Voltage-gated sodium channel modulation by σ-receptors in cardiac myocytes and heterologous systems

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Johannessen M, Ramachandran S, Riemer L, Ramos-Serrano A, Ruoho AE, Jackson MB. Voltage-gated sodium channel modulation by σ-receptors in cardiac myocytes and heterologous systems. Am J Physiol Cell Physiol 296: C1049–C1057, 2009. First published March 11, 2009; doi:10.1152/ajpcell.00431.2008.—The σ-receptor, a broadly distributed integral membrane protein with a novel structure, is known to modulate various voltage-gated K+ and Ca2+ channels through a mechanism that involves neither G proteins nor phosphorylation. The present study investigated the modulation of the heart voltage-gated Na+ channel (Nav1.5) by σ-receptors. The σ1-receptor ligands [SKF-10047 and (+)-pentazocine] and σ1/σ2-receptor ligands (haloperidol and ditolylguanidine) all reversibly inhibited Na+,1.5 channels to varying degrees in human embryonic kidney 293 (HEK-293) cells and COS-7 cells, but the σ1-receptor ligands were less effective in COS-7 cells. The same four ligands also inhibited Na+ current in neonatal mouse cardiac myocytes. In σ1-receptor knockout myocytes, the σ1-receptor-specific ligands were far less effective in modulating Na+ current, but the σ1/σ2-receptor ligands modulated Na+ channels as well as in wild type. Photolabeling with the σ1-receptor photoprobe [125I]-iodoazidococaine demonstrated that σ1-receptors were abundant in heart and HEK-293 cells, but scarce in COS-7 cells. This difference was consistent with the greater efficacy of σ1-receptor-specific ligands in HEK-293 cells than in COS-7 cells. σ- Receptors modulated Na+ channels despite the omission of GTP and ATP from the patch pipette solution. σ-Receptor-mediated inhibition of Na+ current had little if any voltage dependence and produced no change in channel kinetics. Na+ channels represent a new addition to the large number of voltage-gated ion channels modulated by σ-receptors. The modulation of Na+,1.5 channels by σ-receptors in the heart suggests an important pathway by which drugs can alter cardiac excitability and rhythmicity.

σ-receptors were originally identified by their ability to bind opioid receptor ligands (22), but they were subsequently shown not to bind opioid peptides (40). In addition to the natural hallucinogen, N,N-dimethyltryptamine (9), σ-receptors are sensitive to an impressive array of ligands, including opioid receptor ligands such as pentazocine; dopamine receptor ligands such as haloperidol; drugs of abuse such as cocaine and amphetamines; fungicides such as fenpropimorph; class III antiarrhythmics such as amiodarone; vasodilators such as indenprofen; and completely novel compounds such as ditolylguanidine (DTG) (24, 26, 32, 40, 44). Many mammalian tissues express σ-receptors, including heart, brain, liver, kidney, and placenta, as well as numerous forms of cancer (1, 38, 44).

σ- Receptors have been subdivided into two subtypes, σ1 and σ2. The σ1-receptor has been much better characterized: the encoding gene predicts a 25.3-kDa protein (11, 17, 38), with a deduced amino acid sequence that is highly conserved in mammals. It has no vertebrate homologues. When expressed in Xenopus oocytes, the σ1-receptor has two transmembrane segments, with both the COOH- and NH2 termini located in the cytoplasm (2). The σ2-receptor has a distinct pharmacological signature and a lower molecular mass of 19–21 kDa (14, 32), but its molecular structure is unknown.

It is well established that σ-receptors modulate membrane excitability. In rat sympathetic and parasympathetic neurons, σ-receptor ligands modulate N-, L-, P/Q- and R-type Ca2+ channels (48). In rat neurohypophysis, σ-receptors modulate two types of voltage-activated K+ channels (45). Additional studies in rat hippocampal slices, intracellular neurons, and tumor cells have demonstrated modulation of various K+ channels (18, 27, 47). Association of the σ1-receptor and the K1.4 K+ channel has been shown in posterior pituitary nerve terminals and Xenopus oocytes (2), and these proteins colocalize in the focal adhesions of CHO-K1 cells (23). In general, the modulation of ion channels by σ-receptors does not depend on G proteins or phosphorylation (21, 33, 47) but instead results from a direct protein-protein interaction between the receptor and channel (2). σ-Receptors have also been observed in the endoplasmic reticulum, and their association with nascent plasma membrane ion channels may be relevant to their proposed function as a molecular chaperone (12).

