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Blockage of Intermediate-Conductance Ca^{2+}-Activated K^+ Channels Inhibit Human Pancreatic Cancer Cell Growth in Vitro

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ABSTRACT

Ion channels are important in controlling cell cycle progression and proliferation in a variety of cell types. Using the whole-cell recording mode of the patch-clamp technique, functional ion channels were electrophysiologically characterized in PANC-1 (K-*ras* G12D (+/−), p53 R273C, Δp16), BxPC-3 (*smad4*$^-$, p53 Y220C, Δp16), and MiaPaCa-2 [transforming growth factor-β receptor type II defect, K-*ras* G12C(−/−), p53 R248W, Δp16] human pancreatic cancer cell lines. In BxPC-3 and the MiaPaCa-2 cells, we could identify ~600 or ~1200 functional Ca^{2+}-activated K^+ channels (IK) per cell, respectively, whereas PANC-1 cells expressed ~200 functional IK channels per cell. These channels were observed by using pipette solutions buffering $[Ca^{2+}]_i$ to 1 μM. The channels were voltage-independent, blocked by charybdotoxin, clotrimazole, 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34), and blocked by Ba^{2+} in a voltage-dependent manner. In the presence of 10 μM clotrimazole or TRAM-34, proliferation of the BxPC-3 as well as the MiaPaCa-2 cells was completely stopped. In contrast, proliferation of PANC-1 cells was hardly affected by clotrimazole or TRAM-34. Proliferation in all three cell lines could be inhibited in the presence of the Ca^{2+} channel antagonists verapamil, diltiazem, and nifedipine. By quantitative RT-PCR, we could show that MiaPaCa-2 cells exhibit a 2.8-fold and BxPC3 cells a more than 8-fold elevated level of IK mRNA level compared with PANC-1 cells. Interestingly, in primary pancreatic tumors we found a tremendous up-regulation of IK mRNA. In eight of nine (or 89%) primary pancreatic tumor tissues, we found a 6- to 66-fold increase in IK mRNA. Our findings suggest that a certain amount of functional IK channels is crucial for the proliferation of some pancreatic cancer types. The blockade of IK channels may ultimately prove useful as a therapeutic option for some patients with ductal adenocarcinoma of the pancreas with an up-regulated IK channel expression.

There are a lot of indications that ion channels are important for cell cycle progression and proliferation of cells. An excellently documented example for the importance of potassium channels in proliferation and differentiation derived from studies with lymphocytes (DeCoursey et al., 1984; Matteson and Deutsch, 1984). However, this relation between potassium channels and cell proliferation has also been shown for other cell systems, for example in melanoma cells (Nilius and Wohlrab, 1992), breast carcinoma cells (Strobl et al., 1995), fibroblasts (Rane, 1999), and Schwann cells (Pappas and Ritchie, 1998). The role of potassium channels in these cellular events is usually attributed to a regulation in the intracellular calcium concentration. Alternatively, potassium channels could also control cell volume and might therefore modulate cell proliferation (Rouzaire-Dubois and Dubois, 1998). In addition, there are reports that potassium channels are involved in tumorigenesis (Pardo et al., 1999).

IK channels seem to play an important role for epithelial Cl^- because it has been demonstrated that in human intestinal T84 cells, blockage of IK channels by clotrimazole inhibited repressed Cl^- secretion (Rufo et al., 1997). In addition, activation of IK channels stimulated Cl^- secretion in a variety of epithelial tissues (Devor et al., 1996; Singh et al., 2001). The IK channel in erythrocytes, called the "Gardos" channel (Gardos, 1958), is activated in sickle cells because of deoxygenation/sickling and is a major cause of salt loss and dehydration. It has been shown that blocking the Gardos channel with clotrimazole can improve the erythrocyte state of hydration in patients with sickle-cell disease (Brugnara et al., 1996). In addition to the influence of functional IK chan-

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ABBREVIATIONS: IK channel, Ca^{2+}-activated K^+ channel with intermediate conductance; TGF, transforming growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole; DMSO, dimethyl sulfoxide; CTX, charybdotoxin; RT-PCR, reverse transcription-polymerase chain reaction; C_T, cycle threshold; CRAC, calcium release-activated Ca^{2+} channel; ERK, extracellular signal-regulated kinase.

nels in erythrocyte function, these channels also seem to play a role in erythroid differentiation (Vandorpe et al., 1998).

In pancreatic ductal epithelial cells, Ca^{2+}-activated potassium channels have been shown to mediate pancreatic secretion of fluid and electrolytes (Nguyen and Moody, 1998). In combination with the Na^+/H^+ antiport and the Na^+/K^+ ATPase, these channels mediate serosal H^+ secretion, thus balancing luminal HCO_3^- secretion (Nguyen and Moody, 1998). To determine whether potassium channels might also play an important role in the proliferation control of pancreatic carcinoma cells, we first characterized the functionally expressed ion channels in these cells using electrophysiological techniques. In a second step, we measured the increase in cell number of these pancreatic carcinoma cell cultures under conditions in which most of the expressed potassium channels were blocked. We used three different pancreatic carcinoma cell lines that all harbor mutations in p53 and deletions of p16 (Berrozpe et al., 1994; Fujimoto et al., 2000; Moore et al., 2001). In addition to these common mutations and deletions, PANC-1 cells are characterized by a monoallelic activating K-*ras* mutation (Moore et al., 2001), BxPC-3 cells have a *smad*4 deletion (Berrozpe et al., 1994; Fujimoto et al., 2000), and MiaPaCa-2 cells lack a functional transforming growth factor-β (TGF-β) receptor type II in combination with an activating K-*ras* mutation on both alleles (Moore et al., 2001). Although mutations of the K-*ras* gene occur in more than 90% of pancreatic carcinomas (Johnson et al., 2001; Moore et al., 2001), they are not the only alterations that occur (Hruban et al., 2001). Usually, inactivation of tumor-suppressor genes, such as p16 and p53, coupled with activation of oncogenes such as K-ras, are a few of the mutations that trigger the growth of cancerous cells (Hruban et al., 2001).

Here we report the functional expression pattern of functional Ca^{2+}-activated K^+ channels in different pancreatic cancer cells and their possible role in the proliferation control of these cells. Some of the results have been reported in preliminary communications (Ruff et al., 2001).

