Identification of Channels Promoting Calcium Spikes and Waves in HT1080 Tumor Cells: Their Apparent Roles in Cell Motility and Invasion

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ABSTRACT

Intracellular Ca2+ signals have been associated with cell polarization and locomotion. As cell motility underlies metastasis, we have sought to better characterize the Ca2+ signaling events in HT1080 fibrosarcoma cells. We have tested the hypothesis that low voltage-activated (LVA) and nonvoltage-gated (NVG) Ca2+ channel in HT1080 cells participate in dynamic Ca2+ signaling events leading to cell migration and invasion. Immunofluorescence microscopy has shown that HT1080 cells express LVA T-type Ca2+ channels uniformly about the cell periphery, whereas the transient receptor potential-1 (a NVG cation channel) protein appears as punctate spots about a cell’s periphery. HT1080 cells exhibit periodic intracellular Ca2+ spikes. High-speed imaging revealed that the Ca2+ spikes were composed of a single Ca2+ wave traveling unidirectionally about the periphery of the cytoplasm in a clockwise fashion (as viewed from basal to apical surfaces). The T-type Ca2+ channel blocker mibebradil inhibited Ca2+ spikes and waves on cells and, in parallel, inhibited cell motility and invasion in a dose-dependent manner. Similar changes were noted with the NVG cation channel blockers Gd4+ and carboxyamido-triazole. The combination of LVA and NVG blockers further reduced Matrigel invasiveness. However, the Ca2+ channel blockers nicardpine, SKF96365, dilatazem, and verapamil had no effect at appropriate doses. These results indicate that LVA and NVG channels regulate HT1080 cell motility. In addition to providing novel information regarding cancer cell motility, we suggest that it may be possible to design drugs that inhibit a key Ca2+ wave, thereby enhancing the efficacy of emerging therapeutic protocols.

INTRODUCTION

The invasion and metastasis of tumor cells require cell migration (1, 2). Cell migration is a cyclic process involving the repetitive extension of invadopodia/lamellipodia at the leading edge, the formation of adhesion sites, contraction of the cell body, and the release of trailing adhesion sites. The cyclic morphological and adherence changes observed during cell migration are accompanied by repetitive Ca2+ signals, which take the form of Ca2+ spikes or oscillations. Ca2+ transients have been observed in migrating neurons, neutrophils, fibroblasts, eosinophils, tumor cells, and other cell types (3–10). These oscillations are thought to participate in coordinating the cyclic temporal features of cell migration, such as pseudopod extension, actin assembly, integrin regulation, the phosphorylation-mediated regulation of focal adhesion formation, and pericellular proteolysis (1–10). Thus, repetitive Ca2+ signals likely play an important role in the repetitive structural and functional changes required for cell movement.

Intracellular Ca2+ spikes may involve Ca2+ release from intracellular stores as well as Ca2+ entry from the extracellular environment. Ca2+ entry from the extracellular environment is mediated by Ca2+-permeable ion channels of the plasma membrane, of which the voltage-gated Ca2+ channels are best characterized (11). Five types of high voltage-activated (HVA) Ca2+ channels (named L-, N-, P-, Q-, and R-type) and one type of low voltage-activated (LVA) Ca2+ channel (known as T-type) have been identified based on the pharmacological and biophysical characteristics of their currents. HVA channels are broadly distributed on neurons (12), myocytes (13), etc., whereas the transient receptor potential-1 (a NVG cation channel) binds intracellular and/or extracellular messengers, chemical or mechanical stress, and by the Ca2+ level of intracellular stores (16). Among these channels, transient receptor potential (TRP) and TRP-like channels are molecularly identified and distributed in a variety of tissues and species (14). TRPs are likely identical to several NVG channels, such as: (a) store-operated channels (18, 19); (b) mechanosensitive cation channels (20); and (c) receptor-activated cation channels (21). However, the specific physiological functions of TRP and TRP-like channels have not been rigorously established. Nonetheless, carboxyamido-triazole (CAI), an inhibitor of NVG Ca2+ channels, induces tumor cell apoptosis (22) and inhibits tumor cell growth and invasion (23, 24), thus implying that NVG Ca2+ channels are closely related to tumor cell physiology.

