Imatinib-Mesylate Blocks Recombinant T-Type Calcium Channels Expressed in Human Embryonic Kidney-293 Cells by a Protein Tyrosine Kinase-Independent Mechanism

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ABSTRACT

The 2-phenylaminopyrimidine derivative imatinib-mesylate, a powerful protein tyrosine kinase (PTK) inhibitor that targets abl, c-kit, and the platelet-derived growth factor receptors, is rapidly gaining a relevant role in the treatment of several types of neoplasms. Because first generation PTK inhibitors affect the activity of a large number of voltage-dependent ion channels, the present study explored the possibility that imatinib-mesylate could interfere with the activity of T-type channels, a class of voltage-dependent Ca\textsuperscript{2+} channels that take part in the chain of events elicited by PTK activation. The effect of the drug on T-type channel activity was examined using the whole-cell patch-clamp technique with Ba\textsuperscript{2+} (10 mM) as the permeant ion. In conclusion, imatinib-mesylate blocked the cloned Ca\textsubscript{3.3} channels by a PTK-independent mechanism. Specifically, the drug did not affect the activation or the inactivation of the channel but interfered with the ion permeation process.

The 2-phenylaminopyrimidine derivative imatinib-mesylate is a remarkable example of a new class of selective protein tyrosine kinase (PTK) inhibitors that are rapidly gaining a relevant role in the treatment of tumors due to their high specificity, potency, and good tolerability (Fabbro et al., 2002). Imatinib-mesylate is a powerful inhibitor of the PTK abl, an enzyme that is pathologically activated in chronic myelogenous leukemia (Buchdunger et al., 1996; Deininger and Druker, 2003). The drug also blocks c-kit and PDGF receptors (PDGFr) (Buchdunger et al., 1996; Deininger and Druker, 2003). The 2-phenylaminopyrimidine derivative modified neither the voltage dependence of activation nor the steady-state inactivation of Ca\textsubscript{3.3} channels. The decrease in extracellular Ba\textsuperscript{2+} concentration from 10 to 2 mM and the substitution of Ca\textsuperscript{2+} for Ba\textsuperscript{2+} increased the extent of 30 \mu M imatinib-mesylate-induced percentage of channel blockade from 25.9 ± 2.4 to 36.3 ± 0.9% in 2 mM Ba\textsuperscript{2+} and 44.2 ± 2.3% in 2 mM Ca\textsuperscript{2+}. In conclusion, imatinib-mesylate blocked the cloned Ca\textsubscript{3.3} channels by a PTK-independent mechanism. Specifically, the drug did not affect the activation or the inactivation of the channel but interfered with the ion permeation process.

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channels (VDCCs) (Cataldi et al., 1996; Morikawa et al., 1998; Wijetunge et al., 2002), partly explains this phenomenon.

The inhibitory action of PTK inhibitors on VDCCs could be of special relevance in the case of low-voltage-activated (LVA) T-type calcium channels. In fact, these channels, which diverge from high-voltage-activated channels because of their peculiar permeation and gating properties (Cataldi et al., 2002; Perez-Reyes, 2003), are important functional partners of PTKs, for they cooperate with these proteins in a number of physiological processes. For example, T-type channels take part in the chain of events leading to cell proliferation and differentiation (Kuga et al., 1996; Richard and Nargeot, 1996) and are essential for the mitogenic response to the PDGF receptors (Wang et al., 1993). Therefore, the ability to block T-type channels could give a PTK inhibitor additional valuable properties that could optimize its pharmacological activity.

Given the relevance of imatinib-mesylate as an antineoplastic agent, the present article explored its potential effect in modifying the activity of T-type channels. In particular, by using the whole-cell patch-clamp technique on HEK-293 cells, stably expressing the recently cloned CaV3.3 isoform of T-type channels (Lee et al., 1999), specific experimental approaches were adopted to establish whether imatinib-mesylate acts on these channels indirectly through a PTK-dependent mechanism, or whether it directly interferes with channel gating or the permeation process. The results showed that imatinib-mesylate dose-dependently inhibited the activity of cloned CaV3.3 channels. However, this effect was not related to PTK inhibition but rather to the drug's interference with the ion permeation process.

