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Effects of Imipramine on Ion Channels and Proliferation of IGR1 Melanoma Cells

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Abstract. Human IGR1 cells are a model for malignant melanoma. Since progression through the cell cycle is accompanied by transient cell hyperpolarization, we studied the properties of potassium and chloride ion channels and their impact on cell growth. The major potassium current components were mediated by outward rectifying *ether à go-go* (hEAG) channels and Ca^{2+}-activated channels (K_{Ca}) of the IK/SK type. The major chloride channel component was activated by osmotic cell swelling (Cl_{vol}). To infer about the contribution of these channels to proliferation, specific inhibitors are required. Since there is no specific blocker for hEAG available, we used the tricyclic antidepressant imipramine, which blocked all channels mentioned, in combination with blockers for K_{Ca} (charybdotoxin) and Cl_{vol} (DIDS and pamoic acid). Incubation of IGR1 cells for 48 hr in 10–15 μM imipramine reduced DNA synthesis and metabolism without significant effects on apoptosis. hEAG channels were most sensitive to imipramine (IC_{50}: 3.4 μM at +50 mV), followed by K_{Ca} (13.8 μM at +50 mV) and Cl_{vol} (12 μM at −100 mV), indicating that hEAG expression may be of importance for proliferation of melanoma cells. The contribution of K_{Ca} channels could be excluded, as 500 nM charybdotoxin, which completely blocked K_{Ca}, had no effect on proliferation. The impact of Cl_{vol} also seems to be minor, because 500 μM pamoic acid, which completely blocked Cl_{vol}, did not affect proliferation either.

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Introduction

Malignant melanoma is the fastest-rising malignancy of the last four decades, commonly associated with a very poor prognosis for the patient. This aggressive cancer hardly responds to conventional chemo- and radiotherapy due to its high level of drug resistance as well as a successful escape from apoptotic pathways (Satyamoorthy, Bogenrieder & Herlyn, 2001). The tumor progression correlates with reduced growth-factor dependence of the transformed cells, finally leading to malignant melanoma cells, which secrete autocrine growth factors, usually not expressed by melanocytes or benign nevus cells, like the melanoma growth-stimulatory activity protein (Lázár-Molnár et al., 2000). On the molecular level, the functional effectors leading to nonregulated proliferation are only beginning to be understood. Among many other factors, the expression of specific ion channels has been proposed to contribute to enhanced cellular proliferation (Wonderlin & Strobl, 1996).

Functional potassium channels might be required for hyperpolarization, which is necessary for the cell to progress through the G1 phase of the cell cycle (Wonderlin & Strobl, 1996), while upregulation of volume-activated chloride channels was proposed to modulate cell size during the G1/S phase transition (Nilius et al., 1996). Electrophysiological analysis of three melanoma cell lines (IGR1, IGR39, IPC298) revealed the expression of voltage-gated as well as calcium-activated potassium channels in variable

composition (Meyer et al., 1999). The voltage-activated potassium channel *ether à go-go* (hEAG), which is expressed in IGR1 and IPC298 cells, has also been detected in breast carcinoma cell lines (EFM19, MCF-7; Pardo et al., 1999) and neuroblastoma cells (SH-SY5Y, Meyer & Heinemann, 1998). This channel gained increased interest with respect to cell-cycle progression of tumor cells, since Pardo and co-workers (Pardo et al., 1999) showed that inhibition of hEAG expression by means of antisense oligonucleotides caused a significant reduction in cell proliferation in cancer cell lines. In addition, expression of this channel in Chinese hamster ovary cells (CHO) increased both cell proliferation rate and metabolic activity. In a mouse model, these hEAG-transfected cells caused rapidly growing tumors, while control CHO cells were much less aggressive, suggesting an advantage of this channel for cancer cells in vivo (Pardo et al., 1999). While hEAG might influence the cell cycle progression, regulation of this channel through the cell cycle has also been observed. Meyer & Heinemann (1998) showed that native hEAG in SH-SY5Y cells was downregulated by synchronization of the cells in the G0/1 phase. In addition, the electrophysiological properties of hEAG1 undergo changes during the cell cycle, e.g., increased selectivity for potassium ions and increased sensitivity to block by intracellular sodium occurred during the M phase (Brüggemann, Stühmer & Pardo, 1997; Camacho et al., 2000). As putative mechanism for the oncogenic potential of EAG potassium channels, the lowered cellular resting potential caused by these channels has been proposed (Pardo et al., 1999).

A similar function is discussed for calcium-activated potassium channels (K_{Ca}), which have also been implied to promote the cell proliferation process. These channels respond poorly to voltage changes, but open at elevated intracellular calcium concentrations. The resulting potassium efflux could provide the membrane hyperpolarization necessary for cell cycle progression (Nilius & Wohlrab, 1992; Lepple-Wienhues et al., 1996; Wonderlin & Strobl, 1996). In IGR1 melanoma cells, such K_{Ca} currents were observed and mRNA of small- and intermediate-conductance K_{Ca} channels (hIK, hSK1, hSK2) was detected by RT-PCR (Meyer et al., 1999). High levels of IK current were also reported for rat prostate cancer cells as well as for fibroblasts transfected with oncogenic Ras or Raf genes (Rane, 2000). The fibroblast IK channel is shown to be upregulated via the Ras mitogen-activated protein kinase pathway, and its regulatory role for tumor cell growth is under discussion (Peña & Rane, 1999; Rane, 2000).

Apart from potassium currents, chloride channels can also be involved in cell growth and proliferation. In particular, chloride channels activated by cell swelling (Cl_{vol}) were shown to be important for the proliferation process in many cell types (Nilius et al., 1996; Wondergem et al., 2001). Shen and co-workers showed that the arrest of human cervical cancer cells in G0/G1 phase by aphidicolin is accompanied by a significant increase in Cl_{vol} activity (Shen et al., 2000). In turn, the pharmacological blockade of the current by tamoxifen or NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid) inhibited cell proliferation. In IGR1 melanoma cells such Cl_{vol} channels were described by Nilius and co-workers (Nilius et al., 1994).

Here we address the question whether EAG potassium channels promote the proliferation of the human melanoma cell line IGR1. Using the channel blocker imipramine, we could inhibit cellular proliferation at concentrations sufficient to block EAG channels at the normal cellular resting potential. Since imipramine might also block other ion channels expressed in IGR1 cells, we characterized those ion channels that can contribute to cell hyperpolarization (potassium and chloride channels) with respect to their pharmacological properties. Imipramine blocked K_{Ca} channels of the SK/IK type, expressed in this cell line. However, charybdotoxin (ChTX), a specific blocker of these channels, did not affect cell growth. Also Cl_{vol} channels were blocked by imipramine. However, proliferation of IGR1 melanoma cells was not affected by pamoic acid and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) in concentrations sufficient to provide a complete block of Cl_{vol} channels at the membrane potential of proliferating cells. Thus, we show here that the tricyclic antidepressant imipramine, albeit not very specific, blocks human EAG1 channels and that this blockade impairs proliferation of IGR1 melanoma cells.

Material and Methods

Cell Culture

The human melanoma cell line IGR1, Chinese hamster ovary cells (CHO-K1), and human embryonic kidney cells (HEK 293) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All cells were maintained in 250-ml culture flasks in a humidified atmosphere at 37°C and were passaged every 3–4 days. IGR1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum (FCS) in 10% CO_2. CHO-K1 cells were cultured in DMEM-HAM's F-12 (Biochrom, Berlin, Germany) supplemented with 10% FCS in 5% CO_2. HEK 293 cells were grown in 45% DMEM, 45% DMEM-HAM's F-12 and 10% FCS in 5% CO_2.

Transfection

hEAG1 (Schönherr, Löber & Heinemann, 2000) was subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands). Stable transfection of CHO-K1 cells was carried out following the SuperFect method (Qiagen, Hilden, Germany). Two days after plating in 8.8-cm^2 wells (50% confluent), 2 μg linearized DNA, to enhance chromosomal insertion, and 10 μl

SuperFect were incubated with the cells for 4 hours. Cell lines were established by selection through resistance against the antibiotic G-418, encoded in the vector.

