Inhibition of human *ether à go-go* potassium channels by Ca^{2+} /calmodulin

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Intracellular Ca^{2+} inhibits voltage-gated potassium channels of the *ether à go-go* (EAG) family. To identify the underlying molecular mechanism, we expressed the human version hEAG1 in *Xenopus* oocytes. The channels lost Ca^{2+} sensitivity when measured in cell-free membrane patches. However, Ca^{2+} sensitivity could be restored by application of recombinant calmodulin (CaM). In the presence of CaM, half inhibition of hEAG1 channels was obtained in 100 nM Ca^{2+} . Overlay assays using labelled CaM and glutathione S-transferase (GST) fusion fragments of hEAG1 demonstrated direct binding of CaM to a C-terminal domain (hEAG1 amino acids 673–770). Point mutations within this section revealed a novel CaM-binding domain putatively forming an amphipathic helix with both sides being important for binding. The binding of CaM to hEAG1 is, in contrast to Ca^{2+} -activated potassium channels, Ca^{2+} dependent, with an apparent K_D of 480 nM. Co-expression experiments of wild-type and mutant channels revealed that the binding of one CaM molecule per channel complex is sufficient for channel inhibition.

Keywords: calcium/calmodulin/patch-clamp/potassium channel/*Xenopus* oocyte

Introduction

The role of potassium channels (K^+ channels) in the generation of action potentials and the electrical signalling of excitable cells is well documented (Hille, 1992). They contribute to the setting of the resting membrane potential, a function that is not restricted to excitable cells. Many K^+ channel types are subject to various regulation processes that couple them to intracellular signalling pathways. One prominent example is the opening of calcium-activated K^+ channels (K_{Ca}) by cytosolic Ca^{2+} . At least for two classes of K_{Ca} channels (SK and IK), it has been suggested that calmodulin (CaM) forms a constitutive part of these channels (Xia *et al.*, 1998; Fanger *et al.*, 1999). The resulting CaM-gated channels open in situations where the Ca^{2+} concentration is strongly elevated above basal levels. In contrast to K_{Ca} channels, the K^+ channels encoded by the *ether à go-go* (EAG) gene are activated by membrane depolarization and inhibited by intracellular Ca^{2+} (Stansfeld *et al.*, 1996), with a half-maximal inhibition

concentration of ~100 nM. This means that EAG channels are ready to be activated at typical $[\text{Ca}^{2+}]_i$ but undergo Ca^{2+} -mediated inhibition in situations where K_{Ca} channels start to become activated.

Although expression patterns of EAG have been evaluated in different species, the functional role of this channel is only beginning to be understood. The strong expression in rat brain (Ludwig *et al.*, 1994) can be considered an indication for a prominent role in neuronal signalling. Likewise, expression of a recently described isoform (rEAG2; Saganich *et al.*, 1999) was restricted mainly to the brain, but with a pattern distinct from that of rEAG1. Despite the lack of specific pharmacological tools for channels of the EAG family, EAG-mediated currents can be identified reliably based on unique functional properties. Voltage-dependent activation of EAG channels is strongly dependent on the holding potential and is regulated by extracellular divalent cations (Ludwig *et al.*, 1994; Terlau *et al.*, 1996). This feature led to the identification of EAG channels in human myocytes, where they play a role in the formation of muscle fibres (Occhiodoro *et al.*, 1998). Apparently, EAG expression in myoblasts is a prerequisite for these cells to lower their resting potential and thereby to gain fusion competence (Bijlenga *et al.*, 1998).

Another role has become apparent after the identification of EAG channels in various cancer cell lines (Meyer and Heinemann, 1998; Meyer *et al.*, 1999; Pardo *et al.*, 1999). In human neuroblastoma cells, hEAG1 expression is dependent on the cell cycle (Meyer and Heinemann, 1998), and rat EAG channels, expressed in *Xenopus* oocytes, showed cell cycle-related functional changes triggered by the mitosis-promoting factor (Brüggemann *et al.*, 1997; Pardo *et al.*, 1998). These results suggest a role for hEAG in tumour progression, strongly supported by the recent work of Pardo *et al.* (1999), showing enhanced cell growth of hEAG-transfected cells in a mouse model for tumour progression. A somewhat similar function may be assigned to K_{Ca} channels, as inhibition of IK channels in human T lymphocytes leads to inhibition of the proliferative T-cell response after activation (Jensen *et al.*, 1999). Mechanisms by which both K_{Ca} channels and EAG channels influence the cell cycle remain to be elucidated, but the regulation of both channel types by intracellular Ca^{2+} suggests a functional link.

Here we describe the molecular basis for the inhibition of the delayed rectifier channel hEAG1 by Ca^{2+} /CaM.

