Membrane-delimited coupling between sigma receptors and K^+ channels in rat neurohypophysial terminals requires neither G-protein nor ATP

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1. Receptor-mediated modulation of ion channels generally involves G-proteins, phosphorylation, or both in combination. The sigma receptor, which modulates voltage-gated K^+ channels, is a novel protein with no homology to other receptors known to modulate ion channels. In the present study patch clamp and photolabelling techniques were used to investigate the mechanism by which sigma receptors modulate K^+ channels in peptidergic nerve terminals.

2. The sigma receptor photoprobe iodoazidococaine labelled a protein with the same molecular mass (26 kDa) as the sigma receptor protein identified by cloning.

3. The sigma receptor ligands pentazocine and SKF10047 modulated K^+ channels, despite intra-terminal perfusion with GTP-free solutions, a G-protein inhibitor (GDPβS), a G-protein activator (GTPγS) or a non-hydrolysable ATP analogue (AMPPcP).

4. Channels in excised outside-out patches were modulated by ligand, indicating that soluble cytoplasmic factors are not required. In contrast, channels within cell-attached patches were not modulated by ligand outside a patch, indicating that receptors and channels must be in close proximity for functional interactions. Channels expressed in oocytes without receptors were unresponsive to sigma receptor agonists, ruling out inhibition through a direct drug interaction with channels.

5. These experiments indicate that sigma receptor-mediated signal transduction is membrane delimited, and requires neither G-protein activation nor protein phosphorylation. This novel transduction mechanism is mediated by membrane proteins in close proximity, possibly through direct interactions between the receptor and channel. This would allow for more rapid signal transduction than other ion channel modulation mechanisms, which in the present case of neurohypophysial nerve terminals would lead to the enhancement of neuropeptide release.

Sigma receptors modulate the excitability of peptidergic nerve terminals in the neurohypophysis by inhibiting voltage-dependent K^+ channels (I_K) (Wilke *et al.* 1999*a*). The activation of sigma receptors by a variety of ligands reduces current flow through two distinct K^+ channel types, the A-current channel (I_A) and the Ca^{2+}-activated K^+ channel (I_{BK}). Current is reduced by the same proportion over the entire accessible voltage range, with no shift in the voltage dependence of activation or inactivation. Furthermore, the residual unblocked currents inactivate with very similar rates, indicating that sigma receptor modulation entails a shutting down of function rather than a modification of gating behaviour (Wilke *et al.* 1998, 1999*a*, *b*). A comparison of the concentration dependence of I_A reduction with that of I_{BK} reduction indicated that the sigma receptor ligand PPHT inhibits both of these channels with a very similar EC_{50} (Wilke *et al.* 1998); similar results were obtained with haloperidol (Wilke *et al.* 1999*a*). Both I_A and I_K were reduced proportionally by a large number of sigma receptor ligands (including ditolylguanidine, SKF10047, pentazocine, haloperidol, PPHT, U101958, and apomorphine), suggesting that in the rat the same receptor is coupled to two types of K^+ channels. In D_2, D_3 and D_4 dopamine receptor-deficient mice, sigma receptor ligands reduced I_K as effectively as in wild-type mice, indicating that the responses are not mediated by dopamine receptor subtypes known to interact with some sigma receptor ligands (Wilke *et al.* 1999*a*). Many candidate endogenous ligands were tested, including neuropeptides, catecholamines, and steroids, and none altered I_K in this preparation. Furthermore, although the posterior

pituitary contains κ-opioid receptors, which modulate Ca^{2+} channels (Rusin *et al.* 1997), K^+ channels are not modulated by dynorphin in this preparation (authors' unpublished observations), indicating that the reduction of I_K by these ligands is not mediated by opioid receptors.

The sigma receptor binding site was first described over two decades ago. Originally thought to be a novel opioid receptor (Martin *et al.* 1976), subsequent studies demonstrated that the sigma receptor binding site is a distinct pharmacological entity distinguished by unusually promiscuous binding properties (Su, 1993; de Costa & He, 1994). Recent molecular characterization has shown that the sigma receptor is a novel protein with a molecular mass of 26 kDa. Hydropathy analysis has indicated that this protein has a single putative membrane-spanning segment (Hanner *et al.* 1996; Kekuda *et al.* 1996; Seth *et al.* 1997). The deduced amino acid sequence of the sigma receptor has no homology with known mammalian proteins, but a weak homology with fungal sterol isomerase has led some investigators to speculate that sigma receptors may be involved in steroid hormone biosynthesis (Jbilo *et al.* 1997; Moebius *et al.* 1997). Sigma receptors are ubiquitously distributed in both brain and peripheral tissue. Their binding to many different kinds of drugs has made it difficult to determine their function, but sigma receptors have been implicated in a variety of functions, including motor control, psychosis, and a wide range of endocrine and immune processes (Su, 1993; Bowen, 1993).

The unique molecular structure of the sigma receptor has raised intriguing questions about its mechanism of signal transduction. As novel proteins, sigma receptors fall outside the large families of membrane signalling molecules that have been identified in the past two decades. The sigma receptor does not have seven putative transmembrane domains, and so it would appear that this protein lacks the essential structural elements necessary for an interaction with G-proteins. At a topological level, the single putative transmembrane segment of the sigma receptor is reminiscent of many growth factor receptors with protein kinase activity, but at the sequence level no homology was found. There is some evidence that sigma receptor activation stimulates GTPase activity in mouse and rat brain (Itzhak, 1989; Tokuyama *et al.* 1997). GTP analogues have been reported to influence the binding of sigma receptor ligands (Beart *et al.* 1989), but a number of other binding studies led to different conclusions (Bowen, 1994). The modulation of I_K by sigma receptor ligands was abolished by reagents that inactivate G-proteins in some studies (Nakazawa *et al.* 1995; Soriani *et al.* 1998, 1999), but in other studies G-protein perturbation had no effect (Morio *et al.* 1994; Wilke *et al.* 1999*b*). These questions and conflicting results prompted us to use patch clamp techniques to investigate the mechanism by which sigma receptors inhibit I_K in nerve terminals of slices prepared from the posterior pituitary gland. Reagents known to block G-protein and phosphorylation-mediated signal transduction pathways failed to prevent I_K modulation. Sigma receptor ligands modulated channel function in excised outside-out patches in the absence of soluble cytoplasmic factors. In contrast, channels within cell-attached patches could not be modulated by drug application to adjacent membrane outside the pipette tip. These results indicate that sigma receptors modulate membrane function by a novel membrane-delimited mechanism requiring close proximity between receptors and channels.

METHODS

Tissue preparation

Experiments were carried out in accordance with National Institutes of Health guidelines for care and use of laboratory animals. Sprague-Dawley rats were rendered unconscious by rising concentrations of CO_2 and then decapitated. The pituitary gland was removed, and the neurointermediate lobe was separated and glued to a Plexiglas chamber. Slices 75 μm thick were cut with a Vibratome (Technical Products, International, St Louis, MO, USA) while chilling with ice-cold saline (Jackson *et al.* 1991). Tissue was prepared and maintained in artificial cerebrospinal fluid (aCSF) consisting of (mM): 115 NaCl, 4·0 KCl, 1·25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, and 10 glucose, bubbled with 95% O_2–5% CO_2. During recordings the preparation was perfused with aCSF by gravity feed at a rate of 2–4 ml min^{-1}. Slices were stored at room temperature (21–24 °C) in aCSF and used within 3 h of preparation.

