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Cancer Cell Cycle Modulated by a Functional Coupling between Sigma-1 Receptors and Cl^- Channels*

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The sigma-1 receptor is an intracellular protein characterized as a tumor biomarker whose function remains mysterious. We demonstrate herein for the first time that highly selective sigma ligands inhibit volume-regulated chloride channels (VRCC) in small cell lung cancer and T-leukemia cells. Sigma ligands and VRCC blockers provoked a cell cycle arrest underlined by p27 accumulation. In stably sigma-1 receptor-transfected HEK cells, the proliferation rate was significantly lowered by sigma ligands when compared with control cells. Sigma ligands produced a strong inhibition of VRCC in HEK-transfected cells but not in control HEK. Surprisingly, the activation rate of VRCC was dramatically delayed in HEK-transfected cells in the absence of ligands, indicating that sigma-1 receptors *per se* modulate cell regulating volume processes in physiological conditions. Volume measurements in hypotonic conditions revealed indeed that the regulatory volume decrease was delayed in HEK-transfected cells and virtually abolished in the presence of igmesine in both HEK-tranfected and T-leukemic cells. Moreover, HEK-transfected cells showed a significant resistance to staurosporine-induced apoptosis volume decrease, indicating that sigma-1 receptors protect cancer cells from apoptosis. Altogether, our results show for the first time that sigma-1 receptors modulate "cell destiny" through VRCC and cell volume regulation.

Sigma receptors are intracellular proteins that were first postulated as opioid receptors on the basis of pharmacological and behavioral studies (1). Finally, 20 years of pharmacological studies and the cloning of the sigma-1 receptor subtype in 1996 revealed indeed that the "sigma-binding site" corresponded to a 24-kDa protein unrelated to other mammalian proteins and localized in the inner face of the plasma membrane and the membranes of the endoplasmic reticulum and the nucleus (2–5). Although high concentrations of neurosteroids have been shown to interact with brain sigma-1 receptors in behavioral studies (for review see Ref. 6), no high affinity endogene sigma ligand has been identified yet. Nonetheless, exogene compounds from disparate chemical classes ((+)-benzomorphans, cocaine, guanidines, and neuroleptics for example) have been characterized or developed as highly selective sigma ligands (7). The sigma-1 receptors are expressed in various tissues including the brain, the pituitary, and the liver (2, 8–10). Surprisingly, very high levels of sigma receptors have been detected in tumor cells when compared with normal cells (11, 12). Indeed, the expression of sigma receptors in cancer biopsies is correlated with the proliferating state of the cells so that these proteins are now commonly considered to be tumor biomarkers (13, 14). Consequently, many sigma ligands are developed nowadays for imagery (positon emission tomography scan) to detect early stage tumors (15, 16). However, if sigma receptors represent exciting targets to detect cancers *in vivo*, very few data are available on both the function and the action mechanisms linked to these mysterious proteins in tumor cells. Previously, we and others have demonstrated that sigma-1 receptors were functionally coupled with membrane potassium channels in the pituitary (4, 9, 17, 18). Because of the growing amount of evidence involving membrane channels in the control of the cell cycle and tumor growth (19–24), we further explored the coupling of sigma receptors and potassium channels in the field of tumor cell area. We then demonstrated that the activation of sigma-1 receptors by highly selective ligands provoked the arrest of the cell cycle progression in the G_1 phase in cancer cells. This effect was partly linked to the inhibition of voltage-dependent potassium channels (25, 26). In the present work, we have examined in leukemic and SCLC[3] cells a putative interaction between sigma-1 receptors and volume-regulated chloride currents (VRCC) that have been involved in the control of the cell cycle (27–29). We demonstrate for the first time that the sigma-1 receptor modulates VRCC and cell volume regulation properties leading to alterations of cell proliferation and apoptosis mechanisms.

MATERIALS AND METHODS

Cell Culture—SCLC (NCI-H209) and leukemic Jurkat (JA3) cell lines were obtained from CLS (Heidelberg, Germany). The HEK cell line was a gift from Dr. F. Duprat (CNRS U 526, Sophia-Antipolis, France). All of the cell lines were grown at 37 °C with 5% of CO_2 in RPMI 1640 supplemented with L-glutamine (2 mM), sodium-pyruvate (1 mM), penicillin/streptomy-

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[3] The abbreviations used are: SCLC, small cell lung cancer; HEK, human embryonic kidney; NPPB, 5-nitro-2-(phenylpropylamino)-benzoate; VRCC, volume-regulated chloride channel; DTG, 1,3-di-*O*-tolyguanidin; RVD, regulatory volume decrease; AVD, apoptosis volume decrease; TRITC, tetramethylrhodamine isothiocyanate.

cin (100 units/ml), and fetal-bovine serum (10% for SCLC and 5% for Jurkat). The medium was routinely changed three times a week for Jurkat cells and one time a week for SCLC cells. Dead cells were excluded by a Ficoll gradient separation technique (Lymphocyte separation medium; Bio-Whittaker, Verviers, Belgium).

Drugs and Reagents—(+)-Pentazocine, DTG, NPPB, and poly-D-lysine were purchased from Sigma-Aldrich. Igmesine was a generous gift from Dr. F. Roman (Pfizer, Fresne, France). Anti-actin (A2066), anti-cyclin A (C4710), and secondary anti-mouse horseradish peroxidase-coupled antibodies were obtained from Sigma-Aldrich. Santa Cruz anti-p27 (C-19) and anti-$p21^{cip1}$ (C-19) antibodies were from TEBU International (Le Perray-en-Yvelines, France). Secondary anti-rabbit horseradish peroxidase-coupled antibodies (11-035-144; Jackson ImmunoResearch Laboratories) were purchased from Interchim (Montlucon, France).

Electrophysiolology—For whole cell patch clamp recordings, the cells were plated on glass coverslips coated with poly-D-lysine (10 nM) and incubated for 2–4 h in RPMI 1640 medium. SCLC cells need to be mechanically dissociated before being plated. For SCLC and Jurkat cells, the patch clamp experiments were performed at room temperature with an external solution of the following composition: tetraethylammonium Cl, 140 mM; $MgCl_2$, 2 mM; $CaCl_2$, 1 mM; Hepes, 10 mM; glucose, 10 mM (pH adjusted to 7.4 with HCl, 309 mosm/liter). In this case, the hypotonic solution was obtained by omission of 20 mM of triethanolamine-Cl (269 mOsm). For HEK cells, the external solution composition was: NaCl, 140 mM; $CaCl_2$, 2 mM; $MgCl_2$, 2 mM; Hepes, 10 mM (pH adjusted to 7.4 with NaOH; 302 mOsm). The hypotonic solution was obtained by a ¼ dilution (226.5 mOsm). Soft glass patch electrodes (borosilicate glass capillaries GC150TF-7.5; Harvard Apparatus, Edenbridge, Kent) were made on a horizontal pipette puller (P-97; Sutter Instrument Co., Novato, CA) to achieve a final resistance ranging from 3 to 5 MΩ. The internal solution was of the following composition: CsCl, 134 mM; $MgCl_2$, 2 mM; $CaCl_2$, 1 mM; EGTA, 11 mM; Hepes, 10 mM (pH adjusted to 7.2 with CsOH, 298 mOsm). ATP (2 mM) and GTP (100 μM) were extemporaneously added to the internal solution. Electric signals were amplified with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and acquired on an IBM compatible personal computer with a DIGIDATA 1200 interface and pCLAMP 8 software (Axon Instruments). Cl^- currents were recorded at a 5-kHz sampling frequency and filtred at 2 kHz. DTG was dissolved in methanol (final concentration of methanol < 0.1% (v/v)). (+)-Pentazocine was dissolved in methanol/acid (½ methanol + ½ HCl 0.1 M (v/v), final concentration of methanol < 0.1% v/v). Solvent alone had no effect on K^+ currents at this concentration. Igmesine was dissolved in water. Sigma ligand solutions were administered in the vicinity of the cell under study through the use of a gravity-feed system (rate, ~2 ml/mn). The excess of bathing solution was continuously aspired via a suction needle. Current amplitudes were determined with the pCLAMP 8 analysis software (Clampfit). Current/voltage and current/time relationships were fitted by using Microcal Origin analysis software (Sega, Paris, France). The quantitative data are expressed as the means ± S.E.

