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Cloning and functional expression of a TEA-sensitive A-type potassium channel from rat brain

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A rat brain cDNA (Raw3) related to the *Drosophila Shaw* K^+ channel family has been characterized. Raw3 cRNA leads to the formation of TEA-insensitive, fast inactivating (A-type) K^+ channels when injected into *Xenopus laevis* oocytes. Raw3 channels have markedly different properties from the previously cloned rat A-type K^+ channel RCK4. Raw3 channels operate in the positive voltage range.

cDNA cloning, cRNA expression, Potassium channel, A-type K^+ channel, I_A channel

1. INTRODUCTION

Voltage-gated K^+ channels play an important role in the regulation of membrane potential and cell excitability [1]. A-Type potassium channels regulate the firing frequency of neurons because they are usually active in the potential range negative to the threshold of excitation. A-Currents often have been distinguished from other K^+ currents by their sensitivity to 4-AP [2]. Many different mammalian cDNAs encoding K^+ channel forming proteins have been cloned [3, 4]. Functional expression of the derived cRNAs led to K^+ channels having delayed rectifier properties except in the case of RCK4. cRNA encoding RCK4 is, so far, the only one that leads to the formation of fast inactivating (A-type) K^+ channels [5] when injected into *Xenopus* oocytes. A-Channels described in the literature vary widely in their pharmacological profiles and kinetics [2]. Some of them resemble RCK4 channels, other do not. RCK4-mediated A-currents are resistant to TEA and are not very sensitive to blockage by 4-AP. Thus it is most likely that the rat genome encodes other A-type K^+ channel proteins different from RCK4. Here we report the isolation and functional expression of a new A-type K^+ channel cDNA (Raw3).

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Abbreviations, K^+, potassium, Ca^{2+}, calcium, cRNA, cDNA derived mRNA, TEA, tetraethylammonium, 4-AP, 4-aminopyridine, DTX, dendrotoxin

2. MATERIALS AND METHODS

A rat cortex cDNA library (a gift from P. Seeburg, Heidelberg) has been screened with a ^{32}P labelled NGK2 [18] cDNA probe under conditions of low stringency [6, 17]. Two overlapping cDNAs were isolated and sequenced [7, 8]. The combined cDNA sequence was 2858 bp long. The composite raw3 cDNA was cloned into the expression vector pAS2. This vector was constructed of Bluescript KS$^+$ (Stratagene) and pAKS18 [8]. After filling in with DNA-polymerase I (Klenow) an *Ase*I fragment (nt 3320–352) of pAS18, containing the SP6 promoter, the multiple cloning site and the polyA region (266 bp) was cloned into *Pvu*II cut Bluescript KS$^+$. Raw2 pAKS2 was generated by cloning the 5′ *Pst*I/*Stu*I fragment of cDNA 76 (nt −155 to 749) together with the 3′ *Stu*I/*Asp*718 fragment of cDNA 3 (nt 749–2437) into *Pst*I/*Asp*718 cut pAKS2. The resulting raw3-pAKS2 recombinant was linearized by cutting at the *Eco*RI site. Capped run off cRNA for injection into *Xenopus* oocytes was synthesized by a standard protocol [9].

Xenopus laevis oocytes were injected with cRNA and incubated for 2–3 days at 19°C [10]. The kinetic properties of Raw3 channels were determined from macro-patch recordings [11] in the cell-attached configuration of the patch clamp technique. The pharmacological profile was established using a conventional two-microelectrode voltage-clamp. Microelectrodes were filled with 1 M KCl and had a resistance of 200–500 kΩ. Single channels were recorded in the cell-attached configuration. All experiments were done at 20°C in normal frog Ringer solution of the following composition (in mM): NaCl 115, KCl 2, $CaCl_2$ 1.8, Hepes 10, pH 7.2. In some experiments sodium was replaced by 4-AP or TEA (Sigma) or DTX (gift from Dr. F. Dreyer, Giessen, Germany) was added. Patch pipettes were filled with normal frog Ringer solution. Leak and capacitive currents were subtracted digitally using the P/4 method [12].

