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The Sigma Receptor as a Ligand-Regulated Auxiliary Potassium Channel Subunit

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Summary

The sigma receptor is a novel protein that mediates the modulation of ion channels by psychotropic drugs through a unique transduction mechanism depending neither on G proteins nor protein phosphorylation. The present study investigated sigma receptor signal transduction by reconstituting responses in *Xenopus* oocytes. Sigma receptors modulated voltage-gated K^+ channels (Kv1.4 or Kv1.5) in different ways in the presence and absence of ligands. Association between Kv1.4 channels and sigma receptors was demonstrated by coimmunoprecipitation. These results indicate a novel mechanism of signal transduction dependent on protein-protein interactions. Domain accessibility experiments suggested a structure for the sigma receptor with two cytoplasmic termini and two membrane-spanning segments. The ligand-independent effects on channels suggest that sigma receptors serve as auxiliary subunits to voltage-gated K^+ channels with distinct functional interactions, depending on the presence or absence of ligand.

Introduction

The sigma receptor was first described as a novel opioid receptor (Martin et al., 1976), but subsequent studies demonstrated that it is a distinct pharmacological entity distinguished by unusually promiscuous binding to a wide variety of ligands (Zukin and Zukin, 1979; Su, 1993; de Costa and He, 1994). Binding of antipsychotic drugs (such as haloperidol) and psychomimetic drugs (such as pentazocine), along with a genetic linkage to schizophrenia (Ishiguro et al., 1998), implicate sigma receptors in psychosis. Additional functions of sigma receptors in motor, endocrine, and immune systems have also been suggested (Su, 1993; Walker et al., 1993). A sigma receptor photoprobe labels a protein with a molecular weight of 26 kDa (Kahoun and Ruoho, 1992). The gene encoding the sigma receptor was cloned and the protein shown to have a similar molecular weight of 25.3 kDa (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1997). This protein lacks significant homology with known mammalian proteins but possesses weak homology with fungal sterol isomerase. Initially, hydropathy analysis of the deduced amino acid sequence suggested a single transmembrane segment. However, the present study presents evidence that the sigma receptor has two transmembrane segments.

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Sigma receptors have been shown to modulate ion channels in a number of cell types (Morio et al., 1994; Soriani et al., 1999, 1998; Wilke et al., 1999a, 1999b). The novel molecular structure of the sigma receptor raises the question of how signals are transmitted to their channel targets. This question was posed in pituitary nerve terminals, where sigma receptors inhibit voltage-gated K^+ channels (Wilke et al., 1999a). To test the role of G proteins, nerve terminals were perfused internally with GTPγS or GDPβS (Lupardus et al., 2000). Responses to sigma receptor ligands were completely unaffected, indicating that G proteins do not mediate these responses. ATP omission and replacement by AMPPcP in both whole-terminal and outside-out patch recordings also failed to diminish the actions of sigma receptor ligands. Thus, protein phosphorylation also does not play a role. Experiments in cell-free excised patches further ruled out requirements for soluble cytoplasmic factors in sigma receptor signal transduction, and experiments in cell-attached patches indicated that sigma receptors and their target channels must be in the same patch of cell membrane. Thus, signal transduction requires close proximity between the receptor and channel (Lupardus et al., 2000). These studies eliminate the more familiar mechanisms of ion channel modulation involving G proteins and phosphorylation. Since these mechanisms underlie nearly all the known forms of ion channel modulation (Hille, 1992; Jonas and Kaczmarek, 1996; Wickman and Clapham, 1995), these results raise the question of whether the sigma receptor employs a novel mechanism of transduction.

To clarify the molecular basis of signal transduction by sigma receptors, we employed the *Xenopus laevis* oocyte system to express sigma receptors, together with voltage-gated K^+ channels. The reconstitution of sigma receptor-mediated responses in a heterologous expression system allowed us to approach the question of transduction at a molecular level. Immunoprecipitation experiments, together with comparisons of physiological properties between oocytes and neurons, established that sigma receptors interact with K^+ channels and that ligand binding to the sigma receptor modulates channel activity through this interaction.

Results

Reconstitution of Sigma Receptor Function in Oocytes

In oocytes expressing the voltage-gated K^+ channels Kv1.4 or Kv1.5, either alone or with sigma receptor, depolarizing voltage steps evoked large K^+ currents (Figures 1A–1D). In cells coexpressing channels and sigma receptors, the K^+ currents were highly sensitive to sigma receptor ligands. By contrast, the modulation

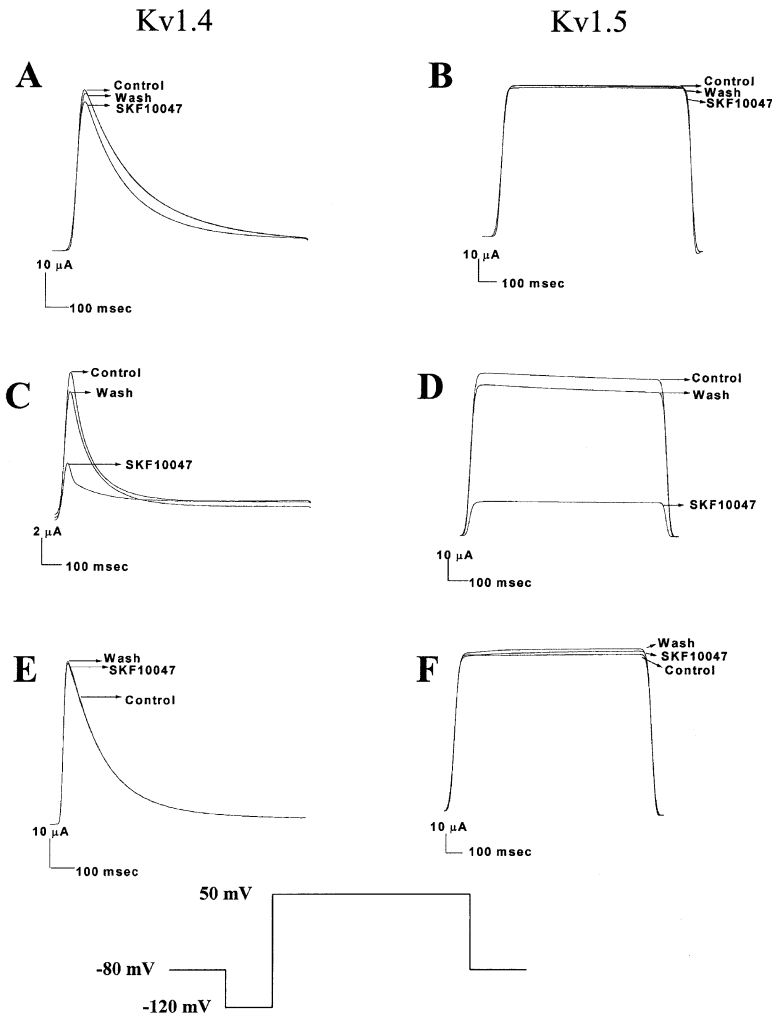

Figure 1. Sigma Receptor-Mediated Modulation of K^+ Channels in Oocytes

Currents through K^+ channels formed by Kv1.4 or Kv1.5 were evoked by 900 ms voltage pulses to 50 mV, following a 200 ms conditioning pulse from −80 mV to −120 mV (pulse sequence shown schematically below). In oocytes expressing only a K^+ channel, 100 µM SKF 10047 slightly reduced outward current in both Kv1.4 (A) and Kv1.5 (B). In oocytes expressing a K^+ channel and the sigma receptor, SKF10047 reduced outward current by more than half in both Kv1.4 (C) and Kv1.5 (D). In oocytes expressing K^+ channels and injected with sigma receptor antisense mRNA, SKF10047 produced little, if any, reduction in outward current in both Kv1.4 (E) and Kv1.5 (F). In all experiments, the drug tests were recorded 2 min after adding 100 µM SKF10047. Washes were recorded 30 min later after perfusing with control solution.

