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Combination of a Rapidly Penetrating Agonist and a Slowly Penetrating Antagonist Affords Agonist Action of Limited Duration at the Cellular Level

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Abstract

The capsaicin receptor TRPV1 (transient receptor potential vanilloid 1) has been an object of intense interest for pharmacological development on account of its critical role in nociception. In the course of structure activity analysis, it has become apparent that TRPV1 ligands may vary dramatically in the rates at which they interact with TRPV1, presumably reflecting differences in their abilities to penetrate into the cell. Using a fast penetrating agonist together with an excess of a slower penetrating antagonist, we find that we can induce an agonist response of limited duration and, moreover, the duration of the agonist response remains largely independent of the absolute dose of agonist, as long as the ratio of antagonist to agonist is held constant. This general approach for limiting agonist duration under conditions in which absolute agonist dose is variable should have more general applicability.

Key Words: TRPV1, Capsaicin, Resiniferatoxin, Vanilloid, Pain, Pharmacodynamics

INTRODUCTION

The capsaicin receptor TRPV1 (transient receptor potential vanilloid 1) is a non-selective cation channel that plays a central role in nociception, thermal perception, and neurogenic inflammation, among other responses (Pingle *et al*., 2007; Bevan *et al*., 2014; Nagy *et al*., 2014). The involvement of TRPV1 in a wide range of pathological conditions such as chronic and inflammatory pain, urge incontinence, and asthma has fueled intense medicinal chemical efforts (Kyle and Tafesse, 2006; Broad *et al*., 2008; Voight and Kort, 2010; Lee *et al*., 2015). Complementary therapeutic strategies are direct TRPV1 inhibition by antagonists and long-term inhibition following desensitization /defunctionalization by potent agonists (Blumberg *et al*., 2011; Szallasi and Sheta, 2012).

The characterization of this emerging wealth of compounds has, not surprisingly, revealed a highly complex pharmacology. TRPV1 is subject to modulation by many effectors in the cellular environment. These include phosphorylation by multiple kinases, dephosphorylation by phosphatases, and binding by various lipids, by other molecules, and by interacting proteins (Winter *et al*., 2013; Bevan *et al*., 2014; Rohacs, 2015). Such factors influence not only ligand binding affinity but also ligand selectivity (Pearce *et al*., 2008).

These contextual elements contributing to TRPV1 pharmacology are complemented by the complex pharmacokinetics of TRPV1 ligands. Elegant studies by Oh and coworkers have argued that the prototypical agonist capsaicin needs to penetrate into the interior of the cell to access the TRPV1 ligand binding site (Jung *et al*., 1999). Such findings are consistent with our current understanding of the TRPV1 ligand binding site structure (Lee *et al*., 2011; Cao *et al*., 2013; Feng *et al*., 2015; Cui *et al*., 2016). Using a fluorescent vanilloid derivative (CHK-884), we found that the half-time for uptake into the cell of this vanilloid derivative was 30 min (Lazar *et al*., 2006). While this might not represent the typical vanilloid, we observed significantly enhanced potencies for the agonist olvanil and the antagonist 5'-iodo-resiniferatoxin (I-RTX) for $^{45}Ca^{2+}$ uptake when assayed at more prolonged incubation times (Lazar *et al*., 2006). Additionally, we have described slow penetration and slow onset of action by protein kinase C ligands closely related structurally to I-RTX and with similar lipophilici-