Proliferation Studies

Cells were trypsinized and plated at a density of $2–4 \times 10^4$ cells/ml in a final volume of 100 µl/well in 96-well plates. Drugs were added to the cells 24 hours after plating. After 48 hours, metabolism and proliferation were estimated. For determination of the cell numbers, cells were cultured in 24-well plates (500 µl/well) and counted in a cell counter (CASY Cell Counter Model DT, Schärfe System, Reutlingen, Germany).

The colorimetric MTT-assay (Roche, Mannheim, Germany) is based on the cleavage of the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to a formazan. This reduction requires the pyridine nucleotide cofactors NADH and NADPH and therefore is a measure for metabolic activity. The assay kit was used according to the manufacturer's instructions. 0.5 mg/ml MTT was added to the cell cultures at the time point indicated. Four hours later the formazan crystals were solubilized for 24 hours and the colored product was measured at 570 nm.

A colorimetric immunoassay (BrdU, Roche) was used for quantification of cell proliferation. This assay determines DNA-synthesis, based on BrdU incorporation, and was applied following the instructions with a labeling period of 24 hours.

FACS Analysis

DNA-content measurements of propidium iodide-stained nuclei were performed on a flow cytometer FACScan (Becton Dickinson, San Diego, CA). Cell cycle phases were calculated using the program Mod FIT LT (Verity Software House, Topsham, ME). For FACS analysis of cell-cycle distribution, cells were detached from the culture dishes with trypsin-EDTA. A nuclear suspension was prepared using a solution of citric acid/Tween 20 in phosphate buffer saline. Fixation was performed with 70% cold ethanol. The suspensions were stored overnight at 4°C. After treatment with RNase for 10 min, the DNA was stained with propidium iodide.

To assess apoptosis, IGR1 cells were trypsinized and plated at a density of 6×10^4 cells/ml in a final volume of 5 ml in 50-ml culture flasks. Cells were cultured in the same medium as mentioned above. Twentyfour hours after plating, imipramine was added at final concentrations of 10, 20, 30 and 50 µM. After 24 hr and 48 hr, supernatant and trypsinized cells were pooled and then aliquots of 10^6 cells/ml were prepared for FACS analysis. The degree of apoptosis was determined using multicolor analysis by Annexin-V-FITC (Pharmingen, San Diego, CA)-binding to phosphatidylserine and DNA-staining by propidium iodide (PI). To reject cell fragments, an appropriate gate was set within the dot plot of forward- and side-scatter light signals. The calculation of the proportion of viable (FITC- and PI-negative), apoptotic (FITC-positive and PI-negative), and late apoptotic (FITC- and PI-positive) cells was performed by quadrant analysis using WinMDI software version 2.8 (written by Joseph Trotter, available as free software on the web). As a control, we also subjected IGR1 cells to a treatment with 22 µM betulinic acid (BioService Halle, Halle, Germany), previously reported to potently induce apoptosis in human melanoma cells (Pisha et al., 1995).

Electrophysiological Measurements and Data Analysis

For electrophysiological recordings, cells were trypsinized and plated in petridishes, 35 mm in diameter. The recordings were performed 1–2 days after plating in the whole-cell patch-clamp configuration using an EPC9 (HEKA Elektronik, Lambrecht, Germany) patch-clamp amplifier. Pulse protocol generation and data acquisition were controlled with the program Pulse (Heka Elektronik). Correction for leak currents and capacitive transients was performed routinely using a P/n method when appropriate (i.e., in the absence of ionic current components at negative membrane voltages). Series resistance errors were compensated in the range of 70–80%. Data were low-pass filtered at 5 kHz. Patch pipettes were fabricated from Kimax-51 glass (Kimble Glass, Vineland, NJ) and were of 1–3 MΩ resistance. All experiments were performed at 19–20°C. The resting potential of IGR1 cells was measured in the current-clamp mode. For data analysis, the programs PulseFit (HEKA Elektronik) and Igor-Pro (Wave-Metrics, Lake Oswego, OR) were used.

Data for each data point shown were obtained from the indicated number (n) of independent measurements, i.e., independent cells, presented as mean ± SEM. Statistical significance ($P < 0.05$) of the difference between two means was estimated with Student's t-test, unless stated otherwise.

To describe drug-channel interaction, a first-order blocking scheme was used. The apparent IC_{50} and Hill coefficient (h) were obtained by fitting the normalized current ($I/I_{control}$) at various drug concentrations [D]:

$$I/I_{control} = (IC_{50}/[D])^h/(1 + (IC_{50}/[D])^h) \quad (1)$$

Solutions and Chemicals

For potassium current measurements, the standard extracellular solution was composed of (mM): 5 KCl, 135 NaCl, 2 $CaCl_2$, 10 HEPES, adjusted to pH 7.4 with NaOH. The standard pipette solution was (mM): 130 KCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES, adjusted to pH 7.4 with KOH. Calcium-activated potassium currents were measured with 800 nM free calcium in the pipette, i.e., 9.3 mM $CaCl_2$ was added to the pipette solution.

For chloride current measurements, the standard extracellular solution contained (mM): 5 CsCl, 110 NaCl, 2 $CaCl_2$, 80 mannite, 10 HEPES, adjusted to pH 7.4 with NaOH, osmolarity 307 mOsm/l as measured with a vapor pressure osmometer (Wescor 5500, Wescor, Logan, UT). In the hypotonic extracellular solution, mannite was not added, yielding an osmolarity of 237 mOsm/l. The pipette solution contained either (mM): 130 CsCl, 2 $MgCl_2$, 5.79 $CaCl_2$, 10 EGTA, 4 NaATP, 10 HEPES, adjusted to pH 7.4 with NaOH; free Ca^{2+}: 100 nM, 301 mOsm/l. For experiments in which pamoic acid and DIDS were applied, the pipette was filled with (mM): 130 TEA-Cl, 2 $MgCl_2$, 4.07 $CaCl_2$, 10 EGTA, 4 NaATP, 10 HEPES, adjusted to pH 7.4 with NaOH; free Ca^{2+}: 50 nM, 298 mOsm/l. To facilitate seal formation, the pipette tip was filled with similar solutions but without NaATP in both cases.

Imipramine and 4-amino-10-methylfolic acid (methotrexate) (Sigma) were kept in water as 100 and 10 mM stock solutions, respectively. Charybdotoxin (ChTX) (Sigma) was kept as 10-µM water solution buffered with 10 mM HEPES to pH 6.9. NPPB, tamoxifen, flufenamic acid and DIDS were obtained from Sigma and dissolved in dimethylsulfoxide (DMSO) as 150, 50, 100, and 100 mM stock solutions, respectively. Final DMSO concentrations applied with the drugs were not higher than 0.1%. 100 mM stock solutions of pamoic acid (Sigma) were prepared in external solutions for electrophysiological measurements for potassium and chloride currents, respectively. All stock solutions were stored at −18°C and were diluted in recording buffer immediately before the experiments.

Drug Stability Test

Some of the drugs were used for proliferation assays, i.e., they were applied to cell culture media at 37°C. In order to test for drug sta-

bility under these conditions, imipramine, ChTX, pamoic acid, and DIDS were dissolved in DMEM containing 10% FCS and were incubated for 48 hr at 37°C with 10% CO_2. The activity of the substances was subsequently tested with electrophysiological methods.

Imipramine, which was incubated in a concentration of 10 μM, was applied to *Xenopus* oocytes heterologously expressing hEAG1 channels. Currents were measured with a two-electrode voltage clamp. The bath solution was changed to DMEM, then to DMEM containing 10% FCS. The application of medium with FCS caused a strong transient decrease of hEAG1 current, which recovered after about 20 min. After that equilibration period, application of 10 μM incubated imipramine resulted in 32 ± 2% (n = 4) block of the current at +20 mV compared with 25 ± 6% (n = 3) for fresh imipramine; i.e., no statistically significant difference was detected ($P > 0.05$).

ChTX was incubated in a concentration of 200 nM. Application to *Xenopus* oocytes expressing mKv1.3 channels (Stühmer et al., 1989) yielded the following block at +30 mV: 1 nM ChTX 7.2 ± 1.3% (n = 3); 5 nM 33.5 ± 4.0% (n = 5); 10 nM 49.1 ± 4.9% (n = 6); 50 nM 66.1 ± 7.9% (n = 3). Application of the fresh ChTX diluted with the same medium blocked the current in a similar manner: 1 nM ChTX 8.0 ± 3.3% (n = 3); 5 nM 38.4 ± 5.5% (n = 4); 10 nM 61.3 ± 9.1% (n = 3); 50 nM 71.6 ± 4.8% (n = 3). The differences between the means for the partial block by the incubated and fresh drug at correspondent concentrations were insignificant ($P > 0.05$).