of recorded current by these ligands was very weak in cells expressing only K^+ channels. In 75% of the 18 oocytes expressing only a voltage-gated K^+ channel, the sigma receptor ligand SKF10047 failed to produce a detectible inhibition of outward current elicited by depolarizing voltage pulses. In the remaining oocytes, a small amount of block was seen (Figure 1A). Similar results were obtained with ditolyl-guanidine (DTG) (data not shown). In oocytes expressing only Kv1.5, the results were similar (Figure 1B). SKF10047 failed to inhibit current in 70% of the oocytes expressing only Kv1.5 ($n = 20$), and in the remaining oocytes, the inhibition was weak. Coexpression of K^+ channels with sigma receptors dramatically increased responses to ligands. SKF 10047 blocked K^+ current by >50% in all oocytes expressing sigma receptors, together with either Kv1.4 (Figure 1C, $n = 22$) or Kv1.5 (Figure 1D, $n = 24$). DTG was equally effective (data not shown). Sigma receptor ligands inhibited K^+ current by an average of ~75% when sigma receptor was expressed but by only ~12–25% without sigma receptor (Figure 2). Thus, K^+ channels show consistent and robust responses to sigma receptor ligands only when the sigma receptor is coexpressed.

The weak effect of sigma receptor ligands in the absence of expressed sigma receptors could be due either to a direct but weak interaction of the ligand with the two K^+ channels we are studying. Alternatively, endogenous *Xenopus* sigma receptor expressed in oocytes could mediate the observed effect. To address this question, we attempted to suppress endogenous sigma receptor production by injecting sigma receptor antisense RNA, together with Kv1.4 or Kv1.5 mRNA. Sigma receptor ligands failed to modulate K^+ channels to a detectible degree in all oocytes tested expressing Kv1.4 channels (Figure 1E). Antisense suppression was also seen in oocytes expressing Kv1.5 channels, but the suppression was not complete, and a small amount of ligand-induced channel modulation was seen in 10% of the oocytes tested (Figure 1F). The residual Kv1.5 channel current inhibition induced by SKF10047 was lower than the inhibition seen without sigma receptor antisense RNA, but this difference was not statistically significant. However, suppression of endogenous sigma receptor reduced responses to levels not significantly different from zero.

Figure 2 summarizes the expression studies for reconstitution of sigma receptor-mediated K^+ channel modulation. The small amount of modulation of Kv1.4 and Kv1.5 in the absence of exogenous sigma receptor is similar to that reported by Suessbrich et al. (1997) for

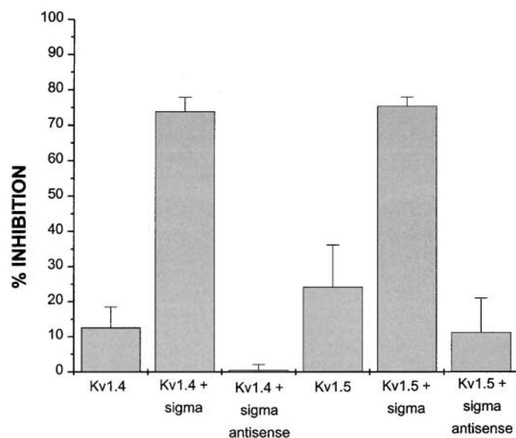

Figure 2. Summary of K^+ Channel Modulation

The bars show the mean percent inhibition by 100 μM SKF10047 from the experiments in Figure 1. When only a K^+ channel was expressed, current was inhibited by 10%–20%. When a K^+ channel was coexpressed with sigma receptor, inhibition was ~75%. Expression of channels with sigma receptor antisense mRNA reduced the amount of inhibition to insignificant levels.

another sigma receptor ligand, haloperidol. In light of the present findings, it is likely that this result can be attributed to the low levels of endogenous *Xenopus* sigma receptor. This interpretation is also consistent with a previous demonstration of low levels of binding activity for a sigma receptor ligand in *Xenopus* oocytes (Yamamoto et al., 1999).

Ligand-Independent Receptor-Channel Interactions

In addition to the ligand-dependent modulation of ion channels by sigma receptors demonstrated above, we found that sigma receptors altered the functional activity of Kv1.4 channels in the absence of sigma receptor ligands. The channels formed by Kv1.4 inactivate in response to sustained depolarization, leading to a reduction in outward current (Figures 1A, 1C, and 1E). Figure 3A superimposes a control K^+ current trace (i.e., no drug) from an oocyte expressing Kv1.4 alone, with a control K^+ current trace from an oocyte expressing Kv1.4 and sigma receptor. This figure shows that the current through Kv1.4 channels inactivates more rapidly in the presence of coexpressed sigma receptor. For voltage pulses to 40 mV, fitting the inactivating current to a single exponential gave a time constant of 61 ± 7 ms (n = 8) in oocytes expressing Kv1.4 alone, and 47 ± 4 ms (n = 6) in oocytes injected with equal amounts of Kv1.4 and sigma receptor mRNA. Increasing the ratio of sigma receptor mRNA to Kv1.4 mRNA to 2:1 further reduced the time constant for inactivation to 26 ± 3 ms (n = 4). The same trend was seen at other voltages (Figure 3B). Increasing amounts of sigma receptor accelerated Kv1.4 channel inactivation at all voltages tested.

Kv1.4 is expressed in nerve terminals (Sheng et al., 1993), and the rapid inactivation of Kv1.4 channels in oocytes resembles the rapid inactivation of the A current in pituitary nerve terminals (Bielefeldt et al., 1992). Furthermore, this A current was modulated by sigma receptors (Wilke et al., 1999a). However, quantitative analysis revealed a significant difference between the A current of pituitary nerve terminals and Kv1.4 in oocytes. The A current in nerve terminals inactivated with a time constant of 21.2 ± 2.1 ms (n = 9) at 40 mV. This was nearly 3-fold faster than inactivation of expressed Kv1.4 channels at the same voltage. However, the inactivation rate of the pituitary A current was very similar to that seen for Kv1.4 in oocytes coexpressing high levels of sigma receptor. This trend was seen for other voltages as well (Figure 3B). Time constants measured from current decays in oocytes injected with sigma receptor and Kv1.4 mRNA at a 2:1 ratio fell very close to the time constants taken from the rapid component of current decay in pituitary nerve terminals. Thus, coexpression of Kv1.4 with sigma receptors produces channels with inactivation behavior in oocytes that is essentially the same as that seen in vivo. These experiments demonstrate that sigma receptors modulate Kv1.4 channels even in the absence of drugs and further suggest that ligand-independent sigma receptor interactions account for the difference between Kv1.4 channel properties in the oocyte system and in vivo.

We compared other biophysical properties of Kv1.4 expressed with and without sigma receptors. Plots of current versus voltage showed that coexpression of the sigma receptor reduced the current at all voltages between −20 and 40 mV (Figure 4A). Normalizing to the maximum current and dividing by the driving force ($V - E_K$, assuming $E_K = -80$ mV) yielded plots of conductance that allowed us to compare the voltage dependence of channel activation (Figure 4B). These plots showed that the presence of the sigma receptor had no effect on the channel activation curve. $V_{1/2} = -9.0 \pm 3.0$ mV for Kv1.4 alone and -7.9 ± 4.0 mV for Kv1.4 + sigma receptor (in a ratio of 1:2). The steepness factors were also unchanged. In summary, Kv1.4 channels interact with sigma receptors in the absence of ligand to accelerate channel inactivation. Net current was also reduced, but the voltage dependence of channel activation showed no detectable change.