A number of σ-receptor ligands influence cardiovascular function, and the heart has binding sites for σ-receptor ligands (6, 7). σ-Receptor ligands alter contractility, Ca2+ influx, and rhythmic activity in cultured cardiac myocytes, but these actions are complex and controversial. σ-Receptor ligands can either increase or decrease the contractile force, with a variable time course that depends on concentration and animal age (7, 8, 28, 29). In myocytes from newborn rats, σ-receptor ligands enhance Ca2+ entry (7), but in adult myocytes the elevation of cellular Ca2+ depends on Ca2+ from sarcoplasmic reticulum (28, 29). Some σ-receptor ligands have proarrhythmic effects, whereas others have antiarrhythmic effects (26). Various σ-receptor ligands inhibit K+ currents and increase the duration of the QT period (15, 27). On the other hand, the σ-receptor ligands haloperidol and chlorpromazine have been shown to block heart Na+ channels in a manner consistent with an antiarrhythmic action (31).

To clarify the effects of σ-receptor ligands on the heart, we have used patch-clamp techniques to study the modulation of...
cardiac Na\textsuperscript{+} channels by \(\sigma\)-receptors. Our results in both myocytes and heterologous expression systems indicate that \(\sigma\)-receptor ligands inhibit the cardiac voltage-gated Na\textsuperscript{+} channel Na\textsubscript{a}.1.5. These results extend the already diverse range of voltage-gated ion channels that are modulated by \(\sigma\)-receptors, and may make for some of the actions of \(\sigma\)-receptor ligands on the cardiovascular system.

**METHODS**

**Ethical approval.** All protocols were approved by the Animal Care and Use Committee of the University of Wisconsin, in accordance with the guidelines of the National Institutes of Health.

**Cell culture and transfection.** Human embryonic kidney 293 (HEK-293) cells stably expressing Na\textsubscript{a}.1.5 were provided by Dr. J. C. Makielski at the University of Wisconsin-Madison (43). COS-7 cells were transiently transfected with recombinant cDNA encoding Na\textsubscript{a}.1.5 using Lipofectamine (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. Both cell types were cultured on glass coverslips at 37°C in 5% CO\textsubscript{2}-air atmosphere and were used for electrophysiological recordings within 3–5 days. HEK-293 cells were cultured in Eagle’s minimum essential medium (Fisher Scientific, Pittsburg, PA) with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, and either 10% horse serum plus 5% fetal bovine serum or 0.5% horse serum plus 0.5% penicillin/streptomycin, and 400 \(\mu\)g/mL gentamicin to select for human cardiac Na\textsubscript{a}.1.5 expressing cells. COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Fisher Scientific) with 10% cosmic calf serum and 1% penicillin/streptomycin.

A \(\sigma_1\)-receptor small interfering RNA (siRNA) construct was designed using the pRNAT-U6.1/Neo plasmid (GenScript, Piscataway, NJ). An siRNA sequence corresponding to nucleotides 130–138 of the human \(\sigma_1\)-receptor open reading frame (PubMed nucleotide ID: NM005866) was inserted into the above plasmid for transfection into mammalian cells. HEK-293 cells stably expressing Na\textsubscript{a}.1.5 were transiently transfected with this construct using TransIT-293 Transfection Reagent (Mirus Bio, Madison, WI) according to the manufacturer’s instructions. Cells were cultured and used as described above.

**Neonatal myocyte isolation and culture.** Neonatal mouse cardiac myocytes were enzymatically isolated and cultured on laminin-coated glass coverslips as previously described (3, 30). Briefly, hearts were aseptically removed from 1- to 3-day-old mouse pups 129/SvEvBrd \(\times\) C57BL/6J, \(\sigma_1\)-receptor knockout (20), and wild type following rapid decapitation. Hearts were minced in digestion enzyme solution consisting of Ca\textsuperscript{2+}-free Hanks’ balanced salt solution with collagenase type II (0.1 mg/ml) (Invitrogen), and an enzyme solution consisting of 5 \(\times\) 10\textsuperscript{5} haloperidol in 50 mM Tris, pH 7.4, for 25 min on ice. The homogenate was centrifuged at 1,000 \(g\) for 10 min, and the pellet was resuspended in the same volume for both spins. The supernatants from both the spins were pooled and centrifuged at 100,000 \(g\) for 1 h. The resulting pellets were suspended in 50 mM Tris-\(\hbox{HCl}\), pH 7.4, assayed for protein using a Bradford protein assay (Bio-Rad, Hercules, CA) aliquoted, and frozen at −80°C until use.

