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# Design of a High-Affinity Competitive Antagonist of the Vanilloid Receptor Selective for the Calcium Entry-Linked Receptor Population

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## ABSTRACT

We describe the synthesis and characterization of *N*-(4-chlorobenzyl) -*N′*-(4-hydroxy-3-iodo-5-methoxybenzyl)thiourea (IBTU), a novel antagonist of the vanilloid receptor 1 (TRPV1 or VR1). IBTU competitively inhibited $^{45}Ca^{2+}$ uptake into CHO cells heterologously expressing rat TRPV1, whether induced by capsaicin or resiniferatoxin ($K_i$ = 99 ± 23 and 93 ± 34 nM, respectively). IBTU was thus somewhat more potent (5-fold) than capsazepine. In contrast to its antagonism of vanilloid-induced calcium uptake, IBTU (30 $\mu$M) inhibited [$^3$H]resiniferatoxin binding to TRPV1 by less than 10%. We hypothesize that these dramatically distinct potencies reflect different fractions of TRPV1 in this system: namely, a minor plasma membrane fraction controlling $^{45}Ca^{2+}$ uptake, and the predominant intracellular fraction that dominates the [$^3$H]resiniferatoxin binding measurements. Intracellular $Ca^{2+}$ imaging supports this explanation. IBTU antagonized the elevation in intracellular $Ca^{2+}$ in response to 50 nM capsaicin with an $IC_{50}$ of 106 ± 35 nM. Likewise, 600 nM IBTU was able to antagonize the elevation in intracellular $Ca^{2+}$ in response to 100 pM resiniferatoxin in the presence of normal (1.8 mM) extracellular $Ca^{2+}$, where the increase in intracellular calcium reflects calcium influx. In contrast, in the absence of extracellular $Ca^{2+}$, where in this system resiniferatoxin induces a modest increase in calcium from intracellular stores, IBTU was unable to block the response to resiniferatoxin, although the TRPV1 antagonist 5-iodoresiniferatoxin was able to do so. In summary, IBTU is a novel, potent TRPV1 antagonist with marked selectivity between subpopulations of TRPV1 and may permit the function of these distinct pools to be explored and potentially exploited.

The vanilloid receptor TRPV1 is a polymodal integrator of noxious stimuli (Caterina et al., 1997), including low pH, heat, and ligands such as capsaicin, the pungent constituent in red pepper. Because of its function as a nociceptor, TRPV1 has attracted much attention as a novel, potential therapeutic target for the treatment of pain and other conditions involving C fiber sensory neurons (Kress and Zeilhofer, 1999; Szallasi and Blumberg, 1999; Caterina and Julius, 2001). Such applications include bladder hyperreflexia, detrusor instability, postmastectomy pain, mucositis, interstitial cystitis, pharyngitis, pancreatitis, enteritis, cellulitis, postherpetic neuralgia, peripheral neuropathy, arthritis, and bony fractures (Robbins, 2000).

The characterization of novel natural products, such as resiniferatoxin, an ultrapotent TRPV1 agonist, and the emerging wealth of synthetic capsaicin analogs are driving a rapidly developing appreciation of the complexities of vanilloid pharmacology. Two general strategies for therapeutic intervention have received much attention. First, activation of TRPV1 by agonists is followed by desensitization/defunctionalization. As illustrated for resiniferatoxin or for olvanil, the acute response of pungency and the chronic response of desensitization/defunctionalization are at least partially dissociable, so the development of potent agonists with minimal pungency represents one therapeutic approach. Because of its long potential duration of action, desensitization/defunctionalization is perhaps most appropriate when a long-term effect is desired, as in bladder hyperreflexia. A second strategy is the development of competitive antagonists. This ap-

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**ABBREVIATIONS:** VR1 or TRPV1, vanilloid receptor 1; BCTC, *N*-(4-*t*-butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropryazine-1(2H)-carboxamide; I-RTX, (4-hydroxy-5-iodo-3-methoxyphenylacetate ester) iodo-resiniferatoxin; IBTU, *N*-(4-chlorobenzyl)-*N′*-(4-hydroxy-3-iodo-5-methoxybenzyl-)thiourea; CHO, Chinese hamster ovary; CHO-TRPV1, CHO cells expressing the rat vanilloid receptor 1; GFP-TRPV1, green fluorescent protein tagged rat TRPV1; CHO-GFP-TRPV1, CHO cells transiently expressing GFP-TRPV1; FBS, fetal bovine serum; DPBS, Dulbecco's phosphate-buffered saline; RTX, resiniferatoxin; JYL-827, *N*-[2-(3,4-dimethylbenzyl)-3-(pivaloyloxy)propyl]-*N′*-[4-(methylsulfonylamino)benzyl]thiourea.

proach has the potential benefit of rapid reversibility. Another characteristic is that antagonism should show greater specificity, because it would not be expected to cause heterologous blockade of other activation pathways for the C fiber neurons.

After the identification of capsazepine (Bevan et al., 1992), which proved that it was possible to develop competitive antagonists of TRPV1, several distinct approaches have afforded antagonists. We have characterized as competitive or partial antagonists derivatives in which the *p*-hydroxy group on the A region of capsaicin analogs has been replaced with a methylsulfonylamino residue (Wang et al., 2002, 2003). Starting with resiniferatoxin, Wahl and coworkers described the *m*-iodo derivative of RTX as a potent (4 nM) TRPV1 antagonist, although the noncompetitive nature of the antagonism in the oocyte model system indicates that its kinetics of action remains to be fully clarified (Wahl et al., 2001). Additional antagonists have also been reported (Park et al., 2003; Yoon et al., 2003, Valenzano et al., 2003). These antagonists now permit evaluation of their potential effects in therapeutic models. In addition to blockade of capsaicin action, antagonists of TRPV1 have been shown to block nociceptive responses in various systems, supporting their potential therapeutic utility (Walker et al., 2003; Pomonis et al., 2003). This inhibition could be visualized as arising either from antagonism of endogenous TRPV1 ligands such as lipoxygenase products or from stabilization of a closed receptor conformation, as implied from the inhibition by *N*-(4-*tert*-butylbenzyl)-*N*′-[3-fluoro-4-(methylsulfonylamino)benzyl]-thiourea (JYL1421 or SC0030) (Wang et al., 2002) of response to heat and pH or the inhibition by BCTC of the response to pH (Valenzano et al., 2003).

The evaluation of the novel vanilloids emerging for the current robust medicinal chemical efforts has been complicated by distinct differences in the structure activity relations as revealed by different in vitro assays. In particular, we have described 20- to 50-fold differences in potencies of vanilloids as assayed by inhibition of [$^3$H]resiniferatoxin binding and by induction of $^{45}Ca^{2+}$ uptake (Acs et al., 1996; Lee et al., 2001). Although these results suggested distinct receptors mediating these responses, cloned, heterologously expressed TRPV1 was able to account both for [$^3$H]resiniferatoxin binding as well as for $^{45}Ca^{2+}$ uptake (Szallasi et al., 1999). Likewise, mice in which TRPV1 was genetically knocked out fully lost response to vanilloids (Caterina et al., 2000; Davis et al., 2000). It seems, then, that the distinct structure activity relations revealed by the [$^3$H]resiniferatoxin binding and $^{45}Ca^{2+}$ uptake assays reflect differently modified fractions of the same TRPV1 receptor.

