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# Functional expression of a rat homologue of the voltage gated *ether á go-go* potassium channel reveals differences in selectivity and activation kinetics between the *Drosophila* channel and its mammalian counterpart

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**We have cloned a mammalian (rat) homologue of *Drosophila ether á go-go* (*eag*) cDNA, which encodes a distinct type of voltage activated potassium (K) channel. The derived *Drosophila* and rat *eag* polypeptides share >670 amino acids, with a sequence identity of 61%, exhibiting a high degree of similarity at the N-terminus, the hydrophobic core including the pore forming P region and a potential cyclic nucleotide binding site. Rat *eag* mRNA is specifically expressed in the central nervous system. In the *Xenopus* oocyte expression system rat *eag* mRNA gives rise to voltage activated K channels which have distinct properties in comparison with *Drosophila eag* channels and other voltage activated K channels. Thus, the rat *eag* channel further extends the known diversity of K channels. Most notably, the kinetics of rat *eag* channel activation depend strongly on holding membrane potential. Hyperpolarization slows down the kinetics of activation; conversely depolarization accelerates the kinetics of activation. This novel K channel property may have important implications in neural signal transduction allowing neurons to tune their repolarizing properties in response to membrane hyperpolarization.**

*Key words: rat eag*/voltage gated K channel/*Xenopus* expression system

## Introduction

The plasma membranes of both excitable and non-excitable cells contain potassium (K) channels. They have an important role in stabilizing and determining the resting membrane potential, shaping the action potential in excitable cells, controlling firing patterns and modulation of neurotransmitter release and secretion (Hille, 1992). Accordingly, K channels comprise a ubiquitous and extremely diverse group of proteins with distinct electrophysiological and pharmacological properties (Chandy and Gutman, 1994).

The analysis of several behavioural mutants of *Drosophila melanogaster* through an elegant combination of classical and molecular genetics has led to the identification of three genes encoding different types of K channel subunits. It has been shown that *Shaker* (and the closely related *Shab, Shal* and *Shaw* genes) codes for proteins which form voltage activated K channels. They are either rapidly inactivating (A-type) or slowly inactivating (delayed-rectifier type) (Wei *et al.*, 1990; Jan and Jan, 1992; Pongs, 1992; Salkoff *et al.*, 1992). The *slowpoke* gene codes for protein(s) which form $Ca^{2+}$ activated K channels (Atkinson *et al.*, 1991; Adelman *et al.*, 1992). Unlike *Shaker* and *slowpoke* mutations, which specifically eliminate the transient voltage activated and the fast $Ca^{2+}$ activated K currents, respectively, mutations in the *ether à go-go* (*eag*) gene (Kaplan and Trout, 1969; Ganetzky and Wu, 1986) affect more than one type of K current in larval muscles and cultured nerve cells. *eag* encodes a protein whose activity may be involved in mediating and/or modulating the expression of voltage and $Ca^{2+}$ activated K currents (Zhong and Wu, 1991). The cloning of *eag* cDNA (Warmke *et al.*, 1991) has shown that the *eag* gene encodes a polypeptide whose structure is closely related to both voltage gated K channel and cyclic nucleotide gated cation channel proteins (Guy *et al.*, 1991). Therefore, it was proposed that the structural similarities between the *eag* polypeptide and voltage gated ion channel subunits on one side, and *eag* and cyclic nucleotide gated ion channel subunits on the other, could mean that *eag* polypeptides participate in the formation of voltage gated K channels that are modulated by second messengers such as cGMP and cAMP. Indeed, the functional analysis of the *eag* gene product in the *Xenopus* oocyte expression system has demonstrated that *eag* protein generates voltage gated ion channels with unusual properties, mediating voltage activated outward K currents and simultaneously a rise in intracellular $Ca^{2+}$. Furthermore, the voltage dependence of *eag* outward currents as well as their amplitude is affected by cyclic nucleotides (Brüggemann *et al.*, 1993).

Considering the importance of wild-type *eag* function for neurotransmitter release in *Drosophila* and the novel properties of *eag* mediated currents in the *Xenopus* oocyte expression system, we searched for an *eag* homologue in the rat central nervous system. In the past, *Drosophila Shaker* and *slowpoke* probes have been successfully used for cloning homologous vertebrate voltage and $Ca^{2+}$ activated K channels (Baumann *et al.*, 1988; Tempel *et al.*, 1988; Butler *et al.*, 1993). Now we have used a *Drosophila eag* cDNA probe for cloning a homologous rat *eag* cDNA. Rat *eag* mRNA elicits voltage activated outward K currents in the *Xenopus* oocyte expression system. They exhibit a novel activation behaviour which has not been described hitherto for cloned K channels. The activation rate of rat *eag* currents is considerably influenced by the membrane

resting potential such that hyperpolarization slows down current activation. This behaviour suggests that rat *eag* type K channels may have a specialized function in nerve membrane repolarization.