Production of a HEK Cell Line Stably Overexpressing the Sigma-1 Receptor (HEK-SIG)—The human sigma-1 receptor (U75283) was inserted in the pCMV-Tag3 vector allowing a N-terminal protein fusion between the c-Myc tag and the receptor (Stratagene; generous gift from Dr. L. Combettes). 2 μg of DNA were transfected in HEK293 cells (300,000 cells/dish) using Jet PEI (Polyplus Transfection, Illkirch, France) according to the manufacturer's recommendations. 60 h latter, the medium was replaced by a medium containing the G418 antibiotic (2 μg/ml). The survival clones were scrapped and cultured in individual dishes.

Microscopy—Micrographs were performed with a Zeiss axiovert microscope with a 63× oil lens coupled to a digital camera and the Zeiss analysis software. Control micrographs were performed in direct light. The nucleus was detected by fluorescence using Hoechst 33342 (100 ng/ml; Molecular Probe). The c-Myc-targeted sigma-1 receptors was detected by immunofluorescence by the use of an anti-Myc tag (Euromedex, France; dilution, 1/500); the secondary andibody was an anti-mouse TRITC conjugate (Sigma; dilution 1/500).

Cell Growth Analysis—To assess cell growth, the cells were seeded at day 0 at a density of 0.25×10^6 cells/ml and counted 24, 48, and 72 h after incubation with NPPB or igmesine. For cell density evaluation, an aliquot of 25 μl of cell suspension was mixed with 25 μl of trypan blue, and the number of cells was counted using a Malassez chamber. Only viable cells (which excluded trypan blue) were counted. This enabled us to differentiate easily between a reduced cell proliferation rate and cell death.

Western Blot—After 3 days of incubation with igmesine, (+)-pentazocine, DTG, or NPPB, the cells were washed in phosphate-buffered saline and then lysed under agitation in ice-cold lysis buffer (50 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, 10 mM NaF, 0.1 mM β-glycerophosphate, 1 mM $NaVO_4$, and a protease inhibitor mixture, Complete® (Roche Applied Science)). The lysate was then centrifuged (11,000 × g, 15 min, 4 °C), and the resulting supernatants were analyzed by immunoblotting. Total protein concentration was determined with a Bio-Rad protein assay with bovine serum albumin as the standard. Proteins (50 μg/lane) were resolved on a 13% acrylamide gel by SDS-PAGE, electrotransferred to nitrocellulose, blocked in 5% nonfat milk, and incubated overnight at 4 °C with a primary antibody directed against either p27 (1:200), $p21^{cip1}$ (1/200), cyclin A (1:750), or actin (1:200) human proteins. The blots were incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabit, 1:15,000; anti-mouse, 1:50,000) for 1 h at room temperature. Labeled proteins were visualized by enhanced chemiluminescence (Pierce SuperSignal West Pico chemiluminescent, Interchim) using Kodak Bio-Max MR film (Sigma-Aldrich).

Cell Volume Measurements—Cell volume was measured by an electronic sizing technique with a CASY 1 (SCARFE SYSTEM). The cells were suspended in CASYton solution (NaCl isotonic solution) for control condition and in 30% diluted CASYton solution in the absence or presence of sigma ligands or staurosporine. These solutions were adjusted to 290 (isotonic solution) or 230 mOsm (hypotonic solution). The values are expressed as percentages of cell volume variations measured during hypotonic shock in the presence or absence of ligand.