Materials and Methods

Cells. BxPC-3, PANC-1, and MiaPaCa-2 human pancreatic cancer cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM *l*-glutamine, and 1 mM sodium pyruvate and maintained continuously in a humidified, 10% CO_2 incubator at 37°C. Tissues from patients with ductal adenocarcinoma of the pancreas and pancreatic tissue from organ donors were provided by the Department of Surgery at the University of Ulm and by the Hungarian Academy of Sciences (Budapest, Hungary). All tissues were obtained after approval by the local ethics committees.

Solutions. All electrophysiological experiments were done at room temperature (21–25°C). The cells under investigation were normally bathed in mammalian NaCl solutions containing 160 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, adjusted to pH 7.4 with NaOH, with an osmolarity of 290 to 320 mOsM. In mammalian KCl solutions, all the NaCl was replaced by an equal amount of KCl. The composition of the internal pipette solution was as follows: 135 mM K^+ aspartate, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, adjusted to pH 7.2 with KOH, with an osmolarity of 290 to 320 mOsM. Different amounts of $CaCl_2$ were added to the pipette solution to yield free $[Ca^{2+}]_i$ of 10 nM (1 mM $CaCl_2$) and 1 μM (8.7 mM $CaCl_2$); $[Ca^{2+}]_i$ was calculated assuming a K_d for EGTA and Ca^{2+} at pH 7.2 of 10^{-7} M (Portzehl et al., 1964).

Clotrimazole was purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany); 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34) was a kind gift from Dr. Heike Wulff (University of California, Irvine, CA). Both substances were dissolved in DMSO as stock solutions and diluted appropriately to yield the final concentrations. The final DMSO concentration in our experiments was less then 0.3%. This concentration had no apparent effect on its own on the property of the IK channels as well as on proliferation. Charybdotoxin (CTX) was purchased from Bachem Biochemica GmbH (Heidelberg, Germany).

Electrophysiology. Experiments were carried out using the whole-cell recording mode of the patch-clamp technique (Hamill et al., 1981; Grissmer et al., 1993; Jäger et al., 2000). Electrodes were pulled from glass capillaries (Science Products GmbH, Hofheim, Germany) in three stages and fire-polished to resistances measured in the bath of 2.5 to 6 MΩ. Membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) interfaced to a Macintosh computer running acquisition and analysis software (Pulse/Pulsefit; HEKA Elektronik). Analysis was also performed using IGOR Pro (Wavemetrics, Lake Oswego, OR) software. Final figures were created using CANVAS (Deneba Systems Inc., Miami, FL). All potentials, because of the liquid junction potential that develops at the tip of the pipette if the pipette solution is different from that of the bath, were < 5 mV and have not been corrected for. Each illustrated response was observed at least three times. The complete pharmacological profiling of the current through Ca^{2+}-activated K^+ channels was done in both the MiaPaCa-2 and the BxPC-3 cell lines. For convenience, only one example of each is shown.

Cell Growth Assay. For determination of cell number, human pancreatic carcinoma cells were seeded in 35-mm tissue culture dishes with 2-mm^2 grids on the bottom (Nunc, Wiesbaden, Germany), incubated overnight in growth medium (DMEM + 10% FCS) and then supplied to assay medium [DMEM + 10% (v/v) FCS + channel blocker and/or DMSO]. Cell numbers from three individual grids were determined every 24 h for at least 4 days. Three to seven independent experiments were performed. Cultures were re-fed every second day (Giehl et al., 2000).

RT-PCR Analysis of IK mRNA in Pancreatic Carcinoma Cell Lines and Tissue. Total RNA was prepared from MiaPaCa-2, PANC-1, and BxPC-3 cells using QiaShredder and RNeasy columns (QIAGEN, Hilden, Germany) with RNase-free DNase treatment according to the manufacturer's instructions. Total RNA from frozen pancreatic tissues was prepared using the RNeasy protocol (QIAGEN). For quantitative PCR, first-strand cDNA was synthesized from 10 μg of total RNA using random primers and reverse transcriptase (SuperScriptII RNase H^-; Invitrogen GmbH, Karlsruhe, Germany) after DNaseI treatment (Roche Diagnostic GmbH, Mannheim, Germany). A quantitative PCR analysis was done using TaqMan ABI PRISM 7700 Sequence Detector System and the SYBR Green PCR master mix kit (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's suggestions. The PCR was performed using sequence-specific primer pairs designed with the PrimerExpress program (Applied Biosystems). In our experience, routinely used housekeeping genes such as *glyceraldehyde-3-phosphate dehydrogenase* or *actin* are significantly differentially expressed in many cancer cells and tissues and are thus not useful as internal standards (Gress et al., 1996). We thus used human ribosomal protein, large, P0 (NCBI Accession no. BC001127) as housekeeping gene. This ribosomal protein was cloned in our laboratory as a ubiquitously expressed gene, specifically not regulated in cancer tissues and cells, and is now used as internal standard in all quantitative real-time PCR experiments or in Northern Blot analyses (Wallrapp et al., 1997). The expression pattern of this ribosomal protein is comparable with that of *Cyclophilin A*, which is frequently used as housekeeping gene for quantitative PCR (data not shown).

The following primer pairs were used for real-time PCR: (1) ribosomal protein: forward (sense), 5′-GCAGCAGAATCTGAGCAGCTC-3′; reverse (antisense), 5′-GCCAGCGTGTCAATCTGTTTC-3′) and (2) KCNN4: forward (sense), 5′-CGTCTCTGCCACTCCTGTCC-3′; reverse (antisense), 5′-CCTTGCACTTGAGGTGCTTTG-3′. To assure optimal and equal amplification, we selected amplicons between 50 and 150 base pairs for SYBR Green I-based real-time PCR. In this system, the SYBR Green I dye binds to the minor groove of double-stranded DNA, generating a fluorescent signal that can be quantified. Quantification is done using the comparative C_T method ($\Delta\Delta C_T$ method) as suggested by the manufacturer (Applied Biosystems). The C_T value represents the cycle in which the signal exceeds the threshold and the exponential increase of the DNA begins. The amount of cDNA is normalized by subtracting the C_T value of the housekeeping gene from the C_T value of the target gene in the same tissue or cell cDNA (= ΔC_T). The ΔC_T values of the different samples can be compared with a common reference sample by calculating the difference in ΔC_T values (= $\Delta\Delta C_T$). The mRNA copy number relative to the common reference sample is then calculated by the formula $x = 2^{(-\Delta\Delta CT)}$. The reactions for the housekeeping gene were carried out in duplicate and for KCNN4 in triplicate. Each cell line was cultured in at least two separate dishes, and total RNA was extracted and used for real-time PCR independently.