Stability test for pamoic acid and DIDS was performed by external application of the drugs, preincubated at a concentration of 1 mM, to the IGR1 cells in whole-cell patch configuration. At +100 mV, 20 μM incubated pamoic acid blocked volume-activated chloride currents by 24.4 ± 1.7% (n = 5), 100 μM blocked by 59.3 ± 2.3% (n = 3). 20 μM fresh pamoic acid blocked the chloride current by 37.7 ± 4.9% (n = 5), 100 μM by 70.1 ± 4.5% (n = 4). The means differed significantly ($P < 0.05$), i.e., the potency of pamoic acid decreased after incubation by about 20%. 20 μM incubated DIDS blocked by 50.2 ± 5.5% (n = 6), 200 μM by 84.1 ± 7.8% (n = 3), while fresh DIDS blocked by 53.0 ± 1.9% (n = 10, 20 μM) and 84.7 ± 2.0% (n = 6, 200 μM), respectively. The differences were not significant.

Thus, imipramine, ChTX, and DIDS were fully stable after 48 hr under cell culture conditions, while the activity of pamoic acid slightly decreased.

Results

Block of Potassium Channels in IGR1 Melanoma Cells

In IGR1 melanoma cells two major types of K^+ channels are expressed. A voltage-dependent outwardly rectifying K^+ current is mediated by hEAG channels, while voltage-independent currents in the presence of intracellular Ca^{2+} are associated with calcium-activated K^+ channels of the IK and SK type (Meyer et al., 1999). A possible contribution of the underlying ion channels to cell proliferation would be ideally tested by the very specific blockade of only a single component. K^+ channels of the EAG family, however, seem to be very resistant to most K^+ channel blockers (Meyer & Heinemann, 1998), and those substances that inhibit EAG currents (e.g., quinidine) also block other K^+ channels. Thus, at present, no substance is known that selectively blocks EAG channels (Bauer & Schwarz, 2001). In a recent report (Weinshenker et al., 1999) it was shown that the tricyclic antidepressant imipramine potently inhibits the related ELK channels from the nematode *Caenorhabditis elegans*. The same study included data on murine EAG channels, which were also blocked by imipramine, albeit at higher concentration. We therefore chose imipramine as a potential blocker for human EAG1 channels in IGR1 cells. Since no perfect specificity for hEAG1 channels was to be expected, we additionally performed experiments with ChTX, which was shown to block K_{Ca} channels in IGR1 cells (Meyer et al., 1999).

Even without pharmacological means, EAG channels are easily identified by their prepulse-dependent opening kinetics (Ludwig et al., 1994; Terlau et al., 1997; Meyer et al., 1999). In Fig. 1*a*, a typical whole-cell patch-clamp recording from an IGR1 cell is shown. In the absence of intracellular Ca^{2+}, depolarizations to +50 mV are applied, preceded by prepulses to either −60 or −120 mV. The kinetics of the elicited currents differ quite strongly—a safe criterion for hEAG channels in these cells. These hEAG-specific outwardly rectifying K^+ currents were not blocked by 500 nM ChTX (Fig. 1*b*). However, increasing concentrations of imipramine blocked the current in a use-dependent manner (Fig. 1*c*). The block reached steady state in less than a second (*not shown*) and was completely reversible. A normalized dose-response curve for hEAG1 block at +50 mV is shown in Fig. 1*d* (filled circles). Fit of a Hill equation (Eq. 1) yielded an apparent IC_{50} value of 3.4 ± 0.5 μM. The block of hEAG1 by imipramine is voltage-dependent: at +10 mV a fit of the Hill equation yielded an apparent IC_{50} value of 8.4 ± 2.5 μM (Fig. 1*d*, open circles).

As the hEAG1 current density in IGR1 cells (5.9 ± 0.1 pA/pF at +60 mV, n = 68) was too small to measure block at weaker depolarizations, we determined the voltage dependence of block in CHO cells stably expressing hEAG1 channels (mean current density at +60 mV; 108 ± 22 pA/pF, n = 35). In Fig. 1*e*, dose-response curves are shown for −30 through +50 mV. The estimated IC_{50} values are plotted in Fig. 1*f* as a function of test voltage, indicating the voltage dependence of block. At the resting voltage of IGR1 cells of about −30 mV (*see below*) an IC_{50} value of about 15 μM was obtained.

Calcium-activated K^+ currents were measured in IGR1 cells in the presence of 800 nM free intracellular Ca^{2+} by application of voltage ramps between −100 and +50 mV (1.5 sec). In the absence of nonspecific leak current, the resulting currents reverse at the K^+ equilibrium potential of about −80 mV (Figs. 2*a, b*). At +50 mV the mean current density was 69.4 ± 12.1 pA/pF (n = 36). Extracellular application of ChTX reversibly blocked these currents in a voltage-independent manner (Fig. 2*a*). The dose-re-

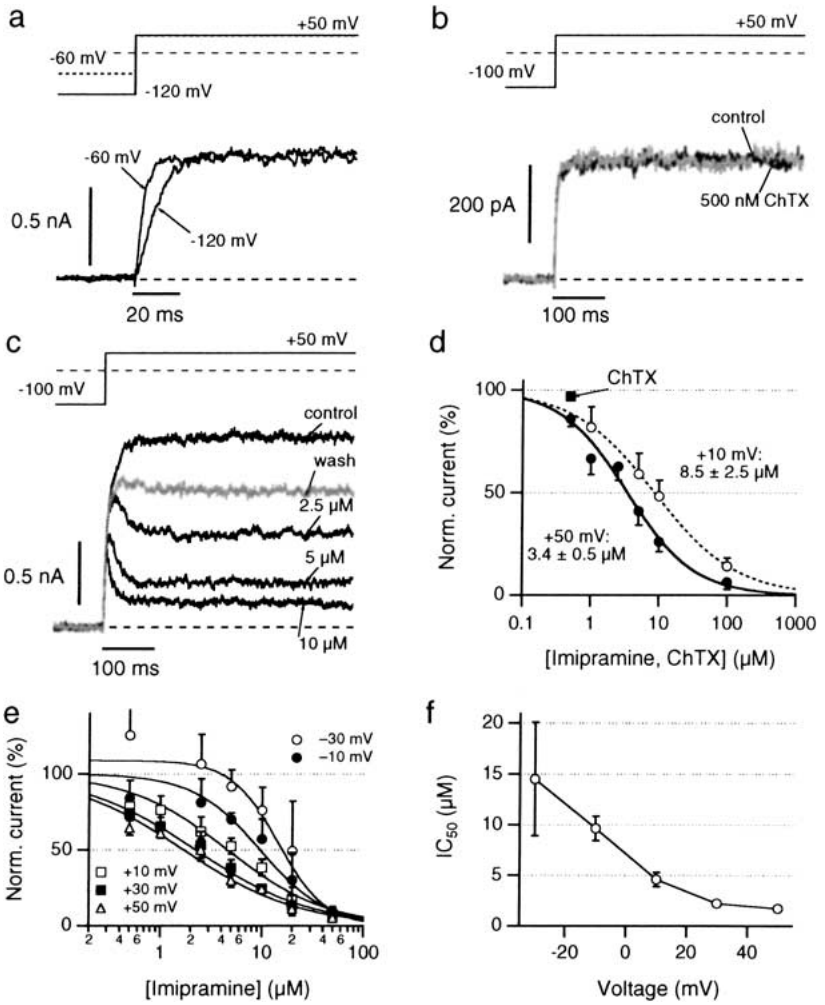

Fig. 1. Sensitivity of hEAG K^+ channels to imipramine and ChTX in IGR1 cells. (*a*) Activation kinetics of EAG channels is dependent on the holding membrane potential preceding the depolarization (Cole-Moore shift). The cell was depolarized to +50 mV after 5 sec holding at −120 mV and −60 mV, respectively. (*b*) Superposition of hEAG current recordings before and after application of 500 nM ChTX. (*c*) Block of whole-cell hEAG current by the indicated concentrations of imipramine. During the course of an experiment the amplitude of hEAG currents sometimes declined (rundown). (*d*) Dose-response plots for hEAG channel block by imipramine (●) and ChTX (■) at +50 mV. Currents elicited as in (*a*) and (*b*) were measured at the end of the depolarization pulse, normalized to the mean of control and "wash" current. The data are presented as mean ± SEM (n = 3–5), fitted with a Hill function yielding at +50 mV an IC_{50} of 3.4 ± 0.5 μM and a Hill coefficient of 0.89 ± 0.10 for imipramine. At +10 mV (○) the IC_{50} was 8.5 ± 2.5 μM, Hill coefficient (0.73 ± 0.14). (*e*, *f*) Imipramine block of hEAG1 channels, heterologously expressed in CHO-K1 cells. (*e*) Dose-response curves at the indicated voltages with Hill fits. (*f*) IC_{50} values estimated from the data shown in (*e*) as a function of membrane voltage.