Location and Membrane Topology of the Sigma Receptor

Various studies have indicated that sigma receptors may be located in the cytoplasm (Hayashi and Su, 2001), on the cell surface (Lupardus et al., 2000), or in both places (Morin-Surun et al., 1999). To investigate this issue, we injected oocytes with mRNA encoding constructs of the sigma receptor fused in frame with GFP at either the N or C terminus. 4 days after injection, confocal fluorescence microscopy revealed bright surface labeling in over 30 oocytes. The fluorescence was clearly localized near the plasma membrane for both N-GFP-Sigma (Figure 5A) and C-GFP-Sigma (data not shown). A cross-section of fluorescence intensity through an oocyte is shown (Figure 5B). The fluorescence observed in the cytoplasm was very low and indistinguishable from a control oocyte. As controls, Kv1.4-GFP-injected oocytes showed a similar fluorescent profile to the Sigma-GFP constructs, while GFP expressed in oocytes showed characteristic cytoplasmic fluorescence (Figure 5C). Thus, the primary location of GFP-tagged sigma receptor is very close to the plasma membrane.

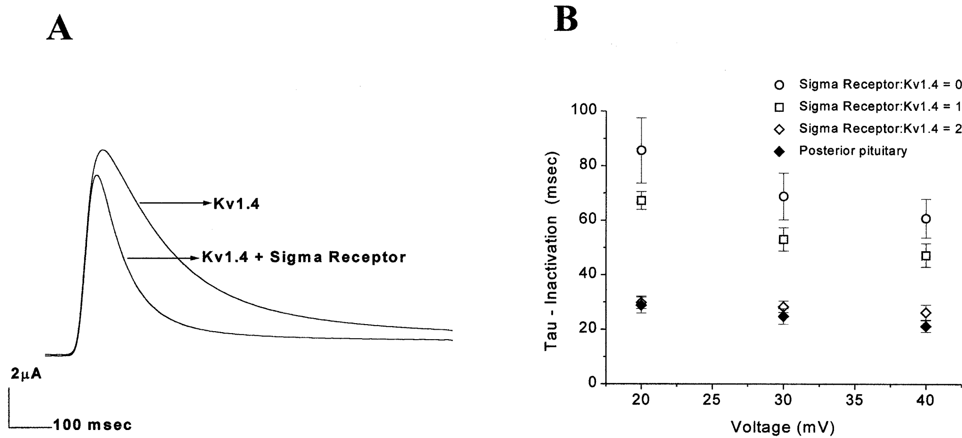

Figure 3. Inactivation Kinetics of Kv1.4

Comparison of current traces for Kv1.4 with Kv1.4 coexpressed with sigma receptor shows that the sigma receptor accelerated the inactivation of Kv1.4 channels. Currents were elicited by voltage pulses, as in Figure 1.

(B) Time constants of inactivation obtained from single exponential fits were plotted versus voltage. The slowest inactivation was seen in oocytes expressing Kv1.4 alone. In oocytes injected with equal quantities of Kv1.4 and sigma receptor mRNA, inactivation was faster. Increasing the ratio of sigma receptor RNA to Kv1.4 RNA (2:1) accelerated inactivation further. Inactivation time constants were also measured for the A current in posterior pituitary nerve terminals from the fast component of a double exponential fit. These values are very close to those obtained from oocytes injected with excess sigma receptor mRNA.

The function of these fusion proteins was tested in oocytes by coexpression with Kv1.4. Both N-GFP-Sigma and C-GFP-Sigma were capable of modulating Kv1.4 channels when SKF10047 was applied (Figures 5D–5F). The magnitude of this inhibition (~45%) was greater than that produced without expressed sigma receptor (~12%) but less than that produced with wild-type sigma receptor (~75%). Furthermore, experiments with the sigma receptor photoprobe iodo-azidococaine (Kahoun and Ruoho, 1992) demonstrated that the N-terminal GFP-sigma receptor fusion can be specifically labeled (Sievert and Ruoho, personal communication). Thus, fusion with GFP at either the C or N terminus yields a protein that retains its physiological activity.

In the original cloning studies of the sigma receptor, the sequence was proposed to harbor a single transmembrane (TM) domain (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1997). We also examined the sequence using a computer program to identify putative TM segments based on homology with a database of known TM segments (at www.isrec.isb-sib.ch) (Hofmann and Stoffel, 1993). The search was conducted for

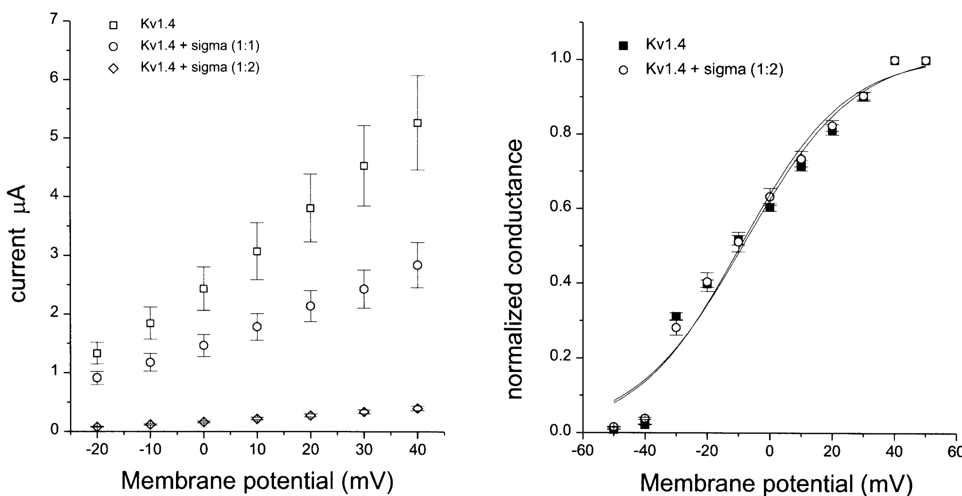

Figure 4. Effect of the Sigma Receptor on Kv1.4 Activation

(A) Current-voltage plots were constructed for peak current using a series of voltage steps with prepulses, as in Figure 1. For Kv1.4 alone, n = 7; for Kv1.4 + sigma receptor 1:1, n = 8; for Kv1.4 + sigma receptor 1:2, n = 7. Increasing amounts of sigma receptor depressed K^+ current at all voltages tested.

(B) Normalized conductance-voltage plots. Current plots from (A) for Kv1.4 alone and Kv1.4 + sigma receptor 1:2 were divided by driving force ($V - E_K$; with $E_K = -80$) to obtain conductance and normalized to the maximum conductance. The plots are essentially superimposable. Fits to the Boltzmann equation gave parameters that were not significantly different (see text).