## Results

It has been shown previously that the primary sequences of proteins like *Shaker* which form voltage gated K channels in *Drosophila* have been highly conserved during evolution (Baumann *et al.*, 1988). The structural conservation between *Drosophila* and mammalian K channels probably holds for many ion channel-forming proteins. The *Drosophila eag* locus (Drysdale *et al.*, 1991; Warmke *et al.*, 1991) has recently been shown to encode a protein, which leads, in the *Xenopus* oocyte expression system, to the formation of a novel type of voltage gated K channel (Brüggemann *et al.*, 1993). This incited us to clone a mammalian (rat) *eag* homologue. The *Drosophila eag* protein sequence shares sequence similarities with voltage gated and cyclic nucleotide gated channels, respectively (Guy *et al.*, 1991), especially with those domains which are proposed to form the ion-conducting pore and a potential cyclic nucleotide binding site. Accordingly, we chose an *eag* cDNA probe that encoded the two domains, and screened a rat genomic DNA library (~$1\times10^6$ recombinant phages) under conditions of low stringency. One positive phage (clone λ4.2) was used for further analysis. λ4.2 DNA contained a 254 nucleotide (nt) long exon within a 1.6 kb *Sph*I–*Pst*I restriction fragment. The protein sequence derived from this exon was very similar to the C-terminal *Drosophila eag* protein sequence, which encodes part of the proposed cyclic nucleotide binding site (Figure 1).

Polymerase chain reactions (PCRs) were carried out with two oligonucleotide primers (G3 and G5, see Materials and methods) corresponding to 5′ and 3′ terminal sequences of the λ4.2 exon, in order to screen several rat cDNA libraries for *eag* cDNA. The DNA templates of hippocampal and cerebellar cDNA libraries yielded a PCR fragment of expected size and sequence indicating the presence of homologous *eag* cDNA clones. Using the amplified PCR fragment as hybridization probe, two cDNA clones (reag01 and reag02) were isolated from the rat cerebellum cDNA library. Reag01 cDNA is 3.1 kb and reag02 cDNA 2.9 kb long. The two sequences overlap for ~1400 nt, thus yielding a combined reag cDNA sequence of 4.6 kb. The longest open reading frame, which was detected in the combined cDNA sequence, codes for a polypeptide (rat *eag*) of 962 amino acid residues (Figure 1) with a calculated molecular weight of 108 kDa. The deduced rat *eag* protein sequence is very similar to that of *Drosophila eag*. The N-terminal *Drosophila* and rat *eag* protein sequences containing the putative membrane spanning segments and the pore forming (P) region, have an identity of 61%. This number increases to 78% allowing for conservative amino acid substitutions. The C-terminal *Drosophila* and rat *eag* protein domains, which correspond to a consensus sequence for binding cyclic nucleotides (see Figure 1), have an identity of 78 and 14% conservatively substituted amino acid residues, respectively. Otherwise, the C-termini of *Drosophila* and rat *eag* proteins have

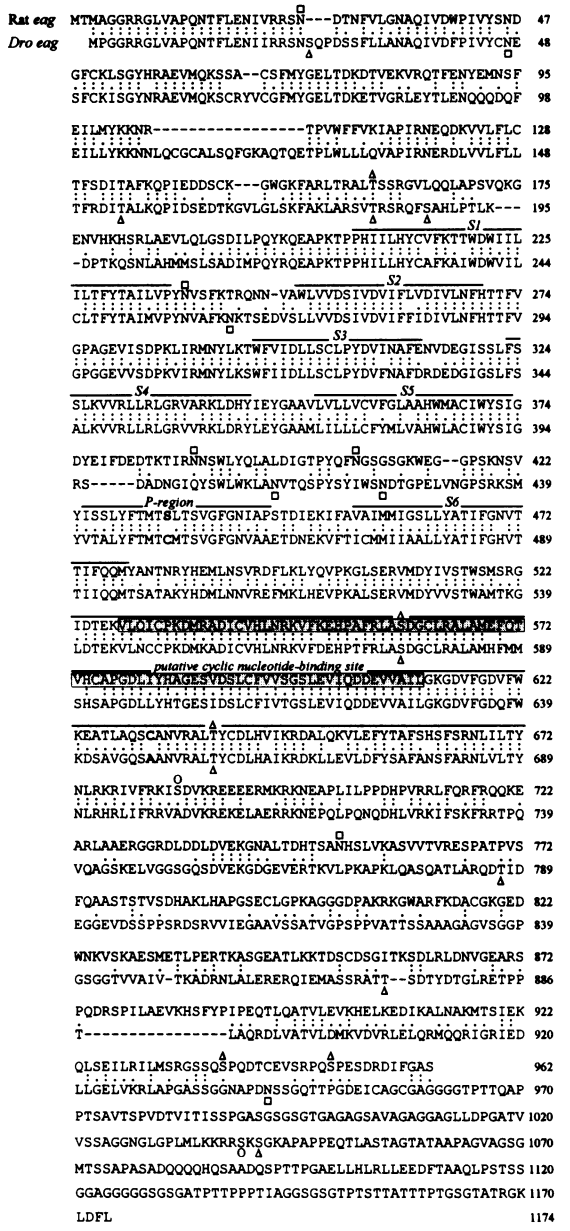


**Fig. 1.** Alignment of the deduced amino acid sequences of *Drosophila eag* and rat *eag*. The rat *eag* protein sequence was derived from reag cDNA as described in the text. The starting methionine of rat *eag* was assigned to the first ATG triplet downstream of stop codons in all frames. A second ATG triplet occurs three nucleotides downstream within a sequence which more closely fits the consensus sequence for eukaryotic translation initiation sites (Kozak, 1986; Cavener and Ray, 1991). The deduced rat *eag* sequence, having an identity of 98% to *m-eag* (Warmke and Ganetzky, 1994), is aligned with *Drosophila eag* (Warmke *et al.*, 1991). Amino acid residues are given in single letter code and are numbered on the right. Identical residues are indicated by colons (:), conservative substitutions by periods (.). Conservative substitutions are exchanges of amino acids belonging to the following groups: A,S,T,P,G; M,I,L,V; D,E,N,Q; H,R,K; W,Y,F (Dayhoff *et al.*, 1978). Gaps (−) were introduced to optimize the alignment. The putative transmembrane segments S1−S6, the pore lining (P) region and the cyclic nucleotide binding region are overlined. Putative N-glycosylation sites (Bairoch, 1992) are marked by squares (□). Consensus sequences for cAMP- and cGMP-dependent protein kinases and CaM kinase II are indicated by ○ and Δ, respectively (Pearson and Kemp, 1991; Bairoch, 1992). The rat *eag* amino acid sequence corresponding to the exon that was isolated from a genomic library (see text) is shaded. The rat *eag* cDNA sequence has been submitted to the EMBL data bank (Accession No. Z34264).