Electrophysiology

An upright DIC microscope (Reichert-Jung Diastar) equipped with either a Zeiss × 40 or Olympus × 60 water-immersion objective was used to locate nerve terminals at the slice surface for recording. Recordings were made using an EPC-7 patch clamp amplifier (Instrutech, Elmont, NY, USA) interfaced to a PC, or an EPC-9 patch clamp amplifier (Instrutech) interfaced to a MacIntosh computer. Stimulus and data acquisition were controlled with the computer program pCLAMP 7 (Axon Instruments, Foster City, CA, USA) on the PC, or PULSE (Instrutech) on the MacIntosh. Patch pipettes were fabricated from thin-walled borosilicate glass (Garner Glass, Claremont, CA, USA) and coated with Sylgard to reduce capacitance (Hamill *et al.* 1981). Terminal capacitance and series resistance were determined by transient cancellation; series resistance was often compensated by ~50%. For whole-terminal and outside-out patch recordings, pipettes were filled with (mM): 130 KCl, 10 EGTA, 2 $MgCl_2$, 10 Hepes, 2 MgATP, 0·1 NaGTP, pH 7·3. In many experiments this solution was modified as indicated in Results and figure legends by omitting ATP and/or GTP, and including β,γ-methylene adenosine triphosphate (AMPPcP, Sigma), γ-thiol-guanosine triphosphate (GTPγS, Sigma), β-thiol-guanosine diphosphate (GDPβS, Sigma) or okadaic acid (Calbiochem). The pipette solution for cell-attached patches contained (mM): 150 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, titrated to pH 7·3 with KOH. Prior to contact with the cell membrane, pipette resistances ranged from 3 to 8 MΩ. Voltage clamp protocols for investigating I_K are indicated in the figure legends, and were similar to those described in previous reports from this laboratory (Bielefeldt *et al.* 1992). Since the modulation of I_K is proportional over a wide range of voltages (Wilke *et al.* 1998, 1999*a*, *b*), the results were insensitive to variations in the pulse protocol employed here.

Photolabelling

Photolabelling follows methods described previously (Wilke *et al.* 1999*b*). The sigma receptor photolabel iodo-4-azidococaine

([^{125}I]IAC) was synthesized according to Kahoun & Ruoho (1992). Posterior pituitary glands were removed from ten rats and homogenized with a Teflon pestle in phosphate-buffered saline (mM: 140 NaCl, 2·7 KCl, 10 Na_2HPO_4, 1·8 KH_2PO_4, at pH 7·3). The homogenate was divided into 100 μl aliquots and incubated in the same saline at 0°C with the indicated concentrations of ligand for 30 min. [^{125}I]IAC (1 nM) was added to each aliquot and the incubation continued for another 7·5 min, at which time the homogenate was illuminated for 5 s with a mercury lamp. Labelled proteins were then resolved by SDS-polyacrylamide gel electrophoresis (12% acrylamide) (Kahoun & Ruoho, 1992) and detected with phosphorimaging.

Drug application

Pentazocine, SKF10047, and ditolylguanidine were obtained from Research Biochemicals, Inc. (Natick, MA, USA), and applied in solutions of aCSF. DMSO was used in some experiments to aid solubilization of drugs, but the final DMSO never exceeded 0·1%, and this level had no effect on I_K. Bath application of drug was made either by direct dilution of stock into the recording chamber or replacement of aCSF perfusing the preparation with aCSF supplemented with the indicated drug concentration. In a few experiments drug was applied from a patch pipette positioned approximately 10 μm from the nerve terminal under recording. Pressure pulses (10 p.s.i. (7 kPa) 5–10 s) were applied by a Picospritzer to eject the drug (General Valve Corp., Fairfield, NJ, USA). Prior to drug addition, the stability of baseline current was verified by recording I_K at 15 s intervals for 1–4 min. In experiments where the patch pipette solution was used to introduce reagents into the nerve terminal interior, a longer predrug baseline was obtained (3–5 min) to allow sufficient time for effective internal perfusion (Pusch & Neher, 1988).

Oocyte expression and recording

Oocytes were collected from female frogs (*Xenopus laevis*, Nasco) anaesthetized by 10–20 min exposure to 3-aminobenzoic acid ethyl ester (1 g l^{-1}). Small abdominal incisions were made above the ovary, first through the skin and then the muscle. Several lobes of ovary were removed with a single scissors cut. The abdominal muscle and the skin were resutured separately and the frog was allowed to recover in shallow water. After the final removal of oocytes the animals were humanely killed. After removal of the follicular membranes, oocytes were cultured at 18°C in storage solution consisting of (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1·8 $CaCl_2$, 5 Hepes at pH 7·4, supplemented with 10 μg ml^{-1} gentamicin. The Kv1.4 K^+ channel clone was obtained from G. N. Tseng (Columbia University) and the Kv1.5 K^+ channel clone was obtained from L. Kaczmarek (Yale University). These genes were subcloned into the pGH19 oocyte expression vector and RNA was transcribed from the T7 promoter using a T7 Message Machine kit (Ambion, Austin, TX, USA). RNA was diluted in sterile water and injected (~10–50 ng per oocyte). K^+ current was recorded using a two-electrode voltage clamp (Warner Gene-Clamp, Hamden, CT, USA) and pCLAMP 7.0 software (Axon Instruments). Glass capillary electrodes had resistances of 0·5–1 MΩ when filled with 2 M KCl. Bathing solution consisted of (mM): 93 NaCl, 5 KCl, 1 $MgCl_2$, 0·3 $CaCl_2$, 5 Hepes at pH 7·4, and recordings were made at room temperature (22–24°C).

Data analysis

Current recordings were analysed on a PC with pCLAMP 7, or on a MacIntosh computer with PULSEFIT. Simple statistical analyses were performed on exported data using Microsoft Excel. Where arithmetic means were computed, they are presented with the standard error of the mean. When statistical significance was determined it was based on Student's t test.

RESULTS

Sigma receptor-mediated inhibition of K^+ current

Nerve terminals of the neurohypophysis have voltage-gated K^+ channels that are activated by depolarizing pulses (Jackson *et al.* 1991; Bielefeldt *et al.* 1992). Figure 1 shows I_K recordings in this preparation, and the reduction of I_K following application of the sigma receptor ligands SKF10047 and pentazocine. A recovery trace recorded ~1 min after SKF10047 removal shows that in this time I_K recovered almost completely. SKF10047 reduced peak I_K to 56 ± 11% of the control; the sustained component of I_K recorded at the end of the 200 ms pulse to 50 mV was reduced to 64 ± 11% of control ($n = 4$). (Here and elsewhere, controls refer to the predrug I_K baseline value obtained with the same pulse protocol prior to drug application.) Pentazocine reduced peak and sustained I_K to 39 ± 8 and 47 ± 9% of control values, respectively ($n = 4$). As with SKF10047, the modulation of I_K by pentazocine reversed after drug removal; the time course of onset and recovery for pentazocine is shown in Fig. 1*C*.

In an effort to relate these physiological responses to a molecularly characterized receptor, experiments were carried out with the sigma receptor photoprobe IAC (Kahoun & Ruoho, 1992). This compound reduced peak I_K to 40 ± 12% of control and sustained I_K to 52 ± 11% of control (Fig. 2*A*, $n = 4$). In contrast to the responses to pentazocine and SKF10047, I_K did not recover following exposure to IAC (Fig. 2*B*). The lack of recovery with IAC may reflect the very high affinity of this drug for sigma receptors ($K_d < 1$ nM; Kahoun & Ruoho, 1992). Alternatively, since IAC is a photoprobe, there could be some photolysis by the microscope illumination while recordings are in progress, leading to chemical modification of residues in or near the sigma receptor binding site.