sponse curve yielded an apparent IC_{50} value of 2.5 ± 0.1 nM at +50 mV (Fig. 2*c*, *filled squares*); the relative block at 0 mV (*open squares*) was virtually identical. Imipramine also blocked K_{Ca} channels in IGR1 (Fig. 2*b*). The dose-response curve at +50 mV yielded an apparent IC_{50} value of 13.8 ± 1.4 μM. The relative block at 0 mV was similar (open circles in Fig. 2*c*), but it appeared that the block at −100 mV was much weaker; fit of the Hill equation yielded an apparent IC_{50} value of 104 ± 23 μM (*data not shown*). Thus, at +50 mV, imipramine blocks hEAG1 channels only about 4 times more potently than K_{Ca} channels, but with the additional aid of ChTX a pharmacological discrimination of both K^+ channel components should be feasible.

Imipramine Inhibits Proliferation and Metabolic Activity of IGR1 Cells

Based on these findings, we studied proliferation of IGR1 melanoma cells in the presence of imipramine and ChTX. To monitor proliferation, we used as parameters the DNA synthesis (BrdU incorporation) and the metabolic activity (MTT assay) (*see* Methods). As shown in Fig. 3*a* (*open circles*), in both assays, increasing concentrations of imipramine reduced the proliferation of IGR1 cells. The MTT assay seemed to be slightly more sensitive, showing a significant effect at 10 μM imipramine. DNA synthesis started to be significantly reduced at a concentration of 15 μM. Apparent half-maximal metabolic activity and DNA synthesis were estimated to occur at 34 and 44 μM imipramine, respectively.

ChTX, applied at a concentration of 500 nM that would completely block K_{Ca} channels, did not influence proliferation of IGR1 melanoma cells. In both proliferation assays, ChTX-treated cells showed at least 90% activity compared to the controls (Fig. 3*a*, filled squares). The stability of ChTX and imipramine during cell culture was verified by blocking mKv1.3 and hEAG1 channels expressed in *Xenopus* oocytes (*see* Methods).

As a control for nonspecific imipramine effects we also measured proliferation of HEK 293 (*downward triangles*) and CHO-K1 cells (*upward triangles*). Up to 50 μM imipramine, the proliferation of these cell lines is

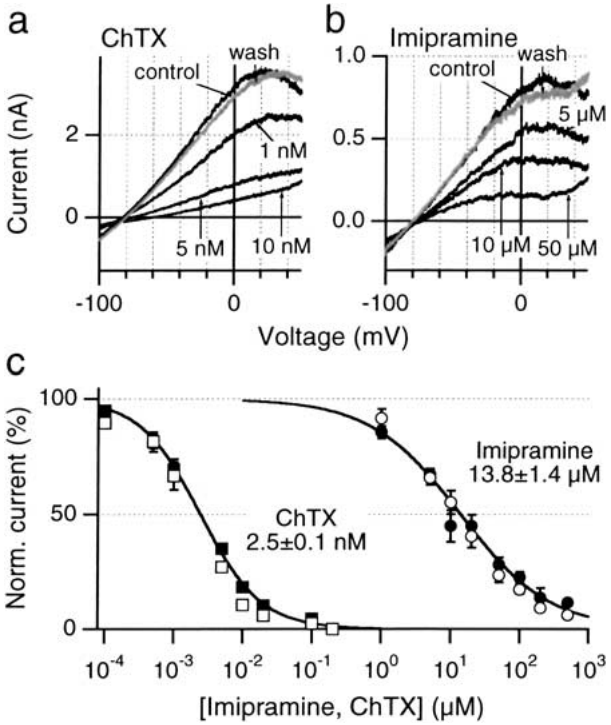

Fig. 2. Sensitivity of calcium-activated K^+ channels in IGR1 cells to ChTX and imipramine. (*a*) Block of calcium-activated K^+ currents by the indicated ChTX concentrations. Currents were measured as responses to voltage ramps (from −100 to +50 mV) with 800 nM free Ca^{2+} in the pipette solution. (*b*) Block of calcium-activated K^+ currents by imipramine. (*c*) Dose-response plots for calcium-activated K^+ currents by external application of imipramine (●, ○) and ChTX (■, □). Open symbols denote normalized currents measured at 0 mV, filled symbols at +50 mV (mean ± SEM, n = 3–7). Data for ChTX block were corrected for currents (leak and chloride current) remaining after application of 200 nM ChTX. Hill functions were fitted to the data at +50 mV, yielding for ChTX: IC_{50} = 2.5 ± 0.1 nM, h = 0.99 ± 0.04 and for imipramine: IC_{50} = 13.8 ± 1.4 μM, h = 0.67 ± 0.04.

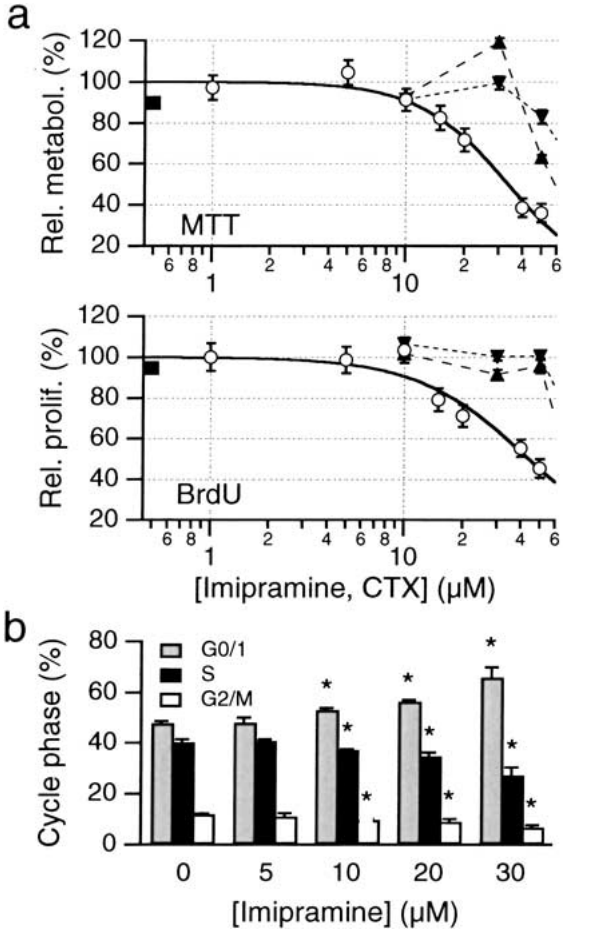

Fig. 3. Effect of imipramine on cell proliferation, metabolic activity, and cell cycle distribution. (*a*) IGR1 cells were treated for 48 hours with the indicated concentrations of imipramine (○) and ChTX (■) before starting the assays. As a control, also HEK 293 (▼) and CHO-K1 cells (▲) were treated with imipramine. The data are relative values compared to the untreated controls. Error bars are SEM values from 5 independent experiments with 5–8 parallel determinations each for MTT (*top*) and BrdU assays (*bottom*). The continuous curves are Hill fits estimating the half-maximal imipramine effect at 34 μM in the MTT assays and at 44 μM in the BrdU assays (Hill coefficients 1.9 and 1.5, respectively). The data points for HEK 293 and CHO-K1 cells are connected by straight dashed lines. (*b*) Cell cycle distribution of IGR1 cells, incubated for 24 hr with the indicated concentration of imipramine. The asterisks indicate significant deviation from the control data ($P < 0.05$) according to a one-sided ANOVA t-test.

much less affected than that of IGR1 cells. At 100 μM imipramine, cell death occurred in all cell types assayed.