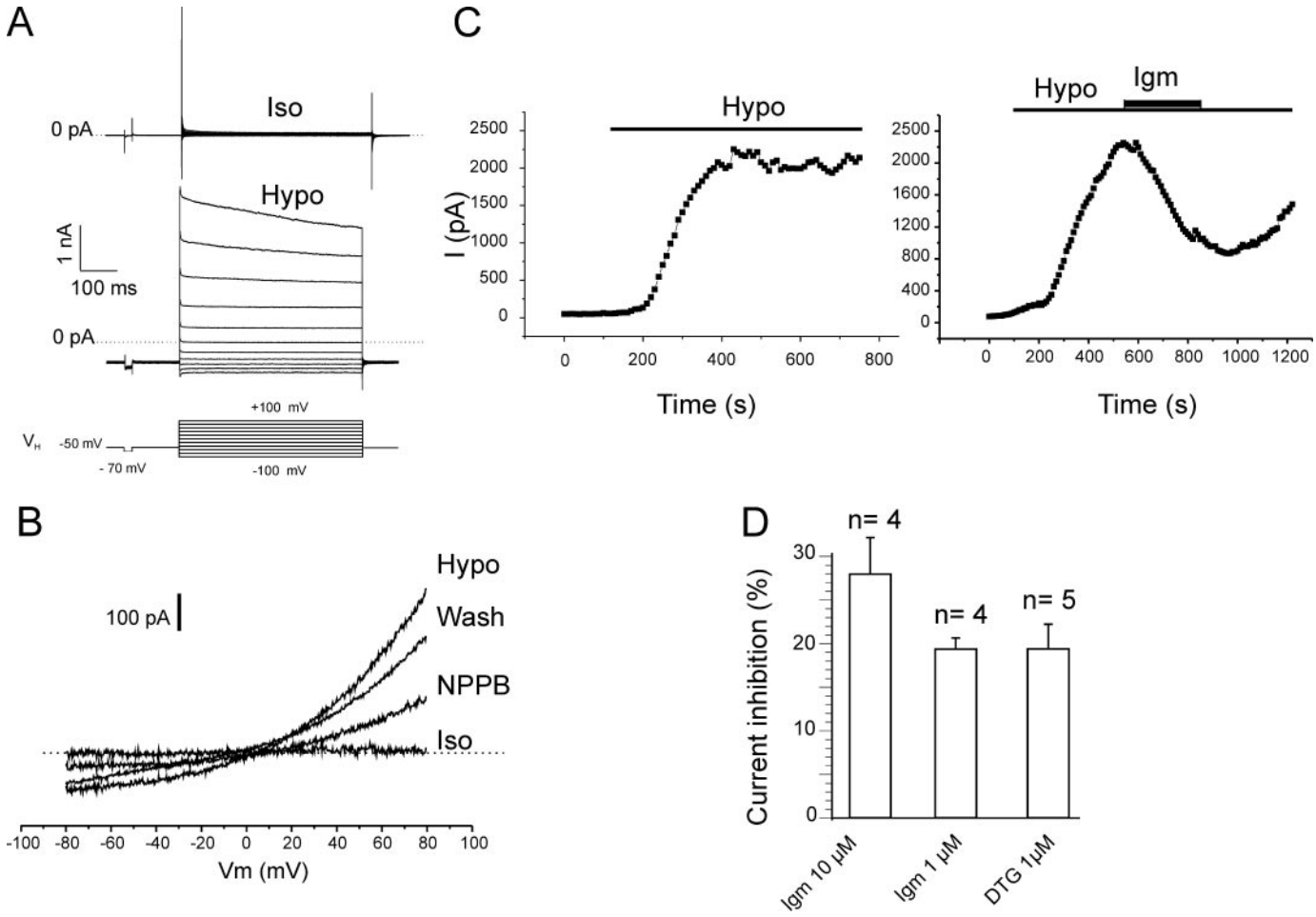

FIGURE 1. **Sigma ligands inhibit VRCC in SCLC cells.** *A*, families of membrane currents recorded from a single cell in the whole cell configuration in isotonic (*Iso*) and hypotonic (*Hypo*) conditions (*upper* and *lower panels*, respectively). The currents are evoked by the voltage step protocol described underneath. *B*, instantaneous *I/V* relationships obtained with voltage ramps (1 s). The currents were recorded in hypotonic conditions successively before (*Hypo*), during (*NPPB*), and after (*Wash*) the application of NBBP (100 μM). A first recording was performed before the hypotonic shock (*Iso*). *C*, evolution of the membrane current during a hypotonic shock in the absence (*left panel*) or in the presence of igmesine (10 μM, *Igm*, *right panel*). The time of application of igmesine is represented by the *thick horizontal bar*. The membrane currents were recorded by voltage steps from −80 to 80 mV. *D*, inhibition of the hypotonic-activated current by igmesine and DTG. The currents were evoked by voltage steps from −80 to 80 mV. The values are the means ± S.E.

RESULTS

Sigma Ligands Inhibit Hypotonic-activated Chloride Currents in SCLC and Jurkat Cells—The experiments were performed using the whole cell variant of the patch clamp technique. The cells were bathed in a Na^+-free extracellular solution. The K^+ currents were cancelled by replacing the intracellular K^+ by Cs^+ (see "Materials and Methods"). In isotonic conditions, no current could be detected in cells (Fig. 1*A*, *upper panel*). The application of an external hypotonic shock by manitol depletion induced the development of a sustained outwardly rectifying current with a reversal potential corresponding to the equilibrium potential for Cl^- ions ($E_{Cl}^- = 3$ mV; Fig. 1, *A* and *B*). This current was sensitive to NPPB (100 μM; Fig. 1*B*), indicating that it corresponds to the VRCC described earlier (30). The current developed progressively to reach a stable value (Fig. 1*C*, *left panel*). The application of a specific sigma-1 receptor ligand such as igmesine (18, 31) produced a reversible inhibition of the current in all tested cells (Fig. 1*C*, *left panel*). The inhibitory effect was dose-dependent and was mimicked by other sigma ligands such as DTG (Fig. 1*D*). The same type of experiments was next performed in jurkat cells. In this latter cell type, the application of a hypotonic shock also induced an outwardly rectifying current that presented a reversal potential corresponding to Cl^- ion equilibrium potential ($E_{Cl}^- = 3$ mV; Fig. 2*A*). This current was inhibited by NPPB (not shown; see Ref. 32) and presented the same characteristics as previously described (33). In contrast to the VRCC observed in SCLC cells, which stabilized at a stable plateau value during the hypotonic shock, the current recorded in jurkat cells showed a noticeable decay after having reached maximum level (Fig. 2*B*). Consequently, we performed cell incubations with sigma ligands (30 min) rather than acute applications to detect a potential modulation of the current by the activation of sigma receptors. The maximum current densities were significantly lowered for cells challenged with either DTG, (+)-pentazocine, or igmesine (Fig. 2, *B* and *C*). Altogether, these results demonstrate for the first time that sigma ligands modulate chloride channels. We next wondered whether the pharmacological blockade of VRCC could provide the same effects as sigma ligands on SCLC and jurkat cell proliferation.

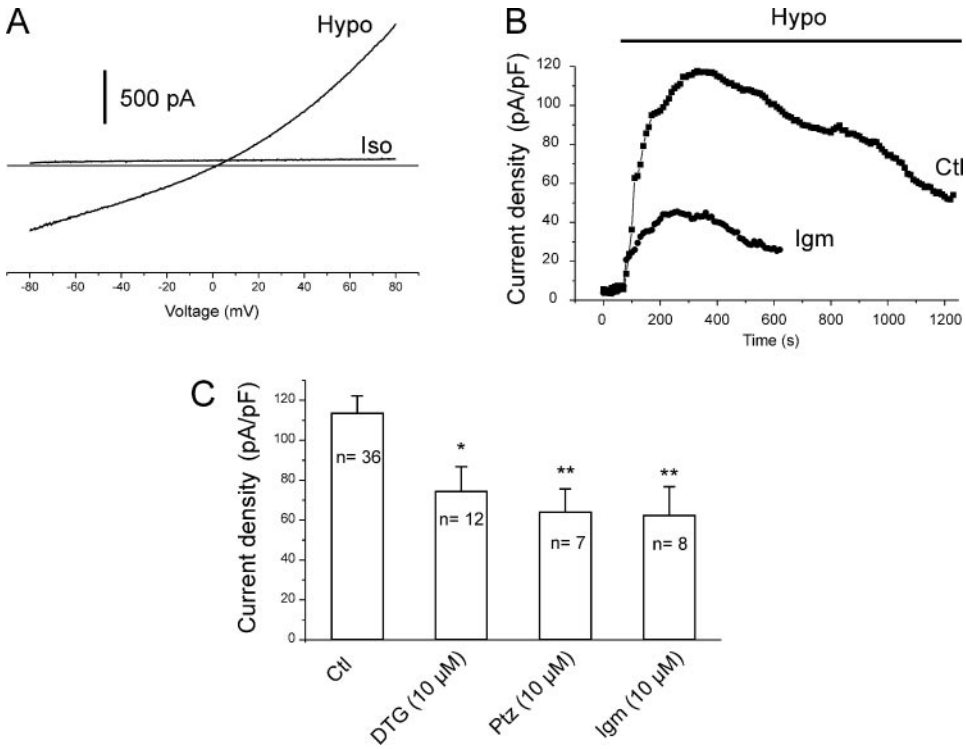

FIGURE 2. **Sigma ligands inhibit VRCC in Jurkat cells.** *A*, instantaneous *I/V* relationships obtained from a single Jurkat cell using voltage ramps (1 s). The currents were recorded before (*Iso*) and during a hypotonic shock (*Hypo*). *B*, evolution of the current density in response to a hypotonic shock in a nonincubated cell (*Ctl*) and in an igmesine-incubated cell (10 μM, 30 mn, *Igm*). In this latter case, igmesine was also present in the bath during the patch clamp recording. *C*, mean current density of the hypotonic-activated current at 80 mV in nonincubated cells (*Ctl*) and cells incubated with DTG (*DTG*, 10 μM), pentazocine (*Ptz*, 10 μM), or igmesine (*Igm*, 10 μM). The experiments were performed using the same protocol as in *B*. The values are the means ± S.E. *, $p < 0.05$; **, $p < 0.005$, Student's *t* test.