Homogenized pituitary was photolabelled with [^{125}I]IAC, and subsequent SDS-polyacrylamide gel electrophoresis revealed that a 26 kDa protein was labelled. The labelling of this protein was selectively blocked by SKF10047 (Fig. 2*C*). This experiment was performed three times, but each experiment required 10 rats, and the small amount of tissue limited how many lanes could be run in one experiment. Similar photolabelling experiments in a clonal lung tumour cell line recently showed that the concentration dependence of block of photolabelling by SKF10047 was similar to the concentration dependence of inhibition of I_K (Wilke *et al.* 1999*b*). These results indicate that the sigma receptor bindings sites of the pituitary (Wolfe *et al.* 1989) and physiological responses to sigma receptor ligands (Wilke *et al.* 1999*a*) are associated with a protein with the same molecular mass as that encoded for by sigma receptor cDNA (Hanner *et al.* 1996; Kekuda *et al.* 1996; Seth *et al.* 1997). The molecular mass of the sigma receptor, 26 kDa, is well below that of the molecularly characterized voltage-gated K^+ channels with properties similar to those modulated in this preparation (see Discussion).

Tests for G-proteins

G-proteins can mediate membrane responses either by directly interacting with ion channels or by activating enzymes to generate second messengers. These two forms of transduction are quite general, and operate in a vast number of systems in which receptor occupancy leads to ion channel modulation (Hille, 1992; Wickman & Clapham, 1995; Breitwieser, 1996; Schneider *et al.* 1997). To test the role of G-proteins in sigma receptor-mediated responses we used the patch pipette filling solution to perfuse nerve terminals internally with a GTP-free solution, the GDP analogue GDBβS, or the GTP analogue GTPγS. All these manipulations have been shown to eliminate responses mediated by G-proteins, with response amplitudes diminished by ~75% after 2 min of internal perfusion (Trussell & Jackson, 1987). Sigma receptor ligands still reversibly inhibited I_K in nerve terminals subjected to each of these three manipulations. The pentazocine response was

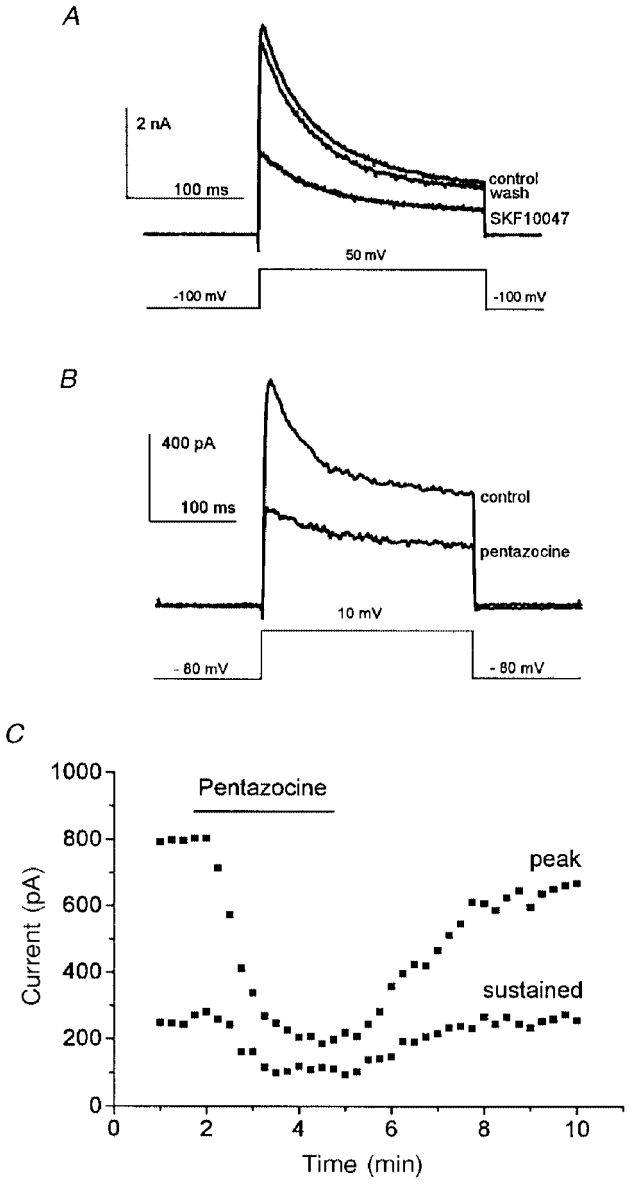

Figure 1. Sigma receptor ligands inhibit I_K

A, nerve terminal membranes were held at −100 mV and stepped to 50 mV for 200 ms to activate I_K. SKF10047 (100 μM) was applied from a picospritzer for 5 s and current was recorded again. Washing with control saline removed the drug and allowed I_K to recover to control levels in 1 min. *B*, nerve terminals were held at −80 mV and stepped to 10 mV for 250 ms to activate I_K. Pentazocine (100 μM) was applied by bath perfusion and current was blocked after 2 min. The drugs reduced both peak and sustained components of I_K. *C*, plot of peak I_K *versus* time as pentazocine was applied and removed (the time of drug application is indicated by the bar).

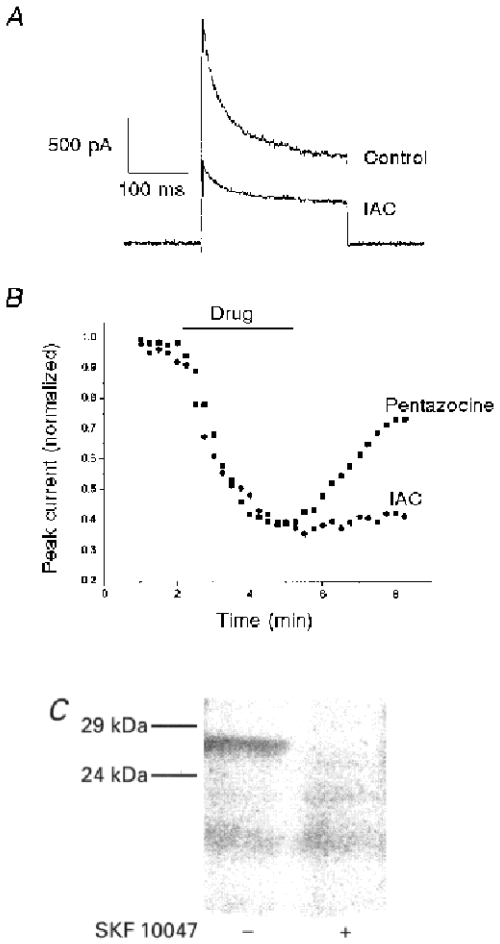

Figure 2. Agonist activity and photolabelling by iodoazidococaine (IAC)

A, IAC reduced I_K evoked by voltage pulses as in Fig. 1. *B*, time course of modulation of I_K while drug was added and removed (the time of drug application is indicated by the bar). Values plotted were normalized to predrug control I_K. Peak I_K was reduced by 100 μM pentazocine and 100 μM IAC. Three minutes after return to control solution I_K partly recovered in the terminal exposed to pentazocine but not in the terminal exposed to IAC. *C*, SDS-polyacrylamide gel electrophoresis was performed on homogenized neurohypophysis after photolabelling with [^{125}I]IAC. Phosphorimaging revealed labelled proteins. A band at 26 kDa was labelled (left), and the labelling of this band was blocked by 100 μM SKF10047 (right).

unaffected by internal perfusion with 1 mM GDPβS (Fig. 3*A* and *C*), and the SKF10047 response was unaffected by internal perfusion with 100 μM GTPγS (Fig. 3*B* and *D*). (In Fig. 3*B* and *D* the drug was applied by pressure ejection from a micropipette positioned about 10 μm from the nerve terminal, so the onset and recovery are much faster than those achieved by bath application of drug shown in Fig. 3*A* and *C*) Even after 10 min of perfusion with GTPγS, the percentage reduction of peak I_K by SKF10047 was the same (Fig. 3*B*). Similar repeat applications after >10 min of internal perfusion with 150 μM GDPβS also produced similar responses (data not shown). Figure 4 summarizes the results of inhibition of I_K by pentazocine with GTP-free patch pipette solutions, and patch pipette solutions containing 150 μM and 1 mM GDPβS. For all these conditions the reduction of both peak and sustained components of I_K by 100 μM pentazocine was the same as control recordings in which cells were perfused with GTP (Fig. 4).