After the cloning of TRPV1 (Caterina et al., 1997), it has become apparent that TRPV1 is localized to internal sites within the cell as well as to the plasma membrane and, indeed, that the internal localization is often predominant. We and others have shown that heterologously expressed green fluorescent protein labeled TRPV1 is predominantly located in the endoplasmic reticulum, with only a minor fraction evident at the plasma membrane (Olah et al., 2001a,b). This pattern of localization depends somewhat on the specific expression system (Jahnel et al., 2001) but is also seen with unmodified, heterologously expressed TRPV1 in various systems as well as with endogenous TRPV1 in dorsal root ganglion cells, as detected with specific TRPV1 antibodies (Guo et al., 1999; Liu et al., 2003). Consistent with internal TRPV1 being functional, resiniferatoxin and, to a lesser degree, capsaicin, can release calcium from internal pools in the absence of external calcium (Eun et al., 2001; Liu et al., 2003; Marshall et al., 2003; Turner et al., 2003; Wisnoskey et al., 2003).

Here, we describe the synthesis and characterization of IBTU, a novel TRPV1 antagonist with a unique pattern of selectivity. Although IBTU displays very weak activity as an inhibitor of [$^3$H]resiniferatoxin binding to TRPV1, IBTU is a potent (100 nM) antagonist of $^{45}Ca^{2+}$ uptake in response to resiniferatoxin or capsaicin. By calcium imaging, IBTU was found to block the increase in intracellular calcium from the medium in response to resiniferatoxin but not that released from internal stores. These results suggest that the basis for the difference in structure activity relations in the assays may be ascribable to differences in the structure-activity relations for TRPV1 at the plasma membrane and internally. In any case, IBTU provides a tool to dissect the roles of those pools of TRPV1 as detected in the different assays and to explore the implications of this selectivity.

## Materials and Methods

**Materials.** Resiniferatoxin was from Biomol (Plymouth Meeting, PA); capsaicin, I-RTX, and other chemicals were from Sigma (St. Louis, MO) if not stated otherwise.

*N*-(4-Chlorobenzyl)-*N*′-(4-hydroxy-3-iodo-5-methoxybenzyl)thiourea (IBTU) was prepared by treating a solution of 4-hydroxy-3-iodo-5-methoxybenzylamine in dimethyl formamide drop-wise with a solution of 4-chlorobenzyl isothiocyanate in dimethyl formamide at room temperature. The mixture was stirred at room temperature for 22 h and poured into water. The product was extracted into ether, and the combined ether extracts were washed with water, dried, and evaporated to dryness. The residue was purified by flash column chromatography (silica gel, $CH_2Cl_2$ followed by 10% MeOH in $CH_2Cl_2$) and recrystallized from a MeOH/ether mixture to provide the product (70 mg, 38%). $^1$H NMR (DMSO-$d_6$) $\delta$ 9.42 (s, 1H), 7.99 (br s, 2H), 7.27–7.39 (m, 4H), 7.17 (s, 1H), 6.93 (s, 1H), 4.68 (br s, 2H), 4.54 (br s, 2H), 3.76 (s, 3H); $^1$H NMR ($CDCl_3$/drop of DMSO-$d_6$) $\delta$ 7.24–7.31 (m, 4H), 7.21 (d, 1H, $J$ = 2.0 Hz), 7.13 (br s, 2H), 6.87 (d, 1H, $J$ = 2.0 Hz), 6.66 (s, 1H, OH), 4.75 (d, 2H, $J$ = 5.4 Hz), 4.65 (d, 2H, $J$ = 5.4 Hz), 3.87 (s, 3H); EI-MS $m/z$ 462 ($M^+$). Further synthetic chemistry details as well as the elaboration of the structure-activity relationship of this particular class of thioureas will be published separately.

**Cell Culture.** The selected stable CHO cell clone expressing TRPV1 (Tet-Off induced CHO-TRPV1 cells) was cultured in maintaining media (F12 supplemented with 10% FBS (USA sourced), 25 mM HEPES, pH 7.5, 250 $\mu$g/ml G-418 (all from Invitrogen, Carlsbad, CA) and 1 mg/l tetracycline (Calbiochem, La Jolla, CA). The derivation of the CHO-TRPV1 cells was described by Szallasi et al. (1999).

The CHO cell line (used for transfections and as control for the measurements) was maintained in Ham's F12 medium supplemented with 10% FBS and 25 mM HEPES, pH 7.5 (all from Invitrogen).

**$^{45}Ca^{2+}$ Uptake Experiments.** CHO-TRPV1 cells were plated in 24-well plates to yield a cell density of 20 to 40% confluence. The next day, the media was changed to remove the tetracycline and induce TRPV1 expression. Experiments were done approximately 48 h after induction when the cultures were confluent to get the highest signal. For assay of $^{45}Ca^{2+}$ uptake, cells were incubated for 5 min at 37°C in a total volume of 500 $\mu$l of serum-free Dulbecco's modified Eagle's medium (Invitrogen) containing 1.8 mM $CaCl_2$ in the presence of 0.25 mg/ml bovine serum albumin (Sigma, St. Louis, MO), 1 $\mu$Ci/ml $^{45}Ca^{2+}$ (ICN Pharmaceuticals, Costa Mesa, CA) and increasing con-

centrations of the compound to be tested. Immediately after the incubation, extracellular $^{45}Ca^{2+}$ was removed by washing the cells three times with ice-cold DPBS (Invitrogen) containing 1.8 mM $CaCl_2$. Then, 400 $\mu$l of radioimmunoprecipitation assay buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) was added to each well to lyse the cells. Plates were shaken slowly for 20 min. Then, 300 $\mu$l of cell lysate was transferred from each well into a scintillation vial and radioactivity was determined by scintillation counting. For each data point in each experiment, four wells were assayed. Data from these experiments were analyzed by computer fit to the Hill equation. Each experiment was performed at least three times.

**[$^3$H]RTX Binding Experiments.** Binding assay mixtures were set up on ice and contained about $5 \times 10^5$ CHO-TRPV1 cells, 0.25 mg/ml bovine serum albumin (Cohn fraction V; Sigma, St. Louis, MO), [$^3$H]resiniferatoxin (37 mCi/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) and nonradioactive ligands. The final volume was adjusted to 400 $\mu$l with the buffer described above. Nonspecific binding was defined as binding occurring in the presence of 100 nM nonradioactive RTX (Alexis Corp., San Diego, CA). Binding was analyzed in the presence of a fixed concentration of [$^3$H]RTX (~40 pM) and various concentrations of competing ligands.

The binding was initiated by transferring the assay tubes into a 37°C water bath and then was terminated after a 60 min incubation period by cooling the assay mixtures on ice. Nonspecific binding was reduced by adding 200 $\mu$g of bovine glycoprotein fraction VI (ICN Pharmaceuticals) to each tube. Membrane-bound RTX was separated from the free and the glycoprotein-bound RTX by pelleting the membranes in a Beckman 12 bench-top centrifuge (15 min; maximal velocity; Beckman Coulter, Fullerton, CA), and the radioactivity was determined by scintillation counting. The binding of [$^3$H]RTX to the membranes of control CHO cells was not detectable, indicating that the heterologously expressed TRPV1 was the only receptor for RTX in the CHO-TRPV1 cells.