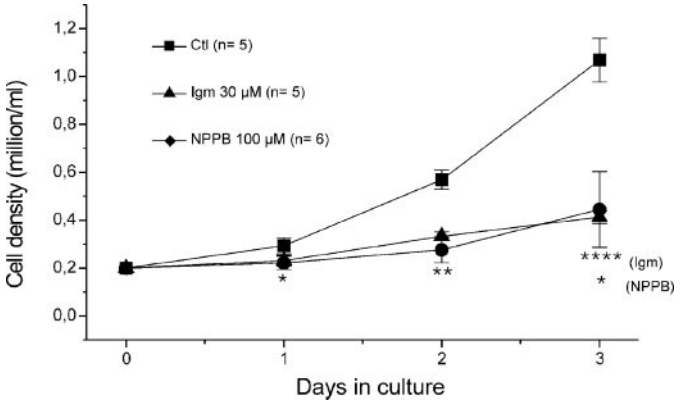

FIGURE 3. **NPPB and igmesine inhibit jurkat cell proliferation.** Plots showing the growth of SCLC cells over three days in the absence (*Ctl*) and the presence of NPPB (*NPPB*) or igmesine (*Igm*). The values are the means ± S.E. *, $p < 0.05$; **, $p < 0.005$; ****, $p < 0.0005$, Student's *t* test. Note that none of the solvents used to prepare igmesine or NPPB had any effect *per se* (not shown).

Igmesine and NPPB Inhibit Cell Proliferation through p27^{kip1} Accumulation—We have previously demonstrated that the pharmacological activation of sigma-1 receptors inhibits cell cycle progress through the accumulation of the CDK inhibitor p27 and the subsequent down-regulation of cyclin A (25). In the present study, we show that the incubation of jurkat cells with either igmesine (40 μM) or NPPB (100 μM) produces a strong inhibition of cell growth over 3 days (Fig. 3). Western blot analysis showed that the cell growth inhibition provoked by NPPB (100 μM) was accompanied by a clear cut increase in p27 and a strong decrease in cyclin A in both Jurkat and SCLC cells (Fig. 4). The same modulation of cell cycle-controlling proteins was also observed for cells challenged with igmesine (Fig. 4) and other sigma ligands (25), suggesting a common pathway.

Functional Expression of the Sigma-1 Receptor in HEK Cells—To verify whether the effects of sigma ligands on VRCC were attributable to a specific activation of sigma receptors and not to a direct action on chloride channels, we produced several stable HEK cell lines expressing the sigma-1 receptor (see "Materials and Methods"). When we established these different cell lines, no efficient antibodies directed against the sigma-1 receptor were available. So we expressed an N-terminal c-Myc-targeted human sigma receptor. This allowed us to detect the expression of sigma-1 receptors with an anti-c-Myc antibody (see "Materials and Methods"). Immunofluorescent micrographs show a strong fluorescent signal in HEK-expressing cells (HEK-SIG) when compared with control cells. The localization of the targeted sigma-1 receptor was diffuse in the cells with a predominant perinuclear labeling (Fig. 5). This result is in a good agreement with previous works that showed the presence of sigma receptors in the nucleus membrane, the endoplasmic reticulum, and the plasma membrane (3, 4, 34). Four different HEK-SIG clones were obtained presenting the same result (not shown). Two of these clones were used to perform growth and patch clamp experiments.

Sigma Ligands Reduce Proliferation and Inhibit the VRCC in HEK-SIG Cells—HEK cell proliferation rate was studied over 3 days. Cell incubation with igmesine (40 μM) had no significant effect on growth (Fig. 6, *upper panel*). Similarly, igmesine failed to slow down the growth of HEK cells expressing the vector without the insert encoding for the sigma-1 receptor. By contrast, HEK-SIG cell incubation with the same dose of igmesine (40 μM) significantly reduced the cell number after 3 days, demonstrating that igmesine modulates cell proliferation through the activation of the sigma-1 receptor (Fig. 6, *lower panel*).

In a second set of experiments, we studied the effects of igmesine on VRCC in HEK and HEK-SIG cells using the patch clamp technique. Volume-activated chloride channels have been extensively described in HEK cells (35). As previously described, application of a hypotonic shock (40 mOsm) induced a slowly developing outwardly rectifying current presenting a reversal potential corresponding to the equilibrium

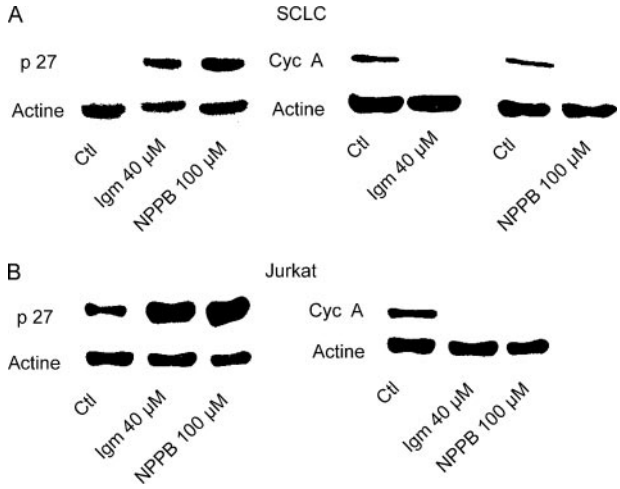

FIGURE 4. **NPPB and igmesine modulate p27 and cycline A levels in SCLC and jurkat cells.** *A*, expression of p27 (*left panel*) and cycline A (*right panel*) in the absence (*Ctl*) and the presence of igmesine (*Igm*) or NPPB (*NPPB*) in SCLC cells. *B*, expression of p27 (*left panel*) and cycline A (*Cyc A*, *right panel*) in the absence (*Ctl*) and the presence of igmesine or NPPB (*NPPB*) in jurkat cells. Actin levels were used as controls in each experiment. The cells were treated with either drug for three days. Immunoblottings show typical examples from three to eight independent experiments.