As previous studies have suggested that: (a) the LVA channel blocker mibebradil inhibits leukocyte adhesion and locomotion (25, 26); (b) the NVG Ca2+ channel blocker CAI inhibits tumor cell invasion (23); and (c) unlike muscle cells, which require strong membrane depolarization and high intracellular Ca2+ elevation, cell adhesion and migration require transient cell activation and rapid inactivation, which is usually the function of T-type Ca2+ channels and TRP/TRP-like channels (27, 28), we have tested the hypothesis that LVA and NVG Ca2+ channels participate in tumor cell signaling. This study provides direct physical evidence that the Ca2+ signal associated with tumor cell migration is a traveling wave, which requires the participation of both NVG and LVA channels for propagation. Our work not only suggests that mibebradil may enhance the antitumor activity of compounds such as CAI, but our studies of signaling waves also provide a new route to understanding basic signaling pathways and a means to search for new drugs.

MATERIALS AND METHODS

Cell Preparation. HT1080 cells were incubated in DMEM supplied with 10% FCS and 1% antibiotics. Cells were passaged by detachment from culture flasks with a trypsin/EDTA solution (Sigma Chemical Co., St. Louis, MO) in PBS for 5 min at 37°C. For high-speed imaging and emission microfluorometry, cells were prelabeled with indo-1-AM at 5 μg/ml for 20 min at 37°C (29).

Materials. Tetramethylrhodamine isothiocyanate, FITC, BAPTA-AM, and Indo-1-AM were obtained from Molecular Probes, Inc. (Eugene, OR). Fibrinectin, verapamil, mibebradil, GdCl3, and nicardpine were purchased from Sigma. EDTA was obtained from Fischer Scientific (Pittsburgh, PA). CAI was the generous gift of Dr. R. Schultz, Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). A polyclonal rabbit anti-TRP-1 antibody was purchased from Chemicon International, Inc. (Temecula, CA).
**Anti-T-type Ca\(^{2+}\) Channel Antibody.** Several previous workers have prepared polyclonal antibodies directed against the T-type Ca\(^{2+}\) channel (30, 31). The antibody was prepared as described by using a peptide corresponding to amino acids 1–22 of the NH\(_2\)-terminal region of human α1G subunit of the low-voltage T-type Ca\(^{2+}\) channel with the addition of a COOH-terminal cysteine: NH\(_2\)-MDEEEDGAGAEESGPRSFMRL(C)-COOH (30). The purified peptide was conjugated to keyhole limpet hemocyanin and then used to immunize rabbits (Bio-Synthesis, Inc., Lewisville, TX). The labeled IgG fraction was found to specifically label cells.

**Cell Motility and Phagokinetic Tracks.** Cell motility was assessed by the Albrecht-Buehler method (32) as modified by Scott et al. (33). Briefly, coverslips were dipped into a 0.17% gelatin solution and then coated with a colloidal gold layer. A suspension of 5000 cells/ml was treated with or without Ca\(^{2+}\) channel blockers and then seeded onto the gold-coated coverslips. After 22 h of incubation, the samples were fixed with 3.7% glutaraldehyde. The tracks produced by the cells were viewed by brightfield microscopy with a ×10 objective. Thirty randomly selected tracks were measured for each slide.