**$Ca^{2+}$ Imaging.** For experiments addressing the pharmacological properties of IBTU by $Ca^{2+}$ imaging, CHO-TRPV1 (Fig. 5) and CHO-GFP-TRPV1 (Fig. 6) cells were used. The CHO-TRPV1 cells were plated on 25-mm round glass coverslips in maintaining media (Ham's F12 supplemented with 10% FBS, 25 mM HEPES, pH 7.5, 250 $\mu$g/ml G-418 and 1 mg/l tetracycline) to yield a cell density about 5 to 10%. The next day, the media was changed to inducing media (maintaining media without tetracycline but containing 1 mM sodium butyrate) to induce TRPV1 expression. Because in these experiments individual cells should be measured (in contrast to the $^{45}Ca^{2+}$ uptake experiments, where the averaged signal is the only measurable value), experiments were done approximately 24 h after induction. At that point, the level of confluence was about 30 to 40%, optimal to find individual cells in the field of the fluorescent microscope, whereas after 48 h of induction, the level of confluence was higher and it was not practical to select individual cells.

The intracellular $Ca^{2+}$ pools of CHO-TRPV1 cells were very limited, as determined by the release of $Ca^{2+}$ from the intracellular pools by thapsigargin. To examine the possible role of IBTU on TRPV1 located in intracellular compartments, we first developed suitable conditions to achieve measurable release from the intracellular $Ca^{2+}$ pools by thapsigargin, using transient transfection of GFP-TRPV1 into CHO cells. For the transfection of GFP-TRPV1, CHO cells were plated onto six-well plates (Corning, Palo Alto, CA) containing 25-mm round, uncoated glass coverslips at the bottom of the wells to reach a confluence about 10 to 15%. The next day, when the confluence was about 20 to 25%, the cells were transfected with GFP-TRPV1 plasmid (1 $\mu$g plasmid into each well) using the LipofectAMINE plus method according to the manufacturer's instructions. Experiments were done approximately 24 h after transfection, when the level of confluence was about 30 to 40%.

For the fura2 loading, the cells were transferred to DPBS containing 1 mg/ml bovine serum albumin and 5 $\mu$M fura2-acetoxymethyl ester (Molecular Probes, Eugene, OR) for 2 h at room temperature. The cells were kept in maintaining media at room temperature until the measurements, which were carried out in DPBS, were performed. The fluorescence of individual cells was measured with an IntCyt Im2 fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH). The cells within a field were illuminated alternately at 340 and 380 nm. Emitted light >510 nm was measured. Data were analyzed with the Incyt 4.5 software and further processed with Excel (Microsoft, Redmond, WA) and Prism 2.0 (GraphPad Software Inc., San Diego, CA) software.

## Results

The two typical assays for measurement of activation of TRPV1 channels are uptake of $^{45}Ca^{2+}$ and elevation of intracellular calcium. These methods, although similar, detect somewhat different pools of TRPV1 (Fig. 1). We routinely use $^{45}Ca^{2+}$ uptake as a functional measure for initial evaluation

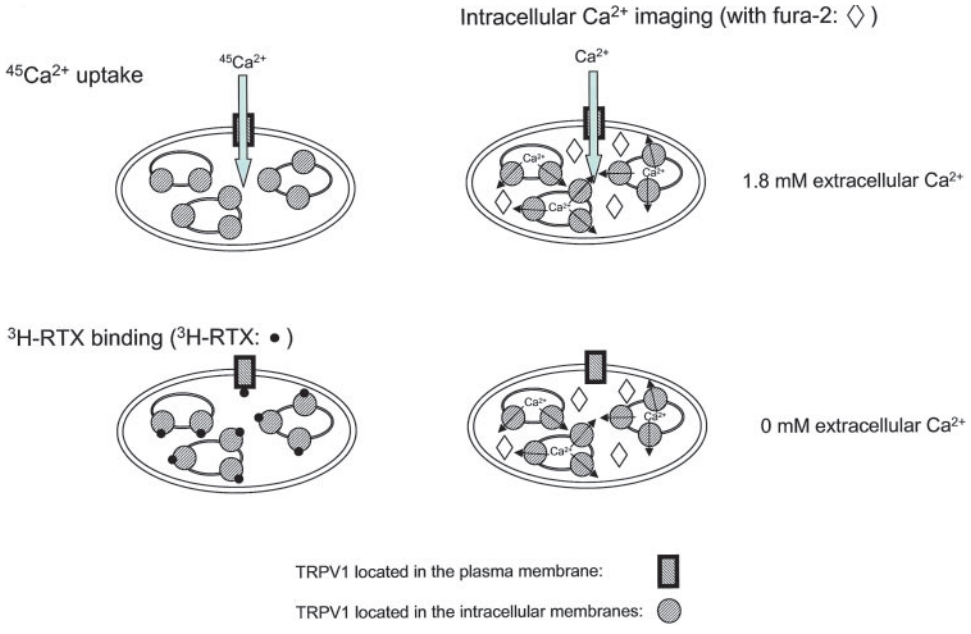


**Fig. 1.** A schematic representation of TRPV1 pools measured by different assays.

of vanilloids. The $^{45}Ca^{2+}$ accumulation in this case depends on the ratio of the $Ca^{2+}$ influx and efflux. In our assay, we used a relatively short incubation time (5 min) in the continuous presence of an agonist (capsaicin or resiniferatoxin) to measure the effects primarily on the influx of $Ca^{2+}$. Intracellular $Ca^{2+}$ imaging, in which fura-2 fluorescence is used to monitor changes in the $Ca^{2+}$ concentrations in the cytosol, provides an alternative measure of TRPV1 function. Upon activation of TRPV1 by agonists in the presence of extracellular $Ca^{2+}$, a rise in the signal will reflect $Ca^{2+}$ influx, $Ca^{2+}$ efflux, and the release of $Ca^{2+}$ from the intracellular pools of $Ca^{2+}$ via activation of TRPV1 located on intracellular membranes. In the absence of extracellular $Ca^{2+}$ (and in the presence of $Ca^{2+}$ chelators extracellularly), the signal will represent the release of $Ca^{2+}$ from the intracellular pools and $Ca^{2+}$ efflux from the cytoplasm. To characterize directly the interaction of vanilloids with TRPV1, inhibition of [$^3$H]RTX binding provides a convenient measure. In this assay, we use membranes from TRPV1 expressing cells (frozen pellet) so that receptor accessibility is not an issue and we incubate for 60 min to insure that we reach binding equilibrium. As discussed in the introduction, most of the TRPV1 receptors are expressed intracellularly; correspondingly, most of the signal detected in the binding experiments is the result of the binding to the TRPV1 that had been located in the intracellular pools.