The time course of whole-terminal I_K shown in Fig. 3 also provides a positive control for the effectiveness of thioguanine nucleotides in this experimental protocol. Previous work from this laboratory has shown that GTPγS activates a protein phosphatase in excised patches of nerve terminal membrane, and this initiates a rapid rundown of activity of the Ca^{2+}-activated K^+ channel responsible for I_{BK} (Bielefeldt & Jackson, 1994). Here we found that adding 100 μM GTPγS to the patch pipette solution caused a decline in I_K

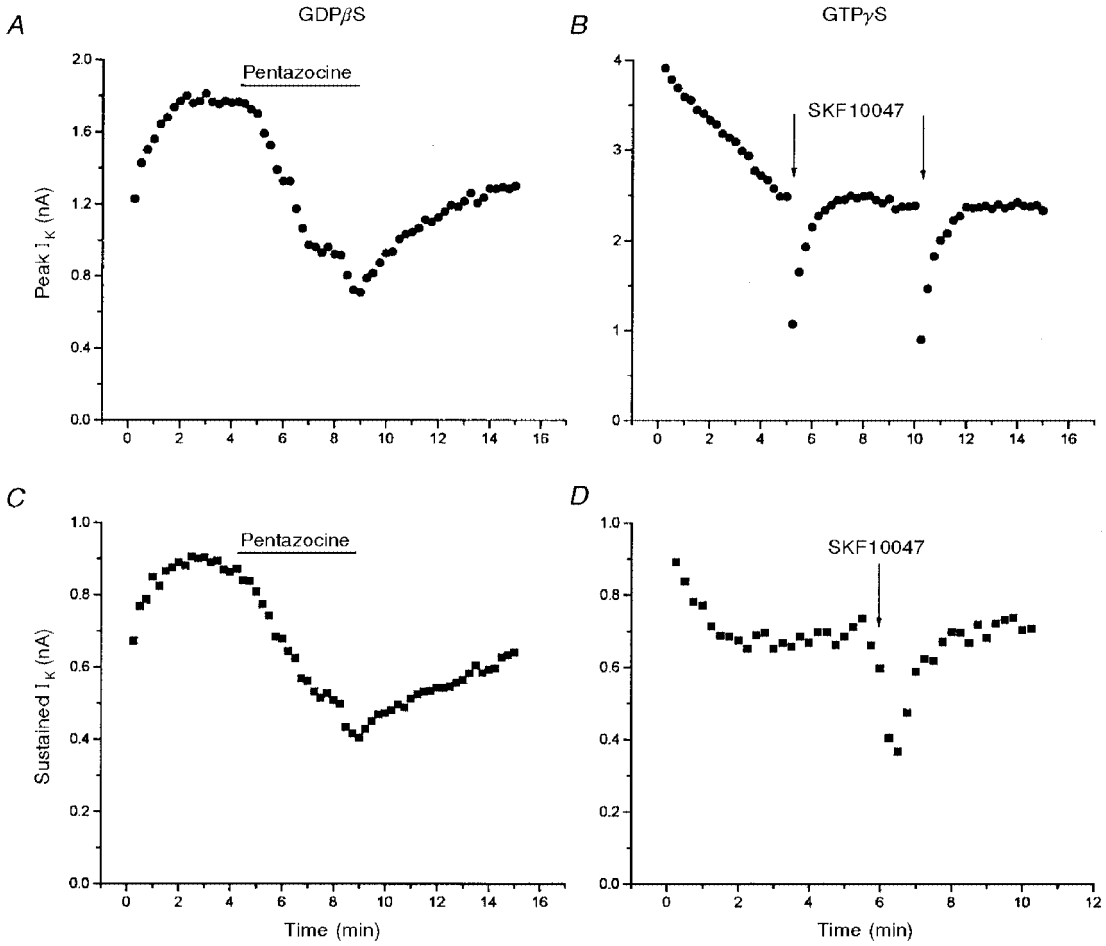

Figure 3. I_K modulation and G-protein perturbation

Pentazocine (100 μM) reversibly reduced both peak (*A*) and sustained (*C*) I_K in a nerve terminal perfused with 1 mM GDPβS (I_K evoked by 250 ms pulses from −100 to 50 mV). SKF10047 (100 μM) reversibly inhibited both peak (*B*) and sustained (*D*) I_K in a nerve terminal filled with 100 μM GTPγS (I_K evoked by 250 ms pulses from −80 to 10 mV). In *A* and *C*, pentazocine was added to the bathing solution; both plots are taken from the same recording. In *B* and *D*, SKF10047 was applied by pressure ejection of an ~2 μm diameter-tipped micropipette positioned within 10 μm of the nerve terminal under recording; the plots are from different recordings. The different time courses of inhibition and recovery reflect the different modes of drug application. GDPβS and GTPγS began to perfuse the cell interior at break-in, shortly before data acquisition was started. The increase (*A* and *C*) in I_K at the beginning of the experiment with GDPβS (prior to drug application) results from block of G-protein-dependent modulation of channels. The decrease (*B* and *D*) in I_K at the beginning of the experiment with GTPγS results from the enhancement of this modulation (Bielefeldt & Jackson, 1994).

in the first few minutes after establishing a whole-terminal recording (Fig. 3*B* and *D*). Likewise, perfusion with GDPβS produced an increase in I_K immediately following break-in (Fig. 3*A* and *C*). Both of these guanine nucleotide effects were seen prior to application of sigma receptor ligands, and show that G-proteins are modulating channel activity independently of receptor activation, presumably by altering the level of protein phosphorylation within the nerve terminal. The decline in peak I_K was variable in time course and extent, and sometimes continued for several minutes. This probably reflects multiple forms of G-protein-mediated modulation of the K^+ channels underlying this current. On the other hand, the rapid changes in sustained I_K are consistent with the finding that membrane-bound G-proteins rapidly activate a membrane-bound protein phosphatase in this system (Bielefeldt & Jackson, 1994).