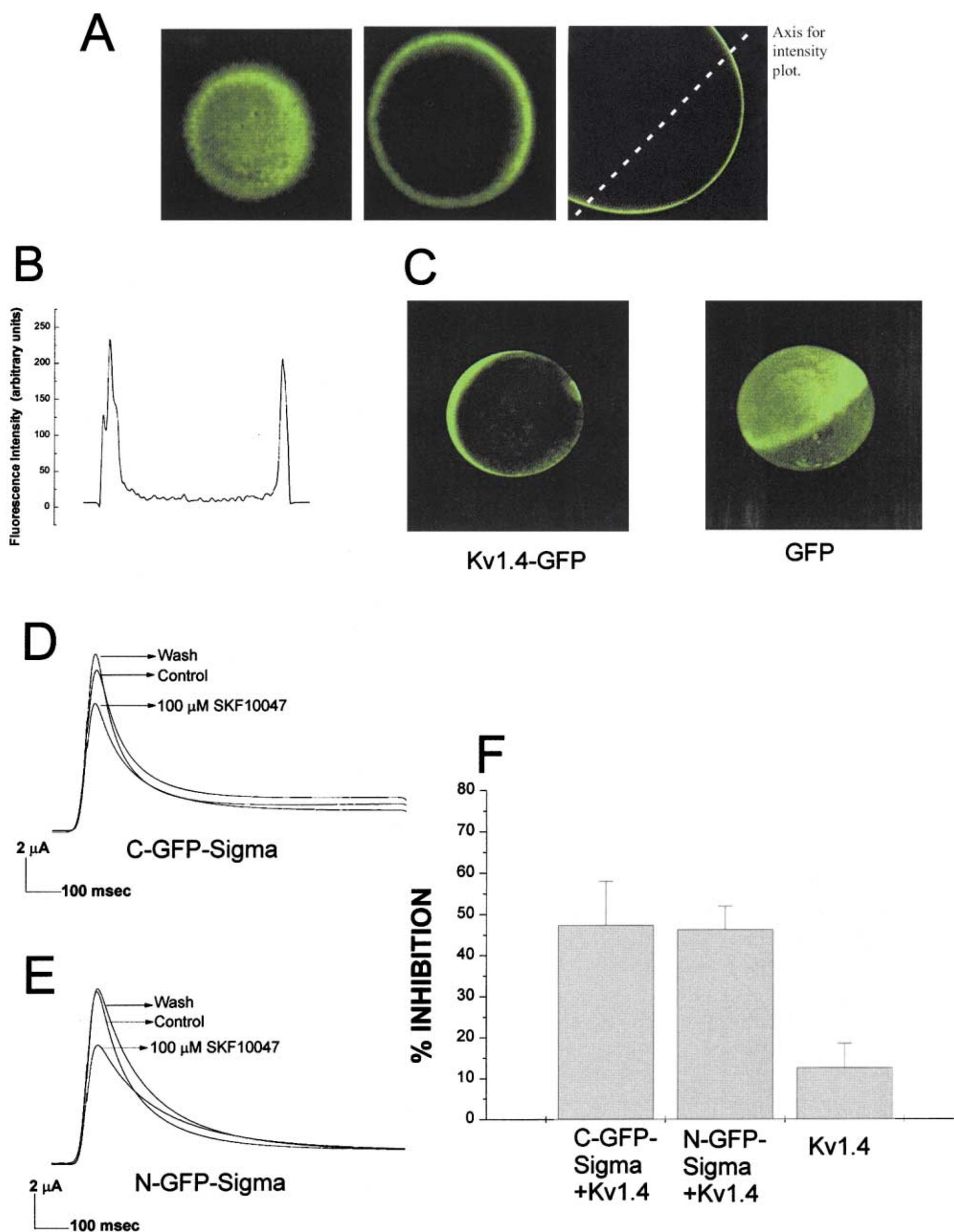

Figure 5. Localization of Sigma Receptors Expressed in *Xenopus* Oocytes

(A and B) Confocal fluorescence micrographs at different focal planes of an oocyte expressing N-GFP-Sigma show cell surface localization. A micrograph is shown with the plane of focus through the middle of the oocyte. The dashed line through the third oocyte shows the path along which fluorescence intensity was plotted (in B). (B) This plot shows that most of the fluorescence is on or near the cell surface.

(C) As controls, Kv1.4-GFP and GFP-expressing oocytes were also examined. Kv1.4-GFP gave a fluorescence profile similar to that of the Sigma-GFP constructs, while GFP showed fluorescence in the cell interior.

(D–F) Voltage-clamp of K^+ current showed that 100 μM SKF10047 reversibly reduced current in oocytes expressing N-GFP-Sigma and Kv1.4 ($n = 10$). Similar results were obtained with C-GFP-Sigma (E), and the average percent inhibition is shown in (F). The percent inhibition of Kv1.4 in oocytes without exogenous sigma receptor is reproduced from Figure 2 to show that the inhibition by the fusion protein is beyond what can be attributed to endogenous *Xenopus* sigma receptor.

segments with lengths in the range of 17–35 amino acids. The results are plotted in Figure 6A and show the original putative TM segment of 20 residues, starting ~80 residues from the N terminus. The plot shows an additional putative TM domain of 21 amino acids starting a few residues from the N terminus. Thus, the sigma receptor may in fact harbor two TM domains rather than one.

To test these models experimentally and to probe the orientation of the sigma receptor in the plasma membrane of the oocyte, we used anti-GFP antibodies to assess the accessibility of the GFP tags in sigma receptor fusion constructs. The anti-GFP antibody was detected with anti-mouse IgG antibody conjugated to Texas red. All oocytes injected with GFP-sigma receptor fusion protein mRNA showed strong fluorescence when

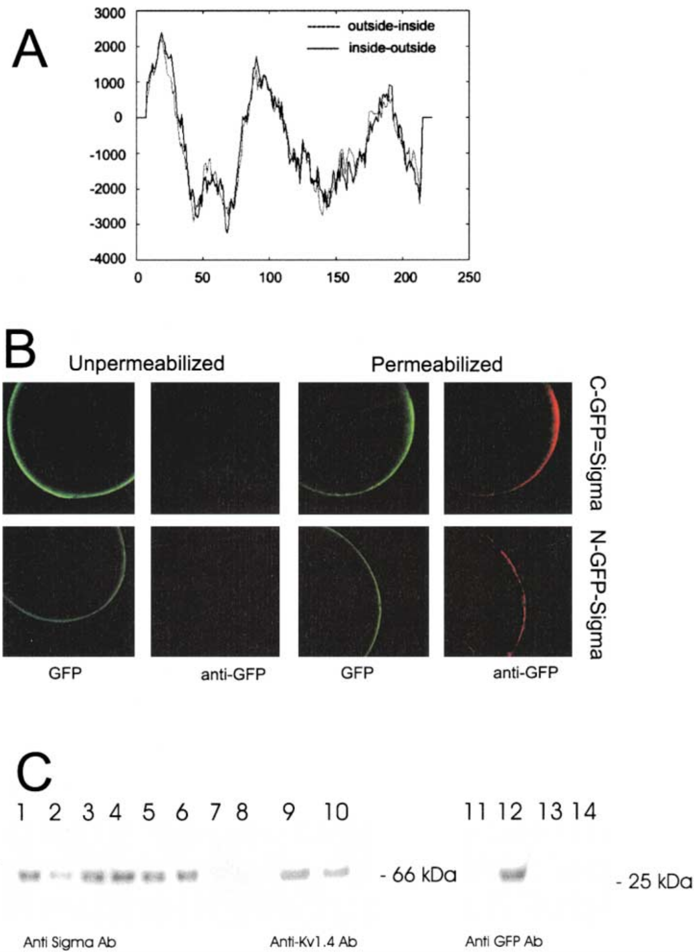

Figure 6. Membrane Topology and Orientation of the Sigma Receptor

(A) TM homology plot based on the deduced amino acid sequence of the sigma receptor. The program TMbase (Hofmann and Stoffel, 1993) (www.isrec.isb-sib.ch) shows two segments with significant homology to TM segments in the database. The orientation of the segments as inside-to-outside or outside-to-inside does not alter the plot. The putative TM segment from residues 80–100 was identified in the original reports of the sigma receptor sequence (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1997). The present plot indicates another putative TM segment in the region from residues 10–30.