The structure of capsaicin and related vanilloids can be divided into three pharmacophoric regions, A, B, and C (Walpole et al., 1993a,b,c), as illustrated in Fig. 2. IBTU exploits the A region found in I-RTX, which conferred antagonistic activity to that RTX derivative (Wahl et al., 2001), together with a B region thiourea, which enhances affinity in the calcium uptake assay (Walpole et al., 1993b), and a C region similar to that of capsazepine (Bevan et al., 1992). As described elsewhere, however, these regions cannot be considered as independent, and in other structural series, the C-region has been clearly shown to modulate the extent of antagonism conferred by the A-region substitution (Wang et al., 2003).

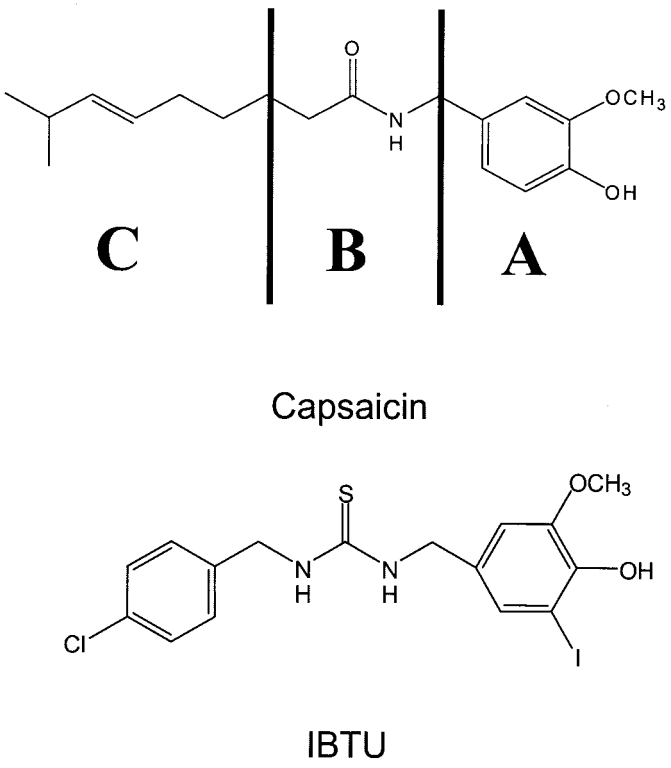


**Fig. 2.** The structures of capsaicin and IBTU. The A, B, and C regions of capsaicin are indicated.

The binding potency of IBTU to TRPV1 was determined by inhibition of [$^3$H]RTX binding (Fig. 3A). There was almost no [$^3$H]RTX binding inhibition (3.3 ± 3.3% inhibition, $n$ = 3 experiments) detected at a 30 $\mu$M concentration of IBTU and none at lower concentrations, suggesting an inactive compound. Surprisingly, in contrast to the inability of IBTU to replace [$^3$H]RTX from the membranes of CHO-TRPV1 cells, it was a potent inhibitor of the $^{45}Ca^{2+}$ uptake induced by capsaicin (Fig. 3B; $K_i$ = 99 ± 23 nM; $p$ = 1.28 ± 0.39, $n$ = 4 experiments). To exclude the possibility that the apparent difference in activity depended on the ligand with which IBTU was competing, we similarly tested the ability of IBTU to inhibit the RTX induced $^{45}Ca^{2+}$ uptake of the CHO-TRPV1 cells (Fig. 3C). The potency of IBTU under these circumstances ($K_i$ = 93 ± 34 nM; $p$ = 1.52 ± 0.12, $n$ = 3 experiments) was almost identical to that determined using capsaicin. Because we have described vanilloids that function as partial antagonists/partial agonists, we also assayed the activity of IBTU as an agonist of TRPV1. At 10 $\mu$M, we detected 1.76 ± 0.88% ($n$ = 3 experiments) of the level of stimulation of $^{45}Ca^{2+}$ uptake over baseline compared with capsaicin (300 nM). Although we can thus conclude that IBTU was a full antagonist under our assay conditions, we have described that the extent of agonism for partial agonists depends on the level of TRPV1 and on the level of modulation by other factors, such as protein kinase C (Wang et al., 2003). It is therefore possible that IBTU would show a low level of agonism under other conditions.

To clarify the mechanism of the IBTU inhibition, we tested whether IBTU was a competitive, noncompetitive, or mixed-type inhibitor of RTX or capsaicin induced $^{45}Ca^{2+}$ uptake. The dose-response curves for capsaicin and RTX were determined in the presence of different concentrations of IBTU (0, 100, 300, 1000 nM). Qualitatively consistent with a competitive mechanism for the antagonism, the maximal responses remained constant, but the dose response curves shifted to the right as a function of IBTU concentration (Fig. 4, A and C). Quantitatively consistent with a competitive mechanism, the apparent $EC_{50}$ values fitted the equation $EC_{50}$ (apparent) = $EC_{50}$ + I/$K_i$, where I = concentration of the antagonist and $K_i$ = dissociation constant for the antagonist. In the case of capsaicin (Fig. 4, A and B), the equation yielded a $EC_{50}$ for capsaicin of 98 nM ($n$ = 3 experiments), similar to our previous determinations, and a $K_i$ value for IBTU of 148 nM ($n$ = 3 experiments), likewise similar to our previously determined $K_i$ of 99 ± 23 nM. For RTX, the equation yielded an $EC_{50}$ for RTX of 32 pM ($n$ = 4 experiments), similar to our previous determinations, and a $K_i$ value for IBTU of 80 nM ($n$ = 4 experiments), likewise similar to our previously determined $K_i$ of 93 ± 34 nM (Fig. 4, C and D). We conclude that IBTU is a competitive antagonist of capsaicin and RTX action on TRPV1 in the $^{45}Ca^{2+}$ uptake assays.

To further explore the mechanism and kinetics of antagonism by IBTU, its activity on TRPV1 was evaluated by intracellular $Ca^{2+}$ imaging. CHO-TRPV1 cells were pretreated