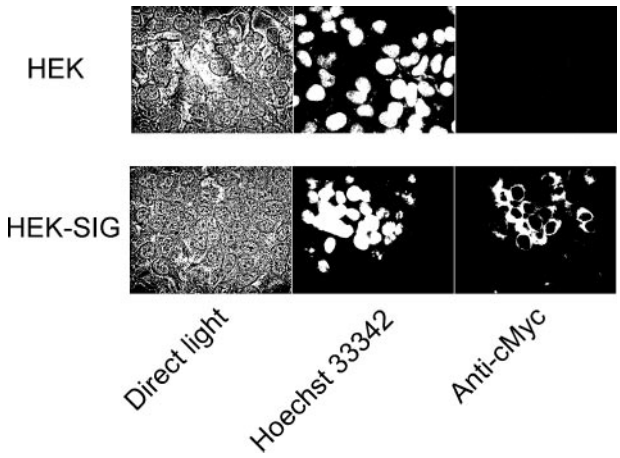

FIGURE 5. **Expression of the sigma-1 receptor in HEK cells.** Micrographs of normal HEK cells (*HEK*, *upper panels*) and HEK cells expressing c-Myc-targeted sigma-1 receptors (*HEK-SIG*, *lower panels*). The pictures of each series show the same area in direct observation (*Direct light*) and the fluorescent counterparts revealing the nucleus (*Hoechst 33342*) or the c-Myc-targeted sigma1 receptor (*anti-cMyc*). Each micrograph was obtained with a 63× oil immersion lens (total magnification, 630×).

potential for chloride anions ($E_{Cl}^- = 6$ mV; Fig. 7*A*). In the 11 tested cells, application of igmesine (10 μM) during the hypotonic shock produced either no effect at all on the current ($n = 8$; Fig. 7*A*) or a slight decrease in amplitude ($n = 3$; not shown). In contrast, igmesine produced a clear cut inhibition of VRCC in the two tested HEK-SIG clones (Fig. 7, *B* and *C*). This demonstrates that the effects of sigma ligands on the current are not the consequence of a direct and nonspecific effect on chloride channels but result in the functional coupling between of sigma-1 receptors and ion channels underlying VRCC.

Sigma-1 receptors have been shown to be specifically overexpressed in tumors. No endogene ligand has been clearly determined yet, so it was interesting to investigate whether the sigma-1 receptor overexpression could produce any effect *per se* on the biophysical properties of VRCC.

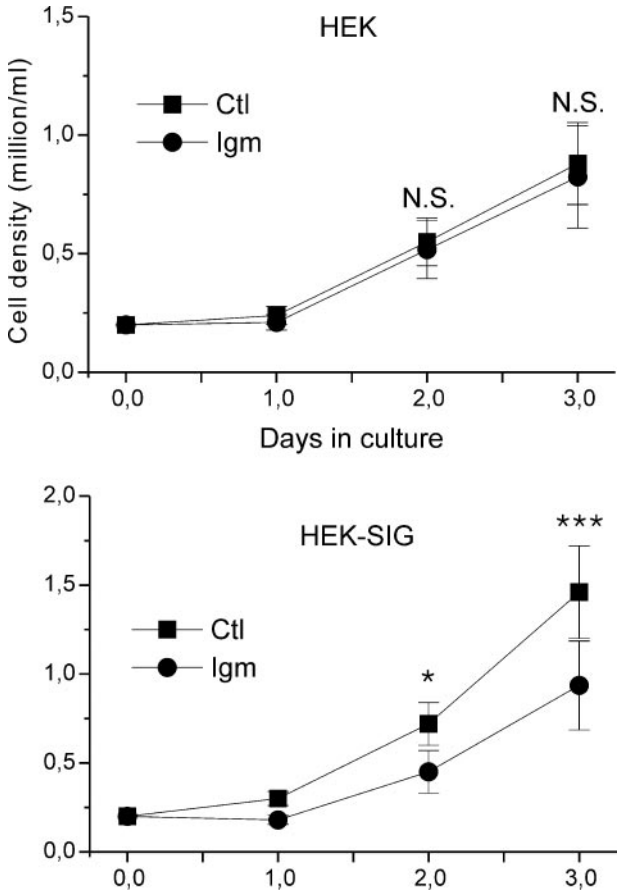

FIGURE 6. **Igmesine decreases proliferation in HEK-SIG cells.** Plots showing the growth rate over three days in the absence (*Ctl*) and the presence of igmesine (40 μM, *Igm*) in HEK (*upper panel*) and HEK-SIG (*lower panel*) cells. The values are the means ± S.E. from three independent experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$. *N.S.*, not significant (Student's paired *t* test).

Sigma-1 Receptor Expression Alters VRCC Activation Properties in HEK Cells—We first analyzed the maximum current density achieved after a hypotonic shock (77 mOsm) in both HEK and HEK-SIG cells. No significant difference could be detected whatever the holding potential (see Fig. 8*A* for a comparison of the mean current densities at −80 mV for HEK and HEK-SIG cells). In a second analysis, we examined the activation kinetic of the current during a hypotonic shock in HEK-SIG compared with normal HEK (Fig. 8*B*). For each tested cell, the activation kinetic could be fitted by a Boltzmann function as previously described (36). Surprisingly, the time for half-maximum activation (T_o) was dramatically augmented in HEK-SIG cells. Additionally, a 2-fold increase in the curve slope (*dx*) could be observed in HEK-SIG cells (Table 1). Altogether, these results demonstrate for the first time that the expression of the sigma-1 receptor modulates VRCC functional properties.

The regulation of cell volume through KCl and water leakage has been shown to be a central event in the control of cell cycle (G_1/S volume set point) and apoptosis signaling pathways (apoptosis volume decrease (AVD)) (37–39). Because cell volume regulation strongly involves VRCC, we next investigated the effects of sigma-1 receptor expression on cell volume regulation in both HEK and HEK-SIG cells.