Results

hEAG1 channels expressed in Xenopus oocytes

hEAG1 channels were expressed in oocytes of *Xenopus laevis*. For an assessment of the effect of intracellular Ca^{2+} on the channels, it was desirable to obtain stable inside-out

Fig. 1. Recording of hEAG1-mediated currents from cell-free membrane patches. (A) Current responses to depolarizing pulses (protocol on top) were recorded from an inside-out patch of *Xenopus* oocytes injected with mRNA coding for hEAG1. (B) The maximum current amplitudes after 500 ms were plotted as a function of the test voltage (open circles). Also shown is the result of current recordings in the on-cell mode of the same patch (filled circles), indicating a small increase in current upon membrane excision. Straight lines connect the data points. (C) Current recordings for assaying the dependence of activation kinetics on the holding voltage (see protocol). The depolarizations to +50 mV activate hEAG1 channels with quite different time courses depending on the holding voltage applied for 5 s.

patch configurations. In solutions containing high concentrations of aspartate instead of chloride (see Materials and methods), we could record from cell-free patches for >30 min without significant rundown. The analysis of the voltage dependence of channel activation (Figure 1A and B) as well as the pre-pulse dependence of channel activation kinetics (Figure 1C) revealed that hEAG1 channels, measured in excised patches of *Xenopus* oocytes, show the same properties as bovine, rat and human EAG channels in mammalian cells (Ludwig *et al.*, 1994; Frings *et al.*, 1998; Occhiodoro *et al.*, 1998). Upon patch excision (Figure 1B), an increase in current amplitude of variable magnitude was often observed, indicating the removal of channel block by intracellular Na^+ (Pardo *et al.*, 1998) and/or Ca^{2+} .

Ca^{2+} sensitivity requires calmodulin

The sensitivity of hEAG1 channels to intracellular Ca^{2+} is easily observed when a membrane patch is excised into bath solutions containing free Ca^{2+} . In Figure 2A, the response of hEAG1 channels to a change from cytosolic $[\text{Ca}^{2+}]$ of an intact oocyte (on-cell patch) to 200 nM free Ca^{2+} (inside-out patch) is shown. Excision of the patch resulted in an almost complete reduction of the current response to depolarizing pulses. The current was restored completely by subsequent perfusion of the patch with an EGTA-buffered Ca^{2+} -free solution. Upon a second application of 200 nM Ca^{2+} to the same patch, however, no current reduction was observed, indicating the loss of a cytosolic factor during the perfusion in the absence of Ca^{2+} . Brief cramming of the patch pipette back into the

Fig. 2. Calmodulin is required for current inhibition. hEAG1-mediated currents were recorded from on-cell or inside-out patches of oocyte membranes in 400 ms depolarizations to +40 mV with a repetition interval of 10 s. Each trace represents the last of five pulses under identical conditions. The cartoons indicate the patch configuration; free Ca^{2+} concentrations are given below the traces. (A) After stabilization of the current amplitude in the on-cell configuration (first trace), the patch was excised into the bath solution, containing 200 nM free Ca^{2+} . Apparently the current was reduced substantially by 200 nM Ca^{2+} , but recovered to the original amplitude after wash with Ca^{2+} -free solution. However, it could not be blocked again by subsequent application of 200 nM Ca^{2+} . Pushing back the pipette tip with the membrane patch into the cytosol of the oocyte restored the Ca^{2+} sensitivity of the channels ($n > 15$). (B) The Ca^{2+} sensitivity could also be restored by application of cell lysate (here lysate from human melanoma cells, IGR1) to the patch. In this panel, all shown traces were obtained from an inside-out patch excised into a bath solution containing 1 μM free Ca^{2+} . The patch had been washed previously without Ca^{2+} to remove the Ca^{2+} sensitivity of the channels. When cell lysate (10 μl) was pipetted close to the patch pipette, channels were inactivated (second trace). Application of the same lysate after pre-incubation with a CaM-binding peptide (CaM kinase II, residues 290–309) at a concentration of 100 μM restored the current, suggesting that CaM is a required factor in the lysate. Re-administration of untreated lysate after a wash without lysate rendered the channels Ca^{2+} sensitive again (last trace) ($n = 2$). (C) Purified recombinant CaM was applied to an excised Ca^{2+} -insensitive patch, resulting in immediate current block by 200 nM Ca^{2+} . The effect of CaM on the channels was Ca^{2+} dependent, as it did not inactivate channels in the absence of Ca^{2+} (fourth trace) ($n = 20$).

oocyte to expose the patch membrane to the cytosol immediately restored the Ca^{2+} sensitivity of the channel and abolished the current. To investigate the nature of the required cytosolic factor, we repeated the experiment in the inside-out configuration and applied cell lysate. The first trace in Figure 2B was recorded in 1 μM Ca^{2+} after perfusion of the excised patch with Ca^{2+} -free solution. Adding the lysate of IGR1 human melanoma cells, which natively express hEAG1 channels (Meyer *et al.*, 1999), in the vicinity of the patch pipette resulted in a strong current reduction. Lysate from *Xenopus* oocytes had the same

effect (data not shown). The lysate could be inactivated by pre-incubation with a synthetic peptide corresponding to the CaM-binding domain of CaM kinase II (Figure 2B). The channels only became sensitive again to the untreated lysate plus Ca²⁺ after thorough washing with Ca²⁺-free solution, indicating that no irreversible channel modification resulted from the application of the CaM-binding peptide. From this experiment, we concluded that CaM must be at least one obligate component of the cell lysate required to confer Ca²⁺ sensitivity to the channels. The experiment shown in Figure 2C illustrates that purified His-tagged CaM is sufficient to cause Ca²⁺-induced inhibition of hEAG1 channels. A membrane patch was excised directly into a solution without free Ca²⁺. The small current increase in the inside-out patch (second trace) compared with the on-cell situation (first trace) reflects the partial inhibition of the channels by cytosolic Ca²⁺ in the intact oocyte. Upon perfusion of this patch with CaM in the presence of 200 nM Ca²⁺, hEAG current was almost completely blocked. The block was relieved by perfusion with solution containing CaM in the absence of Ca²⁺, confirming that both CaM and Ca²⁺ are required.