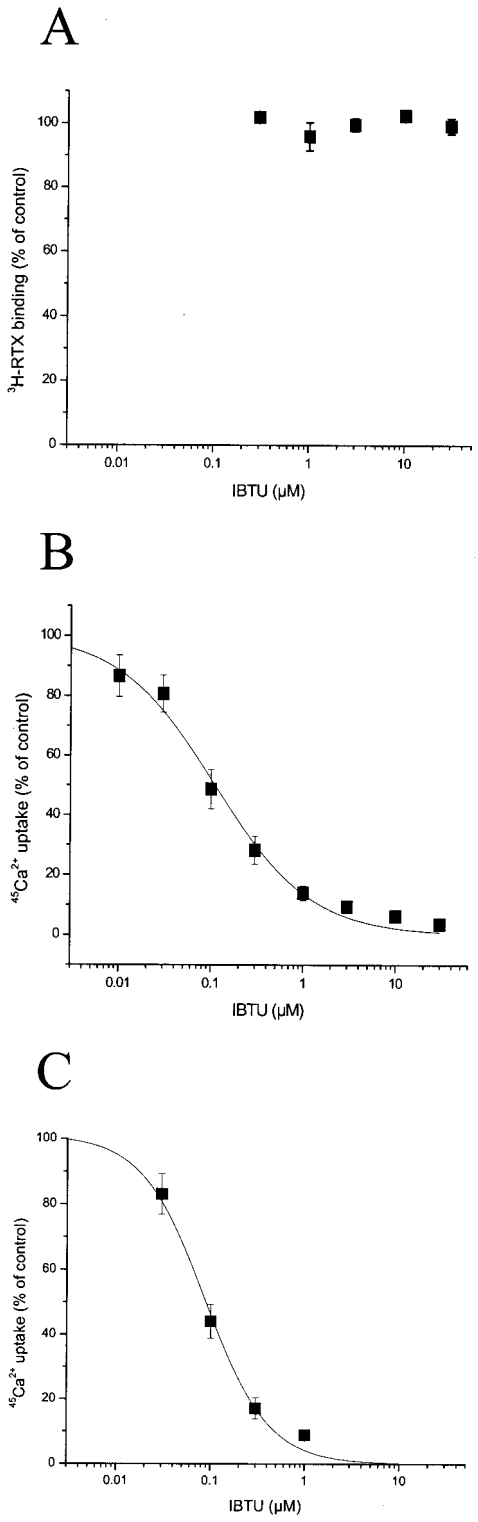


**Fig. 3.** Effect of IBTU on RTX binding and calcium uptake in CHO-TRPV1 cells. A, minimal inhibition by IBTU of [$^3$H]RTX (50–100 pM) binding to CHO-TRPV1 membranes. Binding (percentage of control) was plotted as function of IBTU concentration. A single experiment is shown, bars ± S.E.M.; each point represents triplicate determinations in that experiment. Two additional experiments yielded no inhibition. Antagonism by IBTU of $^{45}Ca^{2+}$ uptake induced by capsaicin (50 nM, B) or resiniferatoxin (100 pM, C). Results are expressed as percentage of stimulation without IBTU as a function of the concentration of IBTU (0.01–30 μmol). The points represent the mean, bars ± S.E.M., from four determinations in a single, representative experiment. Two additional experiments yielded similar results.

with different concentrations of IBTU (0–6500 nM) for 4 min, 50 nM capsaicin was then added, and the intracellular $Ca^{2+}$ concentration was monitored as a function of time (Fig. 5A). The maximal signals (ratio of fluorescence) were plotted as a function of the IBTU concentration (Fig. 5B). Under these conditions, IBTU blocked the elevation in intracellular $Ca^{2+}$ concentration with a similar potency ($K_i$ = 106 ± 36 nM; $p$ = 1.91 ± 0.20, $n$ = 3 experiments) to that in the $^{45}Ca^{2+}$ experiments ($K_i$ = 99 ± 23 nM). Conversely, we tested the ability of IBTU to reverse the action of capsaicin when the IBTU was added after the capsaicin. The cells were treated for 5 min with 50 nM capsaicin, after which 10 μM IBTU was added in the continued presence of the capsaicin (Fig. 5C). The IBTU antagonized the capsaicin, with an immediate onset of action. The reversibility of the capsaicin-induced responses is dependent not only on the inhibition of the vanilloid receptor but also on the ability of the cell to restore its original steady-state $Ca^{2+}$ level. Because capsazepine (10 μM) gave similar kinetics of inhibition (data not shown), we assume that the rate of decrease in $Ca^{2+}$ after addition of IBTU is reflecting this latter parameter.

The vanilloid binding site of TRPV1 is located on the inner face of the membrane. The rapid onset of the antagonism of the capsaicin-induced elevation of the intracellular $Ca^{2+}$ concentrations, as indeed the antagonism per se, provide measures of the penetration of IBTU into the cell (Fig. 5C).

The marked difference (more than 300-fold) between the inhibition of RTX binding and the inhibition of the calcium response to RTX suggested that the two assays are probing different subsets of TRPV1. The $^{45}Ca^{2+}$ experiments monitor TRPV1 function at the plasma membrane, which represents only a small fraction of total TRPV1 in most systems. The [$^3$H]RTX binding assay measures all TRPV1, most of which is intracellular and seems to be located at the endoplasmic reticulum. Because the capsaicin binding site of TRPV1 is located on the inner face of the membrane and our [$^3$H]RTX assay is carried out on membrane preparations, it is clear that lack of penetration of IBTU through the membranes cannot account for the difference in response. It is known that RTX can release calcium from the cytoplasmic stores in the endoplasmic reticulum. Based on the binding assay results, we therefore hypothesized that this internal TRPV1 may not be sensitive to IBTU, unlike the plasma membrane localized TRPV1.

To test this prediction, we used CHO cells transiently transfected with GFP-TRPV1. This system was selected to give a measurable intracellular $Ca^{2+}$ pool as determined by the release of $Ca^{2+}$ induced by thapsigargin. We first measured the response to RTX for cells in the presence of 1.8 mM $Ca^{2+}$ in the extracellular solution. Cells were incubated in buffer for 4 min; then, 100 pM RTX was added. To determine the maximal response under these conditions, a subsequent RTX treatment was applied using a supramaximal dose of RTX (100 nM), which should be sufficient to overcome antagonism by modest concentrations of an antagonist. The response of CHO-GFP-TRPV1 cells to 100 pM RTX was almost maximal (Fig. 6A; three experiments were done, yielding similar results). To determine the sensitivity of the increase in intracellular $Ca^{2+}$ to IBTU, we applied 100 pM RTX after 4-min pretreatment with 600 nM IBTU. This concentration of IBTU fully blocked the response to RTX under these conditions (Fig. 6B; three experiments). On the other hand, the

subsequent application of 100 nM RTX led to a similar maximal response to that seen in the absence of IBTU (Fig. 6A), indicating that the inhibition by IBTU was competitive. The results from these calcium-imaging experiments are thus consistent with the results from the $^{45}Ca^{2+}$ uptake studies.

To address the question of the regulation of the intracellular membrane localized TRPV1, we performed the experiments in the absence of extracellular $Ca^{2+}$ (experiments in the absence of $Ca^{2+}$ and with 100 $\mu$M EDTA in the extracellular solution). First, we confirmed that the intracellular RTX-sensitive $Ca^{2+}$ pool was a part of the thapsigargin releasable $Ca^{2+}$ pool (data not shown), as reported previously (Marshall et al., 2003). We could detect the release of $Ca^{2+}$ into the cytoplasm using 100 pM RTX at 4 min (Fig. 6C, $n$ = 4 experiments; note difference in scale compared with A and B). The effect was about half-maximal compared with the signal upon application of thapsigargin (Fig. 6C), indicating that just half of the intracellular pools were released by RTX. It is noteworthy that the signals were transient in contrast with the signals in the presence of extracellular $Ca^{2+}$, where the elevated intracellular $Ca^{2+}$ concentration was maintained over a long period of time (data not shown). The reason of the difference is presumably the limited $Ca^{2+}$ content of the intracellular pools in the absence of extracellular $Ca^{2+}$. Next, we pretreated the cells with 600 nM IBTU for 4 min before application of 100 pM RTX. (Fig. 6D; $n$ = 3 experiments). Under these conditions, the IBTU failed to inhibit the response to RTX. Once again, we confirmed that RTX was able to induce $Ca^{2+}$ release. The results were indistinguishable from the control experiment in which pretreatment was with buffer alone rather than with IBTU (Fig. 6C). To demonstrate the specificity of the RTX release of $Ca^{2+}$ from the intracellular pools of $Ca^{2+}$, we pretreated the cells with 10 nM I-RTX, reported to be a potent antagonist of TRPV1 with a $K_i$ for inhibition of [$^3$H]RTX binding of 4 nM (Wahl et al., 2001). Unlike IBTU, I-RTX fully antagonized