(B) Localization of the N- and C-terminal GFP tags in oocytes expressing N-GFP-Sigma and C-GFP-Sigma. Oocytes were treated with Texas red-conjugated anti-GFP antibodies. Fluorescence micrographs of GFP (green) in unpermeabilized oocytes show surface localization similar to that in Figure 5A. Fluorescence micrographs of Texas red-conjugated anti-GFP antibodies (red) show no labeling without permeabilization. Antibody labeling was enabled by permeabilization with 0.5% acetone (see Experimental Procedures), indicating that both termini are on the cytoplasmic face of the membrane.

(C) Surface labeling experiments with guinea pig sigma receptor (lanes 1, 3, 5, and 7) and rat sigma receptor (lanes 2, 4, 6, and 8) expressed in oocytes using Sulfo-NHS-SS-biotin. Labeling was tested in unpermeabilized cells (lanes 1, 2, 7, and 8), permeabilized cells (lanes 3 and 4), and total membrane lysates (lanes 5 and 6). Controls (lanes 7 and 8) were conducted on oocytes labeled as in lanes 1–4 but without strepavidin-coated beads. Kv1.4 was also surface labeled both alone (lane 9) and coexpressed with sigma receptor (lane 10). The similar amount of protein recovered in the two experiments indicates that sigma receptor expression does not significantly alter surface expression of Kv1.4. As a control for the surface specificity of Sulfo-NHS-SS-biotin, labeling of GFP was seen in permeabilized cells (lane 12), but not in either unpermeabilized cells (lane 11) or in samples processed without strepavidin-coated beads (lane 13, unpermeabilized; lane 14, permeabilized).

the excitation wavelength was set for GFP. However, when the excitation wavelength was set for Texas red, antibody-treated oocytes expressing N-GFP-Sigma or C-GFP-Sigma revealed no fluorescence if they had not been permeabilized previously (Figure 6B). In oocytes expressing either fusion protein, permeabilization with 0.5% acetone (see Experimental Procedures) permitted anti-GFP antibodies to bind, and red fluorescence could then be seen (Figure 6B). Control oocytes (uninjected or expressing wild-type sigma receptor) showed only a low level of autofluorescence with the GFP or Texas red excitation wavelength in both permeabilized and unpermeabilized oocytes. Thus, both the N and C termini are inaccessible from the outside of the cell. The sigma receptor, therefore, is likely to have two TM segments, with both termini at the cytoplasmic face of the membrane.

To evaluate the surface exposure of proteins in oocytes, we employed the surface biotinylation reagent Sulfo-NHS-SS-biotin. This reagent forms a covalent biotin conjugate with primary amino groups and reacts weakly with arginine guanido groups as well. After treating oocytes, labeled protein was isolated with immobilized strepavidin. In unpermeabilized oocytes expressing the guinea pig sigma receptor, quantitative labeling was obtained (lane 1, Figure 6C); the amount of protein recovered was not significantly increased by permeabilization (lane 3, Figure 6C). Similar quantities of sigma receptor were recovered from total oocyte membrane lysates (lane 5, Figure 6C). These results indicate that most of the guinea pig sigma receptor is in the oocyte plasma membrane, as suggested by the distribution of fluorescence (Figures 5A and 5B).

In contrast to the results obtained with the guinea pig sigma receptor, Sulfo-NHS-SS-biotin labeled only about 20% of the rat sigma receptor in unpermeabilized oocytes (lane 2, Figure 6C). Permeabilization resulted in quantitative labeling, as compared to sigma receptor in membrane lysates (compare lanes 4 and 6 of Figure 6C). The resistance of the rat sigma receptor to labeling prior to permeabilization suggests that this receptor does not present amino groups at the extracellular face of the membrane. In fact, the deduced amino acid sequence of the rat sigma receptor contains no lysines between the two putative transmembrane segments indicated from the homology plot (Figure 6A; see Figure 8 for a

structural model). The guinea pig receptor has only one lysine in this loop, at position 60. This residue is an arginine in the rat sigma receptor. The weak labeling of the rat sigma receptor can be explained by the slow reaction of Sulfo-NHS-SS-biotin with arginine, five of which are present in the putative extracellular loop of the rat sequence. There are two other potential sites of biotinylation in the rat sigma receptor: the terminal amino group and lysine 142. Since these sites are located in the N and C termini, respectively, the weak labeling in unpermeabilized cells indicates that both termini are at the cytoplasmic face of the membrane, corroborating the results obtained with sigma receptor-GFP fusion proteins.

Surface labeling of Kv1.4 was also achieved, consistent with its membrane location. Expressed GFP was not labeled (lane 11, Figure 6C) unless the cells were permeabilized (lane 12), and this is consistent with a cytoplasmic location. Importantly, surface labeling of Kv1.4 was not significantly different when the sigma receptor was also expressed (compare lanes 9 and 10 in Figure 6C). This indicates that sigma receptor expression does not alter the level of expression of Kv1.4.

Sigma Receptor-Kv1.4 Association

Physiological studies had indicated that sigma receptor ligands modulate ion channels without assistance from other proteins commonly involved in signal transduction (Lupardus et al., 2000). Results presented above indicated functional interactions in the absence of ligand (Figure 3). These results could be explained by a direct physical interaction between sigma receptors and channels. To investigate this possibility, immunoprecipitation experiments were performed to detect the association. Membrane lysates were prepared from the posterior pituitary of rats. Duplicate samples were processed with or without mouse anti-Kv1.4 antibody, as described in Experimental Procedures. Samples were then resolved with SDS-PAGE and probed with rabbit antibodies against Kv1.4 or sigma receptor. Bands were visualized with an HRP-conjugated antibody and enhanced chemiluminescence, as described in Experimental Procedures.

Probing the gels with antibodies against Kv1.4 revealed distinct band(s) near 87 kDa in samples immunoprecipitated with anti-Kv1.4 antibodies (Figure 7A). Probing with anti-Kv1.4 antibodies failed to detect bands in control samples processed without the Kv1.4 immunoprecipitating antibody.

Probing with antibodies against the sigma receptor revealed a band at $\sim$25 kDa in the sample immunoprecipitated with antibodies against Kv1.4 (Figure 7A). No sigma receptor bands were observed in the control sample prepared without the anti-Kv1.4 immunoprecipitating antibody. Thus, immunoprecipitation of Kv1.4 from rat posterior pituitary lysate pulls down the sigma receptor. The molecular mass of the sigma receptor band is indistinguishable from the deduced molecular weight of 25.3 kDa. The sequence contains no putative N-glycosylation sites (Kekuda et al., 1996), and the similarity between the deduced and apparent molecular weights suggests that the sigma receptor is not posttranslationally modified by the addition of bulky moieties.