**Cell Invasion.** In vitro tumor cell invasion was assessed using a Biocoat Matrigel invasion chamber (BD Biosciences, Bedford, MA) with cell culture inserts containing an 8-mm pore size membrane with a thin Matrigel basement membrane matrix as described previously (34). One-half milliliter of cells (5 × 10\(^4\) cells/ml) in serum-free DMEM with or without Ca\(^{2+}\) channel blockers and then seeded onto the gold-coated coverslips. After 22 h of incubation, the samples were fixed with 3.7% glutaraldehyde. The tracks produced by the cells were viewed by brightfield microscopy with a ×10 objective. Thirty randomly selected tracks were measured for each slide.

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**Microfluorometry.** Quantitative microfluorometry was performed on single cells using a Peltier-cooled photomultiplier tube D104 system (Photon Technology, Lawrenceville, NJ) attached to a Zeiss Axiosvert 35 (Carl Zeiss, New York, NY) fluorescence microscope (29). A monochromator and fiber- optically coupled xenon lamp were controlled by FeliX software (Photon Technology). For indo-1-labeling experiments, the excitation was set to 350 nm (10-nm bandpass). Emission was detected using a 390LP dichroic reflector and 405DF43 emission filter. The photomultiplier tube output was plotted as a function of time.

**Fluorescence Microscopy.** Fluorescence microscopy was performed with an Axiosvert 135 fluorescence microscope with a high numerical aperture condenser, quartz objective, and an AttoArc mercury lamp (Zeiss). For immunofluorescence microscopy, cells were fixed with 3.7% glutaraldehyde for 20 min at room temperature. Samples were washed and then labeled with the first and second step antibodies. To minimize nonspecific binding, 1% BSA was included in these solutions. Images from multiple focal planes were collected using a z-scan apparatus (Veytek, Inc., Fairfield, IA) and cooled Retiga 1300 camera (Q-Imaging, British Columbia, Canada) or an intensified charge-coupled device camera (Hamamatsu, Hamamatsu City, Japan). Images were managed, processed, and deconvoluted using Supermicro Ultra 320 workstation with the software packages: Image-Pro, Microtome, Image-Scan, and Vox-Blast (Veytek). A narrow bandpass filter set with excitation at 485DF20 and emission at 530DF30 nm and a long-pass dichroic mirror at 510 nm were used. Representative sections of immunostained fluorescent samples were collected.

**High-Speed Fluorescence Microscopy.** High-speed microscopy was performed using an Axiosvert 135 fluorescence microscope with a quartz condenser, quartz objective, and an AttoArc mercury lamp (Zeiss). To detect fluorescence changes in the short wavelength emission region of indo-1, a 355HT15 exciter, 390LP dichroic reflector, and 405DF43 emission filter were used. To increase light collection efficiency, the microscope’s bottom port was used. This port was fiberoptically coupled to the input of an Acton-150 (Acton Instruments, Acton, MA) imaging spectrophotometer. The exit side was connected to a liquid N\(_2\)-cooled intensifier attached to a Peltier-cooled I-MAX-512 camera (∼–20°C; Princeton Instruments, Trenton, NJ; Refs. 34–36). A Get-II tube was used to provide maximal efficiency in the violet-blue region of the spectrum (29), where indo-1 emits. The camera was controlled by a high-speed Princeton ST-133 interface and Stanford Research Systems (Sunnyvale, CA) DG-535 delay gate generator (37). A custom-built computer with dual 3.06 GHz Xenon processors with 1 MB onboard cache each, 3-Gb RAM, 3.2 Tb of hard drive space with 64 MB cache, and a RAID-5 hard drive management system was used. For experiments, 2 Gb of RAM was allocated as a RAM disk. Winspec-32 (version 2.5.14.1; Princeton Instruments) software was used with a PCI communication accelerator. To improve computer acquisition times, the size of the pixel detection array of the charge-coupled device (CCD) chip was adjusted using a Virtual-Chip plug-in. Winspec CPU calls were given system priority to enhance the instrument’s duty cycle. Data were acquired without reporting to the monitor to further improve system speed. Data capture used a software-allocated RAM disk.