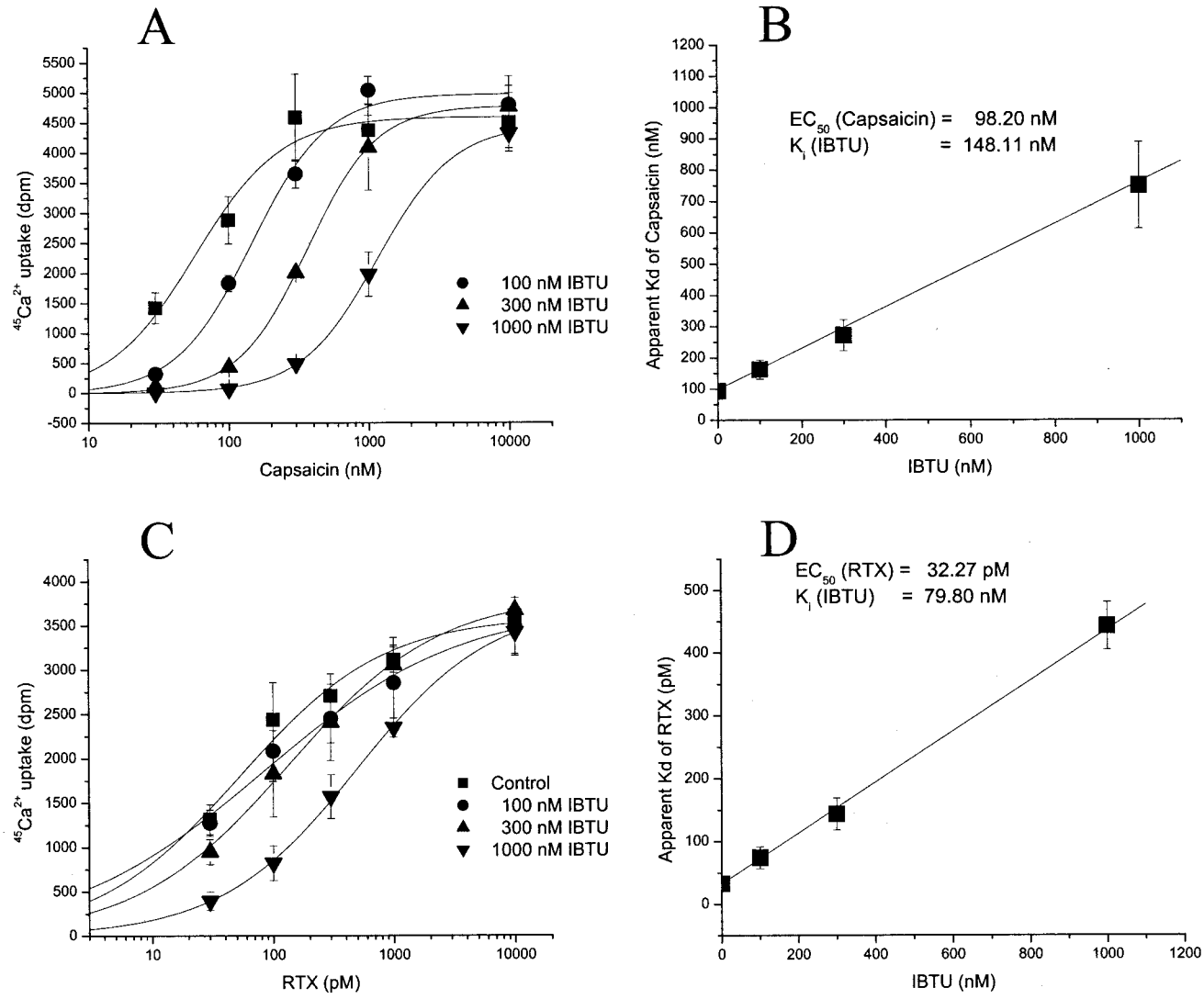


**Fig. 4.** Competitive antagonism by IBTU of the $^{45}Ca^{2+}$ uptake induced by capsaicin or resiniferatoxin in CHO-TRPV1 cells. The stimulation of $^{45}Ca^{2+}$ uptake in CHO-TRPV1 cells in response to capsaicin (A) or resiniferatoxin (C) was determined in the absence or in the presence of IBTU (100, 300, 1000 nM). The points represent the mean ± S.E.M. from four determinations at each point in a single experiment. Two additional experiments with capsaicin and three additional experiments with resiniferatoxin yielded similar results. The apparent $EC_{50}$ values for capsaicin ($n$ = 3 independent experiments) or for resiniferatoxin ($n$ = 4 independent experiments) as a function of IBTU concentration are shown on B (for capsaicin) and D (for resiniferatoxin). Symbols represent mean values, bars ± S.E.M.