3. RESULTS

3.1. *Primary sequence of raw3 K^+ channel protein*

Two overlapping cDNAs, encoding Raw3, were isolated from a rat cortex cDNA library. The longest open reading frame corresponds to an amino acid sequence of 625 residues (Fig. 1). The hydropathy analysis [13]

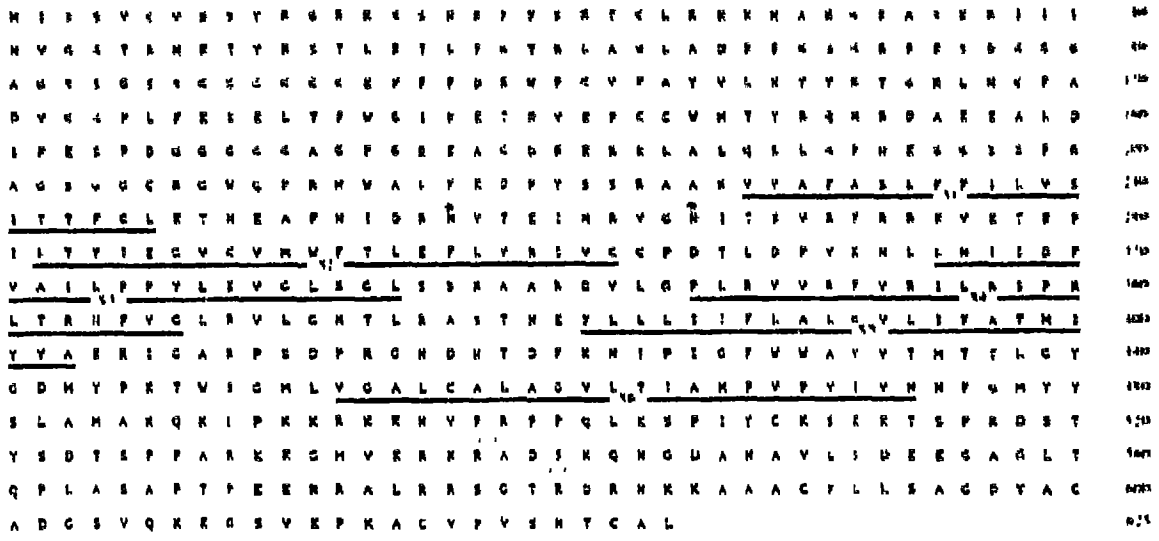

Fig. 1 Amino acid sequence deduced from Raw3 cDNA. Putative membrane spanning segments (S1 to S6) are underlined. Potential *N*-glycosylation sites are marked with an asterisk. Residues 537-541 (KRADS) encode a putative PKA phosphorylation site. The open reading frame was derived from two overlapping cDNAs. The composite Raw3 nucleotide sequence has been submitted to the EMBL data bank.

(not shown) reveals six putative membrane spanning segments (underlined in Fig. 1) similar to other cloned K^+ channel proteins. The segments consist of five hydrophobic (S1, S2, S3, S5 and S6) and one positively charged segment (S4), possibly the voltage sensor of the channel. A leucine zipper motif which may be involved in channel gating connects segments S4 with S5, as for most other K^+ channels [14]. The charge distribution in segments S2 and S3 is similar to that previously found in other K^+ channel protein sequences [15]. Like other K^+ channel proteins Raw3 protein is probably post-translationally modified by *N*-glycosylation between segments S1 and S2 and by phosphorylation in the carboxy-terminus (Fig. 1). The most remarkable difference between the Raw3 protein sequence and other K^+ channel sequences is that the two highly conserved sequence motifs NEYFFD in the N-terminal sequence and MTTVGY between segments S5 and S6 [16] are mutated to QEFFFD and to MTTLGY, respectively. Since the Raw3 protein sequence is highly conserved in the transmembrane spanning region (S1-S6) and in the residues preceding segment S1, Raw3 protein appears as another member of the K^+ channel protein superfamily. When compared with other K^+ channel sequences, that of Raw3 protein is most closely related (78% identity) to those of *Shaw* [17], NGK2 [18] and RK*Sh*IIIA [4] proteins. Obviously, Raw3 is a member of the *Shaw* K^+ channel family.