For subcloning and sequencing the RT-PCR product, 4 μg of total RNA was reverse-transcribed using oligo-dT primer and reverse transcriptase (SuperScript first-strand synthesis system for RT-PCR; Invitrogen GmbH). cDNA was synthesized for 120 min at 37°C. Single-stranded cDNA was amplified by PCR using 2 U of *Taq* DNA Polymerase (QIAGEN) Q-solution and 15 mM $MgCl_2$. The amplification protocol was as follows: 30 cycles at 96°C for 30 s, 61°C for 1 min, 72°C for 1 min, and a final extension time of 10 min at 72°C. The PCR primers were designed based on human cDNA sequences: *hIK1* (*KCNN4*) (GenBank accession number AF022150): forward (sense), 5′-GCCGTGCGTGCAGGATTTAGG-3′; reverse (antisense), 5′-CAGTCAGGGCATCCAGCTTC3–3′. The PCR product was cloned into the pcDNA3.1/V5-HisTOPO TA vector using the TA cloning kit (Invitrogen, Groningen, The Netherlands), and plasmid DNA from individual clones was sequenced.

Results

Expression of Functional IK Channels. To test for the presence of Ca^{2+}-activated K^+ channels in human pancreatic cancer cells, we dialyzed the cytoplasm during whole-cell recording with pipette solutions containing either 10 nM or 1 μM free Ca^{2+} as described previously (Jäger et al., 2000). Although 10 nM free Ca^{2+} was not sufficient to activate Ca^{2+}-activated K^+ channels (Fig. 1A), 1 μM free Ca^{2+} activated IK channels.

Figure 1A shows ramp currents elicited in MiaPaCa-2 pancreatic carcinoma cells. Similar ramp currents were also observed in BxPC-3 cells (for comparison see Fig. 2B). Ramp currents were elicited by 400-ms voltage ramps from −160 to +100 mV. With a pipette solution that buffered $[Ca^{2+}]_i$ to 10 nM, the slope of the ramp current, using an extracellular solution containing 4.5 or 164.5 mM K^+, was very flat at potentials below 0 mV, with a slight increase at potentials above 0 mV. In contrast, with a pipette solution buffering $[Ca^{2+}]_i$ to 1 μM using an identical external bath solution containing 4.5 mM K^+, the slope of the ramp current is much steeper compared with the situation when $[Ca^{2+}]_i$ was buffered to 10 nM $[Ca^{2+}]_i$. In addition, the ramp current changed direction from inward to outward at ~−80 mV. This reversal potential of −80 mV suggested a K^+-selective conductance. This suggestion was confirmed by changing the bath solution around the cell from a solution containing 4.5 mM $[K^+]_o$ to a solution containing 164.5 mM $[K^+]_o$ (using a pipette solution buffering $[Ca^{2+}]_i$ to 1 μM). This solution change resulted in a shift of the reversal potential of the ramp current toward 0 mV as predicted by the Nernst potential for K^+. Because the current-voltage relationship of the ramp current around the reversal potential is almost linear in both 4.5 and 164.5 mM K^+-containing external solution, we conclude that this current is flowing through voltage-independent Ca^{2+}-activated K^+ channels with little if any contribution of voltage-dependence K^+ channels. The slope conductance of the ramp current with 164.5 mM $[K^+]_o$ measured between −100 and −60 mV was 43 ± 14 nS (mean ± S.E.M.; n = 10) for the current in MiaPaCa-2 cells and 21 ± 4 nS (mean ± S.E.M.; n = 11) for BxPC-3 cells. This pattern of functional K^+ channel expression is different in PANC-1 human pancreatic cancer cells, as documented in an example in Fig. 1B, as well as in the scatter plot of conductances obtained for each cell line (Fig. 1C). In the case of PANC-1 cells, the slope of the ramp current was somewhat less compared with the other two cell lines [i.e., 8 ± 3 nS (mean ± S.E.M.; n = 10)]. Therefore, the functional expression of voltage-independent Ca^{2+}-activated K^+ channels in PANC-1 cells is ~3- to 5-fold less than in MiaPaCa-2 or BxPC-3 pancreatic carcinoma cells. From the average whole-cell current, we calculated the whole-cell conductance and could deduce the number of functional channels per cell using the known single channels conductance of the IK channel of ~35 pS (Grissmer et al., 1993). MiaPaCa-2 cells expressed ~1200 ± 400 functional IK channels (mean ± S.E.M.; n = 10) per cell, BxPC-3 cells expressed ~ 600 ± 100 functional IK channels (mean ± S.E.M.; n = 11) per cell and PANC-1 cells expressed ~ 200 ± 90 functional IK channels (mean ± S.E.M.; n = 10) per cell. In addition, the PANC-1

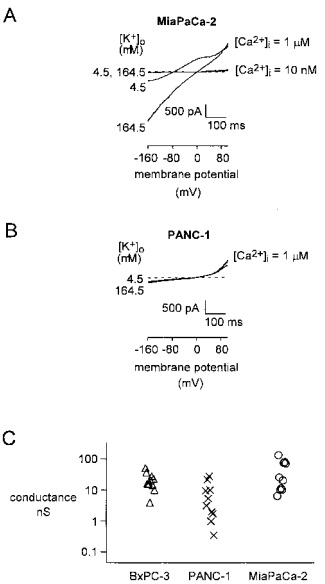

Fig. 1. Expression of IK channels in MiaPaCa-2 (A) and PANC-1 (B) human pancreatic carcinoma cells. Ramp currents were elicited by 400-ms voltage ramps from −160 to +100 mV using two different pipette solutions buffering $[Ca^{2+}]_i$ to either 10 nM or 1 μM. The bath solution around the cell was changed from a solution containing 4.5 mM $[K^+]_o$ to one containing 164.5 mM $[K^+]_o$ using both pipette solution. C, whole-cell conductance in the measured cells was obtained by determining the slope of the ramp current (between −90 and −70 mV in 164.5 mM $[K^+]_o$ and plotted for each cell).