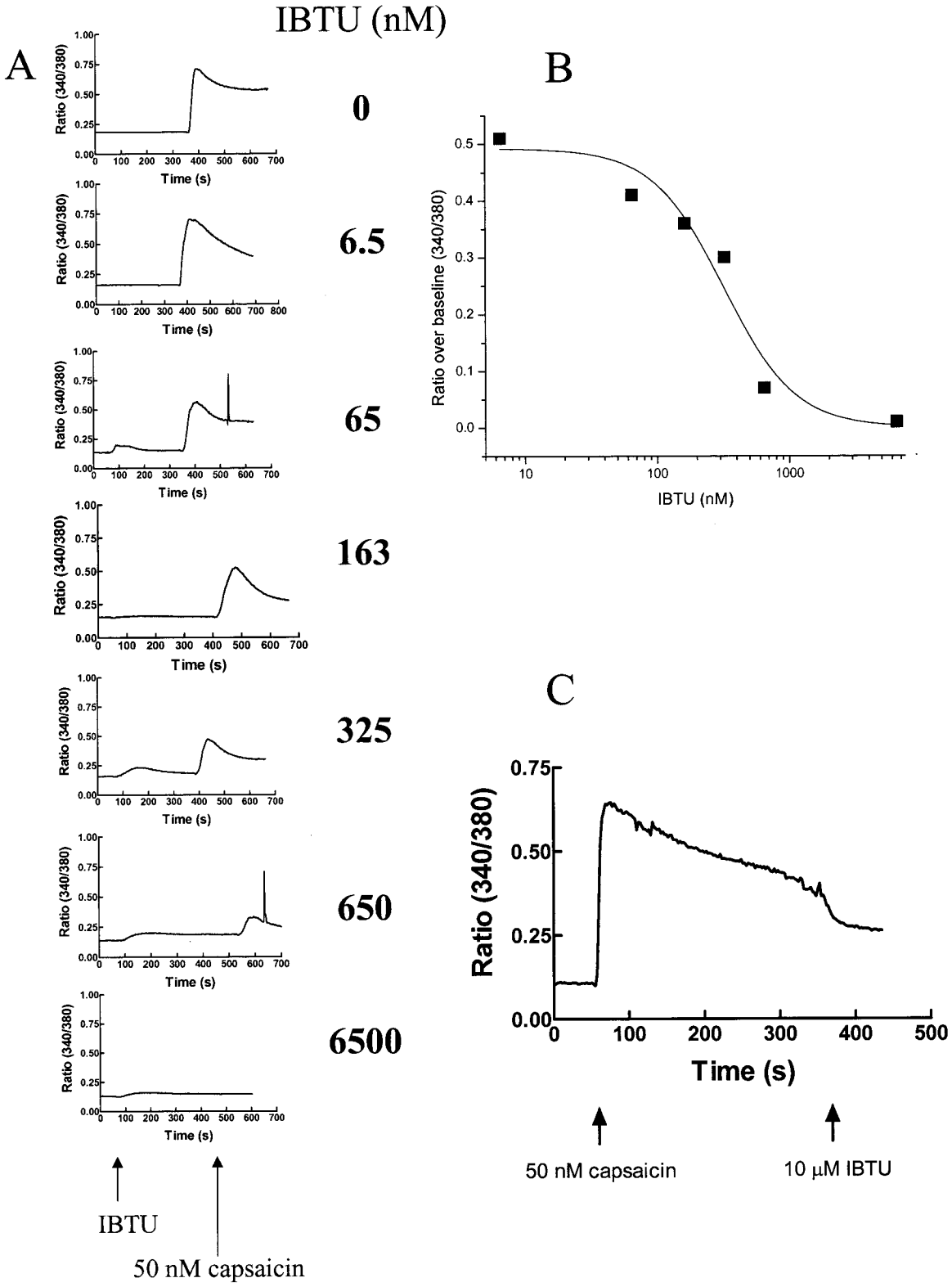


**Fig. 5.** Pharmacological properties of IBTU determined by intracellular calcium imaging. CHO-TRPV1 cells were loaded with fura-2AM. The average fluorescence ratio of the cells (22–49 cells in each experiment) was then determined as a function of time with ligands added as indicated. A, the inhibition of response to 50 nM capsaicin was determined as a function of the concentration of IBTU present. IBTU were added at 1 min, then at 6 min, 50 nM capsaicin was added. B, the maximal elevations in the intracellular $Ca^{2+}$ concentrations induced by 50 nM capsaicin were plotted as a function of the IBTU concentration. Results of a representative experiment are shown. Two additional experiments yielded similar results. C, effect of IBTU addition on the elevation in intracellular calcium induced in CHO-TRPV1 cells by prior treatment with capsaicin. Cells were treated with 50 nM capsaicin at 1 min; at 6 min, 10 $\mu$M IBTU was added. Results of a representative experiment are shown. Two additional experiments yielded similar results.

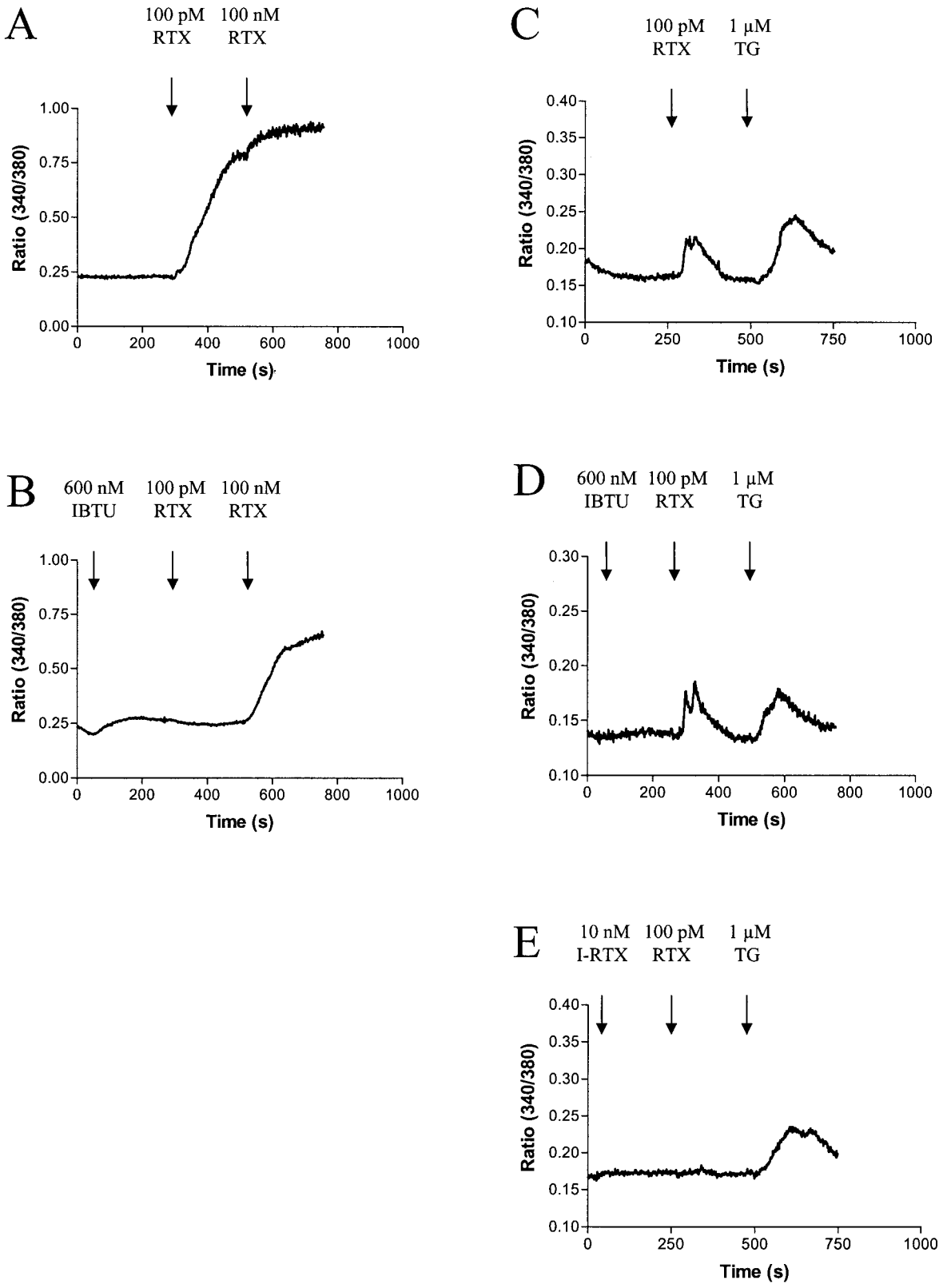


**Fig. 6.** The effect of IBTU on the elevation of intracellular calcium induced by RTX in CHO cells transiently expressing GFP-TRPV1 (CHO-GFP-TRPV1) in the presence or absence of calcium in the extracellular solution. CHO-GFP-TRPV1 cells were treated with resiniferatoxin (100 pM and 100 nM, as indicated) in the absence (A) or in the presence (B) of IBTU (600 nM) under conditions of normal (1.8 mM) $Ca^{2+}$ concentration in the extracellular solution. The effect of resiniferatoxin in the absence of extracellular $Ca^{2+}$ but in the presence of 100 $\mu$M EDTA was also determined. At 1 min, buffer alone (C), IBTU (650 nM, D) or I-RTX (10 nM, E) was added. RTX was added (100 pM) at 4 min, followed by thapsigargin (1 micromol) treatment at 8 min. The average of the responses of 6 to 17 cells are shown from a single, representative experiment. Two additional experiments for each yielded similar results.

the release of $Ca^{2+}$ by RTX under these conditions, confirming that the response to RTX was specific for the TRPV1 (Fig. 6E).