**RESULTS**

**Immunofluorescent Staining of T-Type Ca\(^{2+}\) Channels and TRP1 Cation Channels on HT1080 Cells.** In the present study, we test the hypothesis that LVA (T-type) and NVG channels participate in the signaling mechanism(s) necessary for HT1080 cell migration. Therefore, we sought to confirm that structural elements of these channels are expressed by HT1080 cells using immunofluorescence microscopy. HT1080 cells were stained with a rabbit anti-T-type Ca\(^{2+}\) channel antibody followed by a FITC-conjugated antirabbit IgG antibody. Stacks of fluorescence micrographs were obtained from individual cells followed by deconvolution analyses of the z-scans. Fig. 1, A and B show differential interference contrast microscopy and fluorescence micrographs of an HT1080 cell. The T-type channel is located primarily at the cell’s circumference. Fig. 1, C and D show a similar experiment using a rabbit anti-TRP1 antibody/FITC-conjugated antirabbit IgG staining. Although TRP1 staining was largely located at the cell periphery, the labeling was punctate in appearance. Labeling was often absent from pseudopods and may include a granule fraction near the plasma membrane. In addition, the z-scan/ deconvolution image of Fig. 1D suggests that minor labeling of the endoplasmic reticulum is also observed with the anti-TRP1 reagent. This is consistent with the fact that the fixation protocol results in

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*Fig. 1. Immunofluorescence staining of T-type Ca\(^{2+}\) channels and transient receptor potential 1 cation channels of HT1080 cells. Adherent HT1080 cells were fixed with glutaraldehyde and then labeled with rabbit anti-T-type Ca\(^{2+}\) channel polyclonal antibody (2 μg/ml; A and B) or rabbit antitransient receptor potential 1 cation channel polyclonal antibody (2 μg/ml; C and D), respectively, followed by labeling with a tetramethylrhodamine isothiocyanate-conjugated goat antirabbit antibody (1 μg/ml). Representative differential interference contrast microscopy (A and C) and deconvoluted z-scan fluorescence (B and D) images are shown. Although the T-type channels are largely distributed randomly at the cell periphery, the transient receptor potential 1 channels are found to have a more punctate distribution about the periphery (n = 3; ×980).*
permeabilization of the cell. Although previous studies have shown that tumor cells express T-type channel message, these studies now identify the structural expression of TRP1 and T-type channels by HT1080 cells.