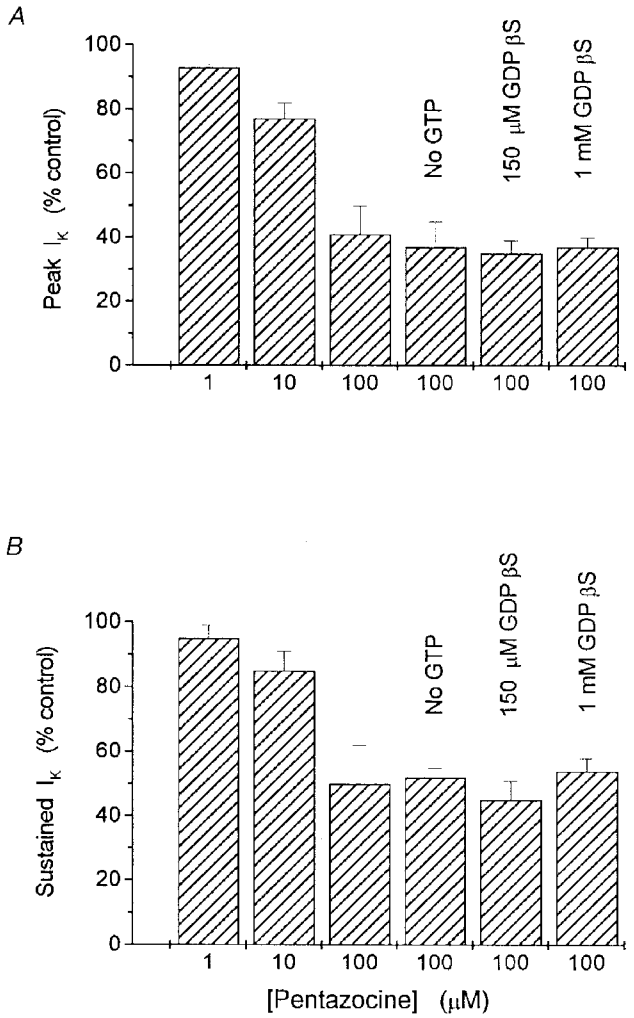

Figure 4. Means of peak (*A*) and sustained (*B*) I_K in the presence of the indicated concentrations of pentazocine (normalized to predrug control I_K level)

Increasing pentazocine from 1 to 100 μM resulted in greater reductions of both components. The reduction by 100 μM pentazocine was the same in recordings with 150 μM or 1 mM GDPβS instead of GTP, or with GTP-free intracellular recording solutions. $n = 3$ to 6 nerve terminals for each result shown; I_K was evoked by 250 ms pulses from −80 to 10 mV.

Based on diffusion, molecules of the size of GTP can be expected to perfuse cells in about 8 s in whole-cell recordings (Pusch & Neher, 1988; Jackson, 1997). This figure in seconds was arrived at using the formula:

$$\tau = 0{\cdot}042\ R_s M^{1/3} C_c,$$

where M was taken as the molecular mass of GTPγS (456 Da), C_c was the capacitance of a typical nerve terminal (5 pF) and R_s was a typical series resistance value (5 MΩ). Since the time taken for current to change during intracellular perfusion is of the order of a minute (Fig. 3), it is probably the kinetics of nucleotide binding and effector activation that is rate limiting, rather than diffusion of the guanosine nucleotides into the nerve terminals.

The fact that neither GDPβS, GTPγS nor GTP-free intracellular solutions reduced responses to pentazocine or SKF10047 indicates that G-proteins are not required for the sigma receptor-mediated modulation of neurohypophysial I_K. Both peak and sustained I_K were reduced by the same amount as in control experiments (Fig. 4). Since peak I_K reflects predominantly I_A and sustained I_K reflects predominantly I_{BK} (Wilke *et al.* 1998), this result indicates that both types of K^+ channels can be modulated independently of G-proteins. Additional experiments with excised patches described below provide further support for this conclusion.

Tests for protein phosphorylation

Protein phosphorylation regulates the activity of many ion channels (Levitan, 1994; Jonas & Kaczmarek, 1996; Breitwieser, 1996). This form of channel modulation may or may not involve G-proteins. The production of second messengers that activate protein kinases generally depends on G-proteins, but many receptors have intrinsic, G-protein-independent protein kinase activity. To investigate the role of protein phosphorylation in the sigma receptor-mediated modulation of K^+ channels, recordings were made with patch pipettes containing the nonhydrolysable ATP analogue AMPPcP in place of ATP, and with 50 nM okadaic acid, a protein phosphatase inhibitor (okadaic acid was included to block protein phosphatase activity and prevent the run-down of K^+ channels that occurs with AMPPcP in place of ATP; Bielefeldt & Jackson, 1994). Under these conditions SKF10047 still reduced both peak and sustained I_K. Figure 5 shows a time course of I_K inhibition and recovery in two successive rounds of drug application. Peak and sustained I_K were reduced to 43 ± 7 and 54 ± 3% of control ($n = 4$), and this inhibition of I_K was indistinguishable from that seen in control whole-terminal recordings ($P = 0{\cdot}18$ and $0{\cdot}21$, for the probabilities of originating from the same distribution with peak and sustained I_K, respectively). In this preparation, AMPPcP and okadaic acid have proven effective in interfering with phosphorylation and dephosphorylation-

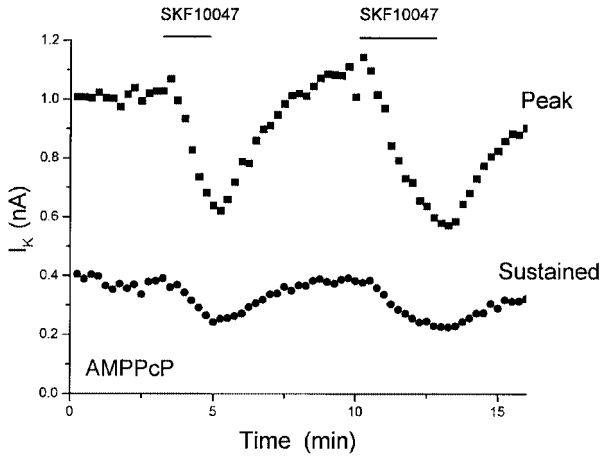

Figure 5. Sigma receptor-mediated inhibition is independent of ATP

ATP (2 mM) was replaced in the patch pipette solution by 2 mM AMPPcP. Protein phosphatase was also blocked by adding 50 nM okadaic acid. SKF10047 (100 μM) was applied twice (indicated by bars) to demonstrate two cycles of response and recovery. As in Fig. 3, internal perfusion began at break-in, ~3 min before the first application of SKF10047. Both peak and sustained I_K were inhibited, and recovered after drug removal (mean reduction given in text).