cells showed functional expression of an outward current at potentials more positive than 0 mV (Fig. 1B). We have not characterized this current in detail, but it is almost completely blocked by 1 mM tetraethylammonium and it is sensitive to $[Ca^{2+}]_i$, indicating that this current might be flowing through Ca^{2+}-activated MaxiK$^+$ channels (data not shown). PANC-1 cells also showed functional expression of voltage-gated, nifedipine-sensitive Ca^{2+} channels that seem to be functionally absent in MiaPaCa-2 as well as BxPC-3 human pancreatic tumor cells (data not shown). It is well known that these types of Ca^{2+} channels are also sensitive to block by verapamil and diltiazem (Catterall et al., 2002). To identify the type of voltage-independent Ca^{2+}-activated K^+ channels functionally expressed in MiaPaCa-2 and BxPC-3 cells, we further characterized this current and determined its pharmacological profile.

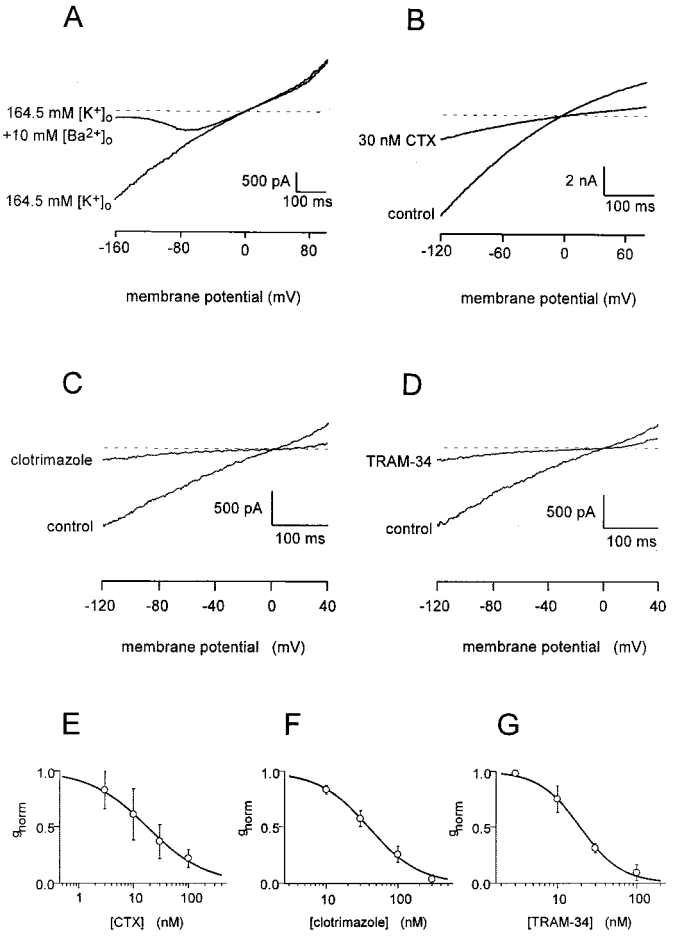

Fig. 2. Block of IK channels in human pancreatic carcinoma cells by Ba^{2+}, CTX, clotrimazole, or TRAM-34. IK channels were activated by whole-cell dialysis with a pipette solution containing 1 μM free Ca^{2+}. A, ramp currents were elicited in a MiaPaCa-2 cell by 400-ms voltage ramps from −160 to +100 mV. Traces shown were obtained in an external solution containing 164.5 mM $[K^+]_o$ in the absence and presence of 10 mM Ba^{2+}. B, ramp currents were elicited in a BxPC-3 cell by 400-ms voltage ramps from −120 to +80 mV. Traces shown were obtained in an external solution containing 164.5 mM $[K^+]_o$ before and after application of 30 nM CTX. C and D, ramp currents were elicited by 400-ms voltage ramps from −120 to +40 mV. Traces shown were obtained in a bath solution containing 164.5 mM $[K^+]_o$ before and after application of 1 μM clotrimazole in C and 1 μM TRAM-34 in D. E–G, dose-response curves for CTX, clotrimazole, and TRAM-34 to block the slope of the ramp current between −90 and −70 mV in BxPC-3 cells from records similar to those shown in B–D. Data point, normalized slope conductance, g_{norm}, from at least three independent experiments obtained by the indicated drug concentration. Mean ± S.D. (n = 3–4) is indicated. The smooth line was fitted to the measured data points ($g_{norm} = 1/\{1 + ([drug]/K_d)^{nH}\}$) and indicates a K_d value for CTX of 18 nM with a Hill coefficient (n_H) of 0.8; a K_d value for clotrimazole and TRAM-34 of 39 and 19 nM, respectively, with a Hill coefficient (n_H) of 1.2 and 1.7, respectively.

Pharmacology of the IK Channels. The ability of a variety of channel blockers to block current through Ca^{2+}-activated K^+ channels provides a good basis for the pharmacologically characterization of these channels.

Figure 2 shows the effect of different compounds on current through these channels functionally expressed in either MiaPaCa2 or BxPC3 human pancreatic cancer cells. The current was elicited with the same voltage ramp protocol as described in Fig. 1, in 164.5 mM $[K^+]_o$. Extracellular Ba^{2+} (10 mM) reduced current through IK channels preferentially at hyperpolarized potentials (Fig. 2A); i.e., the amplitude of the ramp current at −160 mV was reduced by Ba^{2+} to less then 10%, whereas the ramp current was hardly affected by Ba^{2+} at a potential above 0 mV. This voltage-dependent block by extracellular Ba^{2+} (10 mM) is qualitatively similar to results obtained from other voltage-independent, Ca^{2+}-activated K^+ channels expressed endogenously in other cell types, including lymphocytes (Grissmer et al., 1993). Two different types of voltage-independent Ca^{2+}-activated K^+ channels could account for the current in the human pancreatic cancer cells. The first one is the apamin- and scyllatoxin-sensitive SK channel with small conductance that is insensitive to block by CTX up to 100 nM (Jäger and Grissmer, 1997). The second one is the CTX-sensitive IK channel with intermediate conductance that is sensitive to block by CTX with a K_d around 3 nM (Jäger and Grissmer, 1997). To determine which of these two types of Ca^{2+}-activated K^+ channels are expressed in human pancreatic cancer cells, we applied 30 nM CTX to the cells. Figure 2B shows an example of such an experiment using a BxPC-3 cell. CTX (30 nM) reduced the amplitude of the ramp current to about a third of that under control conditions independent of the applied potential. This reduction of current by CTX is typical for an effect on IK channels, indicating that the majority of Ca^{2+}-activated K^+ current in MiaPaCa-2 as well as BxPC-3 human pancreatic tumor cells flows through channels belonging to the IK type rather than SK type (Jäger and Grissmer, 1997). Figure 2E shows a dose-response curve for CTX to confirms that CTX blocks current through IK channels with a 1:1 stoichiometry and a dissociation constant K_d of ~10 nM. A similar dose-response curve with a similar K_d value was also obtained for CTX block of IK current through MiaPaCa-2 cells (data not shown).