For photolabeling experiments, equivalent amounts (500 \(\mu\)g) of cell homogenate (HEK-293 cells, COS-7 cells, or mouse heart membranes) were incubated in the presence (when indicated) and absence of 5 \(\mu\)M haloperidol in 50 mM Tris, pH 7.4, for 25 min on ice. IACOC (1 nM) was then added, and the reaction was continued on ice for 15 min, after which samples were illuminated for 6 s with a high-pressure AH6-mercury lamp. Following photolabeling, the membranes were solubilized with 1% Triton X-100 or 0.2% CHAPS (in some cases), and centrifuged at 14,000 \(g\) for 30 min to separate the extract. \(\sigma_1\)-Receptor polyclonal antibody (36) (2 \(\mu\)g) was added to the solubilized extract and incubated at 4°C for at least 4 h. Immune complexes were captured using protein A Sepharose (GE Healthcare). Proteins were eluted with sample buffer and separated on 12% SDS-PAGE. The gel was placed on a PhosphorImager (445 SI, Molecular Dynamics) exposure cassette for at least 8 h, after which the cassette was scanned to develop the autoradiogram.

**Drug application.** SKF-10047, (+)-pentazocine, haloperidol, and DTG were obtained from Sigma-Aldrich. DTG and occasionally (+)-pentazocine were first dissolved in DMSO, and then diluted in external solution to obtain the desired drug concentration. Final DMSO never exceeded 0.1% (by volume), and control experiments verified that this level of DMSO had no effect on sodium currents. Drugs were applied in recording solution by gravity feed at approximately 1–2 \(\mu\)l/min. In general, currents were recorded at 15-s intervals for ~5 min to obtain a stable baseline, after which the drug was applied. Drug effects typically appeared within 2–4 min of contact with the cell membrane, resistances ranged from 1 M\(\Omega\) to 3 M\(\Omega\). Immediately after breaking in, cell capacitance and series resistance were determined by transient cancellation. Series resistance was compensated 85–95% to reduce the effective series resistance to ~1 M\(\Omega\). When the product of peak \(I_{Na}\) times the series resistance indicated a series resistance voltage error of >5 \(mV\), the data from that recording were discarded. Recordings were made using an Axopatch-200B patch-clamp amplifier (Axon Instruments/Molecular Devices, Foster City, CA), interfaced to a PC. Data acquisition, voltage control, and analysis were carried out with pCLAMP7 software (Axon Instruments/ Molecular Devices).

External solution for recordings in HEK-293 cells (based on Ref. 43) and COS-7 cells (based on Ref. 25) consisted of (in mM) 140 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 10 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH). The pipette solution contained (in mM) 140 KCl, 2 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 5 EGTA, 10 glucose, and 10 HEPES (pH 7.2 KOH). Extracellular solution for recordings from neonatal cardiac myocytes (based on Ref. 30) consisted of (in mM) 100 tetraethylammonium chloride, 40 NaCl, 10 glucose, 1 MgCl\textsubscript{2}, 0.1 CaCl\textsubscript{2}, 1 NiCl\textsubscript{2}, and 10 HEPES (pH 7.3 CsOH). Intracellular solution contained (in mM) 135 CsCl, 5 NaCl, 10 EGTA, and 10 HEPES (pH 7.3 CsOH). Note that ATP and GTP were not included. \(I_{Na}\) was typically elicited with pulses from −80 mV to −10 mV for 25 ms. For current-voltage analysis, cells were held at −80 mV and were depolarized with 25-ms pulses from −70 to +70 mV in 10-mV increments.