To infer about the possibility that imipramine simply reduces the number of vital cells without affecting the cell cycle, we determined the cell cycle distribution after incubating IGR1 cells for 24 hr with various imipramine concentrations. As shown in Fig. 3*b*, starting from 10 μM imipramine, the G0/1 phase was increased significantly at the expense of the S and the G2/M phases, indicating that the cells preferentially accumulate in the G0/1 phase upon imipramine treatment.

Since imipramine was reported to induce apoptosis in eucaryotic cells (Xia et al., 1999), the effect on the proliferation of IGR1 cells could lead to a false interpretation. We therefore subjected IGR1 cultures to comparable protocols, as shown in Fig. 3*a*, and measured the degree of apoptosis by flow cytometry. Imipramine indeed induced apoptosis; however, after 24 hr, apoptosis in 10 and 20 μM imipramine was not greater than the control background level of spontaneous apoptosis (*data not shown*). Even after 48 hr incubation in 10 and 20 μM, the Annexin-V-FITC distributions of treated cells were not significantly different from the control (Kolmogorov-Smirnov test). Fig. 4*a–e* shows the corresponding dot blots of propidium iodide versus Annexin-V-FITC. As a control for the Annexin method, we also measured with the same assay apoptosis induced by betulinic acid (Fig. 4*f*, *g*), which was described as an effective apoptotic stressor for human melanoma cells (Pisha et al., 1995).

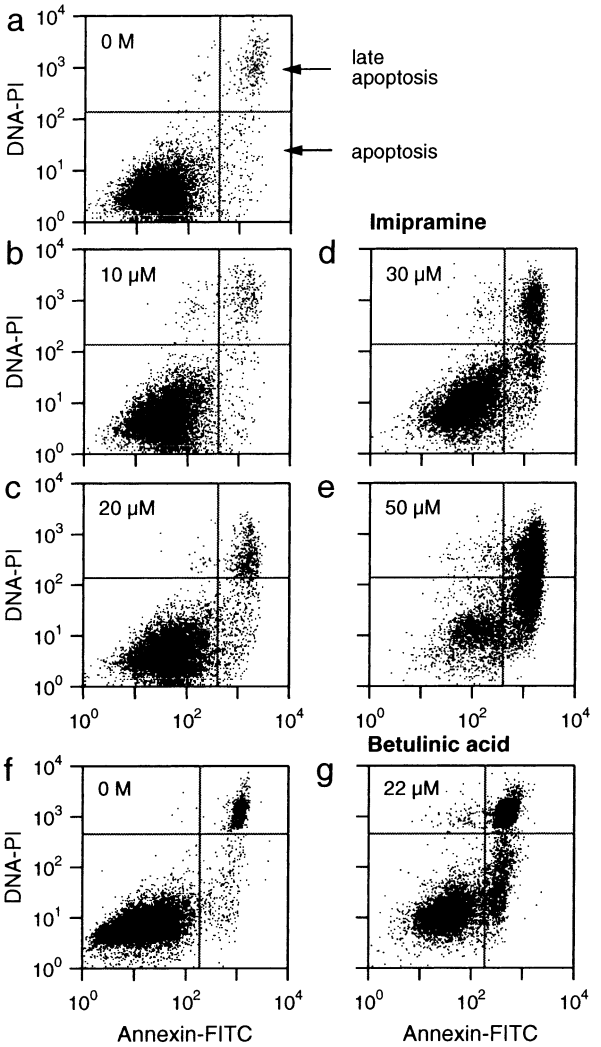

Fig. 4. Effect of imipramine on apoptosis in IGR1 cells as measured by annexin-V (Annexin-FITC) and propidium iodide (DNA-PI) binding. (*a-e*) Cells were treated for 48 hours with the following concentrations of imipramine (*a:* 0 μM *b:* 10 μM; *c:* 20 μM; *d:* 30 μM; *e:* 50 μM). Only at above 20 μM imipramine, annexin-V-FITC distributions were statistically different from the control (Kolmogorov-Smirnov test: 30 *vs.* 0 μM, $P < 0.05$; 50 *vs.* 0 μM, $P < 0.01$). (*f, g*) As a control, another batch of IGR1 cells was treated with betulinic acid, an established apoptotic stressor: *f:* control, *g:* 22 μM betulinic acid.

Block of Chloride Currents in IGR1 Cells

The experiments shown above may indicate that imipramine affects IGR1 proliferation via block of hEAG1 channels. However, other hyperpolarizing ion channels may also be important for proliferation. In particular, chloride channels were reported to play a role in the maintenance of cell volume, pH regulation, and control of membrane potential (Nilius et al., 1996; Wondergem et al., 2001). In IGR1 cells, volume-activated Cl^- currents were described earlier (Nilius et al., 1994). We therefore also addressed the question whether such channels could potentially contribute to the effects shown in Fig. 3*a*.

Cl_{vol} channels were activated in IGR1 cells by changing the ionic strength of the bath solution from 307 to 237 mOsm. About 2 minutes after application of hyposmotic solutions the whole-cell Cl^- currents reached a maximum. At +100 mV the mean current density was 82.7 ± 7.4 pA/pF ($n = 69$). In the presence of hyposmotic solutions these channels were blocked by imipramine in a dose-dependent manner (Fig. 5*a*, *b*). At +100 mV an IC_{50} value of 25 μM was determined; at −100 mV the block was slightly stronger ($IC_{50} = 12$ μM). 1 μM ChTX did not block these currents at all (*filled square* in Fig. 5*b*). Thus, block of Cl_{vol} channels by imipramine is weaker than block of hEAG1 channels. However, significant block occurred in a concentration range in which IGR1 proliferation was affected by imipramine. Therefore, like for K_{Ca} channels, additional specific Cl^- channel blockers are required to discriminate between involvement of hEAG1 and Cl^- channels.

We tested several Cl^- channel blockers aiming to find some that block Cl_{vol} channels, but not hEAG1 and K_{Ca} channels in IGR1 cells. 150 μM NPPB, a concentration that provides a complete block of Cl_{vol} channels (Nilius et al., 1994), also inhibited hEAG1 (98% at +20 mV) and K_{Ca} channels (88%). 300 μM flufenamic acid, which blocked Cl_{vol} channels in different cell lines over 50% (Fransen, Demolder & Brutsaet, 1995; Meyer & Korbmacher, 1996), also blocked K^+ currents of both types: 73% of hEAG1 at +20 mV and 90% of K_{Ca}. Tamoxifen was reported to block Cl_{vol} channels in various cell lines with different potencies (Nilius et al., 1994; Shen et al., 2000; Wondergem et al., 2001). K_{Ca} channels were found to be insensitive to 50 μM tamoxifen ($n = 3$), but hEAG channels were blocked by 92% at +20 mV. However, neither hEAG1 nor K_{Ca} channels were blocked by pamoic acid or DIDS at a concentration of 500 μM ($n = 3$–8). In contrast, pamoic acid and DIDS blocked Cl_{vol} channels in IGR1 cells in a voltage-dependent manner (Fig. 5 *c–f*). At +100 mV, pamoic acid and DIDS inhibited Cl^- currents with IC_{50} values of 34.0 and 12.0 μM, respectively. At −100 mV, the block was less pronounced: $IC_{50} = 189$ μM for pamoic acid and 350 μM for DIDS.

To investigate the potential contribution of Cl_{vol} channels to cell growth, we applied pamoic acid and DIDS to cultured IGR1 cells. The metabolic activity and DNA synthesis were unaffected by pamoic acid in concentrations up to 500 μM. However, DIDS at concentrations exceeding 250 μM inhibited IGR1 cell proliferation with an approximate IC_{50} value of 400 μM (Fig. 6).