Clotrimazole is known for its use as an antimycotic drug, exerting its effect by inhibiting fungal P450-dependent enzymes (Rodrigues et al., 1987; Ayub and Levell, 1990; Maurice et al., 1992). However, clotrimazole also directly blocks current through IK channels either expressed exogenously as the product of the *hIK1* gene (also known as *KCNN4*, *IKCa1*, *hKCa4*, and *hSK4*) (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997; Jensen et al., 2001) or expressed endogenously in human and mouse erythrocytes (Alvarez et al., 1992; Brugnara et al., 1996, Vandorpe et al., 1998) and activated human T lymphocytes (Fanger et al., 1999; Jensen et al., 1999; Khanna et al., 1999). TRAM-34 is a clotrimazole derivative that has been successfully developed to selectively inhibit IK channels without blocking cytochrome P450 en-

zymes (Wulff et al., 2000). Therefore, we also tested these two compounds on their effect on Ca^{2+}-activated K^+ currents in human pancreatic cancer cells.

Figure 2, C and D, show an example of such experiments. The experiments were similarly performed using ramp currents as described for Fig. 2, A and B. Clotrimazole (1 μM) (Fig. 2C) as well as TRAM-34 (1 μM) (Fig. 2D) reduced the amplitude of the ramp current to about 10% of that under control conditions independent of the applied potential. Using different concentrations of clotrimazole and TRAM-34, we obtained dose-response curves for these two blockers as shown in Fig. 2, F and G. This dose-dependent reduction of current by clotrimazole and TRAM-34 is again typical for an effect on IK channels with half-blocking concentrations of ~40 nM for clotrimazole and ~20 nM for TRAM-34. Similar values of 70 and 25 nM, respectively, have been reported (Wulff et al., 2000). These results set the stage for further experiments using clotrimazole as well as TRAM-34 in assays to determine the importance of IK channels for the proliferation of human pancreatic cancer cells. These experiments should demonstrate whether specifically blocking IK channels would have an effect on the proliferation of human pancreatic cancer cells.

Quantitative and Qualitative RT-PCR. The mRNA levels of IK channels in PANC-1, MiaPaCa-2, and BxPC-3 cell lines and primary pancreatic tissue were assessed by quantitative RT-PCR. Total mRNA of exponentially grown MiaPaCa-2, PANC-1, and BxPC-3 cells and primary pancreatic tissue was used to synthesize cDNA for RT-PCR analysis. As shown in Fig. 3A, IK mRNA could be detected not only in MiaPaCa-2 and BxPC-3 cells but also in the PANC-1 cell line. MiaPaCa-2 cells exhibit a 2.8-fold and BxPC-3 cells have a more than 8-fold elevated IK mRNA level compared with PANC-1 cells. This result was partially expected from the electrophysiological measurements, where we found in the PANC-1 cell line a 3- to 5-fold reduced amount of IK current compared with MiaPaCa-2 and BxPC-3 cells.

Moreover, we performed quantitative RT-PCR analysis of primary pancreatic tissue. Figure 3B shows that eight of nine samples (89%) of primary pancreatic tumors contain a 6- to 66-fold increase in IK mRNA (ratio of pancreatic tissue sample and the mean of the control tissues samples). Sample 14 showed a 3-fold elevated IK mRNA level, the lowest IK mRNA content of the primary pancreatic cancer tissue. These results correlate nicely with the IK mRNA level observed in the pancreatic carcinoma cell lines. The identities of the RT-PCR products were verified by subcloning and sequencing of the cDNA (data not shown).

Effect of Blocking IK Channels on the Increase in Cell Number. To test whether the proliferation of human pancreatic carcinoma cells depends on the function of IK channels, we plated out BxPC-3 cells, MiaPaCa-2 cells, and PANC-1 cells and determined daily the cell number while cells were grown under control conditions or in the presence of different concentrations of clotrimazole or TRAM-34.

The results of our experiments are shown in Fig. 4, demonstrating the change in the relative cell number of the respective cells observed under different culture conditions. For all three cell types, the number of cells roughly doubled each day under control conditions. In the presence of 10 μM clotrimazole or 10 μM TRAM-34, the cell number of BxPC-3 and MiaPaCa-2 cell lines did not change for the observed period of time (Fig. 4, A and B) indicating that the normal increase in cell number of these two cell types requires the functional activity of IK channels. In the presence of 1 μM clotrimazole, the proliferation rate is down-regulated by more than 50% compared with control cells (Fig. 6, A and B). In contrast, the increase in cell number of PANC-1 cells could not be significantly modified by 10 μM clotrimazole or 10 μM TRAM-34 (Fig. 4C).