3.2. *Functional and pharmacological properties of currents mediated by Raw3 channels*

Currents mediated by Raw3 K^+ channels were characterized in the *Xenopus laevis* oocyte expression system. Oocytes expressing Raw3 specific cRNA were first

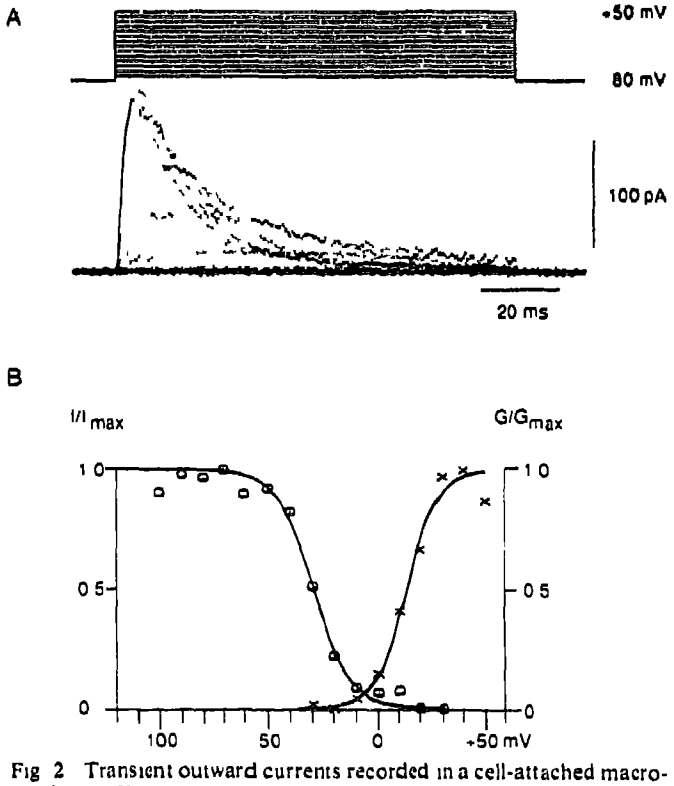

Fig. 2 Transient outward currents recorded in a cell-attached macropatch on a *Xenopus* oocyte previously injected with Raw3 cRNA. (A) Outward currents in response to depolarizing voltage steps. The membrane potential was stepped from a holding potential of −80 mV to test potentials ranging from −70 mV to +50 mV, in 10 mV increments. Intervals between pulses were 25 s. The current was low pass filtered at 1.5 kHz. (B) Voltage dependence of conductance and steady state inactivation (stars). The data points were fitted with single Boltzmann isotherms to get V_{h_1} (13.2 mV) and a_h (7.4 mV) for the activation curve and $V_{h_{1/2}}$ (−29.3 mV) and a_h (8.3 mV) for the inactivation curve.