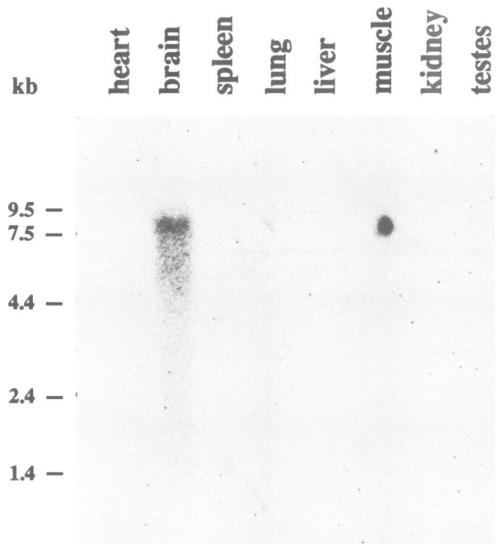


**Fig. 2.** Expression of rat *eag* mRNA in different rat tissues. For Northern blot analysis of rat *eag* mRNA a multiple tissue Northern blot (Clontech, poly($A^+$) RNA, 2 μg each lane) was probed with $^{32}$P-labelled rat *eag* cDNA probe as described in Materials and methods. Tissue origin (heart, brain, spleen, lung, liver, skeletal muscle, kidney and testes) is indicated on top of each lane. The positions of RNA (kb) size markers are indicated on the left.

diverged considerably in both sequence and length, that of rat *eag* being 212 amino acids shorter.

Hydropathicity analysis (Kyte and Doolittle, 1982) suggests that the hydrophobic domain of rat *eag* protein may have in the membrane a similar folding pattern to that of *Shaker* related K channels. It consists of five hydrophobic, probable membrane spanning segments S1–S3, S5, and S6, a positively charged amphipathic segment S4 and the P region. Sequences between segments S1 and S2, S3 and S4, and S5 and S6 may face the extracellular side (Durell and Guy, 1992). Consistent with this hypothesis, the deduced rat *eag* protein sequence contains within the S1–S2 and S5–S6 loops three consensus sequences for N-linked glycosylation. Most likely, these glycosylation sites, which are conserved between the *Drosophila* and rat *eag* polypeptides, are located extracellularly. Note three more N-linked glycosylation consensus sites in the N-terminal, the S6 and the C-terminal rat *eag* sequence, which are not conserved between *Drosophila* and rat *eag* polypeptides (Figure 1). The functional importance of all these sites requires further investigation. The rat *eag* protein sequence contains several putative recognition sites for protein kinases, e.g. cGMP/cAMP-dependent kinases and CaM kinase II (Figure 1).

### *Rat eag mRNA expression*

The expression of rat *eag* mRNA was analyzed for example in rat brain, heart, skeletal muscle and kidney. The results of Northern blot experiments with RNA extracted from the corresponding tissues (Figure 2) indicate that rat *eag* mRNA is predominantly expressed in rat brain. The estimated size of rat *eag* mRNA is ~8 kb. The distribution of rat *eag* mRNA in brain areas was characterized in detail by *in situ* hybridization experiments to horizontal and parasaggittal sections of adult rat brain. Hybridization probes were $^{33}$P-labelled antisense oligonucleotides (nt 1738–1691 and nt 3126–3077) and a sense oligonucleotide for control. The results show that rat *eag* mRNA is most prominently expressed in the hippocampal formation and in cerebellum (Figure 3A and B). The highest levels of rat *eag* mRNA were detected in the granular cells of the dentate gyrus, in the CA3 pyramidal cells of the hippocampal formation and in the cerebellar granule cells; lower levels of rat *eag* mRNA were detected in the hippocampal CA1 field, in the caudate putamen, in the granular cell layer of the olfactory bulb and in neocortical layers.