One possibility to explain how blocking IK channels can influence the increase in cell number is that an increase in $[Ca^{2+}]_i$ is necessary to progress through cell cycle and that this increase in $[Ca^{2+}]_i$ is caused by the influx through Ca^{2+} release-activated Ca^{2+} channels (CRAC). This has been postulated in other cell types (for review, see Lewis and Cahalan, 1995). Such a Ca^{2+} influx is sensitive to the membrane potential because hyperpolarization of a cell, making the inside of the cell more negative, would increase the driving force for Ca^{2+} to enter the cell. In contrast, depolarization of the cell, making the inside more positive, would decrease the driving force for Ca^{2+} to enter the cell. The Ca^{2+} influx per se would lead to a depolarization, therefore limiting the influx itself. A potassium channel that is activated by an

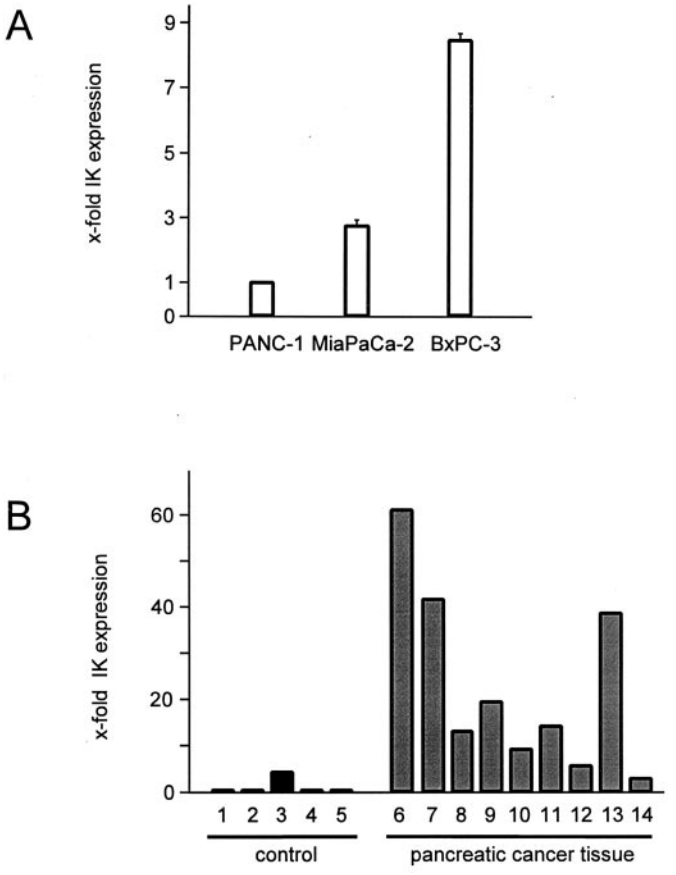

Fig. 3. Quantitative RT-PCR analysis of *hIK1* (KCNN4) mRNA in pancreatic carcinoma cells and tissue. A, total mRNA was prepared from MiaPaCa-2, PANC-1, and BxPC-3 cells and used for quantitative RT-PCR. The IK mRNA level is normalized to the housekeeping gene [human ribosomal protein, large P0 (NCBI Accession no. BC001127)] and shown for each cell line relative to PANC-1. The mean induction and S.D. in at least three independent experiments is shown. B, quantitative RT-PCR was used to determine the IK mRNA amount in primary pancreatic tissue (bars 1–5, control samples of normal pancreatic tissue; bars 6–14, samples of ductal adenocarcinoma of the pancreas.) The IK mRNA level is normalized to the housekeeping gene.

initial increase in $[Ca^{2+}]_i$ would hyperpolarize the cell and could therefore help to stabilize the membrane potential keeping up the driving force for sufficient Ca^{2+} influx. If this hypothesis is correct and the block of proliferation by IK channel blockers is simply caused by a decrease in the Ca^{2+} influx, one might be able to overcome the block of proliferation by IK channel blockers by simply increasing the driving force for Ca^{2+} influx through an increase in extracellular Ca^{2+}.

The average of three to seven such experiments is shown in Fig. 5. The experimental procedure was similar to that described for Fig. 4. Incubation of the cells for 3 days under control conditions increased cell number about 5-fold, whereas treatment of the cells with 10 μM clotrimazole resulted in a complete block of proliferation. This block of proliferation by 10 μM clotrimazole, however, was at least partially overcome by an increase in $[Ca^{2+}]_o$ from the normal 2 mM to 10 mM resulting in a 3-fold increase in cell number under conditions with 10 μM clotrimazole + 10 mM $[Ca^{2+}]_o$. These findings support the above-mentioned hypothesis, suggesting that in MiaPaCa-2 as well as in BxPC-3 cells, an increase in $[Ca^{2+}]_o$ can at least partially overcome the blocking effect of IK channels (Fig. 5, A and B, bar 5). The growth rate of PANC-1 cells was not influenced by clotrimazole with or without $[Ca^{2+}]_o$ (Fig. 5C).

Therefore, PANC-1 cells seem to grow independently of functional IK channels, which could mean that they do not require an increase in $[Ca^{2+}]_i$ to progress through cell cycle. In case they do, this increase might not be mediated through a Ca^{2+} influx through CRAC channels; on the other hand, these cells possess other functionally important potassium