**Membrane preparation and photoaffinity labeling.** Photoaffinity labeling was performed using the \(\sigma_1\)-receptor photolabel [\(^{125}\text{I}\)]-iodoazidococaine (IACOC), as previously described (16, 32). HEK-293 and COS-7 cell homogenates were prepared by passing cells 50 times through a custom-built cell cracker. Mouse heart membranes were prepared as previously described with minor modifications (34). Three to five mouse hearts were homogenized at 4°C in 10 ml of TE buffer (10 mM Tris-\(\hbox{HCl}\) and 1 mM EDTA pH 7.4). The homogenate was centrifuged at 1,000 \(g\) for 10 min, and the pellet was resuspended in the same volume for both spins. The supernatants from both the spins were pooled and centrifuged at 100,000 \(g\) for 1 h. The resulting pellets were suspended in 50 mM Tris-\(\hbox{HCl}\), pH 7.4, assayed for protein using a Bradford protein assay (Bio-Rad, Hercules, CA) aliquoted, and frozen at −80°C until use.

For photolabeling experiments, equivalent amounts (500 \(\mu\)g) of cell homogenate (HEK-293 cells, COS-7 cells, or mouse heart membranes) were incubated in the presence (when indicated) and absence of 5 \(\mu\)M haloperidol in 50 mM Tris, pH 7.4, for 25 min on ice. IACOC (1 nM) was then added, and the reaction was continued on ice for 15 min, after which samples were illuminated for 6 s with a high-pressure AH6-mercury lamp. Following photolabeling, the membranes were solubilized with 1% Triton X-100 or 0.2% CHAPS (in some cases), and centrifuged at 14,000 \(g\) for 30 min to separate the extract. \(\sigma_1\)-Receptor polyclonal antibody (36) (2 \(\mu\)g) was added to the solubilized extract and incubated at 4°C for at least 4 h. Immune complexes were captured using protein A Sepharose (GE Healthcare). Proteins were eluted with sample buffer and separated on 12% SDS-PAGE. The gel was placed on a PhosphorImager (445 SI, Molecular Dynamics) exposure cassette for at least 8 h, after which the cassette was scanned to develop the autoradiogram.
solution change and were recorded until a stable inhibition level was achieved. Reversal of response following drug removal was checked routinely.

Data analysis. Current recordings were analyzed on a PC with pCLAMP7 software. Simple statistical analyses were performed on exported data using Microsoft Excel. Concentration dependence of $I_{Na}$ inhibition was analyzed using Prism (Graphpad Software, San Diego, CA), by fitting to a single-site saturation equation of the form $I = I_{control} / (IC_{50} + [X])$, where $I$ is peak current for a voltage step to $-10$ mV, $[X]$ is drug concentration, and $IC_{50}$ is the concentration producing 50% block. Arithmetic means were computed and presented with the standard error of the mean. Statistical significance was calculated using one-way analysis of variance followed by the post hoc Newman-Keuls test, also using Prism.

RESULTS

HEK-293 cells. Patch-clamp recordings from HEK-293 cells stably expressing the human cardiac Na$^+$ channel Na,1.5 produce large Na$^+$ currents ($I_{Na}$) on the order of several nanoamps in response to voltage steps from $-80$ mV to $-10$ mV (Fig. 1, A and B). $\sigma$-Receptor ligands reversibly inhibited this $I_{Na}$, as illustrated in Fig. 1A for four different cells, each tested with one of the four ligands employed in this study (all at 100 $\mu$M). Two of these ligands, SKF-10047 and (+)-pentazocine, are $\sigma_1$-receptor specific (13, 14), and the other two, haloperidol and DTG, bind to both types of $\sigma$-receptor and are considered $\sigma_1/\sigma_2$-receptor-specific ligands. SKF-10047, (+)-pentazocine, haloperidol, and DTG inhibited $I_{Na}$ by $54 \pm 10, 52 \pm 6, 93 \pm 1$, and $92 \pm 2\%$, respectively ($n = 4–6$). Each panel of Fig. 1 shows the control current prior to drug application, the reduced current in the presence of the drug, and the current following drug removal. Inhibition by all of these ligands was reversible; currents recovered to $>75\%$ of control levels within 10–15 min of the start of superfusion with drug-free solution. The effectiveness of this diverse group of ligands suggests that $\sigma$-receptors can modulate Na,1.5 and that HEK-293 cells express $\sigma$-receptors.