**Materials and Methods**

**Cell Culture.** Stably transfected HEK-293 cells (courtesy of Dr. Perez-Reyes, Department of Pharmacology, University of Virginia, Charlottesville, VA), expressing the rat CaV3.3 subunit of T-type VDCC (Lee et al., 1999), were cultured in a humidified 5% CO₂ atmosphere using Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids; they were kept under constant selection with 1 g/l geneticin. For electrophysiological recordings, cells were plated on poly-L-lysine (30 μg/ml)-coated borosilicate electrodes having a final resistance of 3 to 5 MΩ. The electrodes were backfilled with a CsCl-based internal solution containing 110 mM CsCl, 30 mM tetraethyl ammonium chloride, 10 mM EGTA, 2 mM MgCl₂, 10 mM HEPES, 8 mM glucose, 15 mM phosphocreatine, 5 mM ATP, and 1 mM CaCl₂ (pH 7.4 adjusted with CsOH). Unless otherwise specified, the external solution contained 125 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 10 mM HEPES, and 1 mM MgCl₂ (pH 7.4 adjusted with HCl). The osmolarity of the external solution was adjusted to 300 mOsm by adding an appropriate amount of sucrose.

Test pulses were generated and the ensuing currents were collected with a 200 B patch-clamp amplifier (Axon, Union City, CA) driven by the P-Clamp 6 software running on a PC. Currents were filtered at 2 kHz with the amplifier's built-in Bessel filter, and leak currents were subtracted online with a P/4 protocol. Offline corrections of membrane capacitance and series resistance were routinely performed by the specific commands of the amplifier. Data were stored onto the hard disk of the PC. Offline analyses were then performed with the Clampfit 8.0 (Axon Instruments, Union City, CA) and SigmaPlot 5.0 (SPSS Science, Chicago, IL) software.

Cell capacitance was calculated upon the integration of current traces generated by cell membrane discharges in response to short (5 ms) square pulses of 5-nA amplitude (from −70 to −65 mV), which were delivered immediately after rupturing the patch.

**Drugs.** Imatinib-mesylate was a generous gift from Dr. Buchdunger (Novartis Pharma, Basel, Switzerland). The drug was dissolved in water at a 10 mM stock solution and kept frozen at −20°C until use. Genetin and ATP-sodium salt were obtained from Calbiochem, whereas CsOH was purchased from Aldrich Chemical Co. (Milan, Italy). Dulbecco's modified Eagle's medium, fetal calf serum, penicillin, streptomycin, and nonessential amino acids were purchased from Invitrogen (San Giuliano Milanese, Italy). All the other chemicals were of analytical grade and were purchased from Sigma (Milan, Italy).

**Data Analysis.** All the data have been reported as mean ± S.E.M. Statistical comparisons were performed with ANOVA followed by the Newman-Keuls post hoc test. The threshold for statistical significance was set at p < 0.05. Curve fitting was performed with the SigmaPlot 5.0 (SPSS Science) or N-fit (The University of Texas, Medical Branch at Galveston, Galveston, TX) software.
Results

Imatinib-Mesylate Blocks Ca\textsubscript{\textalpha},3.3 Channels Acting from Outside the Cell. Membrane depolarization, induced by square voltage pulses, elicited large inward currents in Ca\textsubscript{\textalpha},3.3-expressing HEK-293 cells that were bathed with a 10 mM Ba\textsuperscript{2+} solution. These currents displayed all the expected features that clearly differentiated Ca\textsubscript{\textalpha},3.3 channels type from the other cloned members of the Ca\textsubscript{\textalpha},3 family. In fact, they showed relatively slow kinetics with respect to activation and inactivation (Fig. 1, A and B). The fact that these currents were blocked by 500 \mu M Cd\textsuperscript{2+} (Fig. 1A), disappeared when Ba\textsuperscript{2+} ions were omitted from the extracellular solution (Fig. 1B), and were totally lacking in untransfected HEK-293 cells (Fig. 1C), indicated that they were genuine Ba\textsuperscript{2+} currents, flowing through the heterologously expressed T-type channels. Because we were interested in studying the consequences on Ca\textsubscript{\textalpha},3.3 activity of PTK blockade by imatinib-mesylate, preliminary experiments were performed to assess whether Ca\textsubscript{\textalpha},3.3-expressing HEK-293 cells do express the PTKs that are inhibited by this drug. Western blot analysis of total cell lysates showed a strong expression of the c-abl protein in this cell line (Fig. 1D), whereas, despite the strong signal observed in positive controls (serum-stimulated IMR-92 fibroblast), no detectable signal was observed when an anti-PDGFr-\beta antibody was used (data not shown).