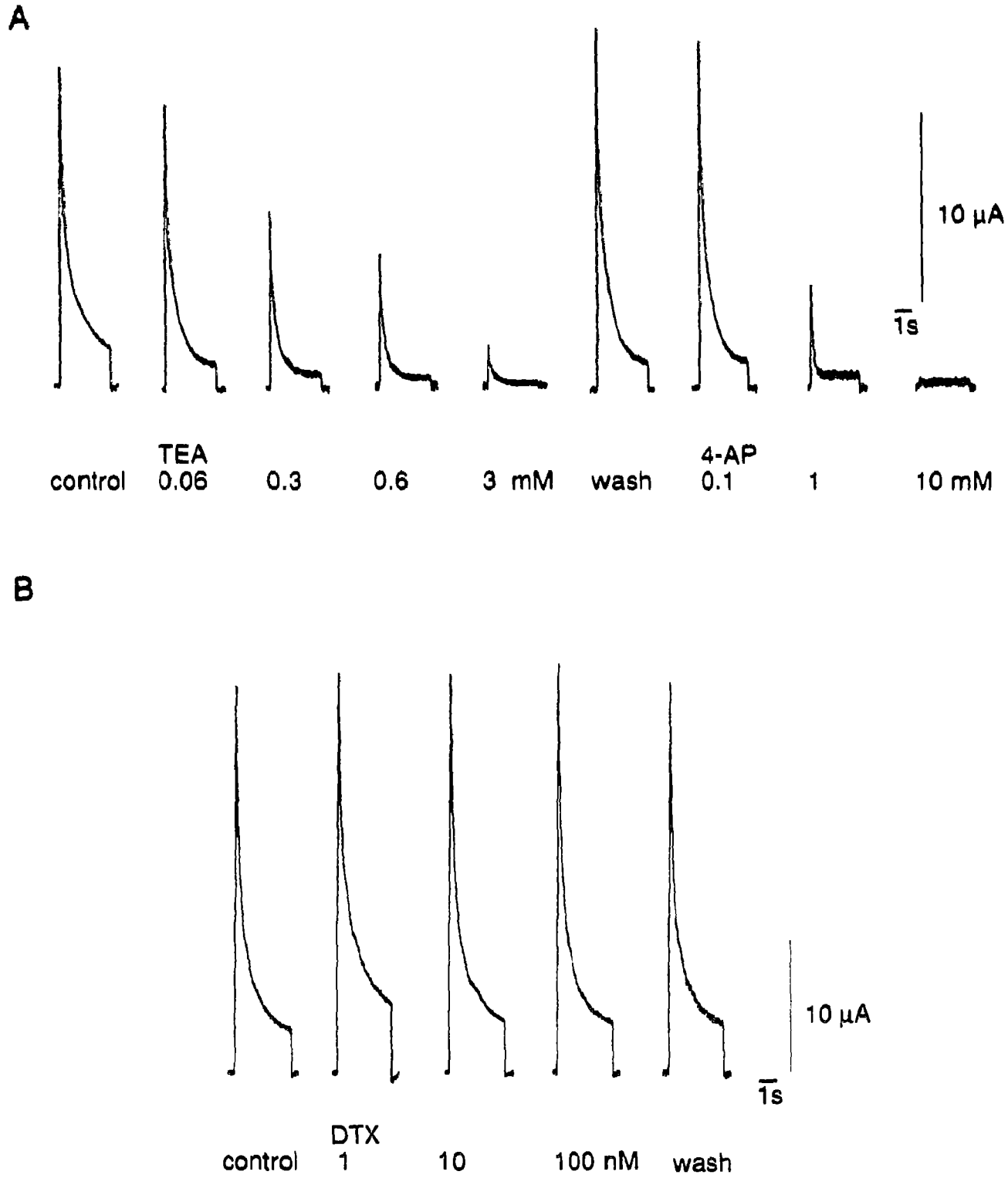

Fig 3 Effect of blockers on Raw3 channels measured in two-microelectrode voltage-clamp Current responses to depolarizing pulses from −80 mV to +20 mV test potential The current was low pass filtered at 1 5 kHz (A) Sensitivity to TEA and 4-AP The control trace shows the current response before the application of drugs and the following four traces illustrate the increasing block of current by increasing concentrations of TEA. The concentration of 50% block (IC_{50}) was about 0 3 mM TEA The sixth trace shows that the action of TEA is reversible The block by 4-AP was tested on the same oocyte after wash-out of TEA. The traces 7-9 show the effect of 4-AP in concentrations between 0 1 mM to 10 mM IC_{50} was 0 5 mM 4-AP and the block was only to 30% reversible (not shown) (B) Sensitivity to DTX tested in another oocyte The first trace shows the current response before addition of DTX The following three traces illustrate that up to the concentration of 100 nM DTX has no effect on the current The last trace shows the unchanged current after wash-out of DTX

tested with two-microelectrode voltage-clamp. Depolarizing steps to positive test potentials elicited transient outward currents with a peak amplitude of 5–20 μA. To determine their exact time course and voltage dependence of activation, ensemble K^+ outward currents were measured in cell-attached macro-patches. Fig. 2A shows a family of current traces in response to depolarizing test pulses ranging from −70 mV to +50 mV. The threshold of activation was at −10 mV. The time course of activation was rapid, rising from 10% to 90% of peak amplitude in 3.4 ± 1.2 ms (n = 8) at +50 mV test potential. Saturation of the current peak amplitude occurred at test potentials positive to +30 mV. The peak current amplitudes of the experiment shown in Fig. 2A were divided by the driving force to obtain the conductance, assuming a K^+ equilibrium potential of −100 mV. The normalized conductance-voltage relation [$G/G_{max}(V)$] is shown in Fig. 2B (crosses). A single Boltzmann isotherm was fitted to the data points to obtain the voltage of half-maximal activation $V_{n,\frac{1}{2}}$ = 14.0 ± 9.2 mV (n = 13) and the voltage change for an e-fold increase in conductance a_n = 9.7 ± 3.0 mV. Inactivation of the current mediated by Raw3 channels was almost complete during the test pulses of 100 ms duration as shown in Fig. 2A. The time constant of inactivation was 15.2 ± 3.2 ms (n = 5) at +50 mV. A steady-state inactivation curve is shown in Fig. 2B (circles). It was measured by responses to test pulses to +20 mV from different prepulse potentials (prepulse duration of 25 s) ranging from −100 mV to +30 mV in 10 mV increments. The data points were fitted with a Boltzmann isotherm from which half-inactivation was at −29.7 ± 6.5 mV (n = 5), with an e-fold decrease of peak current per 12.2 ± 4.2 mV voltage change. Raw3 channels recovered slowly from inactivation after a depolarizing pulse. The time for 50% recovery from complete inactivation was found to be 1.9 ± 0.9 s (n = 6). The reversal potential of K^+ currents mediated by Raw3 channels was tested by short activating pulses of 20 ms duration to +20 mV, followed by a step back to various negative potentials (not shown). The currents measured following the repolarizing step reversed their sign between −80 mV and −100 mV as expected for K^+ currents.