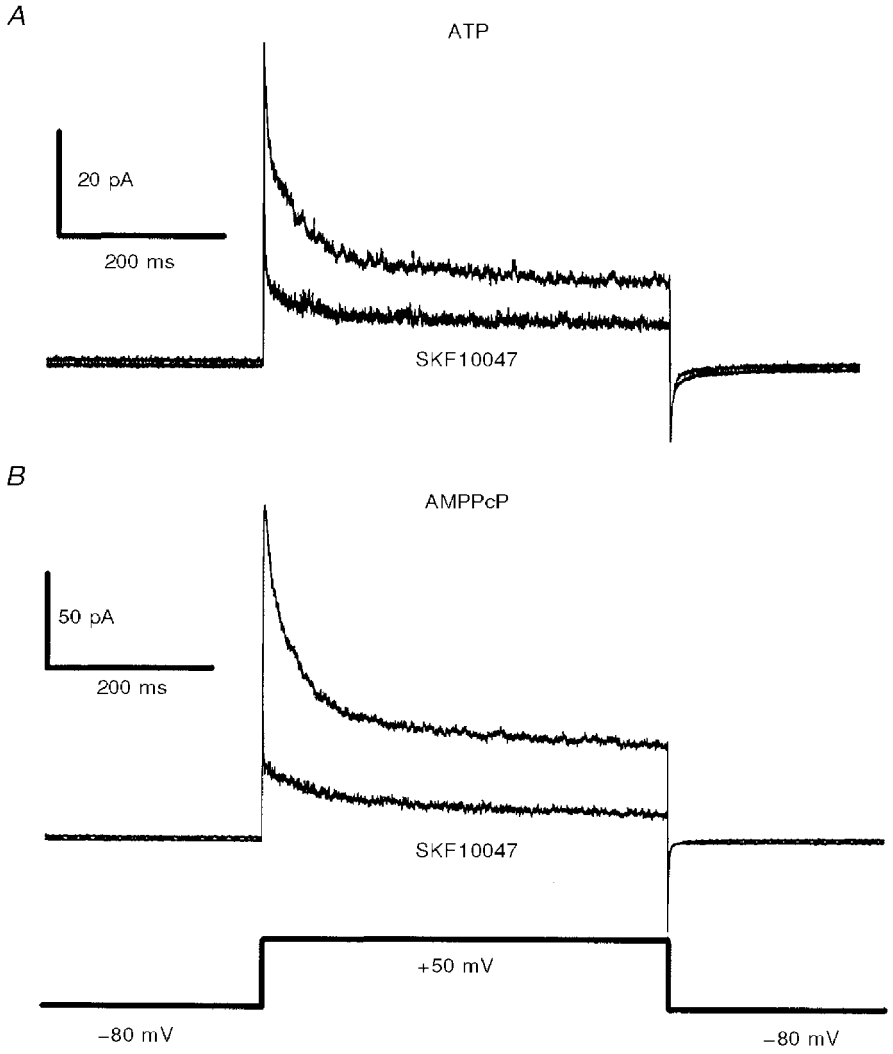

Figure 6. Inhibition of peak and sustained I_K by SKF10047 in outside-out patches

A, I_K reduction in outside-out patches with the control pipette solution containing 2 mM ATP. *B*, I_K reduction in outside-out patches with 50 nM okadaic acid and 2 mM AMPPcP in the patch pipette (no ATP). Voltage pulses 300 ms in duration from −80 mV to 50 mV activated I_K. Averages of ten traces are shown (collected at 15 s intervals) for both predrug control and after 1 min exposure to SKF10047 (100 μM). I_K recovered to predrug levels within 1 min of drug removal (data not shown). I_K recorded from outside-out patches is nearly identical in time course but smaller in amplitude compared with typical whole-terminal I_K (e.g. Fig. 1).

dependent modulation of K^+ channels. These reagents produce significant changes in the whole-terminal I_K within 2 or 3 min of break-in (Bielefeldt & Jackson, 1994). This provides a positive control to verify the utility of these reagents.

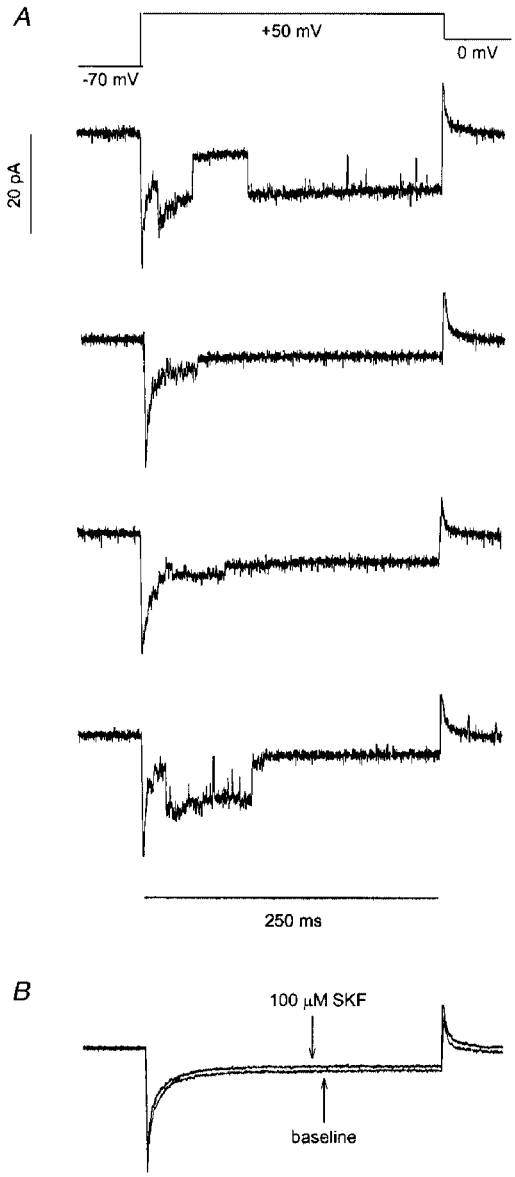

Figure 7. K^+ channels in cell-attached patches
Cell-attached patches were hyperpolarized to −110 mV for 250 ms (to remove inactivation, see Bielefeldt *et al.* 1992), then depolarized to 50 mV for 300 ms to activate K^+ channels. Prior hyperpolarization has been shown to leave the percentage reduction of I_K by sigma receptor ligands unchanged (Wilke *et al.* 1999*b*). Voltage was estimated by assuming a resting membrane potential of −70 mV. *A*, four sweeps show single channel activity through A-current and Ca^{2+}-activated K^+ channels under control conditions. *B*, averages of 25 sweeps were recorded before and after application of 100 μM SKF10047 to the bathing solution. SKF10047 reduced current only slightly in these experiments because the drug could not reach the membrane under the pipette tip in which channel activity was recorded.

These experiments show that sigma receptor-mediated modulation of I_K does not depend on ATP hydrolysis, and presumably does not require protein phosphorylation. Since okadaic acid was present at levels sufficient to block either protein phosphatase 1 or 2a (Shenolikar & Nairn, 1991), it would appear that these enzymes are also not involved. Further, the involvement of other forms of protein phosphatase can be ruled out because recovery from dephosphorylation would depend on protein phosphorylation, and Fig. 5 shows two cycles of response and recovery under conditions where phosphorylation is blocked.

Channel modulation in membrane patches

The formation of excised outside-out patches (Hamill *et al.* 1981) provides a convenient way of isolating ion channels from the cytoplasm and determining the molecular dependence of a membrane transduction process. Figure 6 shows the inhibition of K^+ channel activity in outside-out patches by 100 μM SKF10047. With the normal ATP–GTP-containing control pipette solution (see Methods), peak and sustained I_K were reduced to 42 ± 6 and 60 ± 6% of control values, respectively ($n = 10$). Following replacement of ATP by AMPPcP, and addition of okadaic acid to block membrane-bound protein phosphatase (Bielefeldt & Jackson, 1994), SKF10047 still reduced these components of I_K to 55 ± 9 and 63 ± 12% of control values, respectively ($n = 5$). The inhibition of I_K in excised outside-out patches was statistically indistinguishable from that seen in whole-terminal recordings with the same pipette solution ($P = 0{\cdot}17$ and $0{\cdot}25$ for peak and sustained I_K, respectively), as well as in outside-out patches with control solution ($P = 0{\cdot}13$ and $0{\cdot}40$ for peak and sustained I_K, respectively). These results show that the response to sigma receptor activation does not depend on soluble cytoplasmic factors, and confirm the findings from whole-terminal recordings above, that channel modulation does not depend on protein phosphorylation, dephosphorylation or G-protein activation. Furthermore, these experiments indicate that the sigma receptor binding site is located in the plasma membrane, and this is of interest because the deduced amino acid sequence of the sigma receptor contains an endoplasmic reticulum retention sequence (Kekuda *et al.* 1996; Hanner *et al.* 1996).