Once established that Ca\textsubscript{\textalpha},3.3-expressing HEK-293 cell are an appropriate experimental model, the hypothesis that imatinib-mesylate could affect T-type channel activity was explored monitoring the effect of increasing imatinib-mesylate concentrations (1–300 \mu M) on Ba\textsuperscript{2+} currents, evoked by square pulse depolarization (from –100 up to 0 mV). Test pulses were delivered with a 10-s interpulse interval to allow the inactivated channels a full recovery from the inactivation and, consequently, to prevent the progressive accumulation of Ca\textsubscript{\textalpha},3.3 channels in the inactive state. Imatinib-mesylate induced a marked decrease in current amplitude that was largely reversible upon the drug washout (Fig. 2, A and B). Imatinib-mesylate-induced Ca\textsubscript{\textalpha},3.3 blockade was clearly concentration-dependent. Indeed, when the concentration effect curve was fitted to a Hill function, an estimated IC\textsubscript{50} of 56.9 \mu M and a Hill coefficient of 1.27 were obtained (Fig. 2C).

Moreover, to rule out the hypothesis that the elevated IC\textsubscript{50} needed for Ca\textsubscript{\textalpha},3 inhibition was due to the poor penetration of the drug inside the cytoplasm, we examined whether 3 and 10 \mu M concentrations of imatinib-mesylate, which were closer to the IC\textsubscript{50} for PTK inhibition, affected channel activity after an overnight incubation. At both the concentrations tested, imatinib-mesylate was unable to reduce the amplitude of Ba\textsuperscript{2+} currents expressed as current density (pA/pF) after the normalization for cell capacitance (Fig. 2D).

Moreover, to see whether the effect of imatinib-mesylate on Ca\textsubscript{\textalpha},3.3 channels depended on PTK inhibition (in which case the drug should have acted intracellularly), we examined the impact on channel activity of 500 \mu M imatinib-mesylate concentration dissolved in the pipette solution and dialyzed into the cytoplasm. The results showed that intrapipette imatinib-mesylate neither induced a reduction in Ba\textsuperscript{2+} current density at the beginning of the recording (Fig. 3A), nor determined an acceleration in the rate of its spontaneous decrease over a 500-s period (Fig. 3B).

Imatinib-Mesylate Does Not Affect Ca\textsubscript{\textalpha},3.3 Channel Gating, but It Interferes with the Ion Permeation Process. To identify the molecular mechanism responsible for imatinib-mesylate effect on Ca\textsubscript{\textalpha},3.3 channels, we examined whether the drug could reduce the ability of the channel to open, in response to a given depolarizing step, or increase its tendency to inactivate at a given membrane potential.

First, the effect of imatinib-mesylate on the voltage dependence of Ba\textsuperscript{2+} currents was examined. The delivery of a series of square depolarizing pulses of increasing amplitude elicited inward currents that appeared at membrane potential more positive than –50 mV, reached their maximum around –20 mV, and reverted at approximately +40 mV (Fig. 4A). When the same cells were exposed to 100 \mu M imatinib-mesylate for 180 s, the current amplitude was reduced by approximately 70%, at all voltages tested (Fig. 4A). The fact that the drug did not induce marked changes in the current to voltage (I/V) plots suggested that imatinib-mesylate-in-
the values recorded before drug addition. The number of cells in each
induced by each imatinib-mesylate concentration compared with
with the two tail Student's t-test for unpaired data.