Localization of a CaM-binding domain

To address the question of whether CaM binds directly to the hEAG1 channel, gene fusions were constructed, linking parts of the *heag1* gene to the glutathione S-transferase (GST) gene. In overlay assays, [³⁵S]methionine-labelled human CaM ([³⁵S]CaM) was incubated with various GST fusion proteins immobilized on a polyvinylidene (PVDF) membrane. For this purpose, the fusion proteins were expressed in *Escherichia coli* and loaded directly onto an SDS-polyacrylamide gel without purification (Figure 3C). A construct covering the complete C-terminus of hEAG1 (GST-12345, Figure 3B) showed weak, but reproducible binding of [³⁵S]CaM (Figure 3D, first lane). To narrow down the binding site within the C-terminus, a series of truncated GST fusions was constructed as depicted in Figure 3B. A fragment containing 97 amino acids from the middle of the C-terminus (GST-3), as well as all larger fragments containing this part gave positive signals in the overlay assay (Figure 3C and D). Constructs lacking this part of the C-terminal domain (GST-12 and GST-45) did not interact with CaM. In this experiment, as well as in several independent experiments, the positive fragments resulted in signals of variable intensity. This may reflect variability in the exposure of the binding site within the immobilized polypeptides, which is certainly influenced by the neighbouring domains. The generally weaker signals obtained with the largest fusions (GST-1234 and GST-12345) may, in addition, be caused by a weaker expression and by incomplete transfer to the blotting membrane.

Critical residues of the CaM-binding motif

To characterize the CaM-binding motif within the GST-3 fragment, several point mutations were introduced into this part of the gene. We concentrated on charged residues, replacing them by neutral amino acids, and tested the Ca²⁺ sensitivity of such mutants in excised patches. Out of seven mutations, five (C1-4 and C8, see Materials and methods) did not lead to remarkable changes in the Ca²⁺ sensitivity of the channels (data not shown). The mutations

Fig. 3. Localization of the CaM-binding site in the C-terminus of hEAG1. (A) Transmembrane topology of an α -subunit of hEAG1. (B) Sectioning of the C-terminal domain of hEAG1 and the corresponding names of the GST fusion proteins. Also indicated are the residue numbers of the sites of deletion as well as an indicator of CaM binding ($\pm$, see below). (C) A Coomassie-stained gel of lysates from *E. coli* cells expressing the indicated GST fusion proteins. (D) Autoradiography of a membrane overlay with radioactively labelled CaM. The same samples as shown in (C) were blotted to a PVDF membrane and incubated with [³⁵S]CaM. Clear CaM-binding signals for the constructs GST-3, -34 and -345 and faint signals for the longer constructs GST-12345 (at ~81 kDa) and GST-1234 (at ~70 kDa) are visible. No signals were detected for the constructs GST-12 and GST-45.

C5 and C6 resulted in channels that could not be fully blocked by 200 nM Ca²⁺ in the presence of CaM (Figure 4A and C). The protein sequence in the region of these two mutants fits to a common feature of many thus far determined CaM-binding motifs, which are characterized by amphiphilic helices divided into a basic and a hydrophobic side (James *et al.*, 1995). Figure 4B shows a stretch of 13 residues, which covers the residues replaced in the C5 and C6 mutants, in a helical wheel projection. To test whether hydrophobicity in this segment is really required for CaM interaction with hEAG1, we tested a

Fig. 4. Characterization of a CaM-binding domain. **(A)** Amino acid sequence of hEAG1 between residues 707 and 726 (upper row). Indicated on top of this sequence are the residues that were substituted and the corresponding names of the resulting double site mutants. The mutant C9 is a combination of C5 + C6. The following rows show the alignment of fragments corresponding to *Drosophila* EAG (DDBJ/EMBL/GenBank accession No. M61157), rat EAG2 (DDBJ/EMBL/GenBank accession No. AF185637), as well as postulated CaM-binding sites of hIK and rSK2 (DDBJ/EMBL/GenBank accession Nos AF022150 and U69882) and from the cyclic nucleotide-gated channel rOLFα (DDBJ/EMBL/GenBank accession No. X55519). The first amino acid residues in the fragments shown correspond to the following positions in the full-length proteins: 707, 703, 724, 338, 450 and 61 (from top to bottom). **(B)** The residues of hEAG1 marked by the line in **(A)** (numbered from H1 to Q13) are shown as a hypothetical α -helical wheel (3.6 residues per turn); the mutated residues are shown in bold. The dashed line indicates that such a helix is amphipathic, with one side being hydrophilic, the other hydrophobic. **(C)** hEAG1 wild-type (wt) and the indicated mutants were expressed in *Xenopus* oocytes and the sensitivity of the resulting currents to Ca^{2+} /CaM was assayed by measuring in inside-out patches the reduction of current when the bath solution was switched from Ca^{2+} -free solution to a solution containing recombinant human CaM and 200 nM free Ca^{2+} . Wild-type currents were reduced almost completely, whereas currents mediated by the mutants C7 and C9 were almost insensitive. Mutants C5 and C6 display an intermediate Ca^{2+} /CaM sensitivity ($n = 6$ for all data points). The mutants indicated were also generated in the background of the fusion protein GST-34 (see Figure 3). **(D and E)** The corresponding Coomassie-stained protein gel and the CaM overlay blots, respectively.