A $\sigma_1$-receptor siRNA construct was designed and generated on the basis of the $\sigma_1$-receptor gene sequence (the $\sigma_2$-receptor has not been cloned so there is no sequence on which to base a $\sigma_2$-receptor siRNA). Transfection of HEK-293 cells with this siRNA construct did not alter control $I_{Na}$ but reduced the inhibition of $I_{Na}$ by SKF-10047 to $26 \pm 4\%$ (compared with $54 \pm 10\%$). The siRNA construct had no significant effect on $I_{Na}$ inhibition by DTG ($88 \pm 1\%$ vs. $92 \pm 2\%$) (Fig. 1B; for averages with statistical significance, see Fig. 4). These results are consistent with a selective reduction of $\sigma_1$-receptor protein levels by siRNA. We assayed $\sigma_1$-receptor protein levels biochemically by photoaffinity labeling cell homogenates with IACOC, followed by immunoprecipitation enrichment with a $\sigma_1$-receptor antibody. Resolution with SDS-PAGE and detection by autoradiography revealed strong photolabeling of a 26-kDa protein, the $\sigma_1$-receptor. Expression of the $\sigma_1$-receptor siRNA construct reduced this signal by an average of $33 \pm 9\%$ (Fig. 1C). This reduction observed biochemically parallels the reduction of channel modulation by the $\sigma_1$-receptor-specific ligand SKF-10047.

All four ligands inhibited current in a concentration-dependent manner (Fig. 2A). Plots of peak $I_{Na}$ versus concentration were well fitted by a single-site saturation equation (see METHODS), yielding $IC_{50}$ values of 70, 53, 4, and 10 $\mu$M for SKF-10047, (+)-pentazocine, haloperidol, and DTG, respectively. As shown in Fig. 2A, at saturating concentrations, all four ligands reduced $I_{Na}$ to $<5\%$ of control (data not shown).

These drugs inhibited $I_{Na}$ despite the omission of ATP and GTP from the patch pipette filling solution. In recordings from HEK-293 cells lasting over an hour, modulation of $I_{Na}$ continued unabated. Since G protein and protein kinase-mediated
this further, we examined the voltage dependence of rather than permits activity with altered kinetics. To explore I

idol. (control) and presence of 100

B

SKF-10047(

represent best fits to the data using the single-site saturation equation given in /H11002

80 mV to rundown suggests that these responses to whole cell patch-clamp experiments (35, 42), the absence of responses generally depend on GTP and ATP, respectively, and small molecules generally exchange in a few minutes in whole cell patch-clamp experiments (35, 42), the absence of rundown suggests that these responses to \( \sigma \)-receptor activation do not depend on G proteins or protein kinases.

Normalized currents recorded from HEK-293 cells before, during application, and after removal of 100 

M SKF-10047, (\(+\)-pentazocine, haloperidol, and DTG (all 100 

M) inhibited with the greatest potency, reducing \( I_{\text{Na}} \) by 92 ± 1%. SKF-10047, (\(+\)-pentazocine, and DTG inhibited \( I_{\text{Na}} \) by 18 ± 5%, 28 ± 7%, and 89 ± 4%, respectively (\( n = 4 - 6 \)). Figure 3 shows \( I_{\text{Na}} \) traces from four different COS-7 cells before, during application, and after removal, of SKF-10047, (\(+\)-pentazocine, haloperidol, and DTG (all 100 

M). As in HEK-293 cells, \( I_{\text{Na}} \) inhibition by all of these ligands was reversible, recovering to >75% of control levels within 10–15 min (Fig. 3).

As seen with HEK-293 cells (Fig. 2B), \( I_{\text{Na}} \) current-voltage plots from COS-7 cells revealed no significant shift in voltage dependence following ligand application (data not shown). Furthermore, current traces before, during, and after drug application were superimposable (data not shown), indicating that channel kinetics are not altered. Finally, the modulation continued unabated for more than half an hour despite the omission of ATP and GTP from the patch pipette filling solution.