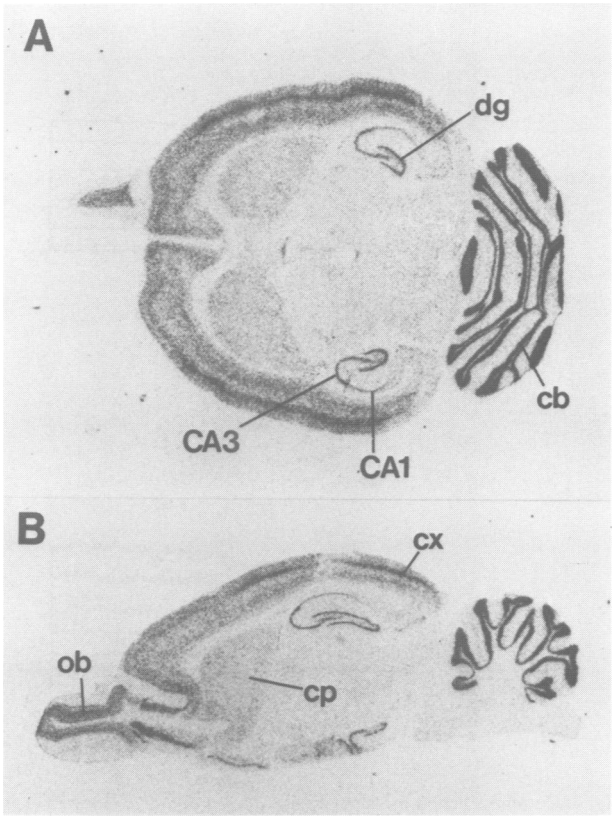


**Fig. 3.** Distribution of rat *eag* mRNA in rat brain. Horizontal (**A**) and parasaggital (**B**) sections of adult rat brain were hybridized with α-$^{32}$P-dATP tailed antisense oligonucleotide specific for rat *eag* (nt 1738–1691). The same hybridization pattern was obtained with a second $^{33}$P-labelled antisense oligonucleotide (nt 3126–3077), but not with a sense oligonucleotide (not shown). Strong signal intensities were detected in granule cells of the cerebellum (cb) and of the dentate gyrus (dg) and in pyramidal cells of the hippocampal CA3 field. Some signal intensity was additionally seen in the hippocampal CA1 field, caudate putamen (cp), olfactory bulb (ob) and some neocortical layers (cx).

### *Functional expression*

The *Xenopus* oocyte expression system was used to study the functional and pharmacological properties of rat *eag*. After injection of *in vitro* synthesized rat *eag* mRNA into *Xenopus* oocytes, non-inactivating outward currents of a delayed-rectifier type were obtained after stepping the membrane potential from −100 mV to depolarizing test potentials under two-electrode voltage clamp (Figure 4A). The corresponding current–voltage relationship is shown in Figure 4B. The currents had a threshold for activation between −40 and −30 mV (Figure 4B). In some oocytes a small fraction of the current (<10%) inactivated during

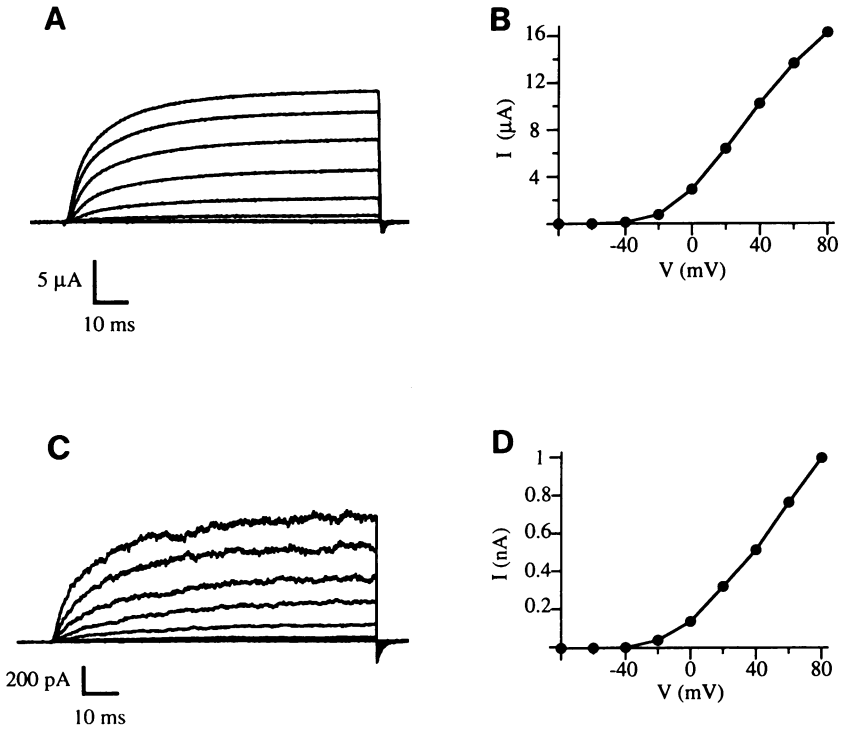


**Fig. 4.** Currents elicited by depolarizing steps in *Xenopus* oocytes expressing rat *eag* mRNA. Oocytes were injected with rat *eag* mRNA (~50 ng). Outward potassium currents were elicited by depolarizing pulses from −80 to +80 mV in steps of 20 mV from a holding potential of −100 mV. Currents were measured in the two-electrode voltage clamp (**A**), where the bath contained normal frog Ringer's, or in the inside-out macropatch clamp (**C**) recording configuration. I−V relationships plotted from the data in (A) and (C) are shown in (**B**) and (**D**), respectively.

longer pulse durations (>1 s) with varying time constants in the hundred millisecond time range (data not shown). In inside-out macropatch recordings we never observed an inactivating component of the current even with pulse durations up to 5 s. Otherwise the currents and the corresponding I−V relationship obtained with inside-out macropatch recordings (Figure 4C and D) were virtually identical to those obtained by two-electrode voltage clamp measurements. Since the evoked currents measured in excised patches rapidly decreased upon rupture of the patch, no long-term excised patch recordings were possible.