mutant in which the phenylalanine residues in positions 714 and 717 were substituted by serines. Indeed, the resulting channels (C7) showed even lower Ca^{2+} sensitivity than C5 and C6, as almost 100% of the initial current in 0 Ca^{2+} was retained after perfusion with 200 nM Ca^{2+} and purified CaM (Figure 4C). The same strong effect was obtained by combination of the replacements used in the mutants C5 and C6. Like C7, this channel mutant (C9) did not respond to perfusion of the patch with Ca^{2+} /CaM. The electrophysiological results obtained with the mutant channels suggest that either binding of CaM to the channel or the inactivation mechanism triggered by Ca^{2+} /CaM must be impaired in these mutants. Therefore, these mutations were transferred into the GST-34 construct that exhibited strong binding of labelled CaM in overlay assays (Figure 3D). Figure 4D and E shows that mutations C5 and C6 strongly reduced the binding of CaM to these GST fusions, whereas the mutants C7 and C9 did not bind CaM. These data suggest that the residues altered in the mutants C5, C6, C7 and C9 are part of a CaM-binding domain in hEAG1 channels that presumably forms a typical amphiphilic CaM-binding helix. The alignment shown in Figure 4A reveals noticeable analogies of this site to corresponding fragments of *Drosophila* EAG and the EAG2 isoform from rat (Saganich *et al.*, 1999), as well as to postulated CaM-binding sites in hIK and rSK2 (Fanger *et al.*, 1999; Keen *et al.*, 1999) and in the cyclic nucleotide-gated (CNG) channel rOLFα (Liu *et al.*, 1994).

Ca²⁺ dependence of channel block and CaM binding

In human melanoma cells expressing hEAG1, we previously determined an IC_{50} value of 99 ± 29 nM for channel inhibition by Ca^{2+}_i (Meyer *et al.*, 1999). To test whether or not the excised patch assay reflects the native situation with respect to the Ca^{2+} dependence, varying Ca^{2+} and fixed CaM concentrations were applied to inside-out membrane patches containing hEAG1 channels. The first trace in Figure 5A shows the current response in the absence of Ca^{2+} and CaM. The following traces were recorded with varying $[\text{Ca}^{2+}]$ and constant $[\text{CaM}]$. The results from 12 patches are summarized in Figure 5C, where we plotted normalized current amplitudes over $[\text{Ca}^{2+}]$ (open circles). Data fits to a Hill function yielded an apparent IC_{50} value of 106 ± 4 nM and a Hill coefficient of 4.2 ± 0.2 . To address the question of whether this Ca^{2+} regulation is determined by the binding properties of CaM, we performed *in vitro* binding assays using the same solutions as in the patch experiments. CaM was incubated with purified GST-34 fusion protein and the amount of CaM that could be co-precipitated on the binding matrix for GST was estimated from SDS-polyacrylamide gels (Figure 5B). The relative intensities of the CaM protein bands in the gels were plotted versus $[\text{Ca}^{2+}]$ in Figure 5C (filled symbols); a Hill function fitted to these data points yielded an apparent K_D value of 480 ± 40 nM Ca^{2+} and a Hill coefficient of 1.7 ± 0.2 .

The weak binding of CaM at physiological Ca^{2+} concentrations suggests a regulatory mechanism mainly controlled by binding and release of the sensor molecule. This is in contrast to other Ca^{2+} -regulated K^+ channels from the SK/IK family, for which a constitutive binding of CaM has been proposed (Xia *et al.*, 1998). For direct

comparison of the CaM-binding properties of hEAG1 and a member of the K_{Ca} channels, we fused the complete C-terminus of the human IK channel (hIK or hSK4) to GST. Figure 5D shows a comparison of this construct with the GST-34 construct containing the binding motif of hEAG1 in an overlay assay. As expected, the hEAG1 fusion only bound CaM at high [Ca²⁺] but not in the absence of Ca²⁺. In contrast, the hIK fusion showed significant binding even in 10 mM EGTA, although a reduction compared with binding in 1500 nM Ca²⁺ was detectable.