**HT1080 Cells Exhibit Ca\(^{2+}\) Oscillations that Are Blocked by Mibefradil and Gd\(^{3+}\).**

We next evaluated Ca\(^{2+}\) signal oscillations in HT1080 cells. Cells were labeled with indo-1-AM as described in "Materials and Methods." HT1080 cells were suspended in HBSS and then incubated at 37°C for 20 min to allow the labeled cells to adhere to glass coverslips. Microfluorometry of untreated polarized cells showed a sustained series of Ca\(^{2+}\) spikes at 20-s intervals (Fig. 2, trace 1). To observe the longitudinal properties of these Ca\(^{2+}\) spikes, a long-time base was used in Fig. 2. However, to observe the temporal details of each Ca\(^{2+}\) spike, data were also acquired using a short-time base. Fig. 3, trace 1 shows a Ca\(^{2+}\) spike of an untreated HT1080 cell. This shows a typical Ca\(^{2+}\) spike profile consisting of a low-intensity shoulder on the leading edge, brief peak, and decay in indo-1 intensity. To confirm that Ca\(^{2+}\) entry participates in Ca\(^{2+}\) spikes, cells were treated with BAPTA-AM or EDTA, which are intracellular and extracellular Ca\(^{2+}\) chelators, respectively. Fig. 2, trace 2 shows an experiment identical to that of trace 1, except that BAPTA-AM was injected into the sample holder to give a final concentration of 50 μM. Within 3 min, the Ca\(^{2+}\) spike amplitude began to decrease, similar to that we have described previously in leukocytes (29). Similarly, rapid
removal of extracellular Ca\(^{2+}\) by the addition of 5 mM EDTA in Ca\(^{2+}\)-free HBSS, to remove Ca\(^{2+}\) in the buffer, also blocked Ca\(^{2+}\) spikes (trace 3). Hence, extracellular Ca\(^{2+}\) participates in the formation of intracellular Ca\(^{2+}\) spikes. No changes in Ca\(^{2+}\) spike frequency or amplitude were observed when cells were treated with 15 \(\mu M\) SKF96365, 1 \(\mu M\) diltiazem, 4 \(\mu M\) verapamil, and 1 \(\mu M\) nicardipine (Fig. 2, traces 4–7, respectively). These concentrations were chosen as they correspond to the levels associated with specific Ca\(^{2+}\) channel blockers; e.g., nicardipine is a HVA (L-type) Ca\(^{2+}\) channel blocker effective in vitro at this concentration (38). The NVG channel blocker and antimetastatic agent CAI, used at 10 \(\mu M\) for these in vitro studies (39), reduced Ca\(^{2+}\) spikes to Ca\(^{2+}\) “bumps,” which was then followed by elimination of the Ca\(^{2+}\) signal (Fig. 2, trace 8). In the experiment of Fig. 2, trace 9, 1.5 \(\mu M\) mibefradil, a T-type channel blocker, was added at the time indicated by the arrow. Within ~3–4 min, the Ca\(^{2+}\) spikes were replaced by very low-intensity bumps, which then disappeared. Similar results were obtained using Gd\(^{3+}\), a specific TRP (40), and stretch-activated cation channel blocker (41). Again, Ca\(^{2+}\) spikes diminished and then disappeared (Fig. 2, trace 10). The Ca\(^{2+}\) bumps were then evaluated using the short-time base settings (Fig. 3, trace 2). These experiments revealed that Ca\(^{2+}\) bumps closely resemble the intensity shoulder noted previously for untreated HT1080 cells (Fig. 3, trace 1).

The HT1080 Ca\(^{2+}\) Spike Is a Perimembrane Ca\(^{2+}\) Wave whose Propagation Is Affected by Mibefradil and Gd\(^{3+}\). Recent studies from this laboratory have shown that Ca\(^{2+}\) spikes of polarized neutrophils and transfectants are associated with certain intracellular Ca\(^{2+}\) waves (29, 34). To visualize these waves, images must be collected using very short shutter speeds to avoid image blurring attributable to wave motion and indo-1 diffusion while the shutter is open. A high-speed imaging method was developed to detect these waves (29, 34–37). High-speed microscopy shows that the Ca\(^{2+}\) spike (Fig. 2, trace 1; Fig. 3, trace 1) is a counterclockwise (in the microscope’s frame of reference) Ca\(^{2+}\) wave (Fig. 4A), e.g., in Fig. 4A, frames 2–16, the bright spot is a region of high Ca\(^{2+}\) concentration brought about by the local opening of Ca\(^{2+}\) channels. Because indo-1 has a relatively high affinity for Ca\(^{2+}\) and a finite amount of time is required for the signaling apparatus to pump down free cytosolic Ca\(^{2+}\), a low-intensity “tail” is observed next to the region of bright Ca\(^{2+}\)-indo-1 fluorescence (i.e., the Ca\(^{2+}\) wave has a low-intensity tail on the side opposite to the direction of wave motion). This wave was found to travel with a velocity of ~200 \(\mu m/s\). However, after the addition of mibefradil, a brief Ca\(^{2+}\) spark was observed at the lamellipodium, although no propagating Ca\(^{2+}\) wave was found (Fig. 4B). Similarly, a Ca\(^{2+}\) wave was observed in cells before (Fig. 4C) but not after (Fig. 4D) the addition of Gd\(^{3+}\). Thus, the...
regular Ca\(^{2+}\) spikes observed in migrating tumor cells are counterclockwise waves, when viewed from the bottom of the cell on a coverslip. Furthermore, the shoulder of the temporal Ca\(^{2+}\) spike likely corresponds to the temporal bump and spatiotemporal spark noted above. These waves are blocked by mibefradil and Gd\(^{3+}\), which suggests that their binding sites, including LVG and NVG channels, participate in wave propagation around the cell or ignition of the wave at the level of the plasma membrane.