HEK-293 cells versus COS-7 cells. Patch-clamp recordings and photolabeling experiments did not yield identical results in COS-7 cells and HEK-293 cells. SKF-10047 and (\(+\)-pentazocine inhibited \( I_{\text{Na}} \) much less in COS-7 cells than in HEK-293 cells. By contrast, haloperidol and DTG inhibited \( I_{\text{Na}} \) very effectively in both cell types. Figure 4A presents the averages for \( I_{\text{Na}} \) modulation in HEK-293 cells and COS-7 cells, including the siRNA-treated HEK-293 cells (from experiments as in Fig. 1B). Figure 4A shows that in control HEK-293 cells, all of

Fig. 2. Concentration and voltage dependence of sodium channel modulation in HEK-293 cells. A: peak \( \text{Na}^+ \) current (\( I_{\text{Na}} \)) amplitude, evoked by steps from −80 mV to −10 mV, was normalized to control and plotted versus drug concentration. Data points represent means ± SE for \( n = 4 - 7 \) cells. Curves represent best fits to the data using the single-site saturation equation given in METHODS. Half-maximal inhibition (IC50) values were 70, 53, 4, and 10 

M for SKF-10047(\( \bullet \)), pentazocine (\( \triangledown \)), haloperidol (\( \bullet \)), and DTG (\( \blacksquare \)), respectively. B: \( I_{\text{Na}} \) amplitude was determined for steps to different voltages, in the absence (control) and presence of 100 

M SKF-10047, (\(+\)-pentazocine, haloperidol, and DTG. \( I_{\text{Na}} \) recorded in the presence of drugs was normalized to controls. Data points represent means ± SE for \( n = 3 \) cells.

Fig. 3. Sodium channel inhibition by \( \sigma \)-receptor ligands in COS-7 cells. \( I_{\text{Na}} \) was evoked by steps from −80 to −10 mV in COS-7 cells transiently expressing Na\(_{\text{a}}\), channels near its reversal potential. Fig. 2B indicates that alterations in the voltage dependence of \( \text{Na}^+ \) channel gating do not play an important role in the modulation by \( \sigma \)-receptor ligands.

COS-7 cells. Patch-clamp recordings from COS-7 cells transiently expressing Na\(_{\text{a}}\),, showed that voltage steps elicit \( I_{\text{Na}} \) (Fig. 3). The same four \( \sigma \)-receptor ligands tested in HEK-293 cells also inhibited \( I_{\text{Na}} \) in COS-7 cells. Again, haloperidol (100 

M) inhibited with the greatest potency, reducing \( I_{\text{Na}} \) by 92 ± 1%. SKF-10047, (\(+\)-pentazocine, and DTG inhibited \( I_{\text{Na}} \) by 18 ± 5%, 28 ± 7%, and 89 ± 4%, respectively (\( n = 4 - 6 \)). Figure 3 shows \( I_{\text{Na}} \) traces from four different COS-7 cells before, during application, and after removal, of SKF-10047, (\(+\)-pentazocine, haloperidol, and DTG (all 100 

M). As in HEK-293 cells, \( I_{\text{Na}} \) inhibition by all of these ligands was reversible, recovering to >75% of control levels within 10–15 min (Fig. 3).

As seen with HEK-293 cells (Fig. 2B), \( I_{\text{Na}} \) current-voltage plots from COS-7 cells revealed no significant shift in voltage dependence following ligand application (data not shown). Furthermore, current traces before, during, and after drug application were superimposable (data not shown), indicating that channel kinetics are not altered. Finally, the modulation continued unabated for more than half an hour despite the omission of ATP and GTP from the patch pipette filling solution.
the tested ligands strongly inhibited $I_{\text{Na}}$. By contrast, in COS-7 cells, SKF-10047 and (+)-pentazocine were much less effective than DTG and haloperidol. The difference between the $\sigma_1$-receptor ligand actions in HEK-293 cells versus COS-7 cells paralleled the difference between control and siRNA-treated HEK-293 cells.

Immunoprecipitation-photolabeling with IACOC provided an assessment of the differences in $\sigma_1$-protein levels between HEK-293 and COS-7 cell homogenates. These experiments again demonstrated strong photolabeling of a 26-kDa protein in HEK-293 cells, and this labeling was blocked by haloperidol (Fig. 4B). By contrast, the photolabeling signal in COS-7 cells, while still haloperidol sensitive, was much weaker. Likewise, note that the low abundance of $\sigma_1$-receptors in COS-7 cells, as demonstrated in $\sigma_1$-receptor siRNA-transfected HEK-293 cells, parallels the very weak effects of the two $\sigma_1$-receptor-specific ligands, tested in patch-clamp recordings [SKF-10047 and (+)-pentazocine]. The results of these photolabeling and patch-clamp experiments indicate that COS-7 cells have much lower levels of $\sigma_1$-receptors than HEK-293 cells.