Resting Membrane Voltage of IGR1 Cells and Cell Synchronization

Resting membrane potential (E_{rest}) was measured in the current-clamp mode of the whole-cell patch-clamp

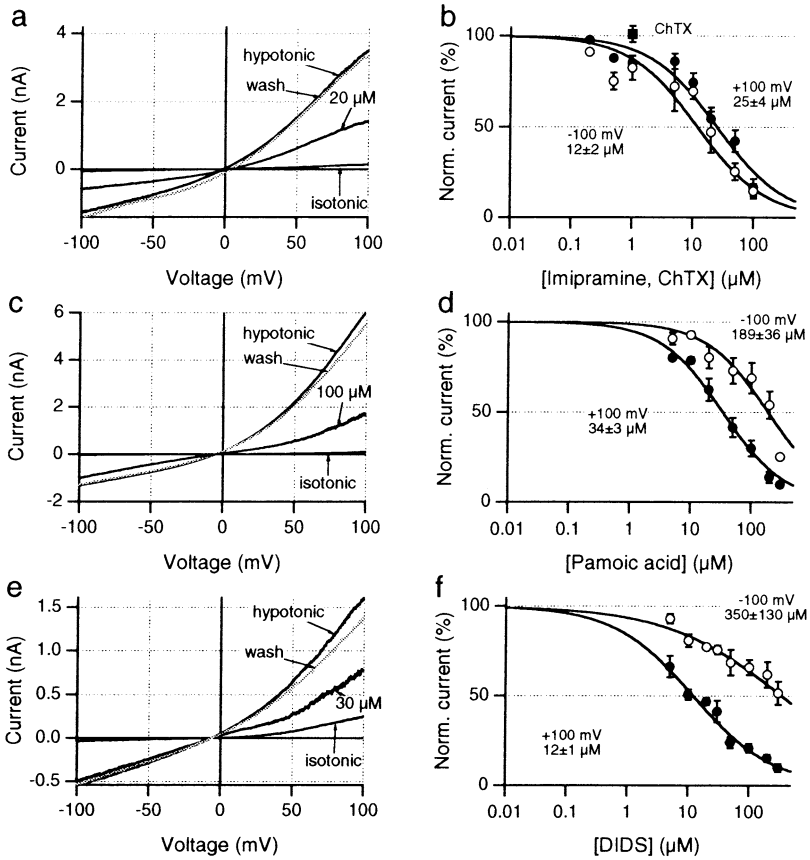

Fig. 5. Sensitivity of volume-activated Cl^- channels in IGR1 cells to imipramine, pamoic acid and DIDS. In the left panels (*a*, *c*, *e*) whole-cell current responses to voltage ramps (−100 to +100 mV) are shown under isotonic conditions, after exchanging the bath to 23% hypotonic solutions, upon blocker application in hypotonic solutions, and after wash with hypotonic, blocker-free solutions. The free Ca^{2+} concentration in the pipette was 100 nM (*a*) and 50 nM (*b*, *c*). The right panels show dose-response data obtained at +100 (*filled symbols*) and −100 mV (*open symbols*) for imipramine (*b*), pamoic acid (*d*), and DIDS (*f*). The superimposed curves are Hill fits yielding at +100 mV for imipramine: IC_{50} = 25.1 ± 3.5 μM, h = 0.78 ± 0.03; for pamoic acid: IC_{50} = 33.9 ± 3.0 μM, h = 0.84 ± 0.05; for DIDS: IC_{50} = 12.1 ± 1.4 μM, h = 0.66 ± 0.05. The fits at −100 mV were generated with the same Hill coefficients, yielding the indicated IC_{50} vales. In panel *b* the insensitivity of volume-activated Cl^- currents to 1 μM ChTX (*filled square*) is also indicated. (n = 3–7).

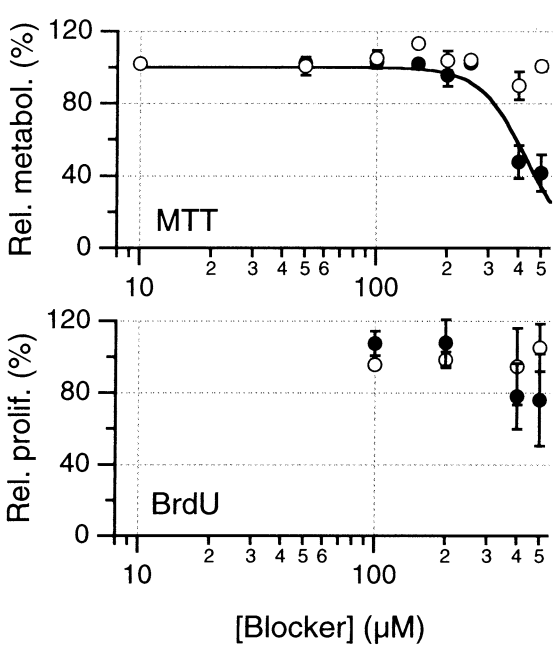

Fig. 6. Influence of Cl^- channel blockers pamoic acid (◯) and DIDS (●) on IGR1 cell proliferation. Starting one day after plating, the cells were treated for 48 hours with the indicated concentrations of channel blockers before carrying out the MTT and BrdU assays. The error bars are SEM values from 3–5 independent experiments.

technique with EGTA in the pipette, i.e., under conditions where hEAG1 channels are active and K_{Ca} channels are inactive. In unsynchronized IGR1 cells, a mean E_{rest} of −28.4 ± 1.4 mV (n = 45) was determined. The mean hEAG1 current density at +60 mV was 5.9 ± 0.1 pA/pF (n = 68). Such unsynchronized cells typically exhibit a cell-cycle distribution such as shown for control cells in Fig. 3*b*: G0/1, 48%; S, 40%, G2/M, 12%. To infer about a possible regulation of hEAG1 channels during the cell cycle and an effect of the cell cycle on E_{rest}, we attempted to synchronize the cells with 1 μM methotrexate. Methotrexate blocks DNA synthesis and populates the G1/S phases (*see* Vogel, Schwepp & Sigwarth, 1978; Sen, Erba & D'Incalci, 1990; Richardson et al., 1994). Acute application of 10 μM methotrexate did not block hEAG1, K_{Ca}, or Cl_{vol} channels (*data not shown*). After methotrexate treatment, 55% of the cells were arrested in the G0/1 phase, 44% in the S, and 1% in the G2/M phase. The resting membrane potential was −26.2 ± 2.3 mV (n = 37). 6 hr after the drug was washed out, the percentage of cells in the S and G2/M phase increased to 85%. Entering the G2/M phase was accompanied by a slight increase of E_{rest} to −21.2 ± 3.2 mV (n = 46) and a decrease of EAG1 current density from 3.5 ± 0.1 pA/pF (n = 50, measured during methothrexate arrest) to 1.7 ± 0.1 pA/pF (n = 79). These data indicate that hEAG1

expression is highest in the G1 phase. In addition, they suggest that hEAG1 expression in the G1 phase contributes to E_{rest} of about −28 mV and, hence, down-regulation of hEAG1 should depolarize the cells during the G1-S transition, not considering the counteracting effect of K_{Ca} channels.

This hypothesis would imply that imipramine should depolarize those IGR1 cells that express functional hEAG1 channels. We therefore measured E_{rest} in the current-clamp mode and applied imipramine. Between the individual measurements of E_{rest} we switched to voltage-clamp mode to record hEAG1 current density. Such an experiment is shown in Fig. 7*a* (*left*) for 20 μM imipramine, illustrating how E_{rest} correlates with the hEAG1 current density. Similar experiments were performed with 800 nM free Ca^{2+} in the pipette to block hEAG1 channels and to activate K_{Ca} channels. Under such conditions E_{rest} was not much affected by 20 μM imipramine, as shown for one example in Fig. 7*a* (*right*). In Fig. 7*b*, shifts in the resting potential (ΔE_{rest}) are plotted versus the hEAG1 current density for 10, 20, and 50 μM imipramine, showing a correlation of ΔE_{rest} and I/C when hEAG1 channels are effectively blocked (i.e., above 10 μM imipramine).