**Mibefradil and Gd\(^{3+}\) Inhibit HT1080 Migration.** The central role of Ca\(^{2+}\) and Ca\(^{2+}\) oscillations in cell motility and ability of mibefradil and Gd\(^{3+}\) to influence these oscillations and waves suggest that these reagents may affect tumor cell motility. The phagokinetic track assay was used to test the ability of mibefradil and Gd\(^{3+}\) to inhibit HT1080 cell motility (32, 33). HT1080 cells were incubated on gold particle-coated glass coverslips for 22 h. As cells migrate on these surfaces, the gold particles are cleared from regions of the surface and frequently picked up by the cells. This makes the cells appear darker and surrounding area appear lighter when viewed by microscopy. Fig. 5 shows representative micrographs of this assay. In the absence of added reagents, large areas of the surface are cleared by the cells, as the cells become darker (Fig. 5A). Control experiments using treatment with the intra and extracellular Ca\(^{2+}\) chelators BAPTA (25 \(\mu\)M; B) and EDTA (5 mM; C) showed an absence of cell motility. Treatment with carboxamido-triazole (CAI, 10 \(\mu\)M; D), mibefradil (1.5 \(\mu\)M; E), or Gd\(^{3+}\) (1.5 \(\mu\)M; F) significantly reduced HT1080 area cleared by the cells. However, SKF96365 (15 \(\mu\)M; G), diltiazem (1 \(\mu\)M; H), verapamil (4 \(\mu\)M; I), and nicardipine (1 \(\mu\)M; J) had no effect on cell migration (\(n = 3\); \(\times 80\)).

![Fig. 5. Effect of Ca\(^{2+}\) channel blockers on HT1080 motility. A suspension of HT1080 cells with or without calcium channel blockers was seeded onto gold-coated coverslips. Brightfield microscopy was performed after incubation at 37°C. The extent of cell motility is indicated by the amount of neighboring area cleared by the cells. An untreated cell is shown in A. Control experiments using treatment with the intra and extracellular Ca\(^{2+}\) chelators BAPTA (25 \(\mu\)M; B) and EDTA (5 mM; C) showed an absence of cell motility. Treatment with carboxamido-triazole (CAI, 10 \(\mu\)M; D), mibefradil (1.5 \(\mu\)M; E), or Gd\(^{3+}\) (1.5 \(\mu\)M; F) significantly reduced HT1080 area cleared by the cells. However, SKF96365 (15 \(\mu\)M; G), diltiazem (1 \(\mu\)M; H), verapamil (4 \(\mu\)M; I), and nicardipine (1 \(\mu\)M; J) had no effect on cell migration (\(n = 3\); \(\times 80\)).](image)

![Fig. 6. Dose-dependent inhibition of HT1080 cell motility by mibefradil and Gd\(^{3+}\). HT1080 cells treated with various doses of mibefradil (A) or Gd\(^{3+}\) (B) were incubated on gold-coated coverslips. The area cleared by each cell was measured using Scion Image software. The swept area ratio was obtained by dividing the total swept area by the cell area. Both mibefradil and Gd\(^{3+}\) effectively inhibited HT1080 cell motility in a dose-dependent manner (\(n = 4\); *, \(P < 0.001\)).](image)
that blockade of LVG and NVG channels, but not L-type Ca\(^{2+}\) channels, diminishes HT1080 cell motility.