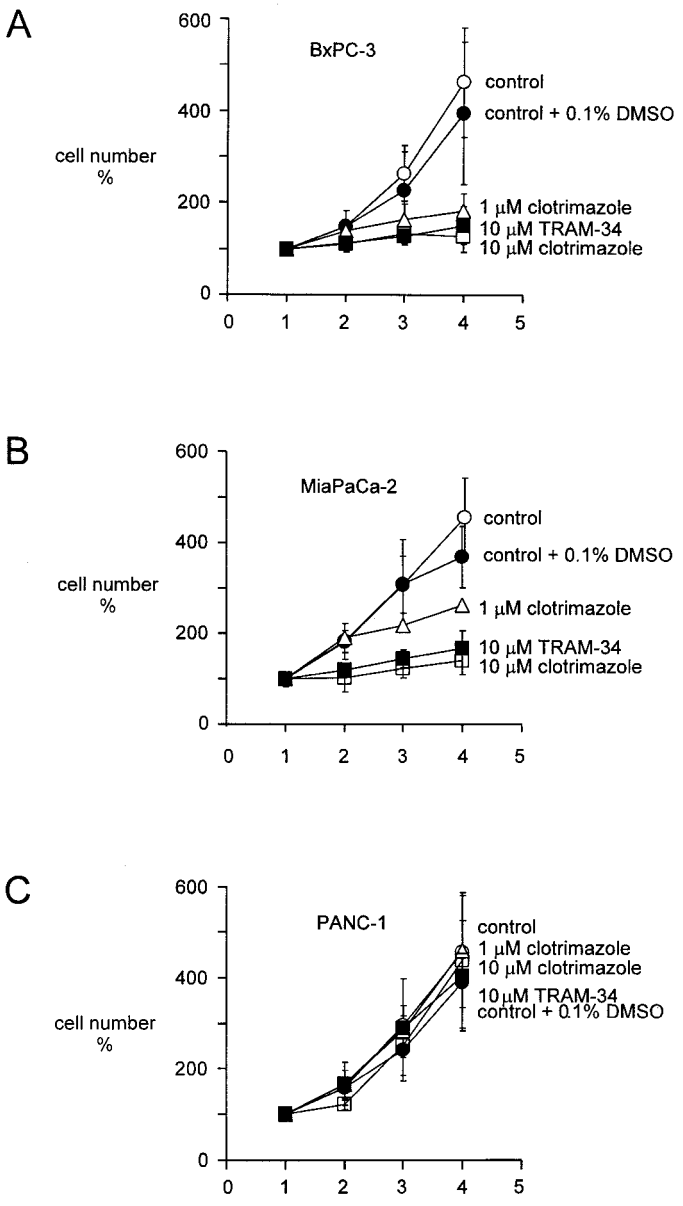

Fig. 4. Effect on increase in cell number of human pancreatic carcinoma cells by externally applied clotrimazole or TRAM-34. BxPC-3 cells (A), MiaPaCa-2 cells (B), or PANC-1 cells (C) were plated out on day 0 under control condition. Cell number was determined every day and normalized to the cell number determined at day 1. Cells were grown from day 1 on in control medium (○) or in medium containing either 0.1% DMSO alone (●) or 0.1% DMSO + 1 μM clotrimazole (△) or 0.1% DMSO + 10 μM clotrimazole (□) or 0.1% DMSO + 10 μM TRAM-34 (■). DMSO was used to dissolve clotrimazole or TRAM-34. Quantification of cells was performed by counting three individual fields of a grid plate. Untreated cells were defined as 100%. Shown are mean ± S.D. of three to seven independent assays.

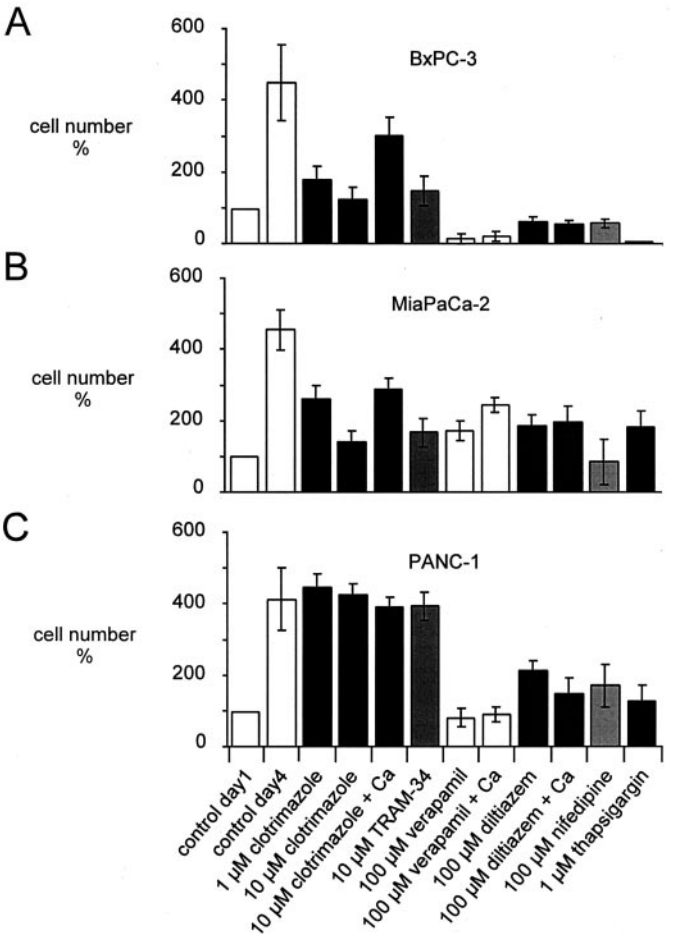

Fig. 5. Block and rescue of the increase in cell number of BxPC-3 (A), MiaPaCa-2 (B), and PANC-1 (C) cells. Cells were plated out on day 0 under control conditions. Cell number was determined at day 1 and used to normalize the cell number determined at day 4. Cells were grown from day 1 for three days in control medium (bar 2) or in medium containing the solutions shown on the bottom of this figure. As a control, cells were treated with the carrier solvent DMSO without an effect on cell growth (data not shown). Quantification of cells was performed by counting three individual fields of a grid plate. Untreated cells were defined as 100%. Shown are mean ± S.D. of three to seven independent assays.

channels that could control the membrane potential independently from IK channels. Both possibilities could be true: (1) they could use an alternative Ca^{2+} influx pathway through the voltage-gated Ca^{2+} channels expressed in these cells (see above) and (2) they might use $MaxiK^+$ channels to control membrane potential (see above). In a first attempt to determine whether proliferation of PANC-1 cells also requires Ca^{2+} signaling, we measured cell number in the presence of the well known Ca^{2+} channel blockers nifedipine, diltiazem, and verapamil. All three substances caused a significant decrease in cell number (Fig. 5C), suggesting that a functional Ca^{2+} channel seems to be required in PANC-1 cells in the proliferation. Surprisingly, these substances also caused a reduction in cell number in BxPC-3 cells and MiaPaCa-2 cells (Fig. 5, A and B). In the case of nifedipine, this could in principle occur through a blockage of IK channels, because nifedipine has been shown to block IK channels with a K_d of 4 μM (Catterall et al., 2002), whereas verapamil and diltiazem have been shown to block current through IK channels with a K_d of 72 and 154 μM, respectively (Catterall et al., 2002). It is therefore unlikely that at least verapamil and diltiazem work through a blockage of IK channels in BxPC-3 and MiaPaCa-2 cells. It is much more likely that the effect of diltiazem and verapamil and possibly also nifedipine is mediated by directly preventing a Ca^{2+} influx pathway, thereby modifying $[Ca^{2+}]_i$ homeostasis. The experiment with the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase blocker thapsigargin, which also inhibits proliferation in all three cell lines, supports this suggestion.