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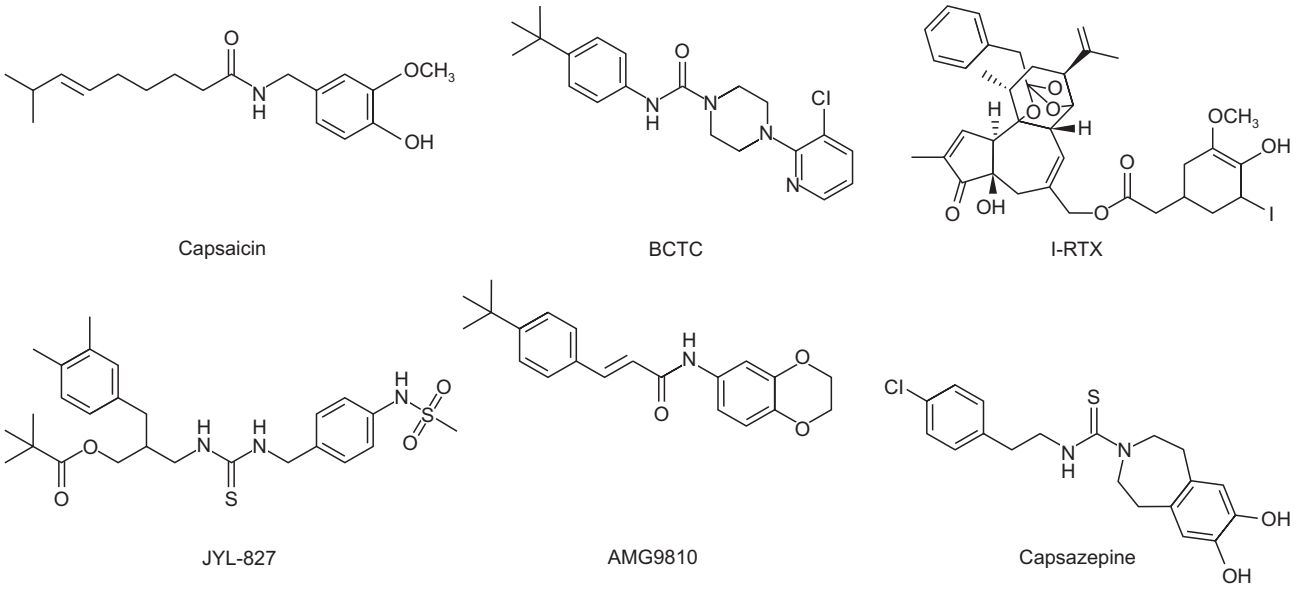

Fig. 1. Structures of the agonist capsaicin and the antagonists BCTC, I-RTX, JYL-827, AMG9810, and capsazepine.

ties (Wang *et al*., 2000; Braun *et al*., 2005). Such observations are not unique. Using other approaches, several groups have reported that olvanil acts more slowly than does capsaicin (Wrigglesworth *et al*., 1996; Liu *et al*., 1997).

As part of our extensive effort to characterize vanilloid structure-activity relations using Chinese hamster ovary cells expressing TRPV1, we have been alert to potential distortions of measured potencies and efficacies as a function of assay conditions. In particular, antagonists are measured through their ability to antagonize an agonist, typically capsaicin. If the compounds are added simultaneously but the antagonist penetrates more slowly than does the agonist, in short term assays the potency of the antagonist will appear weaker than is the actual case. While this phenomenon in many instances may simply be a complication in obtaining reliable structure activity data, it also affords a strategy for manipulating agonist time response profiles. The current paper explores this concept. Depending on the choice of agonist and of antagonist, with appropriate balance between their potencies and rates of (inferred) penetration, the duration of agonist action could be limited and was limited, moreover, in a fashion largely independent of the dose of agonist applied. While our studies were carried out in a cellular system expressing TRPV1, a similar strategy may apply at higher levels of organization where the barrier to uptake is at the level of the tissue. The approach should also be generalizable to other agonist-antagonist pairs targeted to receptors other than TRPV1, provided that ligands with appropriate rates of penetration are available.

MATERIALS AND METHODS

Chemicals

Capsaicin was from Sigma (St. Louis, MO, USA); BCTC (4-(3-chloro-2-pyridinyl)-N-[4-1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide) was obtained from Enzo Life Sciences (Plymouth Meeting, PA, USA); 5'-iodo-resiniferatoxin (I-RTX) was from LC Laboratories (Woburn, MA, USA); AMG 9810 ((2*E*)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide) was purchased from Tocris Bioscience (Ellisville, MO, USA); JYL-827 (*N*-[2-(3,4-dimethylbenzyl)-3-pivaloyloxypropyl]-*N'*-[4-(methylsulfonylamino)benzyl]thiourea) and capsazepine were obtained from Axxora, LLC (San Diego, CA, USA). Their structures are shown in Fig. 1.