Previous studies have shown that fibronectin mobilizes Ca\(^{2+}\) from internal and external stores to the tumor cell cytoplasm (42). We confirmed this fact for HT1080 cells. Coverslips were coated with fibronectin as described (43), and then indo-1-labeled HT1080 cells were allowed to attach to the surface. Ca\(^{2+}\) spikes were observed at a much higher frequency, thus indicating an enhancement of the Ca\(^{2+}\) signaling apparatus (data not shown). No significant change in cell migration was observed in the presence of 25 \(\mu\)g/ml fibronectin for untreated, control, or drug-treated samples (data not shown). Although fibronectin influences the Ca\(^{2+}\) signals, it does not affect the ability of channel blockers to influence cell motility.

**Mibefradil and Gd\(^{3+}\) Inhibit HT1080 Cell Invasiveness.** Because cell motility is required for invasion, we tested the ability of mibefradil and Gd\(^{3+}\) to inhibit HT1080 cell invasion. Biocoat Matrigel invasion chambers were used for HT1080 cell invasion experiments. Cell suspensions were loaded to the top chamber with or without the addition of mibefradil, Gd\(^{3+}\), nicardipine, or other reagents. After a 22-h incubation period, transmigrated cells on the underside of the inserts were fixed, stained, and counted. Both mibefradil and Gd\(^{3+}\) inhibited HT1080 cell transmigration in a dose-dependent fashion (Fig. 7). However, nicardipine, SKF96365, verapamil, and diltiazem had no effect on cell invasiveness at pharmacologically appropriate doses (Fig. 8). Moreover, when the lowest doses of mibefradil and Gd\(^{3+}\) that exhibit maximal inhibition of invasiveness (5 and 6 \(\mu\)M, respectively; Fig. 7) were used simultaneously, the drug combination further augmented inhibition (Fig. 8). Inhibition (95\%) was seen using the combination of 5 \(\mu\)M mibefradil and 20 \(\mu\)M Gd\(^{3+}\). The complementary action of these two reagents is consistent with their ability to act on two different membrane sites contributing to the same physical wave. Similarly, CAI plus mibefradil showed a significant reduction in invasiveness in comparison with mibefradil alone. Therefore, HT1080 cell invasion was regulated by T-type and NVG Ca\(^{2+}\) channels but not L-type Ca\(^{2+}\) channels.

**DISCUSSION**

Previous \textit{in vitro} and \textit{in vivo} studies have suggested that Ca\(^{2+}\) channels are important in tumor cell proliferation and invasion (23, 44–55). Indeed, clinical trials of Ca\(^{2+}\)-active reagents have already been reported (50, 56–58), e.g., verapamil, a HVA Ca\(^{2+}\) channel blocker, has been used \textit{in vitro} and in combined therapy approaches (48–52). At higher doses, verapamil also blocks potassium channels (59), which may lead to some ambiguity in its mechanism of action. CAI is a promising anticancer agent acting on NVG channels, which is in Phase I and II clinical trials (56–58). The present study uses microfluorometry and high-speed imaging to characterize Ca\(^{2+}\) signals in HT1080 cells. Although a recent study has suggested that Ca\(^{2+}\) waves are present in tumor cells (10), we now rigorously demonstrate this fact. Furthermore, inhibitors of this Ca\(^{2+}\) wave, which were identified as NVG and LVA channel blockers, reduce dramatically cell motility and invasiveness. Our studies provide new insights into tumor cell migration and unprecedented detail concerning the cellular effects of Ca\(^{2+}\)-active reagents on tumor cells.