One calmodulin molecule closes the channel

The fact that the IC₅₀ found for hEAG1 channel inhibition by Ca²⁺/CaM is lower than the K_D for CaM binding to the hEAG1 protein (Figure 5) suggests a closing mechanism that requires <4 CaM molecules bound to the channel tetramer. To address this question, we co-injected hEAG1 mRNA with mRNA coding for the C7 mutant into *Xenopus* oocytes and analysed the Ca²⁺ sensitivity of the

resulting currents in inside-out patches. The quality of the two mRNA species was compared, measuring the total current obtained when three different mRNA concentrations for each channel type were injected into *Xenopus* oocytes. As illustrated in Figure 6A, both mRNAs resulted in approximately equal current amplitudes. Using constant amounts of mRNA for the Ca²⁺-insensitive mutant, we then set up a series of co-injections with decreasing content of wild-type mRNA in order to create channels with a reduced number of binding sites per tetramer. In inside-out patches from such oocytes, we first determined for each patch the maximum current amplitude in Ca²⁺-free solution. The current remaining after application of CaM in 200 nM Ca²⁺ was subsequently measured. The fractions of the Ca²⁺-insensitive currents were plotted over the fraction of wild-type subunits in the respective cell (Figure 6B). The calculation of the wild-type fraction is based on the assumption that both mRNA species express equally well and that the subunit assembly is not impaired

Fig. 5. Concentration dependence of current block and CaM binding. (A) The dependence of hEAG1 current inhibition by Ca²⁺/CaM on the concentration of free Ca²⁺ was measured in inside-out patches in various solutions containing identical concentrations of CaM, but different concentrations of Ca²⁺. The current amplitudes were normalized to the control value in Ca²⁺-free solution and were plotted as a function of [Ca²⁺]_i in (C) (open circles). The concentration dependence was described with a Hill equation yielding an apparent IC₅₀ value of 106 ± 4 nM and a Hill coefficient of 4.2 ± 0.2 (*n* = 8–12). The binding of CaM to the GST-34 fusion protein was measured in GST pull-down assays. In (B), a Coomassie-stained gel shows the co-precipitated CaM at the level of the indicated Ca²⁺ concentrations. The binding signal was integrated and plotted in (D) (filled circles) as a function of the Ca²⁺ concentration. Filled squares and triangles represent two more independent experiments. The continuous curve represents a data fit to a Hill equation with the parameters: K_D = 480 ± 40 nM, Hill coefficient = 1.7 ± 0.2. (D) According to the data shown in (B), the binding of CaM to hEAG1 depends on Ca²⁺. This is not the case for the binding of CaM to the Ca²⁺-activated K⁺ channel hIK. The panels show [³⁵S]CaM overlay assays with a C-terminal fragment of hIK [hIK: GST-hIK (286–427)] and the GST fusion protein GST-34 of hEAG1 in 1500 nM Ca²⁺ and in the absence of Ca²⁺.

Fig. 6. Stoichiometry of CaM-hEAG1 interactions. In order to address the question of how many CaM molecules are necessary to inhibit hEAG1 channels, we investigated the Ca²⁺/CaM sensitivity of oocytes injected with mixtures of mRNA coding for the wild-type and mutant C7. In (A), the expression levels measured with the two-electrode voltage clamp method at +20 mV for the two channel types are shown for different amounts of mRNA (*n* = 10–15), indicating that the expression levels in this situation are similar. (B) The Ca²⁺/CaM sensitivity was measured in 200 nM free Ca²⁺. The relative remaining current after Ca²⁺/CaM application was plotted as a function of the expected fraction of wild-type subunits (*n* = 4–6). The thick continuous line indicates the prediction of the Ca²⁺/CaM sensitivity assuming that one wild-type subunit is sufficient to confer Ca²⁺/CaM sensitivity to the channel complex. The dotted curves indicate the alternative predictions for the requirement for two, three or four wild-type subunits. To match the observed data to the model considering two CaM molecules per tetramer, we would have to assume that the wild-type expressed four times more strongly than the mutant. This is not supported by the data shown in (A). The fit to the other models would require an even more biased expression of the wild type, affording correction factors of 11 and 35, respectively.

by the mutation. The curves superimposed on the data in Figure 6B represent the theoretically expected sensitivities, based on a binomial distribution of the channel subunits among CaM-sensitive wild-type and CaM-insensitive C7 subunits (see Equation 1). The solid curve is the prediction of the assumption that only one bound CaM molecule is required to close the channel. The alternative assumptions, namely that either two, three or four bound CaM molecules are required to close a channel, do not describe the data well (dotted curves). Thus, the data clearly support the hypothesis that one CaM molecule bound to hEAG1 channels is sufficient for Ca^{2+} -induced current inhibition.

Discussion

We have identified CaM as the Ca^{2+} sensor for human EAG K^+ channels. CaM binds to the C-terminal domain of hEAG1 in a Ca^{2+} -dependent manner. It thus does not form a constitutive subunit of the channel. Our studies demonstrate that the binding of one Ca^{2+} /CaM complex molecule per channel tetramer is already inducing current inhibition. By means of this stoichiometry, EAG channel availability is regulated just above basal Ca^{2+} levels of resting cells; despite a binding K_D for each individual subunit of ~ 480 nM Ca^{2+} , half-maximal channel inhibition is observed at ~ 100 nM. Exactly the same Ca^{2+} dependence of inhibition was found for EAG channels in human melanoma cells (Meyer *et al.*, 1999). This functional match, as well as the finding that a CaM-specific binding peptide inactivated the Ca^{2+} -sensitive factor in cell lysates, strongly suggests that CaM is a binding partner of hEAG channels under *in vivo* conditions.