Cell culture

The stable Chinese hamster ovary (CHO) cell clone expressing rTRPV1 (Tet-Off) was the generous gift of James E. Krause and Daniel N. Cortwright (Neurogen Corp., Branford, CT, USA). These cells were cultured in maintaining medium (HAM F-12 supplemented with 10% fetal bovine serum (USA sourced, Atlanta Biologicals, Lawrenceville, GA, USA), 25 mM HEPES, pH 7.5, 250 μg/mL geneticin (F-12 medium, HEPES, and geneticin are from Invitrogen, Carlsbad, CA, USA) and 1 μg/mL tetracycline (Calbiochem, La Jolla, CA, USA).

Intracellular Ca^{2+} imaging

CHO-rTRPV1 cells were seeded on 25 mm round glass coverslips in 35×10 mm tissue culture dishes in maintaining medium. After 24 h, the medium was replaced with inducing medium (maintaining medium without geneticin and tetracycline, but containing 1 mM sodium butyrate) to induce TRPV1 expression. Experiments were performed approximately 24 h after induction. For fura-2 AM (Invitrogen) loading, the cells were incubated in Dulbecco's phosphate buffered saline containing calcium and magnesium (Invitrogen), 0.5 mg/mL bovine serum albumin (Sigma), and 5 μM Fura-2 AM for 2 h in the dark at 20°C. After 2 h, the loaded cells were washed 2x with Dulbecco's phosphate buffered saline without calcium and magnesium and immersed in maintaining medium until the measurements. The measurements were carried out in Dulbecco's phosphate buffered saline containing calcium and magnesium plus 0.5 mg/mL bovine serum albumin. The