The sensitivity of Raw3 currents to 4-AP, TEA and DTX was tested in whole-cell current measurements (Fig. 3). Raw3 currents are rather sensitive to 4-AP (IC_{50} at 0.5 mM) and TEA (IC_{50} at 0.3 mM), but insensitive to DTX up to 100 nM. These results contrast with those for RCK4 currents which are about 20-fold less sensitive to 4-AP, and are insensitive to TEA as well as to DTX.

3.5 *Single-channel properties of Raw3 channels*

Single channel currents were recorded in cell-attached patches. Fig. 4A shows a transient current recorded in a patch containing about 30 channels, on a slow time scale. The outward current was activated by a step from −80 mV to +20 mV. The current in Fig. 4A shows rapid inactivation with a time course similar to the macro-patch currents in Fig. 1. After 200 ms of depolarization the current is sufficiently inactivated that single channel openings can be distinguished. Single channel openings are shown on a faster time scale in Fig. 4B. The trace represents the single channel activity in a patch after a long lasting depolarization to 0 mV. The amplitude histogram constructed from the digitized

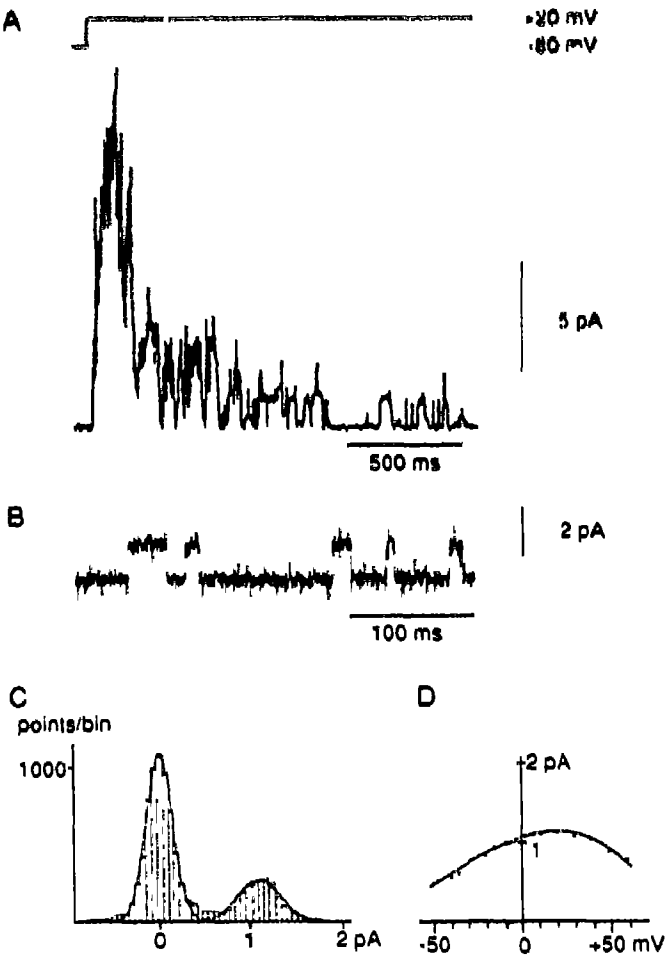

Fig. 4 Single channel currents in cell-attached patches on *Xenopus* oocytes previously injected with Raw3 cRNA (A) Transient current measured in a patch containing about 30 channels in response to a depolarizing pulse from −80 mV to +20 mV (B) Single channel currents in a patch containing more than 40 channels inactivated by a depolarization to 0 mV for about 30 s The currents in (A) and (B) were low pass filtered at 1 kHz and sampled ad 0 5 kHz and 4 kHz respectively (C) Amplitude histogram from the currents shown in (B) The digitized raw data points were directly binned and fitted with the sum of two Gaussians to obtain an amplitude estimate (1 09 pA at 0 mV) (D) Single channel current-voltage (I–V) relation in the range from −50 mV to +60 mV obtained semi-automatically from 3 patches The amplitude at 0 mV is identical with the one obtained from the directly binned histogram shown in (C) The data points were fitted with a polynomial (solid line)

raw data of this recording is shown in Fig. 4C. The single channel amplitude was computed by fitting a sum of two Gaussians to the data (solid curve in Fig. 4C). The amplitude was calculated as the difference between the two peaks (1.09 pA at 0 mV). Single channel current-voltage relations (I-V) were established semi-automatically. The amplitudes were measured from channels that remained open for at least 10 ms. The graph in Fig. 4D summarizes the data obtained from three patches in the voltage range from −50 mV to +60 mV. Each point represents the mean amplitude of at least 30 determinations of the unitary current level at each voltage. The I-V is almost linear in the range from −50 mV to −10 mV. In this range the slope conductance is 14 pS. However, the single channel current reaches a maximum at about +20 mV and the slope conductance becomes negative at more positive potentials.