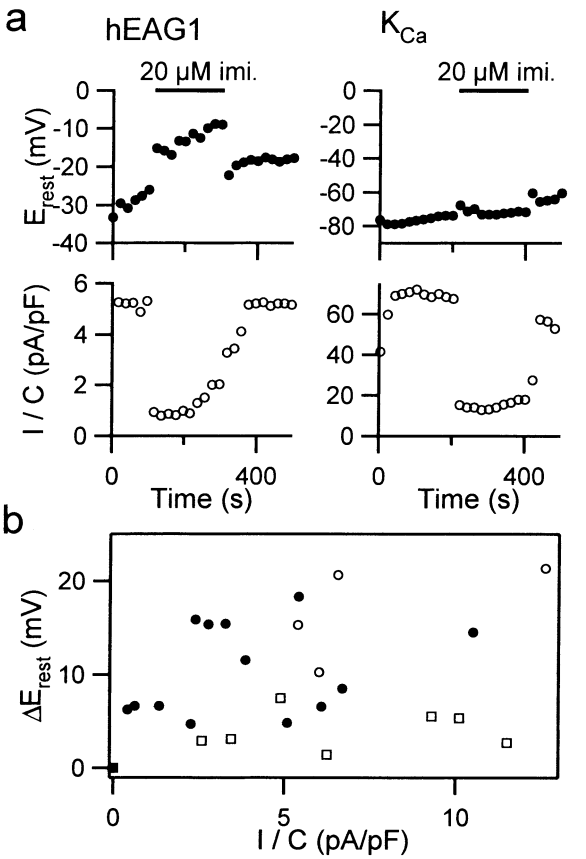

Fig. 7. Resting membrane potential in IGR1 cells, (*a*) Examples of IGR1 cells in which the effect of 20 μM imipramine on the resting potential (E_{rest}), and the current density (I/C) was measured at +50 mV. The left panels show recordings with Ca^{2+}-free pipette solutions, i.e., under conditions where hEAG1 channels are active. For the experiment shown in the right panels, the pipette contained 800 nM free Ca^{2+}, i.e., K_{Ca} channels were active. (*b*) Shift in resting potential induced by 10 (□), 20 (●), and 50 μM (○) imipramine as a function of the initial hEAG1 current density at +50 mV with Ca^{2+}-free pipette solutions.

Discussion

Block of EAG Channels by Imipramine

In a previous characterization of the melanoma cell line IGR1, Meyer et al. (1999) found that the dominant K^+ currents are mediated by EAG and calcium-activated K^+ channels. In the presented paper we demonstrate that the tricyclic antidepressant imipramine blocks voltage-activated K^+ channels hEAG1 in IGR1 cells in a voltage-dependent manner. This block was rapid and reversible, suggesting a binding site at the channel, accessible from the outside. Weinshenker and co-authors (1999) described imipramine as a potent inhibitor of the related ELK channels from the nematode *Caenorhabditis elegans* and of murine EAG channels, expressed in *Xenopus* oocytes assayed by two-electrode voltage clamp. Dose-dependent block at a depolarization to +40 mV suggested a binding stoichiometry of one imipramine molecule to one channel (Hill coefficients 0.84 and 0.89, respectively) with apparent IC_{50} values of 16 μM for the ELK and of 55 μM for mEAG current. For IGR1 cells at +50 mV we determined a Hill coefficient of 0.89, confirming the finding of Weinshenker et al. (1999) for mEAG channels. However, an IC_{50} of 3.4 ± 0.5 μM and block reversibility in IGR1 cells is in contrast to the results obtained in the *Xenopus* oocyte expression system.

Imipramine was shown to block human *eag*-related channels (hERG) transiently expressed in CHO cells (Teschemacher et al., 1999). hERG tail currents following test pulses to +20 mV were inhibited in a reversible manner with an IC_{50} of 3.4 μM, indicating that hERG1 and hEAG1 channels share a similar sensitivity towards imipramine and corroborating the notion that the weak block of mEAG channels found by Weinshenker et al. (1999) is a result of the assay system.

Imipramine and other tricyclic antidepressants have been widely used in psychiatric treatment since the early 1950s even though they cause a great variety of cardiovascular adverse effects (Glassman, 1998). The roles of both, EAG and ERG K^+ channels in neuronal systems are not yet understood. The question therefore arises whether the antidepressive action or neuronal side effects of imipramine and other tricyclics can be related to the inhibition of these channels in neurons. Given the determined IC_{50} value for

hEAG in melanoma cells and the identical value for ERG channels in CHO cells, this seems unlikely, since therapeutic plasma concentrations for imipramine range below 1 μM. However, cardiac arrhythmia, potentially caused by the inhibition of cardiac ion channels, is a known side effect of imipramine overdoses. In fact, cardiac transient K^+ outward currents (I_{to}) and hERG currents (I_{Kr}), providing the early and the late phase of myocyte repolarization (Valenzuela et al., 1994; Casis & Sánchez-Chapula, 1998; Teschemacher et al., 1999), respectively, are inhibited by imipramine. In one extreme-case report, plasma levels up to 20 μM have been observed in a patient, resulting in ventricular fibrillation and cardiac arrest (Sandemann, Alahakoon & Bentley, 1997).

Block of Other Ion Channels by Imipramine

Imipramine is an ion channel blocker with low specificity (Ogata, Yoshii & Narahashi, 1989; Valenzuela et al., 1994; Mathie, Wooltorton & Watkins, 1998). It was reported to block the cardiac sodium current (Ogata, Narahashi, 1989; Bou-Abboud & Nattel, 1998) but also calcium-activated K^+ channels in cultured spinal cord neurons (Kamatchi & Ticku, 1991). Block of hSK3, an apamin-sensitive calcium-activated K^+ channel, by imipramine and other structurally related tricyclic antidepressants has been described by Terstappen et al. (2001). All tricyclic antidepressants tested blocked this channel expressed in CHO cells in a concentration-dependent manner, imipramine with an IC_{50} of 44 μM. K_{Ca} currents in IGR1 cells were shown to be apamin-insensitive (Meyer et al., 1999). The presence of intermediate-conductance (IK) and small-conductance channels (hSK1, hSK2) in these cells was confirmed by RT-PCR analysis. Our experiments now show that this K_{Ca} current is also sensitive to imipramine with an IC_{50} of 13.8 μM at positive potentials.

Like many other cell types, IGR1 melanoma cells express volume-activated Cl^- channels, which help to adapt the cells to conditions of altered osmotic pressure (Nilius et al., 1994). We found these channels to be also sensitive to imipramine, although with a slightly higher IC_{50} value, ranging between 12 and 25 μM at −100 mV and +100 mV, respectively.

Ion Channels and Cell Proliferation

To address the putative role of individual channel types for cellular proliferation, we tested the effects of various channel blockers. However, for a quantitative comparison of the effects of channel blockers on proliferation and on ionic currents in IGR1 cells, it is important to know the mean resting potential of these cells. In unsynchronized IGR1 cultures 48 hr after plating, we measured a resting potential of −28 mV in Ca^{2+}-free intracellular solutions. These data are in accordance with measurements of IGR1 cell membrane potential by Nilius and co-workers (Nilius, Böhm & Wohlrab, 1990), showing that the resting potential of proliferating melanoma cells was between −20 and −30 mV.

Since hEAG1 block by imipramine is voltage-dependent (Fig. 1), an apparent IC_{50} value of about 15 μM can be estimated for −30 mV. Thus, at the normal resting potential of IGR1 cells, imipramine is expected to block hEAG and K_{Ca} currents to a similar extent. In contrast to the lack of specificity of imipramine, ChTX specifically blocked K_{Ca} channels (IC_{50} = 2.5 nM) in a voltage-independent manner, while EAG was not affected by 500 nM ChTX. Since IGR1 cells showed normal proliferation in the presence of high doses of ChTX, we conclude that a lack of K_{Ca} channels in IGR1 does not impair cell proliferation. In addition, it can be speculated that hEAG1 channels exhibit a growth-promoting activity in these melanoma cells. Although a combined role of both K^+ channel types cannot be ruled out, a specific requirement for IK/SK-type channels for IGR1 cell proliferation is very unlikely. Our data are in accordance with a number of other studies, which indicate that a block of ChTX-sensitive K_{Ca} channels does not affect cancer cell proliferation, whereas block of voltage-activated K^+ current does (Lepple-Wienhues et al., 1996; Yao & Kwan, 1999; Fraser, Grimes & Djamgog, 2000).