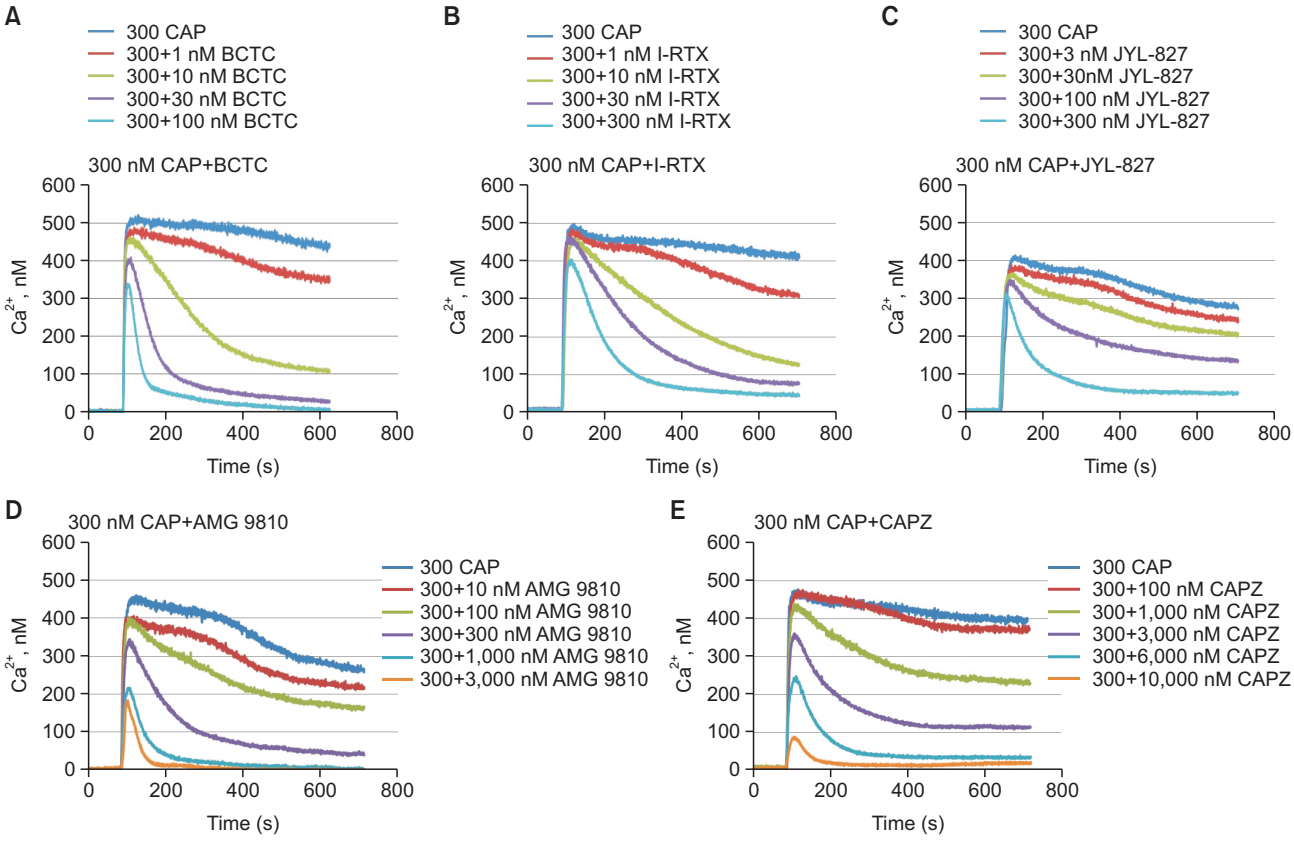

Fig. 2. Calcium signal following simultaneous addition of capsaicin together with an antagonist. At 90 s 300 nM capsaicin was added together with the indicated concentrations of the indicated antagonist, and intracellular calcium levels in CHO cells stably expressing rat TRPV1 were monitored as a function of time using Fura2 as a calcium reporter. The antagonists were (A) BCTC, (B) I-RTX, (C) JYL-827, (D) AMG9810, (E) Capsazepine. Results shown represent the averaged signal from 35-50 cells measured simultaneously in a single experiment and are representative of the 3 experiments performed.

fluorescence of individual cells treated simultaneously with a single dose of agonist and antagonist was measured with an InCyt Im2 fluorescence imaging system (Intracellular Imaging, Cincinnati, OH, USA). The cells within the selected field were illuminated alternately at 340 and 380 nm. Emitted light > 510 nm was measured. To determine the approximate intracellular Ca^{2+} concentrations, the system was calibrated using a fura-2 calibration kit (Invitrogen). Data were analyzed with the Incyt software, tabulated with Microsoft Excel, and graphed with Origin 6.0 (OriginLab Corp., Northhampton, MA, USA) software.

Determination of inhibition values

The TRPV1 agonist (capsaicin) and the indicated TRPV1 antagonist were added simultaneously at the indicated final concentrations to fura-2 AM loaded CHO-rTRPV1 cells 90 s after the beginning of the fluorescent measurements (baseline) and monitored continuously for an additional 10 min. Inhibition values were calculated by comparison with the response to the corresponding dose of capsaicin (100, 300, or 1,000 nM) alone in the same experiment. Experiments at 100 nM and 300 nM were performed three times, each on different days; the experiment at 1,000 nM was performed once. Approximately 35-50 cells were measured in each experiment.

Statistics

The average calcium signals as well as the calcium signals of the 35-50 individual cells in each individual field were calculated by the InCyt 4.5 software (Luminex Corp, Austin, TX, USA) and further analyzed with MS Excel (Microsoft Corp, Redmond, WA, USA) and GraphPad Prism (GraphPad Software, San Diego, CA, USA) for each treatment condition and experiment. The continuous curves in Fig. 2 represent those average signals as provided by the InCyt 4.5 software (Luminex Corp.). In Fig. 3, values were determined as the mean and standard error of the mean of the measurements for the 35-50 cells under each treatment condition in each experiment for the specific times indicated. As indicated, values for multiple experiments were then presented as the mean and standard error of the mean of the average values for the individual experiments.

RESULTS

To analyze the kinetics of response of TRPV1 in the presence of simultaneously added agonist and antagonist, we used an Incyt cellular imaging system to detect changes in intracellular calcium as reported by Fura-2 ratiometric measurement. The system simultaneously detected the responses