## Discussion

The great excitement of the vanilloid field is driven, among other factors, by the combination of important therapeutic targets and the ever growing recognition of the diversity of responses that can be elicited from TRPV1 by different ligands. RTX showed that it was possible to develop ligands that were dramatically more potent than was capsaicin, the lead structure in this area (Szallasi and Blumberg, 1989). RTX likewise illustrated that it was possible to have a compound that was very potent yet in which short-term irritation could be significantly separated from desensitization. Capsazepine established the feasibility of developing antagonists (Bevan et al., 1992) and I-RTX demonstrated that antagonists could again achieve great potency (Wahl et al., 2001). Compounds such as JYL-827 have shown that compounds can greatly change their character from antagonist to agonist as a function of other cellular regulators of TRPV1 (Wang et al., 2003).

The biochemistry of TRPV1 provides ample opportunity for this diversity of response. TRPV1 exists as an oligomer (Kedei et al., 2001). It is subject to phosphorylation in response to multiple cellular signaling pathways, including protein kinase A (Bhave et al., 2002) and protein kinase C (Premkumar and Ahern, 2000; Numazaki et al., 2002; Olah et al., 2002), and to dephosphorylation in response to phosphatases such as calcineurin. A number of these pathways are subject to regulation by calcium, providing feedback loops in response to gating of calcium by TRPV1. Multiple residues on TRPV1 are influenced by pH (Jordt et al., 2000; Welch et al., 2000), endogenous ligands can function as activators (Zygmunt et al., 1999; Hwang et al., 2000), and phosphatidylinositol 4,5-bisphosphate acts as a negative regulator (Chuang et al., 2001; Prescott and Julius, 2003). Localization studies have already clearly established that TRPV1 shows a complicated pattern of distribution between intracellular membranes and the plasma membrane (Jahnel et al., 2001; Olah et al., 2001b). Although the specific pattern of localization may depend on multiple factors including cell type, immunostaining clearly shows TRPV1 to be present on internal membranes in endogenous systems (Guo et al., 1999; Liu et al., 2003). Multiple studies likewise show that TRPV1 in these internal locations is functional and can influence internal calcium.

Before the cloning of TRPV1, our group used [$^3$H]RTX to demonstrate the existence of vanilloid receptors and to characterize their structure-activity relations. Comparison of these structure-activity relations did not agree quantitatively with those obtained from measurements of calcium uptake (Acs et al., 1996). Both these disparities and other functional differences led us to postulate the existence of separate targets for vanilloids that we termed C-and R-type receptors, distinguished by their relative selectivity for ligands as determined in assays for $^{45}Ca^{2+}$ uptake and for inhibition of [$^3$H]RTX binding. Subsequently, the cloning of TRPV1 showed that TRPV1 accounted for both responses, arguing against the existence of separate genes coding for "C-type" and "R-type" receptors and suggesting rather that modulatory mechanisms existed to alter the structure activity relations as detected in these two assays (Szallasi et al., 1999).

For initial evaluation of novel vanilloids emerging from the various synthetic efforts, the relative advantages of different screening assays—$^{45}Ca^{2+}$ uptake, calcium imaging, and inhibition of [$^3$H]RTX binding—have not been clear. Potential advantages of the [$^3$H]RTX binding assay are that its values are not distorted by spare receptors; can be assayed under conditions of longer incubation times, so that kinetics of uptake and equilibration are less of an issue; and are less subject to modulatory mechanisms. It may also be that the relative selectivity of RTX for the binding assay compared with capsaicin suggests that this R-type selectivity might be linked to the therapeutically advantageous profile of RTX for desensitization with limited short-term pain (Szallasi and Blumberg, 1999). On the other hand, the measurements of calcium response are more closely coupled to functional response, and the susceptibility of the calcium response to modulation may be important for predicting its in vivo behavior.

IBTU arose out of our program to develop novel vanilloids, including novel antagonists. IBTU showed 5-fold enhanced potency as an antagonist compared with capsazepine under our conditions (Wang et al., 2002) and is modestly more potent than the *N*,*N*,*N*′-trisubstituted thiourea derivatives (Park et al., 2003) or chain-branched acyclic phenethylthiocarbamates (Yoon et al., 2003), which have also been described as antagonists. Conversely, IBTU is appreciably less potent as an antagonist than BCTC (Valenzano et al., 2003), I-RTX (Wahl et al., 2001), or JYL1421 (also known as SC0030) (Wang et al., 2002). On the other hand, IBTU displays a pattern of selectivity unique among currently described vanilloids in that it is at least 300-fold selective for calcium uptake relative to the [$^3$H]RTX binding assay. It therefore makes it possible to explore and manipulate cellular function coupled to these two different structure activity relations.

Inadequate penetration of IBTU into the cell cannot explain the differences between $^{45}Ca^{2+}$ uptake and [$^3$H]RTX binding assays. In the [$^3$H]RTX binding assay, penetration cannot be limiting because cell membrane pellets are used instead of intact cells to determine the specific binding. Conversely, in case of the $^{45}Ca^{2+}$ uptake and $Ca^{2+}$ imaging experiments the ability and rapidity of IBTU to antagonize the capsaicin mediated response is a measure of penetration because the vanilloid binding site, which is antagonized by IBTU, is believed to be located intracellularly (Jung et al., 1999; Jordt and Julius, 2002). The results therefore suggest that the TRPV1 pools are pharmacologically different rather than simply differentially accessible.

We describe here that IBTU blocks the increase in intracellular calcium from external calcium but fails to block the release of calcium from the intracellular stores in response to vanilloids. It should be emphasized that the release of the $Ca^{2+}$ from the intracellular pools is rather limited under our assay conditions, giving about an order of magnitude less increase in intracellular $Ca^{2+}$ as well as a transient response. In any case, it is already recognized that the TRPV1 at internal locations is functional in engaging intracellular calcium stores. IBTU demonstrates that different pharmacology is associated with these two responses. This conclu-

sion had been already suggested by the relative difference in the responses induced by RTX and capsaicin, where capsaicin was less effective than RTX at causing calcium-linked responses in the absence of external calcium (Olah et al., 2001b, Marshall et al., 2003). The combination of IBTU together with an agonist of limited selectivity should now provide a tool to permit the consequences of TRPV1 mediated release of intracellular calcium to be explored in vivo. Finally, these results emphasize the pharmacological diversity of TRPV1 and thus imply that modulatory factors can dramatically control its structure activity relations. These findings exemplify the concept that ligands may be designed that activate or antagonize TRPV1 only in defined contexts, providing enhanced selectivity for this interesting class of therapeutic molecules.

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