The slope of the plot of the reversal potential against external $K^+$ concentration determined by two-electrode voltage clamp experiments on rat *eag* expressing oocytes was 55 mV per 10-fold change in external $K^+$ concentration, as expected for a highly selective K channel. Relative cation permeabilities for this channel were estimated by measuring reversal potentials under two-electrode voltage clamp conditions using a tail current protocol. The sequence of cation permeabilities is $K^+ \geq Rb^+ > NH_4^+ >> Li^+ \sim Na^+ \sim Cs^+$ (see Table I). In contrast to the *Drosophila eag*, the rat *eag* channel is not permeable to $Cs^+$. A $Ca^{2+}$ activated $Cl^-$ current, which may contribute to the biphasic appearance of *Drosophila eag* mediated outward current (Brüggemann *et al.*, 1993), was not observed in rat *eag* expressing *Xenopus* oocytes. Rat *eag* mediated outward currents were not biphasic (Figure 4A). Unlike *Drosophila eag*, the expression of rat *eag* mRNA in *Xenopus* oocytes may not lead to a rise in intracellular $Ca^{2+}$ sufficient for the induction of $Ca^{2+}$ activated $Cl^-$ currents.

**Table I.** Comparison of pharmacological and permeability properties of rat and *Drosophila eag* channels

| | Rat *eag* | *Drosophila eag* |
|---|---|---|
| Pharmacology ($IC_{50}$)[a] | | |
| TEA | 28 ± 13 mM ($n$ = 3) | 33 ± 11 mM ($n$ = 4) |
| 4-AP | >100 mM ($n$ = 5) | >100 mM ($n$ = 3) |
| Quinine | 0.9 ± 0.3 mM ($n$ = 5) | 0.7 ± 0.3 mM ($n$ = 4) |
| Quinidine | 0.4 ± 0.2 mM ($n$ = 4) | 0.4 ± 0.1 mM ($n$ = 4) |
| Permeability[b] | | |
| $K^+$ | 1 ($n$ = 5) | 1 ($n$ = 9) |
| $Rb^+$ | 0.72 ± 0.26 ($n$ = 6) | 0.75 ± 0.11 ($n$ = 6) |
| $NH_4+$ | 0.13 ± 0.04 ($n$ = 5) | 0.25 ± 0.08 ($n$ = 5) |
| $Na^+$ | <0.01 ($n$ = 5) | 0.11 ± 0.06 ($n$ = 8) |
| $Li^+$ | <0.01 (n = 5) | 0.08 ± 0.06 ($n$ = 5) |
| $Cs^+$ | <0.01 ($n$ = 6) | 0.42 ± 0.14 ($n$ = 5) |

$n$ = number of experiments. Data for *Drosophila eag* are from Brüggemann *et al.* (1993).
[a]$IC_{50}$-values give the K channel blocker concentration required for half-maximal inhibition of outward currents measured in the two-electrode voltage clamp configuration.
[b]Permeability ratios for rat *eag* channels were obtained from reversal potentials determined in two electrode voltage clamp experiments. The bath solution contained 115 mM of the given cation as chloride salt, 1.8 mM $CaCl_2$ and 10 mM HEPES pH 7.2.

*Drosophila eag* channels are modulated by cyclic nucleotides. Application of membrane permeant analogues of either cAMP or cGMP (8-Br-cAMP or 8-Br-cGMP) to *Drosophila eag* expressing oocytes increased the evoked current amplitudes (Brüggemann *et al.*, 1993). However, bath application of 2 mM 8-Br-cAMP or 8-Br-cGMP to rat *eag* expressing oocytes did not affect the outward

currents obtained in two-electrode voltage clamp experiments (not shown). The pharmacological features of rat *eag* channels in comparison with *Drosophila eag* channels are summarized in Table I. The pharmacological profiles, as far as determined, are identical. Both channels are sensitive to quinine and quinidine, but quite insensitive to external tetra-ethyl-ammonium (TEA). In contrast to most other K channels, *eag* channels are insensitive to 4-amino-pyridine (4-AP).

We noticed during our electrophysiological experiments that the activation kinetics of rat *eag* currents varied with the pulse protocol used to elicit outward currents. This was investigated in detail. Rat *eag* expressing *Xenopus* oocytes were kept at different prepulse holding potentials before a depolarizing test pulse to +40 mV was given (Figure 5A). When the prepulse potential was changed from −60 to −140 mV, a gradual deceleration of the rise time to peak current amplitude was observed at the test pulse (Figure 5A). The time required to reach 80% of the maximal current was ~7 ms when the test pulse was given from a holding potential of −60 mV, and ~40 ms when the test pulse was given from a holding potential of −140 mV (Figure 5C). This is reminiscent of the 'Cole and Moore shift' (Cole and Moore, 1960), but was accompanied by a dramatic slowing of the current rise time (Figure 5A). Note that the deactivation kinetics were not altered by a change in prepulse potential (Figure 5A). Most likely these data indicate that the kinetics of *eag* channel activation are controlled by slow, voltage dependent step(s). This activation behaviour is a novel K channel feature, since a comparable dependence of channel activation on prepulse potential has not been found yet for other voltage activated K channels. When the same pulse protocol as in Figure 5A was used to measure *Drosophila eag* mediated outward currents (Figure 5B), a much less pronounced dependence of *eag* channel activation on prepulse potential was observed. The time required to reach 80% of the maximal current was ~20 ms when the test pulse was given from a holding potential of −140 mV, and ~9 ms when the test pulse was from a holding potential of −60 mV (Figure 5C). The pronounced influence of prepulse potential on the activation rate seems to be a unique property of rat *eag* channels.