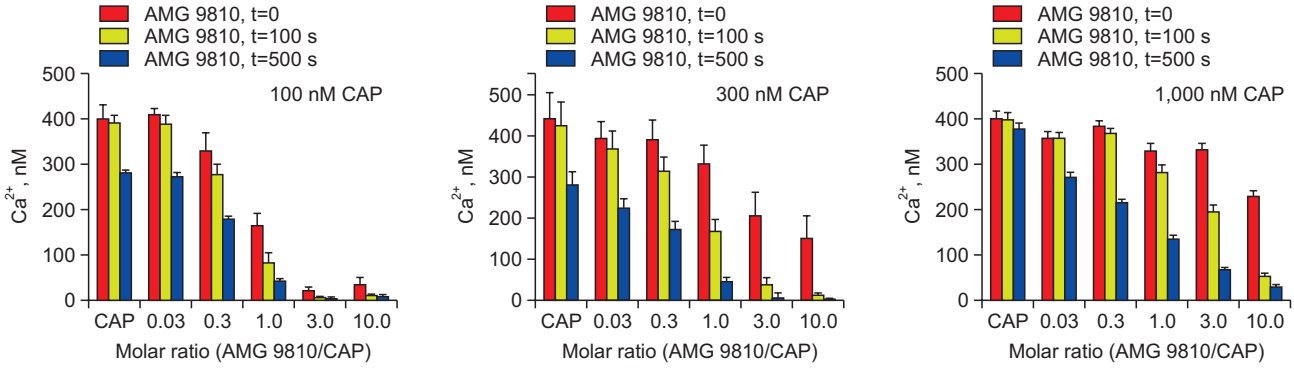

Fig. 3. Comparison of the time course of decrease in calcium signal at different capsaicin concentrations with fixed ratios of antagonist to capsaicin. Capsaicin at 100 nM, 300 nM, and 1,000 nM was added simultaneously with the indicated ratios of antagonist to capsaicin (expressed ratio values rounded to one significant figure). The initial calcium response and that after 100 s and 500 s are plotted. AMG 9810 showed the most rapid rate of apparent penetration, with complete inhibition at T=0 at sufficiently high ratios of antagonist to capsaicin. Values at 100 nM and 300 nM represent the mean ± SE of triplicate experiments. Values at 1,000 nM represent the mean ± SE for the data within the single experiment. Note that the time scale is shifted relative to that in Fig. 2.

of all the individual cells in the field as a function of time and these responses were then averaged for each experimental condition in each experiment. The changes in intracellular calcium were determined for capsaicin in the presence of various concentrations of five previously characterized and commercially available TRPV1 antagonists: BCTC, I-RTX, JYL-827, AMG 9810, and capsazepine. Their CLogP values ranged from 4.0-5.5 (Table 1). For comparison, the LogP value of capsaicin is 2.7. The measured potencies of the antagonists under our usual incubation conditions ranged from 0.35 nM for BCTC to 570 nM for capsazepine.

CHO cells exogenously expressing TRPV1 were incubated for 90 s to establish the basal level of intracellular calcium. 300 nM capsaicin was then added either alone or in combination with increasing doses of one of the TRPV1 antagonists and calcium levels were monitored over the next 10 min. As expected, capsaicin in the absence of antagonist induced an immediate, marked elevation in intracellular calcium which was maintained with a variable, modest diminution over the next 10 min (Fig. 2A-2E). In all cases, the combination of capsaicin together with the highest dose of antagonist led to a time dependent inhibition of the response to capsaicin which had reached a plateau value with complete (BCTC, AMG9810, capsazepine) or largely complete (90%, I-RTX; 82%, JYL-827) inhibition within the 10 min incubation time. In the case of JYL-827, the residual activity presumably reflects the modest but measurable partial agonism of JYL-827 under control conditions as has been previously reported (Wang *et al*., 2003). Combinations with lower ratios of antagonist to capsaicin yielded families of curves characterized by different amounts of immediate response to the capsaicin – antagonist combination and time dependent decreases in calcium signal with both the rate and extent of inhibition reflecting the specific antagonist and the ratio of antagonist to capsaicin. Capsazepine illustrates the behavior of an antagonist where the extent of inhibition at 10 min was associated with substantial immediate inhibition (Fig. 2E). At the other extreme, BCTC (Fig. 2A) and I-RTX (Fig. 2B) yield 80% inhibition at 10 min at doses that caused almost no inhibition of the initial capsaicin response.