$\text{Fig. 3.}$ Imatinib-mesylate does not block Ca$_{\text{v}}$3.3 channels when dialyzed into the cytoplasm through the patch pipette. A, mean ± S.E.M. of the Ba$^{2+}$ current densities measured immediately after reaching the whole-cell configuration and after correcting for the membrane capacitance in a group of six cells patched with standard intracellular solution and in a group of six cells patched with a 500 μM imatinib-mesylate-containing intracellular solution. B, time course of maximal current densities recorded in response to a series of square pulse depolarizations in the same two groups of cells in A. Each data point is the mean ± S.E.M. of the values recorded in the six cells of each group. As reported in the inset, Ca$_{\text{v}}$3.3 channels were activated by delivering a series of 75-ms steps (from −100 up to 0 mV) with an interpulse interval of 10 s. Current densities were obtained by normalizing to cell capacitance (expressed in pF and calculated as reported under Materials and Methods) the maximal Ba$^{2+}$ current amplitudes obtained in response to each step depolarization.

Duced Ca$_{\text{v}}$3.3 channel blockade was not voltage-dependent (Fig. 4B). Given that the voltage dependence of activation curve tends to spontaneously drift leftward upon prolonged patch clamping of Ca$_{\text{v}}$3-expressing HEK-293 cells (Martin et al., 2000), the effect of imatinib-mesylate on the voltage dependence of Ca$_{\text{v}}$3.3 channel activation was determined by the following experiments. I/V plots were obtained from two different groups of cells: one exposed to vehicle and the other to 100 μM imatinib-mesylate. No difference in voltage dependence of activation was observed when the vehicle and imatinib-treated cells were compared ($\bar{V}_m = -25.03 ± 0.8$ in control and $-25.13 ± 1.29$ in imatinib-mesylate-treated cells; $k = 5.79 ± 0.6$ in control and 5.19 ± 0.4 in imatinib-mesylate-treated cells) (Fig. 4C). These results suggested that imatinib-mesylate did not reduce the ability of Ca$_{\text{v}}$3.3 channels to open in response to membrane depolarization.

According to the modulated receptor hypothesis of channel blockade (Hondeghem and Katzung, 1984), another mechanism that might determine a reduction in Ba$^{2+}$ current amplitude via an interference with the gating apparatus is the preferential binding of the drug to the inactivated state of the channel and the consequent stabilization of this state.

The percentage of Ba$^{2+}$ currents blocked by imatinib-mesylate did not increase with progressive increments in step voltages, as expected from a drug interacting with the inactivating state of the channel (Fig. 4D). However, the fact that imatinib-mesylate-induced Ba$^{2+}$ current inhibition was not voltage-dependent does not completely exclude the hypothesis that the drug could act on the inactivated state of Ca$_{\text{v}}$3.3 channels. Its binding kinetics could, in fact, be too slow to yield a significant interaction with those channels undergoing inactivation during the short pulses used to generate the I/V plots. To exclude this possibility, the effect of imatinib-mesylate on Ca$_{\text{v}}$3.3 channel activity was also studied by analyzing the inward Ba$^{2+}$ currents evoked by step depolar-
obtained in the presence and in the absence of the drug are reported. Conductance was estimated as the ratio between the current (I) and the driving force that was calculated as the difference between the voltage of the step and the reversal potential ($V_{\text{rev}}$) (Randall and Tsien, 1997). The values were fitted to the equation $G/G_{\text{max}} = 1/(1 - e^{V_m - V_{\text{rev}}})$, where $V$ is the voltage of the depolarizing pulse, $V_m$ is the midpoint of activation, and $k$ is the voltage step needed to increase an increase of $e$-times in whole cell conductance ($e$ is the base of natural logarithms). C, voltage dependence of activation curves generated from the I/V plots. The I/V plots were obtained with a protocol similar to the one reported in A in a group of seven cells exposed to vehicle for 180 s before patching, and in another group of six cells exposed to 100 μM imatinib-mesylate for the same amount of time. The graph shows the mean ± S.E.M. of the ratios (expressed as a function of step voltages) between the conductance calculated for each step ($G$) and the maximal conductance attained in the whole experiment ($G_{\text{max}}$). Both values obtained in the presence and in the absence of the drug are reported. Conductance was estimated as the ratio between the $I_f$, which is the steadystate inactivation curves, obtained in these conditions, did not differ in these eight cells before and after the exposure to imatinib-mesylate. The data were normalized and expressed as percentage of the maximal inward current ($I_{\text{inward}}$) recorded in the absence of the drug. The ability of 30 μM imatinib-mesylate to block CaV3.3 channels was significantly enhanced in a 2 mM Ba2+ solution. In fact, as opposed to the values recorded before the addition of the drug, Ba2+ current amplitude was reduced by 36.3 ± 0.9 and 25.9 ± 2.4% in 2 mM and in 10 mM Ba2+ solutions, respectively ($p < 0.01$ using analysis of variance followed by the Newman-Keuls test) (Fig. 6, A, B, and D). Because Ca2+ is the permeant ion in physiological conditions and a mechanism of channel blockade involving ion permeation could imply a different ability of the drug in blocking Ba2+ and Ca2+ currents, the effect of 30 μM imatinib-mesylate on the inward currents elicited by step depolarization from −100 to −20 mV with 2 mM Ca2+ in the bath was also assessed. In these experimental conditions, imatinib-mesylate induced a 44.2 ± 2.3% blockade of Ca2+ currents that was significantly different from what was observed with 2 mM Ba2+ ($p < 0.05$ using analysis of variance followed by the Newman-Keuls test) (Fig. 6, C and D).