To investigate the need for close proximity between sigma receptors and the target ion channels modulated in nerve terminal membranes, experiments were conducted in cell-attached patches. This experimental configuration isolates the patch of membrane under recording from the rest of the membrane of the nerve terminal. Thus, receptors outside a patch can be presented with drug, and the effect on channels within a patch can be monitored. In the case of responses mediated by a soluble intracellular second messenger, the modulation of ion channels in cell-attached patches can be elicited by the bath application of receptor ligands (Siegelbaum *et al.* 1982). Single K^+ channel currents are readily recorded in cell-attached patches in neurohypophysial nerve terminals (Bielefeldt *et al.* 1992), and superfusion of the preparation with SKF10047 produced

only a small reduction in channel activity (Fig. 7). I_K measured 10 ms after the start of a depolarizing pulse was reduced to 87 ± 6 % of the predrug control I_K ($n = 10$). This is much less than the reduction in I_K at this same time point seen in outside-out patches with ATP (41 ± 6 %, $n = 9$) or okadaic acid + AMPPcP (57 ± 9 %, $n = 5$), and these differences were statistically significant ($P = 0{\cdot}0005$ and 0·007, respectively). Figure 7 shows that there was little reduction in I_K for the duration of the depolarizing pulse. Due to their different activation and inactivation kinetics (Bielefeldt *et al.* 1992), inhibition of either I_A or I_{BK} would have reduced I_K either early or late in the pulse, respectively. Thus, in cell-attached patches neither of these K^+ channels was appreciably modulated by externally applied drug. The slight reduction of I_K in these experiments is probably due to the lipophilic nature of SKF10047, enabling a small amount of drug leakage through the cell membrane and into the region sealed off by the patch pipette.

The failure to see channel modulation in cell-attached patches indicates that receptors in the cell membrane located outside the walls of the patch pipette tip cannot modulate channels inside the patch of membrane sealed off by the electrode. The wall thickness of a typical patch electrode is generally of the order of < 1 μm. Thus, separating sigma receptors from target K^+ channels by this distance prevented the transmission of signals between these two membrane proteins.

Failure of ligands to modulate K^+ channels in the absence of sigma receptors

The findings that G-proteins, ATP and cytoplasmic factors were not required for ion channel modulation by sigma receptor ligands raise the possibility that ligands interact directly with the ion channel protein itself. The molecular structure of sigma receptors strongly suggests that it is incapable of forming ion channels (Hanner *et al.* 1996; Kekuda *et al.* 1996; Seth *et al.* 1997). The existence of antagonists that can block the action of sigma receptor agonists without inhibiting K^+ currents on their own (Wilke *et al.* 1998) argues against a mechanism of K^+ current inhibition involving open channel block. As an additional test that the modulation of K^+ channels by sigma receptor ligands does indeed depend on the presence of the sigma receptor we expressed voltage-gated K^+ channels in *Xenopus* oocytes and applied sigma receptor ligands to see if these K^+ channels were inhibited.

For these experiments we selected the mammalian homologue of the *Drosophila Shaker* K^+ channel, Kv1.4. This channel has biophysical properties that are similar to those of the A-current channel of the posterior pituitary, and has been shown to be localized in nerve terminals (Sheng *et al.* 1993). Neither SKF10047 (Fig. 8; $n = 8$; 100 μM) nor ditolylguanidine ($n = 6$; 100 μM) had any effect on K^+ current in oocytes expressing Kv1.4. Further experiments with the related K^+ channel Kv1.5 produced the same result in five experiments with SKF10047 and seven with ditolylguanidine. For both these channels, K^+ current was inhibited when the K^+ channels were expressed together with sigma receptors, and the amount was somewhat greater than that seen in nerve terminals (Aydar *et al.* 2000). Thus, the modulation of K^+ channels by these drugs requires the sigma receptor, and does not result from direct interactions between the ligand and the channel protein.

DISCUSSION

This study has shown that signal transduction by sigma receptors does not depend on any of the well-established molecular systems known to operate in the receptor-

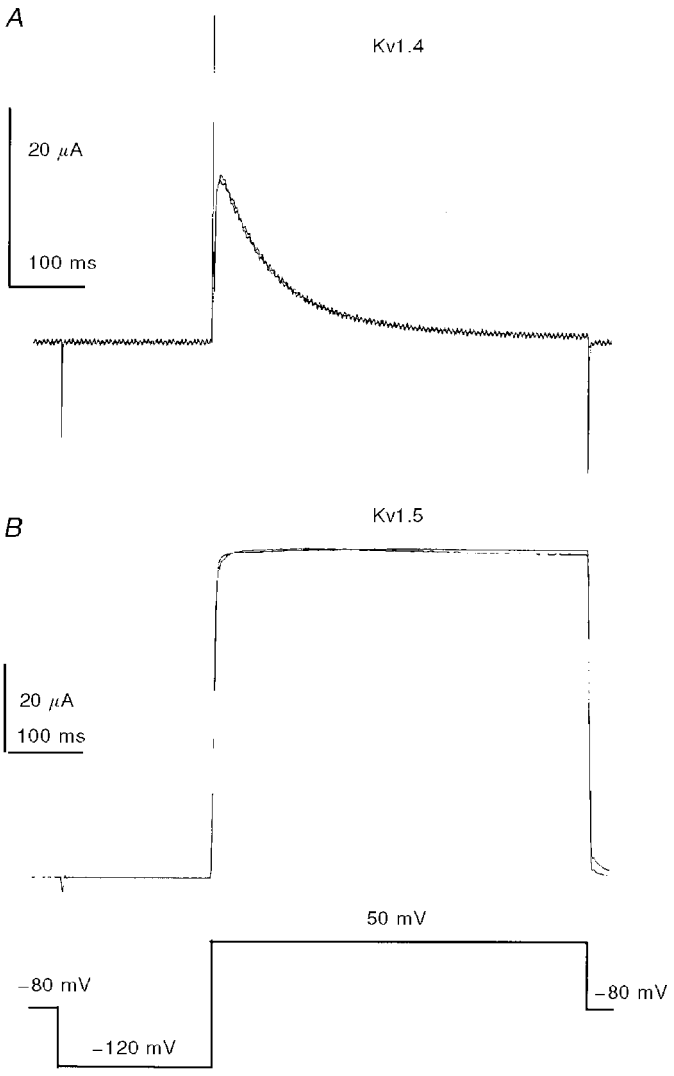

Figure 8. Lack of modulation of K^+ channels in the absence of sigma receptor

The voltage-gated K^+ channels Kv1.4 and Kv1.5 were expressed in *Xenopus laevis* oocytes by injecting mRNA encoding for these proteins. Voltage steps from −120 to +50 mV activated K^+ current. Addition of 100 μM SKF10047 left the current essentially unchanged in 8 oocytes expressing Kv1.4 (*A*) and 5 oocytes expressing Kv1.5 (*B*). Another sigma receptor agonist, ditolylguanidine, also failed to modulate K^+ current (data not shown).

mediated modulation of membrane excitability. Although the basic phenomenon of modulation of voltage-gated channels has been encountered many times, in every instance where the transduction mechanism was investigated, G-proteins or protein phosphorylation have been shown to play a role (Hille, 1992; Levitan, 1994; Wickman & Clapham, 1995; Breitwieser, 1996; Jonas & Kaczmarek, 1996; Schneider *et al.* 1997). However, we found that neither GTPγS nor GDPβS interfered with sigma receptor-mediated modulation of I_K, even as initial changes in current amplitude provided a report of the effectiveness of these reagents in manipulating G-proteins. Likewise, removal of ATP and blockade of protein phosphorylation left signal transduction intact. These findings therefore imply a novel mechanism of signal transduction, in keeping with the novel structure of the sigma receptor protein.