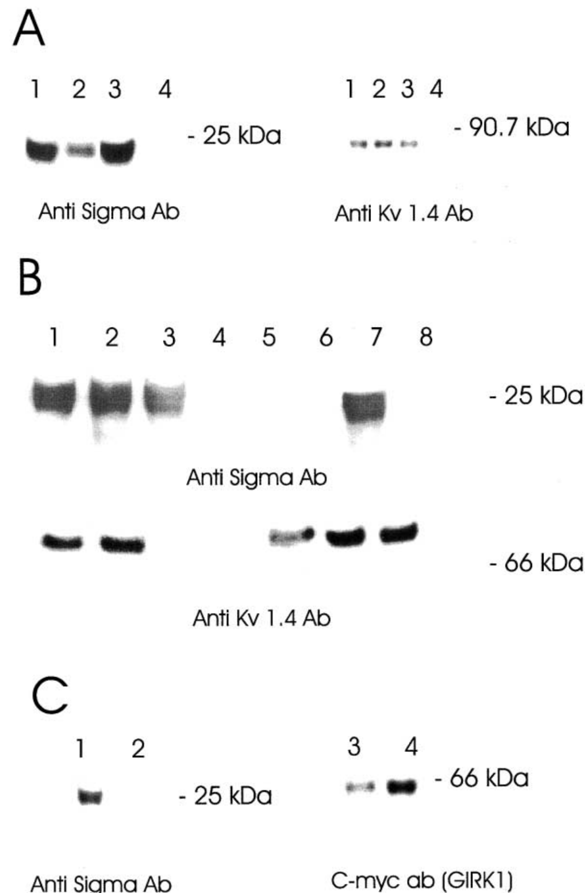

Figure 7. Coimmunoprecipitation of Sigma Receptors and Kv1.4 Channels

(A) Membrane lysates were prepared from rat posterior pituitary, immunoprecipitated with anti-Kv1.4 antibody, and resolved with SDS-PAGE. Immunoprecipitated samples were run on duplicate gels and the blots probed with either anti-sigma receptor or anti-Kv1.4 antibodies, as labeled. Total membrane preparations were run in lanes 1 and 3, and the eluates from the coimmunoprecipitations were run in lanes 2 and 4.

(B) Membrane lysates were prepared from *Xenopus* oocytes expressing Kv1.4 (lanes 5 and 6), sigma receptors (lanes 3 and 4), and both Kv1.4 and sigma receptors (lanes 1, 2, 7, and 8). The lysates were immunoprecipitated with anti-Kv1.4 antibody and resolved with SDS-PAGE. Immunoprecipitated samples were run on duplicate gels, and blots were probed with either anti-sigma receptor or anti-Kv1.4 antibodies, as labeled. Total membrane lysates (lanes 1, 3, 5, and 7) and the coimmunoprecipitated eluates (lanes 2, 4, 6, and 8) are shown. The oocyte eluates were concentrated 4-fold, compared to the total membrane lysates. Control samples without an immunoprecipitation antibody are included (lane 8).

(C) Sigma receptors were coimmunoprecipitated with Kv1.4 channels. Again, membrane lysates were prepared and coimmunoprecipitation was attempted with an anti c-*myc* antibody. The samples, both total membrane lysates (lanes 1 and 3) and eluates (lanes 2 and 4), were separated on duplicate SDS-PAGE gels, blotted, and probed with anti-sigma receptor or anti-c-*myc* antibodies, as labeled. Molecular weight markers are shown to the right of all blots.

A similar experiment was carried out in oocytes expressing Kv1.4 and sigma receptor (see Experimental Procedures). Here, Kv1.4 ran in a single band with a molecular mass very close to the predicted value (Figure 7B). As with the posterior pituitary, samples immunopre-

cipitated with anti-Kv1.4 antibodies and control samples were divided into duplicate, processed, and resolved with SDS-PAGE. Western blots revealed the sigma receptor at ∼25 kDa in the sample immunoprecipitated with anti-Kv1.4 antibody, but not in the control sample (Figure 7B). The interaction between Kv1.4 and sigma receptor can thus be detected both in posterior pituitary nerve terminals and in the oocyte heterologous expression system.

Based on the intensities of these bands, we estimate that our antibodies against Kv1.4 precipitated 92% of the Kv1.4 protein in oocytes and coprecipitated 26% of the total sigma receptor. In the posterior pituitary, antibodies against Kv1.4 precipitated the same percentage of Kv1.4 protein (92%) but only coprecipitated 8% of the total sigma receptor. The intensity of the Kv1.4 bands was12% and 16% of the intensity of the sigma receptor bands in gels of oocyte and pituitary membrane lysates, respectively. Thus, the sigma receptor is in 6- to 8-fold excess over Kv1.4 in the two systems. This is likely to be one factor in the incomplete coprecipitation of sigma receptors with Kv1.4. However, the lower percentage of sigma receptor coprecipitation with Kv1.4 in pituitary versus oocytes (8% and 26%) probably reflects the presence of additional ion channel targets in the pituitary, such as the BK channel (Wilke et al., 1999a).

The immunoprecipitated Kv1.4 from posterior pituitary has a higher molecular mass (∼87 kDa) than that expressed in oocytes (∼73 kDa). This is thought to be due to differences in glycosylation (Shi and Trimmer, 1999). In some blots of Kv1.4 from the posterior pituitary, multiple bands are observed at around 87 kDa, and this probably also reflects differences in glycosylation (data not shown). The sigma receptor has a molecular mass of ∼25 kDa in both posterior pituitary and *Xenopus* oocytes.

To evaluate the specificity of sigma receptor-Kv1.4 coimmunoprecipitation, we tested the GIRK1 (G protein-coupled receptor activated channel) channel for its ability to coimmunoprecipitate with the sigma receptor in *Xenopus* oocytes. GIRK1 expression induced small K^+ currents in oocytes, and these currents were not modulated by sigma receptor ligands, either in the presence or absence of sigma receptors (data not shown). In oocytes expressing the sigma receptor and GIRK1, the sigma receptor was not coprecipitated by antibodies against the myc tag of GIRK1 (Figure 7C).

Discussion

The results presented here support the hypothesis that sigma receptor signal transduction is mediated by a protein-protein interaction. This form of transduction was proposed recently based on negative results with reagents and manipulations that eliminate or alter G protein function and protein phosphorylation (Lupardus et al., 2000). The present study supports this hypothesis with two additional forms of evidence. (1) Reconstitution of sigma receptor-mediated responses in oocytes was achieved with the sigma receptor and Kv1.4 or Kv1.5 channels as the only two heterologously expressed proteins. (2) The sigma receptor was precipitated by antibodies against the Kv1.4 channel from extracts of either nerve or oocytes. Additional support for this hypothesis derives from the finding that the sigma receptor alters channel function in the absence of ligand. The picture that emerges from these studies is that the sigma receptor associates with Kv1.4 channels, and within this complex, functionally relevant interactions take place between the two proteins. These interactions take on two forms. In the absence of a sigma receptor ligand, the sigma receptor accelerates voltage-dependent channel inactivation by a factor of ∼3. In the presence of ligand, the interaction between the sigma receptor and Kv1.4 reduces peak current flow by ∼70%. The ligand-independent interactions also reduce current amplitude through Kv1.4 channels, and since Sulfo-NHS-SS-biotin labeling indicated that Kv1.4 surface expression was not reduced by coexpression with sigma receptor (lanes 9 and 10, Figure 6C), it is likely that the lower current amplitude is a consequence of the interaction between these proteins.

The functional changes brought about by coexpression and the demonstration that sigma receptors are precipitated by antibodies against Kv1.4 indicates that these two proteins are part of a complex. Whether the two proteins adhere through a direct interaction or through interactions with additional proteins cannot be answered at present. In *Xenopus* oocytes, the coprecipitation of the two exogenous proteins might be taken as evidence that the interaction does not require additional proteins. However, it is possible that homologous *Xenopus* proteins substitute for additional signal transducing proteins. This possibility is consistent with the finding that endogenous *Xenopus* sigma receptors support low levels of channel modulation without exogenous sigma receptor (Figures 1 and 2). The fact that the *Xenopus* sigma receptor can modulate rat channels indicates that other *Xenopus* proteins could also substitute for rodent proteins in this transduction process. The involvement of additional proteins in the stabilization of the sigma receptor-K^+ channel complex is suggested by the recent report of a ternary complex between sigma receptors, IP_3 receptors, and ankyrin (Hayashi and Su, 2001), but this complex was observed in the endoplasmic reticulum and much of our data suggests that the sigma receptor-Kv1.4 complex resides in the plasma membrane. The close agreement between the channel inactivation rate of the expressed sigma receptor-Kv1.4 complex and the A current of posterior pituitary nerve terminals (Figure 3) would require that additional *Xenopus* ersatz proteins have exactly the same functional activity as the rat protein.