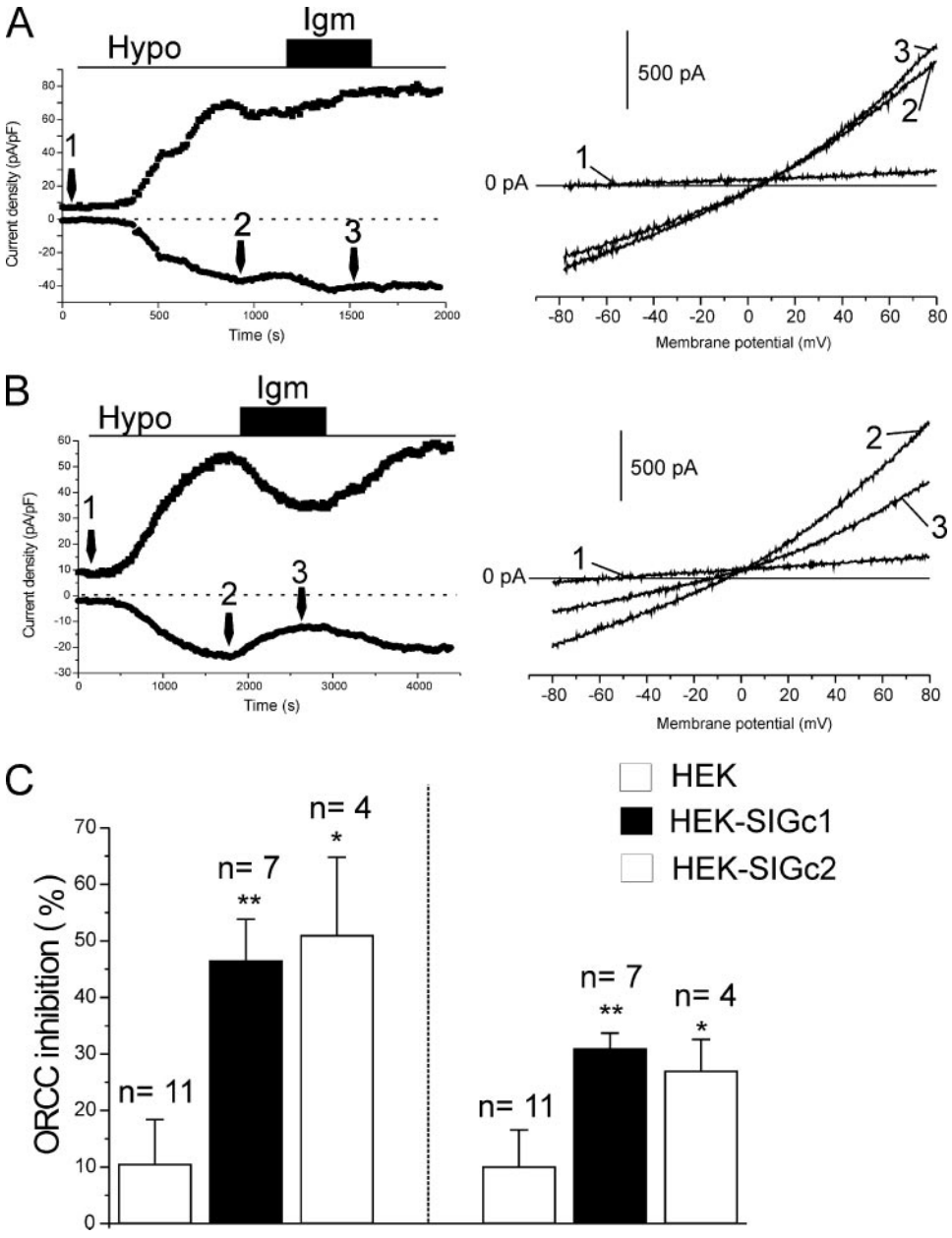

FIGURE 7. **Igmesine decreases VRCC amplitude in HEK-SIG cells.** *A*, evolution of VRCC density during a hypotonic shock in a single nontransfected HEK cell. *Left panel*, currents were evoked by 1-s voltage ramps from −80 to 80 mV. Current densities were measured at −80 (*black circles*) and 80 mV (*black squares*). Igmesine application time (10 μM, *Igm*) is represented by the *thick black bar*. The *thin bar* represents the hypotonic shock (*Hypo*). *Right panel*, corresponding instantaneous *I/V* relationships recorded in isotonic (*line 1*), hypotonic (*line 2*), and hypotonic + igmesine (10 μM, *line 3*) conditions. *B*, evolution of VRCC density during a hypotonic shock in a single HEK-SIG cell. *Left panel*, currents were evoked by 1-s voltage ramps from −80 to +80 mV. Current densities were measured at −80 (*black circles*) and +80 mV (*black squares*). Igmesine application time (10 μM, *Igm*) is represented by the *thick black bar*. The *thin bar* represents the hypotonic shock (*Hypo*). *Right panel*, corresponding instantaneous *I/V* relationships recorded in isotonic (*line 1*), hypotonic (*line 2*), and hypotonic + igmesine (10 μM, *line 3*) conditions. *C*, histogram representing the igmesine-induced inhibition of VRCC in HEK and sigma-1 receptor-transfected HEK cells at −80 (*left panel*) and 80 mV (*right panel*). Two different clones of sigma-1 receptor-transfected cells were used: HEK-SIGc1 and HEK-SIGc2, respectively. Currents were evoked by the same voltage ramps as in *A* and *B*. The values are the means ± S.E. *, $p < 0.05$; **, $p < 0.005$ (Student's *t* test).

Sigma-1 Receptor Expression and Sigma-1 Receptor Pharmacological Activation Alter the Regulatory Volume Decrease (RVD) and the AVD in HEK Cells—In the present set of experiments, we have measured the volume in HEK cells during a hypotonic shock. Both HEK and HEK-SIG cells swelled during the first 2 min following the hypotonic shock and reach up to 119.2 ± 1.1 and 120.2 ± 1.1% of the initial volume, respectively ($n = 3$, each; Fig. 9*A*). Then a decrease occurred within 9 min for HEK cells so that the initial volume was totally recovered as predicted by the model of the RVD mechanism (Fig. 9*A*). However, HEK-SIG cells presented a significantly delayed RVD process because the volume was still of 106.6 ± 1.5% after 10 min ($n = 3$; Fig. 9*A*). Interestingly, the incubation of HEK-SIG cells with igmesine (40 μM) dramatically enhanced the cell volume increase ($n = 3$; 129.4 ± 2.2%) and virtually abolished the RVD because the volume reached a rather high and stable volume (117.8 ± 2.7%) 10 min after the onset of the hypotonic shock ($n = 3$; Fig. 9*A*). In very much the same way (Fig. 9*B*), the incubation of Jurkat cells with igmesine (40 μM) enhanced the volume increase (124.9 ± 1.2% and 129.3 ± 0.9% for control and incubated cells, respectively; $n = 3$, each) and provoked a clear cut inhibition of the RVD; 7 min after the onset of the hypotonic shock, the initial volume was recovered in control cells (101.6 ± 0.4%, $n = 3$), whereas it was still at 118.3 ± 1.0% after the same time delay ($n = 3$). These results demonstrate that in the absence of ligand, the presence of sigma-1 receptor modulated the RVD process. Activating the sigma-1 receptors by specific ligands further amplified the phenomenon, which leads to a blockade of the RVD.