To find out whether imipramine effects on cell proliferation could also be attributed to Cl_{vol} channels, we used an approach similar to that with ChTX, which eliminated the contribution of IK/SK channels. We found that pamoic acid and DIDS could be used to block Cl^- channels, as neither of them blocked hEAG currents. Pamoic acid blocked Cl_{vol} channels in IGR1 cells at −30 mV with an apparent IC_{50} of 69 μM and DIDS, with an apparent IC_{50} of 92 μM. In proliferation experiments, pamoic acid did not affect cell growth at 500 μM, i.e., at a concentration that almost completely blocked Cl^- currents, indicating that Cl^- channels are not of prime importance for the proliferation of IGR1 cells either. The growth-inhibiting effect of high concentrations of DIDS may be caused by its known effect on the intracellular ATP concentration (Wondergem et al., 2001). However, based on our data we cannot safely exclude the contribution of Cl_{vol} channels, as imipramine might cause a combined block of K^+ and Cl^- currents in IGR1 cells. A cell-cycle transition from G1 to the S phase is accompanied by an increase in cell volume, and a net efflux of KCl is necessary for the further regulatory volume decrease (Nilius et al., 1996). In some cells a combined block of K^+ and Cl^- channels suppresses regulatory volume decrease, while blocking K^+ or Cl^- alone provides no effect (Fatherazi

et al., 1994). Since the size of IGR1 cells increases during the G0-S transition (*data not shown*), this situation may also apply here.

Thus, either the exclusive block of hEAG1 current by imipramine or the combined block of K^+ and Cl^- currents by this drug may provide inhibition of IGR1 cell growth and metabolism. However, imipramine is a well-known inducer of apoptotic events in eukaryotic cells (Xia et al., 1999). It uncouples the processes of oxidative phosphorylation, inhibits membrane-associated ATPase characteristics (Weinbach, Costa & Wieder, 1985; Weinbach et al., 1986) and causes proteolytic degradation of lysosomal sphingomyelinase (Albouz et al., 1981). Our apoptosis test shows that excess apoptotic events in IGR1 melanoma cells become detectable at concentrations above 20 μM. Thus, we suppose that the reduction in the number of cells and the inhibition of metabolic activity and DNA synthesis in IGR1 cells, observed under imipramine treatment, is due to block of ion channels controlling the cell-cycle progression. However, imipramine-induced apoptosis contributes to cell death and therefore complicates a quantitative analysis. At this point it cannot be concluded whether or not the induction of apoptosis by imipramine is partly related to channel blockade. It should be noted that in other cell types anti-proliferative effects of imipramine have also been reported (Fu et al., 1996; Manev et al., 2001).

To infer about a specific effect of imipramine on hEAG1 channels, we also performed proliferation experiments with HEK293 and CHO-K1 cells, i.e., well-established cell lines that do not express hEAG1 channels. These cells were much more resistant to imipramine, supporting the notion that hEAG1 in IGR1 cells is a likely target.

Regulation of hEAG Channels during the Cell Cycle

The effect of imipramine on IGR1 cell number, metabolism, and DNA synthesis in our experiments could at least partially be caused by the block of hEAG1 channels. This notion is very reasonable because hEAG1 channels were found to be expressed in different tumor cell lines but are absent or significantly less expressed in correspondent non-tumor cells (Pardo et al., 1999). Pardo and co-workers also showed imipramine's oncogenic role for tumor cell proliferation and tumor progression in experimental systems.

Considering the unique EAG channel properties, namely the prepulse-dependent activation kinetics and the block by intracellular Ca^{2+}/calmodulin (Schönherr et al., 2000), we suppose the channel might play an important role providing K^+ efflux and maintaining the resting membrane potential in the absence of intracellular Ca^{2+}. There is evidence for different cell types that the concentration of intracellular Ca^{2+} is the lowest in the early G1 phase (Pande, Kumar & Manogaran, 1996) and the cell membrane is depolarized (Wonderlin & Strobl, 1996). The early G1 phase is considered to be "the window of sensitivity" for many K^+ channel blockers with antiproliferative effect (Wonderlin & Strobl, 1996; Klimatcheva & Wonderlin, 1999).

To test such a scenario we synchronized IGR1 cells by methotrexate in the G1/S phases and measured hEAG current density as well as the resting membrane potential upon release of synchronization. Unsynchronized cells expressed hEAG currents at a density of 5.9 pA/pF. Upon synchronization the resting potential was −26 mV and the hEAG current density 3.5 pA/pF. Six hours after washout of the drug re-entry of the S/G2 phases was accompanied by a slight increase of the resting potential up to −21 mV and a decrease of hEAG current to 1.7 pA/pF. These data support the notion that hEAG1 currents are predominantly expressed in the G1 phase and that this expression pattern is accompanied by a slight hyperpolarization of the cells. Therefore, expression of hEAG1 channels in the G1 phase may help to hyperpolarize the cell membrane and to progress in the cell cycle. Furthermore, by transition to the middle and late G1 phase, the rising content of intracellular Ca^{2+} and the activation of mitosis-promoting factor suppress EAG current (Brüggemann et al., 1997). Cytoskeletal reorganizations during the M phase change the channel's electrophysiological properties (Camacho et al., 2000).

The role of hEAG current in progression through the G1 phase in breast cancer cells MCF-7 was recently studied by Ouadid-Ahidouch et al. (2001). Their experiments showed the cells arrested in G0/G1 were depolarized and hEAG density was small compared to cells progressing in the G1 phase. hEAG mRNA level was two times higher in the cells progressing through the G1 phase than in cells arrested in G0/G1. K^+ channel blockers TEA and astemizole inhibited both hEAG current and cell proliferation, while other K^+ channels present in MCF-7 cells, Ca^{2+}-activated and ATP-sensitive, were insensitive to TEA at the concentrations tested.

Based on these data we propose a possible interpretation of the role of EAG channel in the cell cycle of the IGR1 melanoma line. Cells in the early G1 phase express EAG1 channels at high density. K^+ efflux through the channel hyperpolarizes the cells, providing an increased driving force for Ca^{2+} influx. The mechanism of hyperpolarization-induced Ca^{2+} entry in IGR1 cells was described by Nilius, Schwarz & Droogmanns (1993). Elevation of $[Ca^{2+}]_i$ would inhibit hEAG1 (Stansfeld et al., 1996; Schönherr et al., 2000), but would also activate K_{Ca} channels that then could hyperpolarize the cells even further. While the feedback regulation of K_{Ca} channels by

intracellular Ca^{2+} has been studied in detail, the nature of the Ca^{2+} influx pathway in IGR1 cells still remains to be elucidated (Nilius et al., 1993).

The role of intracellular Ca^{2+} for cell-cycle progression is well characterized. Non-tumor cells require higher Ca^{2+} concentration in the growth medium for G1/S transition, during the S phase and at the exit from mitosis (Santella, 1998). Severe Ca^{2+} deprivation arrests the cells in the early G1 phase (Whitfield et al., 1979). Ca^{2+} signaling throughout the cell cycle is tightly coupled to changes of the membrane potential and K_{Ca} channel activity (Wonderlin & Strobl, 1996; Kamouchi et al., 1999). Cancer cells, in contrast, can proliferate with much less Ca^{2+} in the culture medium (Takuwa, Zhou & Takuwa, 1995). Tumor cells show a significantly smaller response to changes in $[Ca^{2+}]_i$ (Binggeli, Weinstein & Stevenson, 1994). Such cells undergo a slight hyperpolarization in the G1 phase to -20 to -40 mV, even earlier than the related non-tumor cell lines, but then their membrane potential remains unchanged over the cell cycle. This supports the hypothesis that tumor cells possess a mechanism allowing the initial hyperpolarization in low Ca^{2+}-concentration. In fact, a number of studies report on a role of voltage-activated K^+ channels in cell-cycle progression of different cancer cell lines (Lepple-Wienhues et al., 1996; Yao & Kwan, 1999; Fraser et al., 2000). In case of IGR1 cells, hEAG1 may be such a voltage-activated channel providing initial hyperpolarization even if the contribution of K_{Ca} channels is diminished, e.g., by ChTX application.

In summary, we found IGR1 melanoma cells to be a useful system for studying physiology and pharmacology of hEAG channels. Our data show that all K^+ channels as well as Cl_{vol} channels present in these cells are sensitive to imipramine in the micromolar range. According to the proliferation studies, we conclude that hEAG channels contribute to cellular proliferation, whereas the other channel types did not have a major impact in our assays.

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