4. DISCUSSION

K^+ channels are particularly diverse, and are probably present in all excitable and nonexcitable cells [2]. The first K^+ channel gene cloned was the *Shaker* K^+ channel gene in *Drosophila* [15;19-21]. The transcription of this gene leads to the synthesis of several mRNAs expressing either rapidly or slowly inactivating K^+ channels when injected into *Xenopus* oocytes. Subsequently, the *Shaker*-related K^+ channel cDNAs *Shab*, *Shaw*, and *Shal* were identified in *Drosophila* [17]; these code for slowly- or noninactivating K^+ channels [22]. Similar vertebrate K^+ channel cDNAs were identified in the mammalian brain, most notably the *Shaker*-related RCK K^+ channel family in rat brain [5]. Expression of RCK cRNA in *Xenopus* oocytes produces several types of voltage-gated K^+ channels, of both the delayed rectifier (slowly-inactivating) or A-type (rapidly-inactivating). Members of the *Shaw*-related K^+ channel family in rat brain also resemble delayed-rectifier type K^+ channels (NGK2, RK*Sh*IIIA) [4,18] or A-type K^+ channels (Raw3). These observations suggest that A-type and delayed-rectifier K^+ channels were both derived from a prototype voltage-gated K^+ channel and that within a given K^+ channel family small sequence variations obviously suffice to convert an A-type K^+ channel into a delayed rectifier type K^+ channel.

The first mammalian A-type K^+ channel to be described was the RCK4 K^+ channel [5]. Here, we have described the properties of a second A-type K^+ channel. The Raw3 K^+ channel differs in several ways from the RCK4 K^+ channel. Raw3 currents activate in a more positive potential range, inactivate more rapidly and the steady state inactivation curve lies 35 mV more positive. The single channel conductance of Raw3 K^+ channels is about 3-fold higher than of RCK4 RCK4 channels do not saturate at positive potentials whereas Raw3 channels have a negative slope conductance The negative slope conductance is possibly caused by a magnesium block, and will be further investigated. Finally, the pharmacological profile is remarkably different between the two K^+ channels.

K^+ channel proteins are capable of forming heteromultimers with other K^+ channel subunits. The formation of heteromultimers changes conductance, inactivation and pharmacological properties of K^+ channels [23,24]. Therefore, RCK4- and Raw3-containing heteromultimeric K^+ channels might show the properties of a number of different A-type channels reported for excitable cells [2]. A-Type K^+ channels like RCK4 start to activate negative to the threshold of sodium channel activation. Raw3 K^+ channels, on the other hand, have a more positive threshold of activation. They apparently operate in the voltage range of Ca^{2+} current activation. Thus, Raw3 K^+ channels might be involved in the modulation of Ca^{2+} inward currents.

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