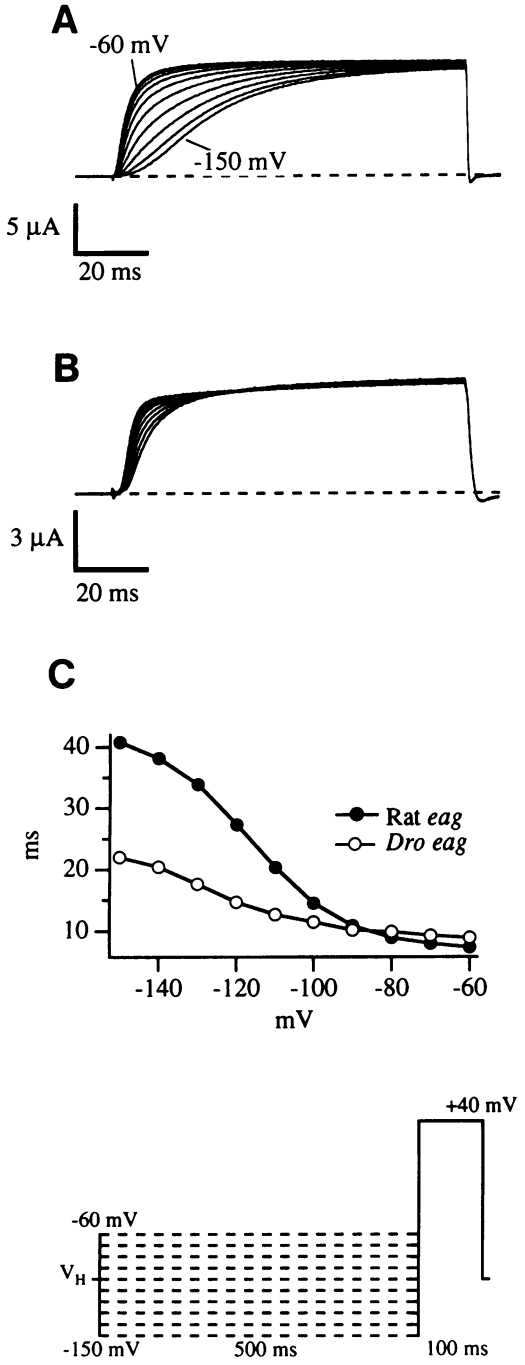


**Fig. 5.** Dependence of *eag* mediated outward currents on prepulse potential. Rat (**A**) and *Drosophila eag* (**B**) mediated outward currents were elicited in *Xenopus* oocytes by stepping the membrane voltage from different prepulse potentials to a test pulse of +40 mV. Prepulse potentials were varied from −150 mV to −60 mV. Prepulse durations were 500 ms. The pulse protocol is indicated at the bottom. Currents were measured in the two-electrode voltage clamp recording configuration. Rise times to 80% of the maximal currents (at the end of the test pulse) were estimated from the current traces shown in (A) and (B) and plotted against the prepulse potential (**C**). Note that the activation kinetics of rat *eag* channels depend on the prepulse potential much more strongly than *Drosophila eag* channels.

## Discussion

### *eag*—an emerging new family of distinct voltage activated K channels

Using a *Drosophila eag* cDNA probe we have cloned a homologous rat *eag* cDNA. The derived protein sequences indicate that *eag* polypeptides are highly conserved from *Drosophila* to mammals. During the preparation of our manuscript, mouse, *Drosophila* and human *eag* related cDNA sequences (*m-eag*, elk and h-erg) have been published (Warmke and Ganetzky, 1994), which show a high degree of similarity. A dendrogram of the derived *eag* related polypeptides constructed on the basis of the hydrophobic core sequences, suggested that *eag* related genes belong to a new family of K channel structural genes (Warmke and Ganetzky, 1994). Our results show that *Drosophila* and rat *eag* mRNAs give rise to distinct voltage gated K channels. Although the functional properties of *m-eag*, elk and h-erg channels are not known, the *eag* gene family will obviously further extend the known diversity of K channels.

The N-termini, the hydrophobic core regions and the putative cyclic nucleotide binding sites of the *Drosophila*

**Table II.** Comparison of the signature sequence of *Shaker*, *Drosophila eag* and rat *eag* K channels

| | 1 | | | | | | | 8 |
|---|---|---|---|---|---|---|---|---|
| | ● | | | | ● | ● | | ● |
| *Shaker* | T | M | T | T | V | G | Y | G |
| *Drosophila eag* | C | M | T | S | V | G | F | G |
| Rat *eag* | S | L | T | S | V | G | F | G |

The signature sequence corresponds to highly conserved sequence in the P region of K channels (Heginbotham *et al.*, 1994). Amino acid residues are given in single letter code. Mutations of the amino acid residues marked by dots lead to non-selective channels which are markedly permeable to $Na^+$, $Cs^+$ and $Li^+$.

and rat *eag* polypeptide sequences are very similar. Despite this high degree of similarity, the corresponding K channels exhibit remarkable differences when expressed in *Xenopus* oocytes, notably in their ion selectivity. It has been proposed that the 'signature' sequence of K channels, a highly conserved stretch of eight amino acids in the P region (Table II) is a major determinant of ion selectivity in K channels. Mutations of four amino acids (T1, V5, G6 and G8) in the TMTTVGYG signature sequence of *Shaker* voltage activated K channels result in loss of $K^+$ selectivity (Heginbotham *et al.*, 1994). The *eag* signature sequences differ at the first amino acid, which is a cysteine (C1) in *Drosophila eag* and a serine (S1) in rat *eag* (see Table II). Rat *eag* channels are highly selective for $K^+$, like other voltage activated K channels. Note in this context that a change of GYGD, which occurs in most K channel signature sequences, to GFGN in rat *eag* channels does not alter $K^+$ selectivity. On the other hand, *Drosophila eag* channels are less selective for $K^+$ than other voltage activated K channels, exhibiting unusually high permeability ratios (against $K^+$) for $Li^+$, $Na^+$, $Rb^+$, $Cs^+$ and $NH_4^+$. Future *in vitro* mutagenesis experiments will have to show whether the presence of C1 in the *Drosophila eag* signature sequence may be responsible for the reduced selectivity of *Drosophila eag* channels.