A comparable Ca^{2+} regulation of K^+ channels has thus far only been observed for K_{Ca} channels. These channels show no or only weak voltage dependence and are activated by elevated intracellular $[\text{Ca}^{2+}]$. EAG channels, however, belong to the large group of voltage-activated K^+ channels that exhibit delayed rectifier characteristics. Our results show that the functional principle of CaM-mediated ion channel regulation must be extended to delayed rectifier channels and indicate a unique function of EAG channels within this group. Interestingly, the same sensor protein that is known to activate K_{Ca} channels in response to rising $[\text{Ca}^{2+}]$ can reduce the availability of EAG channels. This reverse regulation can take place in the same cell, as both EAG and channels of the SK/IK type were identified in human melanoma cells (Meyer *et al.*, 1999). This apparent paradox is explained by the different regulatory mechanisms in both systems. hEAG1 channels are closed by binding of only one CaM molecule, whereas the requirement for four Ca^{2+} -loaded CaM molecules for the activation of IK and SK channels has been reported (Fanger *et al.*, 1999; Keen *et al.*, 1999). The CaM-mediated regulation mechanisms in both classes of channels are compared in Figure 7. This simplistic model implies that cells expressing hEAG1 and IK/SK channels, such as IGR1 melanoma cells (Meyer *et al.*, 1999), should exhibit a minimum of K^+ conductances at intermediate Ca^{2+} concentrations.

Despite these different stoichiometries, similarly steep Ca^{2+} dependencies have been determined for both systems. For rSK2, a Hill coefficient of 4.6 has been reported,

Fig. 7. Schematic comparison of the CaM-dependent gating mechanisms of hEAG1 and IK/SK potassium channels. Channel tetramers (grey) are depicted from the cytosolic side with the central pore in the open (white) or closed (black) conformation. Up to four CaM molecules can bind to the channel complexes. In IK/SK channels, Ca^{2+} binding to pre-bound CaM triggers channel opening. Release of CaM from IK channels at extremely low $[\text{Ca}^{2+}]$ may occur according to overlay experiments (see Figure 5D) but is not required for gating. In hEAG1 channels, Ca^{2+} controls the dynamic binding and release of CaM. Channels can only open when no CaM is bound. The model shows how Ca^{2+} regulates K^+ conductances in an opposite direction at different critical Ca^{2+} concentrations using only one type of Ca^{2+} -sensing molecule.

indicating a pronounced cooperativity in Ca^{2+} binding (Keen *et al.*, 1999). Reducing the number of functional Ca^{2+} -binding sites in the CaM protein from four to one reduced this value to 2.8 (CaM234) or 2.5 (CaM134), respectively, showing that both partners, CaM and the channel subunits, contribute to a complex cooperative effect. For hEAG regulation in excised patches from oocytes, we now determined a Hill coefficient of 4.2. For EAG in melanoma cells, a value of 3.3 (Meyer *et al.*, 1999) and for EAG in human myoblasts a value of 4.5 (Bijlenga *et al.*, 1998) has been reported. Thus, CaM-mediated channel inhibition shows a steeper Ca^{2+} sensitivity than CaM binding to purified hEAG1 protein, where we estimated a Hill coefficient of 1.7. This may reflect a more complex mechanism of channel inhibition; CaM binding may only be a first step towards channel closure that requires higher Ca^{2+} loading of CaM.

Within the identified CaM-binding domain of hEAG1, a short stretch of residues (708–721) fits to a common feature of many CaM-binding sites (James *et al.*, 1995), amphiphilic α -helices with a hydrophobic and a hydrophilic, positively charged face. The functional relevance of this motif in hEAG1 was demonstrated by point mutations affecting both Ca^{2+} sensitivity in membrane patches and CaM binding *in vitro*. The mutants confirmed the importance of both hydrophobicity and basic residues. The potential binding motif fits neither to the described consensus for IQ motifs (IQXXRGXXRR), nor to other patterns defined by the spacing of hydrophobic residues (1-8-14 motif, 1-5-10 motif; Rhoads and

Friedberg, 1997). However, the alignment shown in Figure 4A implies that CaM-binding motifs used by EAG and SK/IK channels form a distinct group. Although CNG channels share higher sequence similarity with EAG channels than with SK/IK channels, CaM-binding sites are less related. Only a CaM-binding site in the N-terminus of the rat olfactory CNG α -subunit appears similar to the motif defined in EAG channels (Liu *et al.*, 1994). Further sites identified in both the N- and C-terminal domains of rod CNG β -subunits (Grunwald *et al.*, 1998; Weitz *et al.*, 1998) or in the N-terminus of the cone CNG α -subunit (Grunwald *et al.*, 1999) are not related to the EAG motif. Other CaM-regulated channels, such as L-type and P/Q-type Ca²⁺ channels (Lee *et al.*, 1999; Peterson *et al.*, 1999; Qin *et al.*, 1999; Zühlke *et al.*, 1999) or N-methyl-D-aspartate (NMDA) receptors (Ehlers *et al.*, 1996) do not contain EAG-type CaM-binding sites. Despite the similarities in the identified binding motifs of EAG and K_{Ca} channels, a striking functional difference between these channels is the Ca²⁺-regulated binding of CaM to EAG in contrast to its constitutive incorporation into K_{Ca} channels. In addition, for *Drosophila* EAG analysed under conditions identical to those shown in Figure 2B, we did not observe current reduction by CaM in the presence of 1 μ M Ca²⁺ ($n = 7$; data not shown). Thus, small structural differences within or outside the identified binding domain may result in considerable functional changes.