**Discussion**

The main findings emerging from the present paper indicated that imatinib-mesylate blocks CaV3.3 T-type channels by acting in a PTK-independent mechanism. Specifically, it interfered with their permeation pathway without affecting the activation or the inactivation of CaV3.3 channels.

Several arguments support the conclusion that PTK inhibition is not responsible for the T-type channel blockade...
The voltage needed to reduce e-times the amplitude of inward Ba\(^{2+}\) that determined a decrease in CaV3.3 channel activity was acting on an extracellular site. In particular, the main factor exerted by imatinib-mesylate. First, the IC\(_{50}\) value for channel blockade was more than 100 times higher than the one needed for PTK inhibition (Buchdunger et al., 1996). This latter result could be explained by the inefficient intracellular diffusion of the drug owing to the short exposure of the cells during the brief patch-clamp experiments. However, the fact that micromolar concentrations of imatinib-mesylate were still ineffective when the cells were exposed to the drug for a prolonged time interval clearly excludes this possibility and strongly implies a mechanism that was independent of PTK inhibition. This idea is further supported by the evidence that imatinib-mesylate acted from outside the cell and remained ineffective when introduced into the cytoplasm through the recording pipette. As shown by the strong expression of c-abl found in these cells by Western blot analysis, the lack of efficacy of intrapipette imatinib-mesylate cannot be explained with the absence in the cytoplasm of Ca\(_{\text{a},3.3}\)-expressing HEK cells of the PTKs susceptible to the blocking action of this drug, and suggests that imatinib-mesylate was acting on an extracellular site. In particular, the main factor that determined a decrease in Ca\(_{\text{a},3.3}\) channel activity was the drug’s reversible binding to an extracellular site that caused the block of the permeation path of the channel itself. Because imatinib-mesylate is a charged organic cation, this notion is consistent with the classical evidence, which states that charged drugs and toxins may impinge on the outer vestibule of VDCC and disturb the access of Ba\(^{2+}\) ions to the pore region of the channel. The key role played by imatinib-mesylate in blocking Ca\(_{\text{a},3.3}\) channel ion permeation was highlighted by the experiments performed with low Ba\(^{2+}\) concentrations. In fact, when an extracellular solution containing 2 mM Ba\(^{2+}\) was used, the extent of imatinib-induced Ca\(_{\text{a},3.3}\) channel blockade was significantly higher than the one at 10 mM Ba\(^{2+}\). The channel blockade sensitivity to a decrease in the permeant ion concentration was as expected from a drug that competes with Ba\(^{2+}\) ions for the access to the pore region of the channel. A further argument supporting the idea that imatinib-mesylate exerts its Ca\(_{\text{a},3.3}\) channel block activity due to an interference with the permeation process was provided by experiments performed using a 2 mM Ca\(^{2+}\)-containing extracellular solution. In this experimental conditions the extent of drug-induced channel blockade was significantly higher, clearly suggesting that the nature of the ion competing with imatinib-mesylate could determine the effectiveness of the drug-induced channel blockade. Interestingly, Martin et al. (2000) obtained similar findings with the T-type blocker mibefradil that, besides interfering with other channel functions, displays similar permeation-blocking properties. Given a similar mechanism for imatinib-mesylate-induced Ca\(_{\text{a},3.3}\) channel blockade, a relevant issue that emerges is that of specificity. In fact, it could be argued that, not differently from what observed for mibefradil, this drug could also interact with the pore region of other VDCC channel types. Indeed, preliminary observations from our laboratory confirm this idea because imatinib-mesylate proved to be effective also in blocking recombinant L-type channels in stably transfected Chinese hamster ovary cells (courtesy of Dr. F. Hofmann, Technische Universitat Munchen, Munich, Germany) coexpressing the rabbit Ca\(_{\text{a},1.2}\) subunits (M.C., unpublished data).