It is likely that the functional interaction between sigma receptors and Kv1.4 channels depends on contacts between these two proteins. The original analysis of the sigma receptor failed to turn up sequences that might mediate contacts with other proteins (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1997). We repeated this analysis using the computer programs ProfileScan and Smart, using the most current sequence data, and confirmed the absence of recognizable protein-protein interaction motifs, such as SH3, PDZ, or WW motifs. Since ligand binding to sigma receptors alters K^+ channel function, the contacts between these two proteins must be malleable. The domains responsible for these interactions have yet to be identified, but

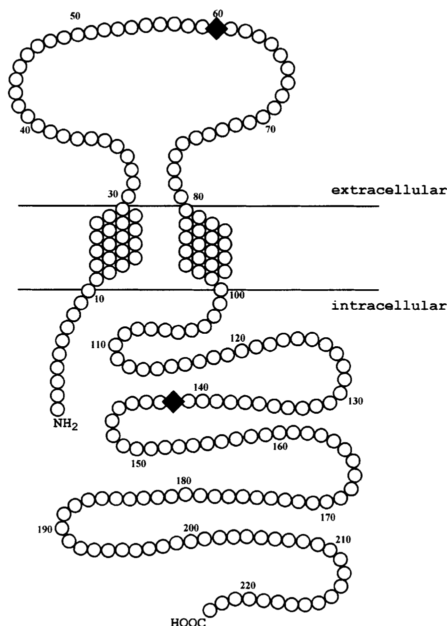

Figure 8. A Structural Model for the Sigma Receptor

This model contains two transmembrane segments in the regions indicated by the TM homology plot (Figure 6A). The N and C termini are shown on the intracellular side of the membrane, as indicated by the inaccessibility of GFP tags (Figure 6). The two lysines in the guinea pig sigma receptor are indicated by closed diamonds. In the rat sigma receptor, residue 60 is arginine, so the only primary amino groups are lysine 142 and the terminal amino group. Their intracellular location in this model is consistent with poor biotin labeling prior to permeabilization and efficient labeling thereafter.

the present studies on receptor topology suggest a structural model of the sigma receptor with domains that can be probed for interactions with channels (Figure 8). Based on lack of antibody access to GFP labels at the two termini, surface labeling experiments with Sulfo-NHS-SS-biotin, and analysis of homology with TM segments, we propose that the sigma receptor has two TM segments, a ~50 amino acid extracellular loop and a ~125 amino acid intracellular C terminus. The C terminus contains the region homologous with fungal sterol isomerase. The N terminus is also intracellular but is relatively short (~10 amino acids). This domain map of the sigma receptor should serve as a useful guide for more detailed studies of the interaction between sigma receptors and their regulatory targets.

The finding that the sigma receptor forms a stable complex with Kv1.4 channels and that sigma receptors can modify channel function calls to mind the β subunits of voltage-gated channels (Adelman, 1995; Pongs et al., 1999). Although the sigma receptor has no sequence homology with these proteins, there are some interesting parallels. Some of the β subunits specifically accelerate inactivation (Rettig et al., 1994), as did the sigma receptor (Figure 3). However, β subunits accelerate inactivation with an inactivating N-terminal ball domain, a motif absent from the sigma receptor sequence. Another interesting parallel is that both sigma receptors and β subunits show sequence homology with enzymes. Sigma receptors are homologous with fungal sterol isomerases, and β subunits are homologous with aldoketo reductases. Indeed, consideration of its cofactor binding site prompted the suggestion that β subunits (Gulbis et al., 1999), like sigma receptors, might be sensitive to ligands or chemical signals. The sensitivity to O_2 conferred by a β subunit was offered as a case in point (Perez-Garcia et al., 1999). The β subunits associated with members of the Kv1 family are peripheral membrane proteins with no putative TM segments. The structurally distinct β subunit of the Ca^{2+}-activated K^+ channel *Slo*β has two putative TM segments and a proposed topology (Knaus et al., 1994) very similar to that proposed here for sigma receptors (Figure 8). *Slo*β alters channel activity in a manner quite different from that described here (Dworetzky et al., 1996). However, Dworetzky et al. (1996) also noted that *Slo*β modifies the drug sensitivity of the channel, and the sigma receptor clearly has that capability. The inactivation kinetics of the sigma receptor-Kv1.4 complex (Figure 3) may indicate that this complex produces the A current of nerve terminals. Considering the broad properties and structures of proteins designated as β subunits of voltage-gated channels, it may be worth considering sigma receptors as candidates for this grouping.

The idea of a sigma receptor-K^+ channel complex casts some earlier puzzling pharmacological results in an interesting light. In rodent posterior pituitary, the sigma receptor modulates two distinct K^+ channels (Wilke et al., 1999a). In mouse, one particular ligand, U101958, only modulated the function of a slowly inactivating Ca^{2+}-activated K^+ channel and failed to modulate the A current. Another sigma receptor ligand, PPHT, modulated both channels. In rat, several different sigma receptor ligands modulated both channels with equal efficacy (Wilke et al., 1999a), but the sigma receptor ligand NE-100 only inhibited the Ca^{2+}-activated K^+ channel (M.J., unpublished data). These findings recall the differential coupling of PACAP receptors to different effectors when different agonists were used to activate the receptor (Spengler et al., 1993). A three-state allosteric mechanism was invoked to explain this behavior (Leff et al., 1997). However, if the receptor and channel form a complex, then the channel could contribute to the drug binding site and allow different channels to be preferentially modulated by different drugs. Sigma receptors exhibit extremely promiscuous binding activity. Because of this, they have been confused with phencyclidine receptors, opioid receptors, and dopamine receptors (de Costa and He, 1994). If the binding activity of this molecule depends on the presence and identity of an associated channel, this could account for some of the confusion in the literature.

Recognition of the importance of protein-protein interactions in membrane signaling has grown in recent years. The β subunits discussed above provide many examples. Another example is the receptor-activity-modifying proteins, which modify the function of the calcitonin receptor-like receptor (McLatchie et al., 1998). The $GABA_A$ receptor and D5 dopamine receptors form a complex with reciprocal functional interactions (Liu et al., 2000). The present findings indicate that the sigma receptor also performs a membrane signaling operation based on protein-protein interactions and possibly belongs to an extended family of auxiliary β subunits of

voltage-gated channels. Determining the relative importance of the ligand-dependent and ligand-independent forms of ion channel modulation by sigma receptors presents an interesting new direction in the study of the role of protein-protein interactions in membrane signaling.