Further insight was provided when we varied the concentration of the agonist capsaicin (100 nM, 300 nM, 1,000 nM) and maintained fixed ratios of antagonist to agonist (Fig. 3-5). Levels of stimulated calcium uptake were compared for the initial peak after ligand addition (indicated as t=0 in Fig. 3-5) and after t=100 s and t=500 s (Note that in Fig. 2 the times there include an additional 90 s before capsaicin addition and 10 s to allow maximal capsaicin response). The idealized model is that the capsaicin dose response curve will be shifted to the right, with the extent of the shift depending on the ratio of capsaicin to antagonist, the potency of the antagonist, and the intracellular level of antagonist as a function of time and rate of penetration compared to its level after full penetration.

AMG 9810 illustrates the behavior of an antagonist that penetrates relatively rapidly, although not as rapidly as capsazepine (Fig. 3). The response to 100 nM capsaicin at t=0 s diminished dramatically as the ratio of AMG 9810 to capsaicin (ratio range from 0.03-10, *i.e.* 3 nM to 1,000 nM AMG 9810, ratio values in figure rounded to one significant figure) increased, indicating that the rate of penetration of AMG 9810 was sufficiently rapid compared to that of capsaicin and its potency was sufficiently strong so that an effective antagonistic dose could enter the cell at this early time with the high ratio of antagonist to capsaicin. At the highest dose of capsaicin tested, 1,000 nM, the effect of slower penetration of the an-

Table 1. CLogP and *in vitro* activity of TRPV1 ligands

Compound[a]	CLogP[b]	K_i or EC_{50} (nM)
Capsaicin	2.7	94.8 ± 8.4
BCTC	4.5	0.35 ± 0.07
I-RTX	4.7	1.42 ± 0.27
JYL-827	4.8	67 ± 25
AMG 9810	5.5	33.0 ± 0.6
Capsazepine	4.0	570 ± 30

[a]Reference: BCTC (Valenzano *et al*., 2003), I-RTX (Seabrook *et al*., 2002), JYL-827 (Wang *et al*., 2003), AMG9810 (Gavva *et al*., 2005), Capsazepine (Bevan *et al*., 1992).

[b]CLogP values calculated by ChemDraw (PerkinElmer, Waltham, MA, USA).

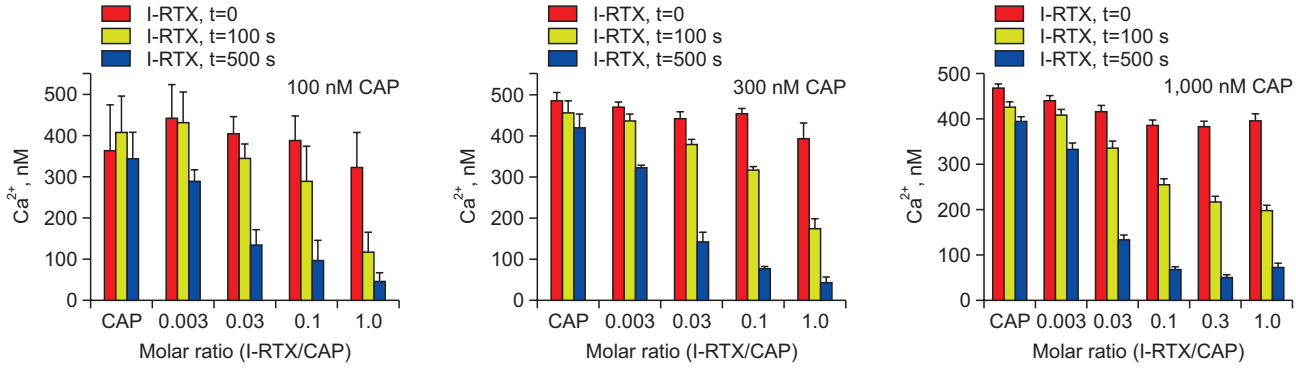

Fig. 4. Comparison of the time course of decrease in calcium signal at different capsaicin concentrations with fixed ratios of antagonist to capsaicin. Capsaicin at 100 nM, 300 nM, and 1,000 nM was added simultaneously with the indicated ratios of antagonist to capsaicin (expressed ratio values rounded to one significant figure). The initial calcium response and that after 100 s and 500 s are plotted. I-RTX showed the apparent slowest rate of penetration, with little decrease at T=0 at any ratio of antagonist to capsaicin. Values at 100 nM and 300 nM represent the mean ± SE of triplicate experiments. Values at 1,000 nM represent the mean ± SE for the data within the single experiment. Note that the time scale is shifted relative to that in Fig. 2.