Another remarkable difference between the *Drosophila* and rat *eag* polypeptide sequences is found within the putative cyclic nucleotide binding sites. One particular amino acid position being crucial for cyclic nucleotide binding specificity has been identified in cAMP- or cGMP-dependent protein kinases as well as in cyclic nucleotide-gated channels (Weber *et al.*, 1989; Altenhofen *et al.*, 1991). This position is usually occupied by either a threonine or an alanine. The presence of a threonine is associated with a higher selectivity for cGMP rather than cAMP, whereas the presence of an alanine can be correlated with a preference for cAMP (Weber *et al.*, 1989; Altenhofen *et al.*, 1991; Shabb *et al.*, 1990, 1991). The *Drosophila eag* polypeptide contains an alanine at the equivalent position in its putative cyclic nucleotide binding site (A648 in Figure 1). However, the rat *eag* polypeptide, on the other hand, contains a cysteine at the equivalent position (C631 in Figure 1). This substitution may be responsible for the lack of both cAMP and cGMP effects on the evoked rat *eag* outward currents.

It has been suggested that CaM kinase II may modulate *eag* dependent K currents in larval *Drosophila* muscle (Zhong and Wu, 1993). Both the *Drosophila* and rat *eag* polypeptides contain several possible target sites for CaM kinase II dependent phosphorylation. Interestingly, two such sites which are located in the putative nucleotide binding site (see Figure 1) and a third site within the cytoplasmic N-terminus have been conserved from *Drosophila* to mammals and therefore may be of functional importance. This suggestion obviously has to be clarified in further experiments. Similarly, the significance of the single consensus site for cAMP/cGMP-dependent kinase phosphorylation in the C-terminus of *Drosophila eag* (S1039) and rat *eag* (S684) polypeptides remains to be shown.

### *Novel activation behaviour of rat eag channels*

The gating of voltage activated K channels involves voltage dependent and voltage independent steps which control the transition of a closed channel at resting stage to an activated channel upon membrane depolarization (Hille, 1992). Channel activation gives rise to a gating current because charges must move in response to changes in membrane potential to open the channels. Usually, gating currents and macroscopic channel activation have a similar voltage range, which is more positive than the membrane resting potential. It has been shown that certain mutations in *Shaker* channels shift (and reduce) the voltage dependence of activation along the voltage axis to positive potentials (McCormack *et al.*, 1991; Schoppa *et al.*, 1992). The effect of these mutations on *Shaker* channels may be explained by the existence of several components of gating charge movement, one of which has its voltage dependent equilibrium shifted by the mutation(s). Accordingly, charge movements and the accompanying transitions during channel activation may occur in several separate steps, which have different voltage dependencies. Therefore, the dependence of the kinetics of activation of rat *eag* channels on the holding potential may be explained by the occurrence of several voltage dependent transitions during *eag* channel activation. A change in holding potential may induce a shift in the equilibrium of one of the early voltage dependent transitions and thus lead to the observed retardation in channel activation.

The pronounced hyperexcitability of *eag* mutants in *Drosophila* (Ganetzky and Wu, 1985) demonstrates that *eag* channels play an important role in maintaining normal neuronal excitability. By analogy, rat *eag* channels may be of similar importance in the mammalian nervous system and be involved in action potential repolarization, e.g. in hippocampal neurons, where rat *eag* mRNA is expressed. The unique activation properties of rat *eag* channels suggest that they may have a specialized function in repolarization mechanisms. Possibly rat *eag* channels are able to adapt their activation kinetics to changes in membrane resting potential. This adaptation may depend on both the membrane resting potential and post-translational modifications affecting the voltage range and kinetics of rat *eag* channel activation. In this context an attractive hypothesis is that rat *eag* channels may slow or accelerate repolarization and thereby aid neurons to modulate their signalling capabilities.

## Materials and methods

### *Screening genomic and cDNA libraries*

A genomic library prepared from Spraque Dawley rat testes in λ-DASHII (Stratagene) was screened at low stringency using a *Nhe*I restriction

fragment from the cloned *eag* cDNA (nucleotides 1155–2329, numbers refer to the coding region of *eag* cDNA ch20, Warmke *et al.*, 1991). Plaque DNA was transfered to Nylon filters and cross-linked to the membrane by UV irradiation. Filters were equilibrated in 6× SET (1× SET is 150 mM NaCl, 1 mM EDTA, 2 mM Tris−HCl, pH 7.4), 30% formamide, 1% SDS, 5× Denhardt's solution (1× Denhardt's is 0.2 g/l each of Ficoll, polyvinylpyrrolidone and bovine serum albumin) and 0.1 mg/ml denatured DNA from herring testes at 37°C. To this pre-hybridization solution randomly primed radioactively labelled DNA (Feinberg and Vogelstein, 1983) was added at $1\times10^6$ c.p.m./ml for hybridization. The final wash was carried out in 1× SET, 0.1% SDS at 56°C.