Neonatal cardiac myocytes. The above studies showed that $\sigma$-receptor ligands modulate Na$_v$1.5 in heterologous expression systems. To evaluate this modulation in native tissue, we tested $\sigma$-receptor ligands on $I_{\text{Na}}$ in neonatal cardiac myocytes. Figure 5 shows $I_{\text{Na}}$ recordings from wild-type and $\sigma_1$-receptor knockout myocytes before, during, and after application of SKF-10047, (+)-pentazocine, haloperidol, and DTG (all 100 $\mu$M). These ligands inhibited $I_{\text{Na}}$ by 46 ± 4, 49 ± 5, 90 ± 3, and 89 ± 3%, respectively (n = 3–4). In knockout myocytes, these drugs inhibited $I_{\text{Na}}$ by 19 ± 5, 9 ± 2, 83 ± 1, and 90 ± 3%, respectively (n = 3–4). Figure 6A summarizes the percent inhibition of these drugs in myocytes. SKF-10047 and (+)-pentazocine produced much less inhibition in knockout compared with wild-type myocytes (P < 0.05). In fact, the comparison of the profiles of inhibition for the various drugs between wild-type and knockout myocytes shows a striking parallel to the comparison of profiles between HEK-293 cells and COS-7 cells (Fig. 4A). Immunoprecipitation photolabeling experiments further demonstrated this parallel. Identical experiments to those carried out with HEK-293 and COS-7 cell homogenates were performed on wild-type and $\sigma_1$-receptor knockout mouse heart membranes. These experiments demonstrated the presence of $\sigma_1$-receptors in wild-type mouse heart membranes and verified their absence in the knockout (Fig. 6B). Again these profiles indicate that $\sigma_1$-receptors play a significant role in the inhibition of $I_{\text{Na}}$ by the $\sigma_1$-receptor-selective compounds SKF-10047 and (+)-pentazocine, but that in the absence of $\sigma_1$-receptors, $\sigma_2$-
receptors, for which binding sites have been reported in the heart (6), can still mediate responses to haloperidol and DTG. As in HEK-293 cells (Fig. 2B), $I_{Na}$ peaked between $-10$ and $-20$ mV in the absence or presence of various $\sigma_1$-receptor ligands, in both wild-type and $\sigma_1$-receptor knockout myocytes (data not shown). Channel kinetics also remained unchanged (insets of Fig. 5, A and B), as noted above for HEK-293 and COS-7 cells (inset of Fig. 1A and data not shown). In a further parallel with HEK-293 and COS-7 cells, the modulation of $I_{Na}$ in myocytes does not require the inclusion of ATP or GTP in the patch pipette filling solution. Responses were seen in recordings lasting more than half an hour, and there was no decline in the magnitude of the response over time.

Fig. 5. Sodium channel inhibition by $\sigma$-receptor ligands in cardiac myocytes. $I_{Na}$ was evoked by steps from $-80$ mV to $-10$ mV in neonatal cardiac myocytes from wild-type (A) and $\sigma_1$-receptor knockout (B) mice in the absence (control, black), presence (drug, red), and after washout (recovery, blue) of 100 $\mu$M SKF-10047, (+)-pentazocine, haloperidol, and DTG. Insets: normalization revealed that inhibition occurred without a change in channel kinetics [pentazocine (A) and DTG (B)]. Note that SKF-10047 and (+)-pentazocine, two $\sigma_1$-receptor-specific ligands, inhibit $I_{Na}$ by $\sim 50\%$ in wild-type mice (A) and 20% or less in knockout (B) mice. By contrast, haloperidol and DTG, two nonspecific $\sigma$-receptor ligands, inhibited $I_{Na}$ by $\sim 90\%$ in both wild-type and knockout mice (see Fig. 6).