VRCC and the cell volume regulation process are known to be involved in the apoptosis signaling through the mechanism of AVD. For example, in Jurkat cells, apoptosis is accompanied by a membrane shrink that is linked to the activation of VRCC (40). This membrane shrink is considered as an early signaling step in cell death. Thus, we examined whether the expression of sigma-1 receptors could have an influence on the AVD induced by staurosporine, a pro-apoptosis agent. When challenged with staurosporine (100 μM), HEK cells lost up to 65% of their initial volume after 5 h. Interestingly the response of HEK-SIG cells to staurosporine was significantly lowered after 5 h when compared with control cells

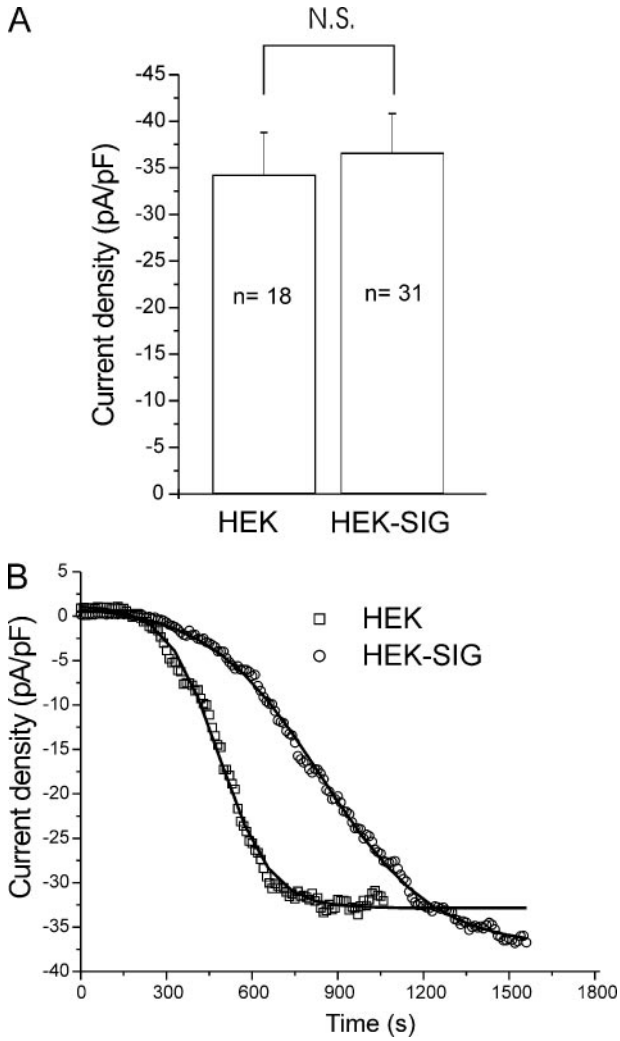

FIGURE 8. **The expression of sigma-1 receptors modulates the activation rate of VRCC in HEK cells.** *A*, histogram comparing the maximum current densities at −80 mV in HEK and HEK-SIG cells challenged with hypotonic shock. The values correspond to the plateau reached by the current during the hypotonic shock. The values are the means ± S.E. *N.S.*, not a significant difference (Student's *t* test). *B*, representative activation rates of the VRCC during a hypotonic shock in single HEK and HEK-SIG cells. The *continuous lines* represent the result of the Boltzman fit performed for each cell (see "Materials and Methods"). Current densities were measured at −80 mV.

TABLE 1

Kinetic properties of the VRCC activation in HEK and HEK-SIG cells

The activation rate of VRCC was measured at −80 mV during a hypotonic shock (see Fig. 8*B*). The current activation was fitted with the Boltzman function $I = [(I_{max} - I_{min})/(1 + e^{(T-T_0)/dx})] + I_{min}$. where T_o and dx represent, respectively, the time for half-maximum activation and the rate of current activation.

	HEK	HEK-SIG	Student's *t* test
dx ($pA^{-1}{\cdot}pF^{-1}{\cdot}s^{-1}$)	97.16 ± 19.20	201.3 ± 18.84	$p < 0.0005$
$T_{½}$ (s)	515.09 ± 90.93	877.11 ± 69.34	$p < 0.005$
n	18	32	

(25,6%; Fig. 10). Incubation of HEK-SIG cells with igmesine (40 μM) further extended the protection against AVD (45.8%; Fig. 10). These data indicate that sigma-1 receptors protect cells from AVD even in the absence of ligand. This suggests that the expression on these receptors in tumors may protect cells from apoptosis through a VRCC- and volume-dependent pathway.

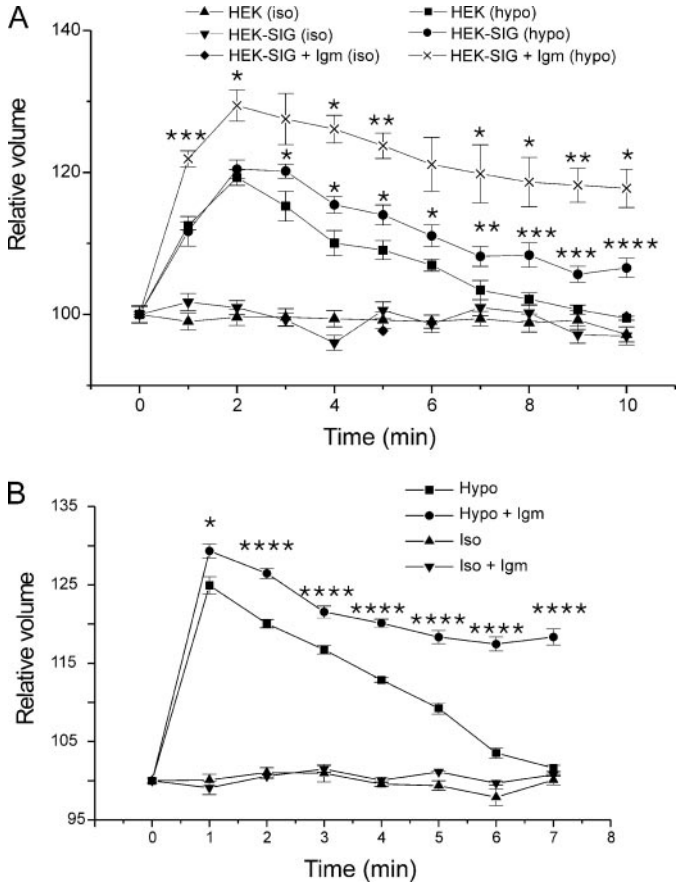

FIGURE 9. **The sigma-1 receptor modulates RVD in HEK and Jurkat cells.** *A*, evolution of cell volume during a hypotonic shock in HEK, HEK-SIG, and HEK-SIG cells in the presence or the absence of igmesine (40 μM). *B*, evolution of the cell volume during a hypotonic shock in Jurkat cells in the presence or the absence of igmesine (40 μM).Control experiments were performed in the isotonic medium in the absence or the presence of igmesine (40 μM).

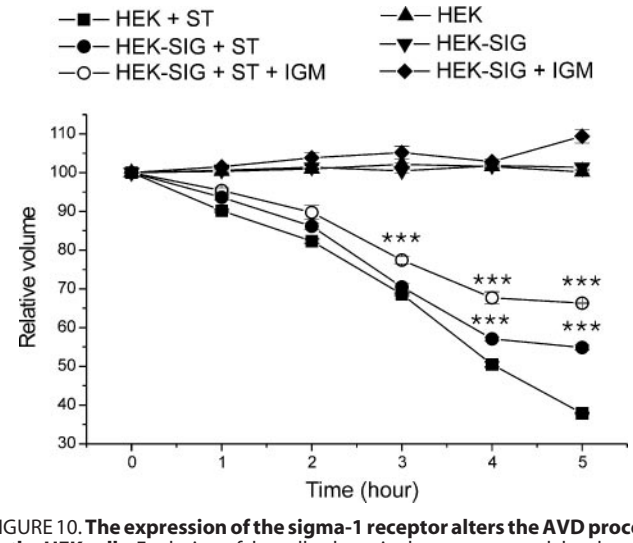

FIGURE 10. **The expression of the sigma-1 receptor alters the AVD process in the HEK cells.** Evolution of the cell volume in the presence and the absence of staurosporine (100 μM, *ST*) or igmesine (*Igm*, 40 μM) in HEK and HEK-SIG cells. The values are the means ± S.E. ***, $p < 0.001$ (Student's *t* test).