Contrary to other T-type channel blockers, i.e., mibefradil (McDonough and Bean, 1998; Gomora et al., 2000) and the neuroleptics pimozide and penfluridol (Santi et al., 2002), which do interfere with the channel inactivation process, imatinib-mesylate apparently does not. In fact, contrary to what is expected from drugs that bind to the channel in its inactive state, the extent of channel blockade did not increase despite the progressive increments in the step voltages that increased the inactivation rate of Ca\(_{\text{a},3.3}\) channels. Furthermore, the hypothesis that the interaction of the drug with the inactivated state of the channel could be precluded by its slow binding kinetics can also be discarded, because no leftward shift in steady-state inactivation was observed when Ca\(_{\text{a},3.3}\) channels were inactivated by long prepulses in the presence of the drug. Similarly, the activation process seems to be equally unaffected by imatinib-mesylate, for this 2-phenylaminopyrimidine derivative did not induce any changes in the channel voltage dependence of activation.

Therefore, it is worth underscoring the fact that PTK first generation inhibitors (genistein, lavendustin, and herbimycin) differ from imatinib-mesylate, for they reduce the activity of naive T-type channels in NG108-15 cells by a PTK-dependent mechanism (Morikawa et al., 1998).

Because T-type channels have a role in a number of physiological processes that are also regulated by the activity of PTKs, such as the control of cell growth (Kuga et al., 1996; Richard and Nargeot, 1996) and neuronal excitability (Huguenard, 1996; Perez-Reyes, 2003), the availability of compounds provided of T-type channel- and PTK-blocking properties could be of valuable clinical interest. For example, a pharmacological approach targeting T-type channels and

Fig. 5. Lack of effect of imatinib-mesylate on the steady-state inactivation of Ba\(^{2+}\) currents in Ca\(_{\text{a},3.3}\)-expressing HEK-293 cells. A, steady-state inactivation curves obtained in two groups of five cells, one bathed with 10 mM Ba\(^{2+}\) and the other exposed to a 100 mM imatinib-containing Ba\(^{2+}\) solution. The current traces, obtained in a representative cell of each group, are reported in B. Steady-state inactivation curves were generated using the protocol described in the inset of B. In particular, Ca\(_{\text{a},3.3}\) channels were inactivated by applying a series of long (3-s) prepulses of increasing amplitude (from -100 to +20 mV) in 10-mV increments. The amount of ion channels still available for opening at the end of the prepulse was estimated by looking at the amplitude of the inward currents elicited by a brief depolarizing step up to 0 mV, delivered 5 ms after setting the membrane potential back to the holding potential value. Each data point is the mean ± S.E.M. of the maximal inward Ba\(^{2+}\) currents recorded in each cell during each step depolarization up to 0 mV. The data points were fitted to the Boltzmann function \(I_{\text{max}} = I_{\text{m}} \times \frac{1}{1 + e^{(V - V_m)/k})} \), where \(I_{\text{m}}\) is the amplitude of the inward Ba\(^{2+}\) current recorded after each step, \(I_{\text{max}}\) is the maximum value of inward Ba\(^{2+}\) current amplitude, reached during the entire experiments; \(V_m\) is the midpoint of inactivation; and \(k\) is the voltage needed to reduce e-times the amplitude of inward Ba\(^{2+}\) current amplitude (where e is the basis of natural logarithms).
PTKs simultaneously could be useful to prevent vascular remodeling as it occurs in arterial hypertension. The rationale behind this approach could be represented by the relevance of both PTKs and T-type channels in the pathogenesis of this condition. In fact, specific PTKs, as the PDGF receptors, which are activated by shear-stress (Hu et al., 1998), contribute to myointimal cell proliferation (Balasubramaniam et al., 2003) and represent the target of novel antivascular remodeling therapies (Waltenberger et al., 1999). On the other hand, T-type channels are expressed in proliferating myointimal cells (Kuga et al., 1996; Richard and Nargeot, 1996), and their pharmacological blockade with mibefradil prevents the development of neointimal hyperplasia in spontaneous hypertensive rats (Li and Schiffrin, 1996).