As we have emphasized (29, 35, 60), intracellular Ca\(^{2+}\) waves, which are the Ca\(^{2+}\) signal, can be very heterogeneous in their physical
properties (e.g., ignition, shape, location, direction, velocity, number, etc.); thus, a Ca$^{2+}$ signal is not an amount but rather a great variety of time-varying chemical patterns (60). The Ca$^{2+}$ wave associated with HT1080 cells travels about the periphery of the cytoplasm. These results are similar to our recent analysis of Ca$^{2+}$ spikes and counterclockwise Ca$^{2+}$ waves of morphologically polarized neutrophils (29). Ca$^{2+}$ signals of both neutrophils and HT1080 tumor cells were not sensitive to HVA type Ca$^{2+}$ channel blockers (29). Although HVA channels have been reported to regulate insect hemocyte adhesion (61) and mammalian neuronal migration (62), we demonstrated that they had no effect on HT1080 cell motility (Fig. 5) and invasion (Fig. 7). We demonstrated that the counterclockwise Ca$^{2+}$ wave could be blocked by mibebradil and Gd$^{3+}$, suggesting the association of LVA and TRP channels with Ca$^{2+}$ signaling in HT1080 cells. Furthermore, these two reagents apparently act early in wave propagation as a temporal “bump,” or a spatio-temporal spark of Ca$^{2+}$ is observed. Thus, the timing mechanism regulating cell motility seems to be intact (as suggested by the occurrence of these bumps), at least for a few minutes, although wave propagation and motility are not. This change in Ca$^{2+}$ signaling is likely to be the underlying biophysical mechanism accounting for the cellular effects of mibebradil and Gd$^{3+}$. As these reagents act on different proteins participating in the formation of the same chemical wave, we tested their ability to complementarily inhibit cell function. Although neither of these reagents completely blocked cell movement and invasion, the combination of mibebradil and Gd$^{3+}$ inhibited ~95% of HT1080 cell invasion.

The studies reported above provide some insight into the nature of Ca$^{2+}$ signaling associated with cell motility. On the basis of these and earlier (29, 35) studies, we suggest that the initial Ca$^{2+}$ “flash” or “bump” is associated with the endoplasmic reticulum or a component of the endoplasmic reticulum closely associated with the plasma membrane. Alternatively, the TRP channel could be responding to cytoskeletal interactions or changes in membrane stress (63, 64). In either case, we speculate that TRP or TRP-like channels communicate this signal in a Gd$^{3+}$-sensitive fashion to the plasma membrane. T-type Ca$^{2+}$ channels propagate this Ca$^{2+}$ signal at the plasma membrane, as suggested by mibebradil inhibition. T-type channels (LVA) are activated by small depolarizations of the plasma membrane, which promote a Ca$^{2+}$-permeable state and its accompanying depolarization. This depolarization allows the signal to propagate about the cell periphery. This Ca$^{2+}$ signal may participate in regulating cell migration through its effects on integrins, the cytoskeleton, membrane recycling, and other cellular processes. Our in vitro studies of tumor cell Ca$^{2+}$ signaling and behavior lead to several points of potential clinical relevance: (a) CAI, a subject of clinical trials (56–58), is likely acting on the same Ca$^{2+}$ pathway and cell functions described in this study; (b) because mibebradil is also acting on this same physical Ca$^{2+}$ signal, and augments the inhibition of cell motility and invasiveness in vitro, it may be possible to improve clinical outcomes with CAI by combining treatment protocols with mibebradil. However, mibebradil was removed from the market because of dangerous drug interactions. Mibebradil analogues, which do not exhibit such drug interactions, is a viable alternative; (c) because this dynamic microscopic technique could be used with tissue samples or isolated leukocytes from patients (which display a similar pattern of NVG and LVG channels), it might be possible to adjust doses for each patient. This controls for potential genetic variations in the Ca$^{2+}$ signaling apparatus among patients. Moreover, the temporal features of tumor cell signaling may be central in patient evaluation and devising new treatment strategies; and (d) because the concentration of Gd$^{3+}$ used in the present in vitro studies is lower than the doses leading to illness in experimental animals (65), it might provide another therapeutic route. Thus, high-speed imaging is useful in evaluating the underlying dynamic biophysical properties of tumor cells and may significantly contribute to translational medicine.

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REFERENCES