A rat cerebellum cDNA library in λ-ZAPII (Stratagene) (provided by F.Mastiaux) was screened under high stringency conditions (5× SET, 1% SDS, 5× Denhardt's solution and 0.1 mg/ml denatured herring testes DNA at 65°C). Radioactively labelled probe was generated by PCR (Saiki *et al.*, 1988) with [α-$^{32}$P]dCTP using a fragment of genomic clone λ4.2 as a template and primers corresponding to the 5′ and 3′ ends of the exon from genomic clone λ4.2 (G3, nucleotides 1604–1624; G5, nucleotides 1812–1793; numbers refer to the coding region of reag cDNA). Filters were washed in 0.5× SET, 0.1% SDS at 65°C. Filters were exposed to X-ray films (Kodak XAR 5) for 8–48 h with intensifying screens (Dupont Cronex hi$^+$) at −70°C.

'Subcloning' of positive λ-clones was carried out by *in vivo* excision of pBluescript with the cDNA insert from λ-ZAPII according to the manufacturer's protocol (Stratagene).

### *DNA sequencing*

Both strands of reag cDNA were sequenced with the dideoxy chain termination method (Sanger *et al.*, 1977). Sequencing reactions were carried out with Sequenase 2.0 sequencing kit (USB) and either M13-universal, M13-reverse or internal primers, using double stranded plasmid DNA as a template.

### *Northern blot*

For Northern blot analysis a rat multiple tissue Northern blot (Clontech) was hybridized at 45°C in 5× SSPE (1× SSPE is 150 mM NaCl, 1 mM EDTA, 10 mM $NaH_2PO_4$/NaOH, pH 7.4), 50% formamide, 1.5% SDS, 5× Denhardt's solution and 0.1 mg/ml herring testes DNA. As probe an [α-$^{32}$P]dCTP labelled *Eco*RI restriction fragment from preag (nt 1963–3′-cloning site) was added to a concentration of $2\times10^6$ cpm/ml. The final wash step was carried out at 68°C in 0.1× SET, 0.1% SDS.

### *In situ hybridization*

Cryostat (15 μm) sections of rat brain were mounted on polylysine-coated slides, dried and fixed for 5 min in 4% paraformaldehyde. Slides were rinsed in 1% PBS for 1 min, transferred to 70% ethanol and subsequently stored in 95% ethanol at 4°C. Hybridization was carried out with [α-$^{33}$P]dATP-tailed antisense oligonucleotides as described previously (Wisden *et al.*, 1990; Kues and Wunder, 1992; Rettig *et al.*, 1992). The sequence of the oligonucleotides (GTGATAGATGAGGTCC-CCTGGGGCGCAGTGTACTGTCTGGAACTCCAT and ACAACTG-TCAATGGCTAGCCCAACACAGCTGCCATCTTGCATGGTAGGGT) corresponds to nucleotides 1738–1691 and 3126–3077 of reag cDNA.

### *mRNA synthesis*

For rat *eag* mRNA synthesis the reag01 and reag02 cDNAs, subcloned in pBluescript-SK, were combined using a unique *Sph*I restriction site (nt 2365 in reag cDNA) within the overlapping region of both cDNA sequences. Subclone preag01 was digested with *Eco*RV(5′ polylinker) and *Sph*I. Subclone preag02 was linearized with *Xba*I (3′ polylinker). Protruding 5′ ends were filled in with Klenow DNA polymerase I. The linearized clone was then digested with *Sph*I. The resulting 5.2 kb long, blunt/*Sph*I preag02 restriction fragment was ligated with the 5′ *Eco*RV−*Sph*I preag01 restriction fragment to yield preag-SK. An *Avi*II (nt −67 in reag cDNA)−*Xho*I (3′ polylinker) restriction fragment of preag-SK was subcloned into *Eco*RV−*Xho*I cut pNKS2 (Stocker *et al.*, 1991) to yield preag-NKS2. Rat *eag* mRNA was synthesized *in vitro* (Melton *et al.*, 1984) from *Not*I linearized preag-NKS2 using SP6 RNA polymerase. Transcription was primed with the cap dinucleotide 7-methylguanosine (5′)-triphospho (5′)-guanosine (Konarska *et al.*, 1984). *Drosophila eag* mRNA was prepared as described (Brüggemann *et al.*, 1993).

### *Electrophysiological measurements*

mRNA was injected into *Xenopus laevis* oocytes (50 ng/oocyte) as described (Methfessel *et al.*, 1986). Currents were recorded 1–5 days after injection. Electrophysiological recordings on whole oocytes were performed under conventional voltage clamp conditions using a Turbo-TEC amplifier (NPI, Tamm, FRG). Intracellular electrodes had a resistance of 0.6–1.0 MΩ and were filled with 2 M KCl. To obtain identical conditions all pulse protocols were designed with 5 s intervals between pulses, thus avoiding effects of one pulse on the activation kinetics during the next pulse. Current records were sampled at 10 kHz and low-pass filtered at 3 kHz. Leak and capacitive currents were corrected on line by using the P/4 method.

Inside-out patch clamp recordings were performed as described (Hamill *et al.*, 1981). In these measurements the pipettes had resistances between 0.8 and 1.0 MΩ and contained normal frog Ringer's (composition in mM: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES−NaOH, pH 7.2). The bath solution was (in mM): 115 KCl, 1.8 EGTA, 10 HEPES−KOH, pH 7.2. Current records were sampled at 20 kHz and low-pass filtered at 5 kHz. They were corrected for leak and capacitive currents on line by using a P/4 method. All electrophysiological experiments were performed at room temperature (19–22°C). Pulse patterns were generated and currents were sampled with an Mac-Quadra 700/EPC-9 system using the pulse protocol software (Heka, FRG).

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