Regulation of ion channels by Ca²⁺ can be considered an important principle for converting Ca²⁺ signals into electrical signals. Does this common mechanism also indicate similar physiological functions for the different ion channel systems? For three types of Ca²⁺-permeable channels, a feedback regulation has been implied that limits the rise of intracellular Ca²⁺: the activities of CNG channels (Molday, 1996) as well as NMDA receptors (Ehlers *et al.*, 1996) are reduced in elevated [Ca²⁺]_i, and inactivation of L-type Ca²⁺ channels can be augmented by Ca²⁺/CaM (Zühlke *et al.*, 1999). A similar feedback function for Ca²⁺ regulation of hEAG1, presumably underlying long-term effects in neuronal signalling, appears possible, as its inhibition would reduce the electrochemical driving force for Ca²⁺ influx.

Apart from their putative function in neuronal cells, EAG channels are tightly linked to the process of cell proliferation and display an oncogenic potential (Meyer and Heinemann, 1998; Pardo *et al.*, 1999). Since it is well documented that changes in [Ca²⁺]_i have a regulatory function during the cell cycle (Santella, 1998), it will be important to elucidate whether the described Ca²⁺/CaM regulation is a critical feature linking EAG channels to cell proliferation and tumour progression.

Materials and methods

Electrophysiological measurements

Stage V oocytes were prepared from *X. laevis* as described previously (Stühmer *et al.*, 1992) and 50 nl of *in vitro* transcribed mRNA (5–500 ng/ μ l) were injected. Currents were recorded at 20–23°C 2–7 days after injection. Patch-clamp measurements were performed with a pipette solution composed of (in mM): 103.6 Na-aspartate, 11.4 KCl, 1.8 CaCl₂, 10 HEPES pH 7.2 with NaOH. The concentration of free Ca²⁺ ions in standard bath solution and solutions used for patch perfusion was calculated according to Tsien and Rink (1980). The solutions were made from two stocks, composed of (in mM): 115 K-aspartate, 10 EGTA, 8.73

CaCl₂, 10 HEPES pH 7.2 with KOH and 100 K-aspartate, 15 KCl, 10 EGTA, 10 HEPES pH 7.2 with KOH. For solutions with higher concentrations, we used 10 mM HEDTA (up to 5 μ M free Ca²⁺) as Ca²⁺ buffer. To measure the concentrations of free Ca²⁺ ions in the solutions up to 500 nM, we used the fluorescent dye Fura-2 according to Neher (1995). Higher concentrations were verified with a Ca²⁺-selective electrode (Kwik-Tip™; World Precision Instruments Inc., Sarasota, FL), calibrated according to Tsien and Rink (1980). Change of solutions was performed using a 10-channel perfusion system with the inside-out patch placed directly in the middle of the streaming solution. For solutions containing His-tagged CaM, the purified protein was first transferred to HEPES buffer (in mM: 100 K-aspartate, 15 KCl, 10 HEPES pH 7.2) via PD10 columns (Amersham-Pharmacia GmbH, Freiburg, Germany). The protein was then diluted in the desired bath solution (1:25) resulting in a final CaM concentration of ~30 μ g/ml. Dithiothreitol (DTT, 1 mM) was added to stabilize CaM. Application of IGR1 cell lysate to excised patches was performed by pipetting the lysate into the bath. The lysate was obtained from ~10⁷ IGR1 melanoma cells. These were trypsinized, washed once with phosphate-buffered saline (PBS; in mM: 140 NaCl, 2.7 KCl, 10 Na₂HPO₄, 1.8 KH₂PO₄ pH 7.2), homogenized on ice in a Dounce homogenizer and centrifuged for 30 min at 38 000 g (4°C). The supernatant was transferred into 3 ml of HEPES buffer through a PD10 column and stored at –20°C. Aliquots of 10 μ l were used for patch experiments.

The channel inhibition in Ca²⁺/CaM solutions of heteromeric wild-type-C7 channel complexes (Figure 6B) was predicted according to a binomial distribution, with p indicating the probability for wild-type subunits and j indicating the minimal number of wild-type subunits required for CaM-mediated channel inhibition. $I(wt)$ and $I(C7)$ are the relative currents in Ca²⁺/CaM solutions under control conditions, i.e. for the respective homomeric channel complexes.

$$I(j) = I(wt) + \{I(C7) - I(wt)\} \sum_{i=j}^4 \binom{4}{i} p^i (1-p)^{4-i} \quad (1)$$

For two-electrode voltage clamp, a Turbo-TEC 10CD amplifier (NPI electronic; Tamm, Germany) was used, and electrodes filled with 2 M KCl had a resistance between 0.5 and 0.9 M Ω . The bath solution contained (in mM): 115 NaCl, 2.5 KCl, 1.8 CaCl₂, 10 HEPES pH 7.2 (NaOH). As patch-clamp amplifier we used an EPC9 (HEKA elektronik, Lambrecht, Germany). The patch pipettes were fabricated from aluminium silicate glass with resistances of 1–2 M Ω . Experiment control including pulse generation and data recording was performed with the Pulse+PulseFit software package (HEKA elektronik). For leak correction, a P/n method, supported by Pulse+PulseFit, was used. Data analysis was performed with IgorPro software (WaveMetrics, Lake Oswego, Oregon). Pooled data are represented as means $\pm$ SEM (n = number of independent experiments).