Experimental Procedures

Oocyte Expression

Oocytes were removed from anesthetized frogs (*Xenopus laevis*, Nasco) through a small abdominal incision, according to procedures approved by the University of Wisconsin Research Animals Resource Center and the NIH. The follicular membranes were removed following collagenase treatment (Collagenase B, Boehringer Mannheim) and, in some cases, following osmotic shock (Pajor et al., 1992).

mRNA was synthesized from a T7 promoter using the mMESSAGE mMACHINE kit (Ambion, Austin, TX). Sigma receptor antisense RNA was generated using a pGH19 vector and synthesized from an SP6 promoter using the same kit. RNA was diluted in sterile water to different ratios to give upon expression an optimal 5–50 μA outward current for depolarizing steps to 40 mV. Oocytes were cultured at 18°C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, and 10 μg/ml gentamicin [pH 7.4]). The clone for Kv1.4 was provided by G.N. Tseng of Virginia Commonwealth University (Tseng-Crank et al., 1990). The clone for Kv1.5 was provided by L. Kaczmarek of Yale University (Pragnell et al., 1990). The clone for the rat sigma receptor was provided by V. Ganapathy of the Medical College of Georgia (Kekuda et al., 1996). The clone for the guinea pig sigma receptor was provided by A. Ruoho. Except for the indicated biotinylation experiments presented in Figure 6, all results reported here were obtained with the rat sigma receptor clone. The clone for GIRK1 (Kir3.1) was provided by H.H. Van Tol of University of Toronto, and a c-*myc* tag was attached to the C terminus by PCR.

Current Recording and Data Analysis

Current was recorded using a two-electrode voltage clamp (Model 725A, Warner Instruments, Hamden CT) and pClamp 7.0 software (Axon Instruments, Foster City, CA). Oocytes were impaled with two glass microelectrodes filled with 2 M KCl and having resistances of 0.5–1 MΩ. Recordings were performed at room temperature (∼25°C) with oocytes bathing in a solution consisting of: 93 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, and 5 mM HEPES (pH 7.4). Drugs used in these experiments were obtained from Sigma (St. Louis, MO). Compounds were dissolved in bathing solution and either superfused onto the preparation at a rate of 2–4 ml/min by a gravity-feed system or added directly to the bathing solution by pipette injection. Prior to adding drugs, K^+ current was recorded at 15 s intervals for 1–3 min to obtain a stable baseline. Current was also recorded after drug removal to demonstrate reversibility. The highly lipophilic drug ditolyl-guanidine (DTG) was first dissolved in DMSO and then diluted into bathing solution. PClamp and Origin 5.0 (Microcal Software Inc., MA) were used for data analysis and generating plots.

K^+ current was recorded from nerve terminals of the posterior pituitary by techniques described previously (Lupardus et al., 2000; Wilke et al., 1999a). The time constant for inactivation of the A current was taken as the fast component of a double exponential fit (Bielefeldt et al., 1992).

Sigma-GFP Fusion Constructs

Sigma receptor-GFP fusion constructs were prepared by PCR, with six extra bases between the sigma receptor and the GFP sequences (encoding glu-phe). GFP was fused either to the N terminus of the sigma receptor (N-GFP-Sigma) or the C terminus (C-GFP-Sigma) and subcloned into the pGH19 *Xenopus* oocyte RNA transcription vector. GFP was fused to the C terminus of Kv1.4 by the same procedure. Constructs were confirmed by DNA sequencing and restriction enzyme digestion. These fusion proteins were expressed by procedures described above, and the oocytes were used for experiments 4 days after injection.

Confocal Fluorescence Microscopy

Oocytes expressing GFP-sigma receptor fusion proteins, GFP-Kv1.4 fusion proteins, or GFP were viewed with a Bio-Rad MRC 1024 confocal laser scanning microscope using 20× or 4× objectives. GFP and Texas red fluorescence were selected with the prescribed optical methods of this microscope. All images were recorded at the same settings of laser power and photo-multiplier sensitivity (30% laser, no low signal). Images were processed with Adobe Photoshop (ADOBE Systems, Mountain View, CA) with identical values for contrast and brightness.

Immunohistochemistry of GFP-Sigma Receptor Fusion Proteins

Oocytes expressing N-GFP-Sigma and C-GFP-Sigma were permeabilized by treatment for 30 min with 0.5% acetone in ND96. Permeabilized or unpermeabilized oocytes were washed five times in ND96 and then incubated in mouse anti-GFP antibody (Molecular Probes, Eugene, OR) for 1 hr in ND96, with 1% BSA at 18°C. The oocytes were then washed five times in ND96 and incubated in donkey anti-mouse IgG Texas red conjugate (Jackson ImmunoResearch) for 1 hr in ND96 with 1% BSA at 18°C. The oocytes were washed five more times in ND96, after which Texas red fluorescence was visualized by confocal microscopy.

Immunoprecipitation

The posterior pituitary gland was removed from ten 2-month-old rats and homogenized at 4°C with a 10 ml dounce homogenizer in 200 μl 1% Triton X-100 in IP buffer (100 mM NaCl, 10 mM Tris, 5 mM EDTA [pH 7.4]) with a proteinase inhibitor cocktail (CytoSignal, Irvine, CA). The homogenate was spun twice at 13,000 rpm for 15 min at 4°C in microtubes, and the supernatant was removed and split into two 100 μl aliquots. Mouse anti-Kv1.4 antibody (1 μg) (Upstate Biotechnology, Lake Placid, NY) was added to one aliquot, but not to the other. Samples were incubated for 18 hr at 4°C with gentle rocking. The immunoprecipitation was processed using an IMMUNOcatcher kit (CytoSignal, Irvine, CA). Eluates and total membrane preparations were loaded on duplicate 10% SDS-PAGE gels and subsequently blotted to a PVDF membrane. Following blocking in 4% BSA in IP buffer with 0.01% Tween 20, each blot was incubated with a rabbit anti-Kv1.4 antibody (Alomone Labs, Jerusalem) or a rabbit anti-sigma receptor antibody (Yamamoto et al., 1999). A detection system, consisting of an anti-rabbit IgG horseradish peroxidase conjugate (Jackson ImmunoResearch, West Grove, PA) and an ECL detection kit (Amersham Pharmacia, Piscataway, NJ) was used to visualize immunoprecipitated proteins, followed by detection on XOMAT film or detection and quantification using a LUMI-Imager detection system (Roche). Immunoprecipitated bands were compared to protein markers of known molecular size run in parallel on the SDS-PAGE gel (Amersham rainbow markers).

Membrane lysates were prepared from *Xenopus* oocytes 4 days after injection with Kv1.4 and Sigma receptor mRNA (Connor et al., 1998). The lysates were processed by the same procedure described above for the posterior pituitary. The immunoprecipitated proteins were separated, blotted, and detected as described above. As a control, coprecipitation experiments were carried out with GIRK1 containing a c-*myc* tag and using an anti c-*myc* antibody for immunoprecipitation and Western detection.

Biotin Labeling

Oocytes expressing guinea pig sigma receptor, rat sigma receptor, Kv1.4, or GFP (4 days postinjection) were incubated for 30 min in either ND96 or ND96 + 0.5% acetone (permeabilization agent) at 16°C, followed by three washes in ND96. The oocytes were subsequently incubated in 10 mM EZ link Sulfo-NHS-SS-biotin (Pierce Biochemicals) in ND96 for 10 min at 16°C, followed by three washes in IP buffer to neutralize the surface label. Ten oocytes each were suspended in 400 μl of fresh IP buffer + proteinase inhibitor cocktail (used in immunoprecipitation), and membrane lysates were prepared as described above. The preparations were divided in two and either 20 μl of strepavidin immobilized to beaded agarose (Sigma) was added or agarose beads alone were added, and the preparations were incubated at 4°C for 4 hr with gentle rocking. These preparations were subsequently processed using IMMUNO-

catcher columns (CytoSignal) and washed once with both IP buffer and IP buffer with 1% SDS. Subsequent elution of the biotin-labeled proteins was achieved by incubation in IP buffer with 2% mercaptoethanol heated to 80°C for 10 min. The preparations were separated on 12% SDS-PAGE gels, Western blotted, and probed with anti-sigma receptor antibody, anti-Kv1.4 antibody, or an anti-GFP antibody, as described above.

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