Discussion

BxPC-3 and MiaPaCa-2 pancreatic carcinoma cells express ~600 to 1200 functional K^+ channels per cell. These K^+ channels were observed using pipette solutions buffering $[Ca^{2+}]_i$ to 1 μM. They were voltage-independent, were blocked by CTX, and were blocked by Ba^{2+} in a voltage-dependent manner, indicating that these channels belong to the Ca^{2+}-activated K^+ channels with intermediate conductance (i.e., IK channels). Blocking these IK channels with either clotrimazole or TRAM-34 resulted in an almost complete stop of an increase in cell number. Thus, these results indicate that functional IK channels may be required for cell proliferation of these pancreatic carcinoma cells. Although PANC-1 cells also express functional IK channels (~200 functional channels per cell), their proliferation pattern could not be modified by blocking these IK channels. A comparison between the mRNA expression of IK in normal and tumorigenic pancreatic tissue revealed that the majority of patients with ductal adenocarcinoma of the pancreas has a 6- to 66-fold increase in IK mRNA level compared with normal pancreatic tissue.

Proliferation and Up-Regulation of IK. Normal mitogen-induced cell proliferation requires stimulation of receptor and nonreceptor tyrosine kinases by growth factors such as TGF-β, epidermal growth factor, and basic fibroblast growth factor. IK channels seem to take part in proliferation as well as differentiation of fibroblasts after mitogenic stimulation. IK channels were suggested to keep the cell hyperpolarized to drive an influx of Ca^{2+} via CRAC-like channels, resulting in subsequent proliferation of the cells (for review see Rane, 1999). The expression of IK channels in fibroblasts is up-regulated as measured by current density in response to mitogenic activation of the Ras/ERK signaling pathway by basic fibroblast growth factor and TGF-β (Pena et al., 2000). Similarly, up-regulation of IK channels from about 10 functional IK channels per cell to about 500 functional channels in activated T lymphocytes (Grissmer et al., 1993) was shown to be caused by stimulation of the PKC pathway to enhance IK channel expression via transcriptional activation of the *hIK1* promoter (Ghanshani et al., 2000). A reporter gene assay defined the minimal active promoter region of the *hIK1* gene, demonstrating the usage of an AP-1 promoter, which is the PKC-dependent binding site for Jun/Fos heterodimers (Ghanshani et al., 2000). An elevated IK mRNA amount is found in all primary pancreatic tissues tested. Eight of nine samples show a 6 to 66-fold increase in the IK mRNA level. This result correlates nicely with the IK mRNA level we found in the pancreatic cancer cell lines and demonstrates that MiaPaCa-2 and BxPC-3 cell lines are good model systems to examine ductal adenocarcinoma of the pancreas.

It has been demonstrated that ERK activity in MiaPaCa-2 cells was up-regulated (Seufferlein et al., 1999), and in the BxPC-3 cell line, a hyperactive MEK activity is found (Yip-Schneider et al., 2001). Therefore, both cell lines have a deregulated Ras signaling pathway in common, leading finally to up-regulated IK gene expression. That PANC-1 cell lines show lower IK gene expression, as shown by RT-PCR and patch-clamp measurements (current study), might be a result of the heterozygous K-*ras* genotype.

Functional Consequences of IK Channel Block. Clotrimazole as well as TRAM-34 have been demonstrated to directly block current through IK channels (Wulff et al., 2000). Our experiments clearly show that the use of these drugs can completely inhibit the increase in cell number in two pancreatic carcinoma cell lines. This effect is not a result of the known effect of clotrimazole on inhibiting cytochrome P450 enzymes, because TRAM-34, lacking this effect, has an identical effect on the increase in cell number. We therefore conclude that clotrimazole and TRAM-34 inhibit cell growth (or division or proliferation) by blocking IK channels. The simplest explanation for this observation is to assume that IK channels control the membrane potential. By doing so, they might stabilize the driving force for Ca^{2+} influx through CRAC channels. This hypothesis is supported by our experiments, in which we could at least partially overcome the IK channel block by increasing the Ca^{2+} influx through an increase in extracellular Ca^{2+} (see Fig. 5). This suggests that in MiaPaCa-2 as well as in BxPC-3 cells, an increase in $[Ca^{2+}]_i$ is essential for the increase in cell number and that this $[Ca^{2+}]_i$ can be controlled through the activity of functional IK channels, presumably by controlling the Ca^{2+} influx through CRAC channels. This relationship does not seem to be the case for the PANC-1 cells. Several possibilities could account for this: (1) PANC-1 cells might not require a $[Ca^{2+}]_i$ signal to progress through cell cycle; the experiments with the Ca^{2+} channel blocker diltiazem, nifedipine, and verapamil, as well as the experiments with thapsigargin, suggest that PANC-1 cells also require a possibility to control $[Ca^{2+}]_i$. (2) PANC-1 cells might have a different Ca^{2+} influx pathway not requiring cell hyperpolarization. We have been able to record functional voltage-gated Ca^{2+} channels in PANC-1 cells that seem to be absent in MiaPACa-2 and BxPC-3 cells. The observation, however, that the growth rate of all three cell

lines can be blocked by diltiazem, nifedipine, and verapamil suggested to us that their Ca^{2+} influx pathway might be identical. Therefore, we conclude that the expression of a functional voltage-gated Ca^{2+} channel in PANC-1 is not the reason for the unchanged growth rate in the presence of IK channel blockers. (3) PANC-1 cells might express a functional potassium channel different from IK that could control membrane potential. The electrophysiological experiments (Fig. 1) as well as the quantitative RT-PCR experiments (Fig. 3) showed that in PANC-1 cells, the mRNA of IK channels is much lower than in MiaPaCa-2 and BxPC-3 cells and that the effectiveness of IK blockers to inhibit growth of pancreatic cancer cell lines actually correlates with the amount of IK current and IK mRNA found in the respective cell lines. One explanation for the failure of IK channel blockers to modify proliferation in PANC-1 cells could be the smaller amount of IK current and, on top of that, the functional expression of another K^+ channel, i.e., the MaxiK$^+$ channel (see Fig. 1B) that would be able to control membrane potential. Experiments with specific MaxiK$^+$ channel blockers might be helpful to elucidate this hypothesis in the future.

Because pancreatic cancer makes up about 15% of all gastrointestinal cancers in developed countries and is characterized by poor survival rates and resistance to radiotherapy and chemotherapy, novel therapeutic strategies are needed. They could arise from a better understanding of the signaling mechanisms that regulate the growth of pancreatic cancer cells.

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