-Jentgens, I., Baumann, A., Kiltz, H.H., Canal, I., Llamazares, S. and Ferrus, A. (1988) EMBO J. 7, 1087–1096.
[3] Kamb, A., Tseng-Crank, J. and Tanouye, M.A. (1988) Neuron 1, 421–430.
[4] Tempel, L.B., Jan, Y.N. and Jan, L.Y. (1988) Nature 332, 837–839.
[5] Timpe, L.C., Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. and Jan, L.Y. (1988) Nature 331, 143–145.
[6] Baumann, A., Grupe, A., Ackermann, A. and Pongs, O. (1988) EMBO J. 7, 2457–2463.
[7] Voellmy, R. and Rungger, D. (1982) Proc. Natl. Acad. Sci. USA 79, 1776–1780.
[8] Rungger, D. and Türler, H. (1978) Proc. Natl. Acad. Sci. USA 75, 6073–6077.
[9] Krieg, P. and Melton, D.A. (1984) Nucleic Acid Res. 12, 7057–7070.
[10] Gundersen, C.B., Miledi, R. and Parker, I. (1983) Proc. R. Soc. London B220, 131–140.
[11] Methfessel, C., Witzemann, V., Takahashi, T., Mishina, M., Numa, S. and Sakmann, B. (1986) Pflügers Arch. Ges. Physiol. 407, 577–588.
[12] Stühmer, W., Methfessel, C., Witzemann, V. and Sakmann, B. (1986) in: Membrane Control of Cellular Activity (Lüttgau, H.C. ed.) Fortschritte der Zoologie, vol. 33, pp. 155–159, Gustav Fischer, Stuttgart.
[13] Romey, G., Hugues, M., Schmidt-Antomarchi, H. and Lazdunski, M. (1984) J. Physiol. (Paris) 79, 259–264.
[14] Blatz, A.L. and Magleby, K.L. (1984) J. Gen. Physiol. 84, 1–23.
[15] Petersen, M., Penner, R., Pierau, F.-K. and Dreyer, F. (1986) Neurosci. Lett. 68, 141–145.
[16] Hodgkin, A.L. and Huxley, A.F. (1952) J. Physiol. 117, 500–544.
[17] Stanfield, P.R. (1983) Rev. Physiol. Biochem. Pharmacol. 97, 1–67.
[18] Weller, U., Bernhardt, U., Siemen, D., Dreyer, F., Vogel, W. and Habermann, E. (1985) Naunyn Schmiedeberg's Arch. Pharmacol. 330, 77–83.
[19] Penner, R., Petersen, M., Pierau, F.-K. and Dreyer, F. (1986) Pflügers Arch. 407, 365–369.
[20] Stansfeld, C.E., Marsh, S.J., Parcej, D.N., Dolly, J.O. and Brown, D.A. (1987) Neuroscience 23, 893–902.
[21] Harris, G.L., Henderson, L.P. and Spitzer, N. (1988) Neuron 1, 739–750.
[22] Llano, I., Webb, C.K. and Bezanilla, F. (1988) J. Gen. Physiol. 92, 179–196.
[23] Iverson, L.E., Tanouye, M.A., Lester, H.A., Davidson, N. and Rudy, B. (1988) Proc. Natl. Acad. Sci. USA 85, 5723–5727.
[24] Timpe, L.C., Jan, Y.N. and Jan, L.Y. (1988) Neuron 1, 659–667.