DISCUSSION

Sigma-1 receptors are known as tumor markers (11, 13, 41), but the function of these enigmatic proteins in cancer cells has not been clearly described yet. The aim of the present study was

to examine a possible interaction between sigma-1 receptors and VRCC, a class of membrane currents that have been involved in the control of tumor cell cycle progression and apoptosis (27–29, 39, 42). Herein, we demonstrate for the first time that sigma-1 receptors modulate VRCC properties leading to alterations of both cell division and apoptosis signalization in leukemic and SCLC cells.

Volume-regulated chloride channels underlying VRCC have been recently involved in the control of the cell cycle. For example, the inhibition of these currents by blockers such as NPPB stops cell proliferation in liver cells (43). Moreover, the expression of chloride channels is tightly regulated along the cell cycle progression in carcinoma cells (27). Thus, we first studied the effects of highly specific sigma-1 receptor ligands on VRCC. The patch clamp experiments we performed showed the presence of VRCC in both SCLC and jurkat cells upon external hypotonic shock and demonstrated for the first time that these currents are inhibited by sigma ligands. In the case of Jurkat cells, a stable current after hypotonic shock was difficult to achieve, so we have preincubated cells with sigma ligands: the comparison of the maximum current densities in control and incubated cells showed that sigma ligands significatively lower VRCC (more than 40% for igmesine or (+)-pentazocine, 10 μM). At this stage, it could not be excluded that sigma ligands directly interacted with chloride channels without involving sigma-1 receptors. To run out this possibility, we studied the effects of sigma ligands on two different HEK293 cell types. The first one is the normal cell line, whereas the other one stably overexpressed the sigma-1 receptor (HEK-SIG). In the control cells, igmesine produced none or very slight effects on VRCC. By contrast, the sigma-1 receptor ligand strongly inhibited the VRCC in the HEK-SIG cells, clearly demonstrating that the sigma ligands modulated chloride channels through the activation of sigma-1 receptors. The slight effects detected in the control cells can be explained by a low basal expression of the protein, revealed by a newly developed antibody (generous gift from Drs. L. Combettes and F. Monnet; not shown).

In a previous work, we have demonstrated that the pharmacological activation of these receptors by igmesine blocked tumor cell cycle through a mechanism involving the inhibition voltage-dependent K^+ channels (25). Altogether, our data showing the modulation of both potassium and chloride flux across the membrane by sigma ligands indicate that the sigma-1 receptors modulate the RVD process. This mechanism is widely shared among animal cells, allowing them to recover a normal volume after a membrane swelling through the loss of water and KCl. This process is tightly regulated by potassium and chloride channels such as VRCC and voltage-gated potassium channels (44, 45). In the present work, we demonstrate that igmesine strongly inhibits RVD in both Jurkat and HEK-SIG cells. Interestingly, the RVD process has been shown to participate to the control of the cell cycle progression because its inactivation arrests cells at the end of the G_1 phase (37, 46). A cycling cell encounters osmotic stresses that are the consequence of the metabolite accumulation in the cytoplasm or the depolymerization of the actin cytoskeleton, both leading to a cell swelling. It has been proposed that the cell volume is a key factor that is controlled at the end of the G_1 phase to allow the transition toward the S phase (volume set point). A constant volume may help to maintain the correct concentrations of cell cycle-controlling proteins; it may also avoid the destabilization of the cytoskeleton in response to membrane stretches that may in turn alter the ROCK/mDia balance (ROCK and mDia are two downstream effectors of Rho-A) and thus lead to p27 accumulation (47). In the light of these data, we showed that the cell incubation with NPPB, a classical blocker of VRCC and RVD, inhibits proliferation in SCLC and jurkat cells. The cell cycle arrest provoked by NPPB is underlined in both SCLC and Jurkat cells by an accumulation of the CDK inhibitor p27, but not $p21^{cip1}$ (not shown), and the subsequent decrease in cyclin A, which is consistent with a blockade in the G_1 phase (25, 26, 48, 49). Interestingly, we had previously demonstrated that the sigma receptor ligands and K^+ channel blockers provoke a cell cycle arrest with the same modulation profile, *i.e.* a p27 accumulation, a decrease in cycline A, but no effect on $p21^{cip1}$ (25), indicating that sigma ligands and potassium and chloride channels blockers share a common pathway to modulate p27. Altogether, these results strongly suggest that the activation of the sigma-1 receptors alters the G_1/S transition through the inhibition of potassium and VRCC channels and the subsequent inhibition of the RVD process.

A question arises from our observations. Why do tumor cell overexpress sigma-1 receptors? A striking clue came from the observation of the kinetic properties of the VRCC in HEK-SIG cells in the absence of any sigma ligand. Although the expression of the sigma-1 receptors did not alter the current density, we observed that it provoked a huge delay in the current activation kinetics. In good agreement, we report that the RVD process is not inhibited but delayed in HEK-SIG cells when compared with control cells. We then wondered why a delayed VRCC would be an advantage for tumor cells. Some recent studies have nicely demonstrated that the cell shrink (AVD) was an early stage of the apoptosis signaling cascade in various tumor cell types (20, 39). This volume-related signaling event is linked to the activation of both potassium and VRCC currents and the subsequent KCl-coupled water efflux (20, 39, 50). In the light of these data, we propose that the strong expression of the sigma-1 receptors increases the apoptosis resistance in tumor cells through the down-regulation of the VRCC activation. In support of this, we demonstrate here that the staurosporin-triggered AVD is diminished in HEK-SIG cells when compared with control HEK. This hypothesis is reinforced by a recent study showing that sigma-1 receptor expressing cells such as tumor cells are less resistant to apoptotic stress than normal cells and that this resistance is abolished by sigma receptor antagonists (51). Moreover, Aydar *et al.* (4) have recently demonstrated that the expression of sigma-1 receptors was able to produce a tonic down-modulation of voltage-dependent potassium channels in *xenopus* oocytes, suggesting that potassium channels also participate in the sigma-1 receptor-dependent apoptosis resistance we propose here. In conclusion, our study demonstrates for the first time that sigma-1 receptors modulate chloride channels and the cell volume regulation through an interaction with sigma receptors. In the absence of exogenous sigma ligands, the overexpression of sigma-1 receptors limits VRCC activity so that the protection against AVD is reinforced

but not enough to block RVD and the cell cycle progression. By contrast, when tumor cells are challenged with sigma ligands, this balance is strongly altered and the RVD is inhibited, leading to an arrest of proliferation. In conclusion, our study brings very new elements in the understanding of both function and action mechanisms of sigma-1 receptors and may lead to the development of ion channel targeted cancer therapy through sigma-1 receptor modulation.

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Membrane Transport, Structure, Function, and Biogenesis:

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