Molecular biology and recombinant proteins

The full-length cDNA encoding hEAG1 was cloned from a human brain cDNA phage library (Clontech GmbH, Heidelberg, Germany) and inserted into the oocyte expression vector pGEM-HE (Liman *et al.*, 1992). The complete open reading frame was sequenced using the LI-COR 4000 system (MWG-Biotech AG, Ebersberg, Germany) and the Thermosequencing cycle sequencing kit (Amersham-Pharmacia GmbH, Freiburg, Germany). The derived sequence was identical to a clone previously described by Occhiodoro *et al.* (1998; DDBJ/EMBL/GenBank accession No. AJ001366). Capped mRNA was synthesized *in vitro* with T7 RNA polymerase (mMESSAGE mMACHINE kit; Ambion, Austin, Texas). Point mutations were generated by overlap extension PCR (Ho *et al.*, 1989). Sequences of the subcloned DNA fragments were confirmed by cycle sequencing. The following amino acid replacements in hEAG1 were carried out: C1, K547N-K550N; C2, K676N-R677N; C3, R681Q-K682N; C4, E690Q-E691Q; C5, R711Q-R712Q; C6, R716Q-R718Q; C7, F714S-F717S; C8, K503N; and C9, R711Q-R712Q-R716Q-R718Q. The full-length cDNA encoding hCaM (DDBJ/EMBL/GenBank accession No. M27319) was generated from a human brain cDNA library (Clontech) as a PCR product flanked by *Bam*HI and *Xho*I restriction sites and cloned in-frame with the His₆-coding sequence of the pQE30 vector (Qiagen GmbH, Hilden, Germany). According to the Qiagen protocols, His-CaM was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) in *E. coli* M15, harvested after 4 h induction at 30°C and purified using Ni-NTA agarose. To

generate GST fusions of hEAG1, the relevant gene fragments were generated as PCR products, flanked by *Bam*HI and *Xho*I restriction sites and cloned in-frame with the GST-coding sequence of pGEX-5X-2 (Amersham-Parmacia Biotech GmbH). The 3' end of the human IK channel gene (DDBJ/EMBL/GenBank accession No. AF0022150) was cloned by PCR from a human brain cDNA phage library (Clontech GmbH). A fragment encoding amino acids 286–427 was cloned in-frame into the *Bam*HI–*Xho*I sites of pGEX-5X-2. Fusion proteins were induced with 0.1 mM IPTG in *E.coli* BL21 according to the manufacturers' protocols. For overlay assays, *E.coli* cells were harvested after 3 h induction, boiled for 5 min in 2× Laemmli's loading buffer and separated on a denaturing SDS–polyacrylamide gel (13% acrylamide). Samples were loaded in duplicate and in one half of the gel the proteins were visualized by Coomassie staining. The other half was blotted onto a PVDF membrane (Millipore GmbH, Eschborn, Germany) using Towbin buffer (in mM: 25 Tris, 192 glycine, 20% methanol, 0.05% SDS). The membrane was blocked with 1% bovine serum albumin (BSA) in HBS-Tween (in mM: 137 NaCl, 3 KCl, 10 HEPES, 0.05% Tween-20 pH 7.4), washed once with HBS-Tween containing 1 mM CaCl₂ and incubated for 1 h at room temperature in 5 ml of HBS-Tween with 20 µl of [³⁵S]CaM. The blots were washed twice for 10 min with 1 mM CaCl₂ in HBS-Tween, air-dried and radioactive signals were detected by autoradiography. The human CaM gene was subcloned into the pGEM-HE vector for *in vitro* synthesis of mRNA. CaM was synthesized and labelled with [³⁵S]methionine during *in vitro* translation using the Flexi-Rabbit Kit (Promega, Heidelberg, Germany). A 20 µl aliquot of mRNA was translated in a 100 µl reaction volume for 1 h, and the reaction mixture was applied without further treatment to the overlay membrane. For GST pull-down assays, cleared lysate of *E.coli* BL21 expressing the fusion protein GST–34 was loaded onto glutathione–Sepharose. Sepharose beads were washed twice with 10 vols of PBS buffer and divided into aliquots. Each Sepharose aliquot was washed with K-aspartate buffers with different Ca²⁺ concentrations (see above), using either 10 mM EGTA (0–500 nM free Ca²⁺) or 10 mM HEDTA (100–5000 nM free Ca²⁺) as Ca²⁺ buffer. Finally, Sepharose was equilibrated in 1 ml of the individual buffer, and ~15 µg of His–CaM were dissolved in 20 µl of HEPES buffer (in mM: 100 K-aspartate, 15 KCl, 10 HEPES pH 7.2) were added to each aliquot. After 5 min incubation at room temperature and centrifugation at 500 g for 5 min, the supernatant was discarded. The remaining glutathione–Sepharose was boiled in 50 µl of 2× Laemmli's loading buffer and separated on a denaturing SDS–polyacrylamide gel.

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