Fig. 6. Comparison of $I_{Na}$ inhibition in wild-type (WT) and $\sigma_1$-receptor knockout (KO) myocytes. A: average inhibition by each ligand at 100 $\mu$M was determined from $I_{Na}$ traces in Fig. 5. Bars represent means $\pm$ SE for $n = 3-4$. There is a significant difference between current inhibition by SKF-10047 and (+)-pentazocine in the two systems ($^*P < 0.05$). B: immunoprecipitation-photolabeling experiment with [125I]-iodoazidococaine demonstrated $\sigma_1$-receptor expression in wild-type mouse heart membranes and confirmed the elimination of $\sigma_1$-receptors in knockout mouse heart membranes. Haloperidol (5 $\mu$M) reduced photolabeling in wild-type mice; however, in knockout mice, there was no photolabeling either with or without haloperidol.
DISCUSSION

This study has shown that σ-receptor ligands modulate the voltage-gated Na\(^+\) channel Na\(_{1.5}\). Experiments demonstrated this modulation in two different heterologous expression systems, as well as a native preparation (mouse cardiac myocytes). The myocyte experiments demonstrate this modulation in mice and the HEK-293 cell experiments demonstrate this modulation in humans. Thus, the present study makes the case for σ-receptor modulation of Na\(_{1.5}\) channels in two different species and shows that, in each, the pharmacological profiles are similar (Figs. 4 and 6).

The four ligands tested here, SKF-10047, (+)-pentazocine, haloperidol, and DTG, all have well-documented actions on σ-receptors (40, 44, 45). Haloperidol also interacts with dopamine receptors, but as an antagonist. The minus stereoenantiomers of SKF-10047 and pentazocine interact with dopamine receptors, but as an antagonist. The minus stereoenantiomer of SKF-10047 targets include M-current, A-current, and Ca\(^{2+}\)-activated K\(^+\) channels in sympathetic neurons (18), inward rectifying K\(^+\) channels in the heart (27); M-current, delayed rectifier, and Ca\(^{2+}\)-activated K\(^+\) channels in intracardiac neurons (47); and A-current and Ca\(^{2+}\)-activated K\(^+\) channels in neurohypophysis (45). σ-Receptors also inhibit a variety of Ca\(^{2+}\) channels, as demonstrated in hippocampal (5) and parasympathetic (48) neurons. The present study extends the σ-receptor ion channel interface by demonstrating the modulation of voltage-gated Na\(^+\) channels. If σ-receptors modulate Na\(^+\) channels by a direct association, as has been suggested for the modulation of the K\(^+\) channel K\(_{v1.4}\) (2) and the L-type Ca\(^{2+}\) channel (41), then these receptors have a remarkable ability to associate with channel proteins from very different families. As the number of different ion channels targeted by σ-receptors increases, the question of the structural basis for the implied association assumes greater prominence. Elucidating the basis for this association may also shed light on how this modulation can be achieved either with (48) or without (2, 45) (present work) alterations in channel kinetics and shifts in voltage dependence.

The modulation of cardiac I\(_{Na}\) by σ-receptors is clearly relevant to the actions of drugs on the heart. σ-Receptors regulate intracellular Ca\(^{2+}\) and modulate cardiac contractility (26), with different effects in neonatal and adult rat cardiac myocytes (7, 8, 28, 29). σ-Receptor ligands have been shown to inhibit both inwardly rectifying K\(^+\) channels and human ether-a-go-go-related gene K\(^+\) channels in the heart (19, 27). Given the modulation of Ca\(^{2+}\) channels in neurons (5, 48), it will clearly be important to assess the effect of σ-receptor activation on cardiac Ca\(^{2+}\) current. The inhibition of I\(_{Na}\) described in the present study would be expected to inhibit contractility and rhythmicity, while the inhibition of various K\(^+\) channels could have the opposite effects. How these two opposing actions are resolved will be of critical importance in understanding how σ-receptor ligands alter the cardiac action potential to act either as antiarrhythmics, or to produce acquired arrhythmias such as acquired long QT, Brugada, and Timothy syndromes (4, 37, 39).

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REFERENCES

24. Moebius FF, Reiter RJ, Hanner M, Glossmann H. High affinity of sigma 1-binding sites for sterol isomerization inhibitors: evidence for a pharmacological relationship with the yeast sterol C8 –C7 isomerase. Br J Pharmacol 121: 1–6, 1997.