Similarly, the combined blockade of T-type channels and PTKs could also be useful in controlling pathological neuronal excitability, because they are both involved in this process. Specifically, T-type channels play a relevant role in regulating neuron excitability (Huguenard, 1996; Perez-Reyes, 2003) and take part in the process of epileptogenesis. The crucial involvement of T-type channels in seizures is, in fact, demonstrated by the finding that the antiseizure drug ethosuximide blocks these channels (Coulter et al., 1990; Gomora et al., 2001) and by the observation that the thalamic neurons of genetic absence epilepsy rats of Strasbourg, an experimental model of absence epilepsy, show higher T-type current density than average (Tsakiridou et al., 1995). By contrast, CaV3.1 T-type channel knockout mice have shown to be resistant to thalamic spike-and-wave discharges, normally induced by the administration of GABAb receptor agonists (Kim et al., 2001). The involvement of PTKs in the process of epileptogenesis is supported by the evidence that specific PTKs, such as PYK-2, are activated in response to seizures (Tian et al., 2000) and by the fact that the overexpression of selected PTK, such as Fyn (Kojima et al., 1998), raises the tendency to develop seizures in vivo, whereas the

**Fig. 6.** Effect on imatinib-induced CaV3.3 blockade of replacing Ca²⁺ for Ba²⁺ ions or changing Ba²⁺ concentration in the extracellular solution. A to C, representative current traces obtained in three different CaV3.3-expressing HEK cells bathed with an extracellular solution containing either 10 mM Ba²⁺ (A), 2 mM Ba²⁺ (B), or 2 mM Ca²⁺ (C). As shown in the inset, currents were elicited by a series of 75-ms step depolarizations, from −100 to −20 mV, delivered with a 10-s interpulse interval. For each cell, we reported a current trace acquired during the baseline perfusion with a drug-free extracellular solution, a current trace obtained at the nadir of 30 µM imatinib-induced current decrease, and a current trace recorded during the drug washout. The inset of each panel reports the time course of maximal inward current amplitude obtained in the respective cell. The traces reported in A to C are representative of at least five other cells for each group recorded with the same experimental conditions. D, mean ± S.E.M. of the percentage of decreases in inward current amplitude induced by 30 µM imatinib-mesylate respect to the values recorded before the addition of the drug attained in each of the three experimental groups are compared. The single asterisk indicates *p* < 0.05 versus 2 mM Ba²⁺, whereas the double asterisk indicates *p* < 0.01 versus 10 mM Ba²⁺.
knocking out the same kinases has opposite effects (Cain et al., 1995). Furthermore, the susceptibility of hippocampal slices in vitro to epileptic discharges, in response to electrical stimulation, can be raised by adding the PTK src into the recording pipette and can be decreased with the use of the src inhibitor PP2 (Sanna et al., 2000). Therefore, a drug that can simultaneously block T-type channels and specific PTKs could constitute a promising antisiezure agent.

Last, because T-type channels are selectively expressed in proliferating tumor cells, whereas their expression is lost after cell differentiation (Hirooka et al., 2002; Mariot et al., 2002), it has been suggested that they are also involved in tumor growth. Although it is still matter of controversy, this hypothesis suggests that T-type channel blockade could synergize with PTK inhibition and consequently determine an antineoplastic effect. Actually, drugs provided with T-type blocking properties such as mibebradil and pimozone have already shown to exert an antiproliferative effect on retinoblastoma and breast cancer cell lines in vitro (Bertolisi et al., 2002).

In conclusion, imatinib-mesylate has the ability to block Ca,3.3 channels by a PTK-independent mechanism when used in the high micromolar range. More importantly, its T-type channel blocking ability could add to this important drug additional and useful pharmacological properties that could be exploited in the development of a new class of derivatives, which in addition to having the capability of blocking T-type channels could also retain the remarkable PTK inhibitory properties of imatinib-mesylate.

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