Our finding of G-protein-independent transduction by sigma receptors is consistent with previous work from this laboratory on the modulation of I_K in a lung tumour cell line (Wilke *et al.* 1999*b*). Another group reported similar results for sigma receptor-mediated modulation of K^+ channels in a neuroblastoma cell line (Morio *et al.* 1994). However, a number of other studies have suggested that sigma receptors may interact with G-proteins and regulate protein phosphorylation (Su, 1993; Nakazawa *et al.* 1995; Soriani *et al.* 1998, 1999). Some of these effects could be interpreted as Ca^{2+}-dependent processes that are indirect consequences of ion channel modulation (Brent *et al.* 1997). The mechanism of signal transduction may also depend on what is chosen experimentally as the physiological endpoint. Sigma receptors may have other physiological functions and it has been suggested that they initiate processes other than the modulation of ion channels (Moebius *et al.* 1997). It is possible that some sigma receptor-mediated responses employ other transduction mechanisms. With regard to the reports suggesting an involvement of G-proteins in ion channel modulation (Nakazawa *et al.* 1995; Soriani *et al.* 1998, 1999) it is also possible that sigma receptor variants differ in their coupling to G-proteins (Bowen, 1994), and this has been proposed as a resolution of the differences in the literature regarding effector pathways employed by sigma receptors (Soriani *et al.* 1999).

The photolabelling data from the neurohypophysis presented here (Fig. 2*C*) suggest that sigma receptor ligands interact with a 26 kDa protein similar in molecular mass to the sigma receptor protein identified by molecular cloning. Further, in DMS-114 human lung tumour cells, where better photolabelling signals were obtained, the concentration dependence of blockade of photolabelling by SKF10047 was found to be very similar to the concentration dependence of inhibition of I_K (Wilke *et al.* 1999*b*). These results indicate that the receptor that modulates K^+ channels in tumour cells also has a molecular mass of 26 kDa, thus identifying it with the cloned sigma receptor.

Ordinarily, the observation that ion channels can be modulated in excised outside-out patches, with G-proteins and ATP hydrolysis blocked (Fig. 6), is diagnostic of a ligand-gated channel in which the receptor and channel are part of the same protein. However, the sigma receptor does not have the amino acid sequence or the membrane topology of an ion channel (Hanner *et al.* 1996; Kekuda *et al.* 1996; Seth *et al.* 1997), and the photolabelling results obtained with IAC suggest that this protein does indeed mediate the modulation of K^+ channels. Furthermore, the photolabelling results indicate that the actions of sigma receptor ligands are mediated by a protein with a molecular mass smaller than the channels that are modulated. The two K^+ channels inhibited by sigma receptor ligands produce the A-current and a Ca^{2+}-activated K^+ current (Bielefeldt *et al.* 1992). The molecular mass deduced from the genes of members of these protein families are 73 and 134 kDa, respectively. Accessory or auxiliary subunits of voltage-gated channels are smaller, and if these drugs interact with such a protein, then it would coincidentally have the same molecular mass as the cloned sigma receptor.

The size of the photolabelled protein makes it unlikely that the inhibition of I_K by sigma receptor ligands results from direct blockade of open channels (such as that produced by local anaesthetics). The hypothesis of open channel block is also inconsistent with the fact that in this same preparation two antagonists, eticlopride and RBI257, were shown to block the modulation of I_K by sigma receptor agonists, without producing any inhibition of current on their own (Wilke *et al.* 1998). Finally, when K^+ channels are expressed in oocytes in the absence of sigma receptors, there is no modulation (Fig. 8). Only when both channel and receptor proteins were expressed in oocytes could the K^+ current be inhibited by sigma receptor ligands (Aydar *et al.* 2000). Thus, the inhibition of K^+ channels by sigma receptor agonists does not arise from direct interactions of these ligands with the channel itself.

Since sigma receptors and ion channels are distinct proteins, there must be a relay mechanism to transmit the signal of binding site occupancy to the target ion channels. Since G-proteins, protein kinases, protein phosphatases and soluble cytoplasmic molecules were ruled out by the results of the present study, and since the results with cell-attached patches indicate a requirement for close proximity between the receptor and channel, the most parsimonious explanation is that the transduction signal is mediated by protein–protein interactions within the cell membrane. These interactions could be through a direct association between the receptor and channel, and this mechanism is especially appealing in its simplicity. It is also possible that the coupling between the receptor and channel is mediated by other proteins, which would then also be membrane bound. Thus, the sigma receptor could either behave like, or activate, a minK-like protein. With a single membrane-

spanning segment this protein resembles the sigma receptor in overall topology. It cannot form a channel by itself but can modify the activity of other voltage-gated K^+ channels (Sanguinetti *et al.* 1996; Barhanin *et al.* 1996). Auxiliary β-subunits are another example of membrane proteins that do not form channels themselves, but can modify ion channel activity (Adelman, 1995; England *et al.* 1995). Sigma receptor activation could lead to a similar modification of K^+ channels, but in a ligand-dependent manner. The recently described receptor-activity-modifying protein (RAMP) has a single membrane-spanning segment and regulates the transport and functional activity of the calcitonin-receptor-like receptor (McLatchie *et al.* 1998). From this perspective the sigma receptor may act in an analogous manner as a channel-activity-modifying protein. Direct functional interaction between integral membrane proteins such as these appears to be an emerging theme in physiology, as similar mechanisms have been proposed for neurotrophin receptor-mediated modulation of Na^+ channels (Kafitz *et al.* 1999), and mutual modulation between $GABA_A$ receptors and dopamine D_5 receptors (Liu *et al.* 2000).

Since the molecular characterization of the sigma receptor has occurred only recently, it is difficult to speculate whether its mode of signal transduction is widely used. Sigma receptor homologues may or may not exist, but to date none have been described. The sigma receptor itself is ubiquitously distributed in vertebrates, and has been implicated in a wide range of biological functions (Bowen, 1993; Su, 1993). The neurohypophysis has a high density of sigma receptors (Wolfe *et al.* 1989), and in this preparation the sigma receptor-mediated reduction of I_K would be expected to enhance the release of neuropeptide hormones. Future work will clarify the physiological processes that depend on sigma receptors, and as this work progresses it will be interesting to see if a biological principle emerges for functions that are better served by the sigma transduction process.

Endogenous ligands that activate sigma receptors have not been identified. Although the sigma receptor binds progesterone (Su *et al.* 1988), concentrations of this steroid of up to 1 mM were tested and found to have no impact on the modulation of I_K in the neurohypophysis (Wilke *et al.* 1999*a*). A number of additional candidate ligands, including catecholamines, peptides and other steroids, were also tested with negative results. Given the potent modulation of I_K in every nerve terminal tested, it would appear that if endogenous chemical signals enter the neurohypophysis and activate sigma receptors, the release of the two neurohypophysial peptides, oxytocin and vasopressin, would both be enhanced. The inhibition of I_K would prolong individual action potentials (Jackson *et al.* 1991) as well as increase the duration of action potential bursts (Bielefeldt & Jackson, 1993), and both of these would increase the release of hormone. Thus, the transduction mechanism employed by sigma receptors is likely to play a role in endocrine functions, both in the neurohypophysis and other endocrine systems in which sigma receptors are found.

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