tagonist was more evident, with maintenance of the capsaicin response at t=0 s just beginning to be reduced at the highest ratio of antagonist to agonist and with marked antagonism at later time. The explanation of course is that in all cases the increasing ratios of antagonist would be predicted to shift the dose response curve for capsaicin to higher levels. A decrease in response as a result of this shift will become evident when the shift was great enough so that the capsaicin was no longer at a full effective dose, which would happen at a lower ratio of antagonist when the absolute concentration of the capsaicin was lower. At later times (100 and 500 s) the response diminished with increasing ratios of antagonist to capsaicin (as well as to a modest extent due to desensitization to the capsaicin). For example, at 1,000 nM capsaicin with a 3:1 ratio of AMG 9810 to capsaicin the t=0 s response remained within 80% of that for capsaicin alone whereas the response at 100 s was reduced to 25% and that at 500 s was reduced to 10%. If one compares the responses for different capsaicin concentrations (100 nM, 300 nM, and 1,000 nM) at a dose of AMG 9810 (a ratio of 0.3 for AMG 9810 to capsaicin, i.e. 30 nM, 100 nM, and 300 nM , respectively; ratio values in Figure rounded to one significant figure) that has limited effect on the T=0 response, relatively similar levels of inhibition were observed at T=500 s.

I-RTX represents the other extreme for the series of antagonists examined (Fig. 4). Over the range of ratios of antagonist to capsaicin (ratio range 0.003:1-1.0:1) examined there was no diminution of the immediate response to capsaicin at any of the capsaicin concentrations (100-1,000 nM). Response was reduced by increasing extents with increasing ratios of antagonist to capsaicin and with increasing time (600 s versus 200 s). At 500 s, a ratio of 0.3:1 yielded 80% inhibition or greater over the capsaicin range examined. As expected, relatively little difference was observed between the 300 and 1,000 nM doses of capsaicin, provided the antagonist concentrations were expressed as the ratio of concentrations of antagonist and agonist. For example, compare the levels of inhibition at 500 s for I-RTX at a ratio of 0.03 or 0.1 of I-RTX to capsaicin (3, 10, and 30 nM I-RTX and 100, 300, and 1,000 nM capsaicin, respectively; or 10, 30 and 100 nM I-RTX and 100, 300, and 1,000 nM capsaicin, respectively; ratio values in Figure rounded to one significant figure).

Finally, BCTC afforded intermediate behavior (Fig. 5), with a quite modest drop in the t=0 s level of stimulation by capsaicin at 100 nM but with retention of stimulation at higher concentrations. At 100 s, ratios of antagonist to agonist of 0.03:1 gave similar, greater than 90% inhibition over the range of capsaicin concentrations examined.

It should be noted that the simple scheme of control of kinetics of response by differential rates of penetration of agonist and antagonist will be influenced by real world constraints. For example, at a concentration of the agents high enough to exceed the solubility limits the rate of penetration will no longer be determined by the amount but rather by the solubility limit of the agent. Likewise, the action of very high affinity antagonists may be determined by their on rates rather than by their equilibrium K_i values.

DISCUSSION

In the present paper, we describe how a fixed combination of a vanilloid agonist together with a vanilloid antagonist can achieve a unique pattern of response, limiting the duration of action of the agonist largely independent of the dose of agonist administered, provided that the agonist is present at an effective dose. This approach complements other, distinct strategies involving agonist – antagonist combinations that have been described previously.

Agonist-antagonist combinations have been described to achieve a variety of pharmacological objectives. Treatment of several cancer types with high dose methotrexate followed by subsequent rescue with N^5-formyltetrahydrofolate (leucovorin) is the classic therapeutic example, although properly regarded methotrexate is an enzyme inhibitor and the N^5-formyltetrahydrofolate restores the enzymatic product downstream of the block (Bertino *et al*., 1971). The rationale for this approach is that methotrexate causes thymidine deficiency. The cancer cells are largely in the S-phase of the cell cycle and susceptible to killing in consequence of the thymidine deficiency, whereas the stem cells in the bone marrow are more slowly cycling. The “rescue” with N^5-formyltetrahydrofolate restores thymidine synthesis before most of the bone marrow cells en-

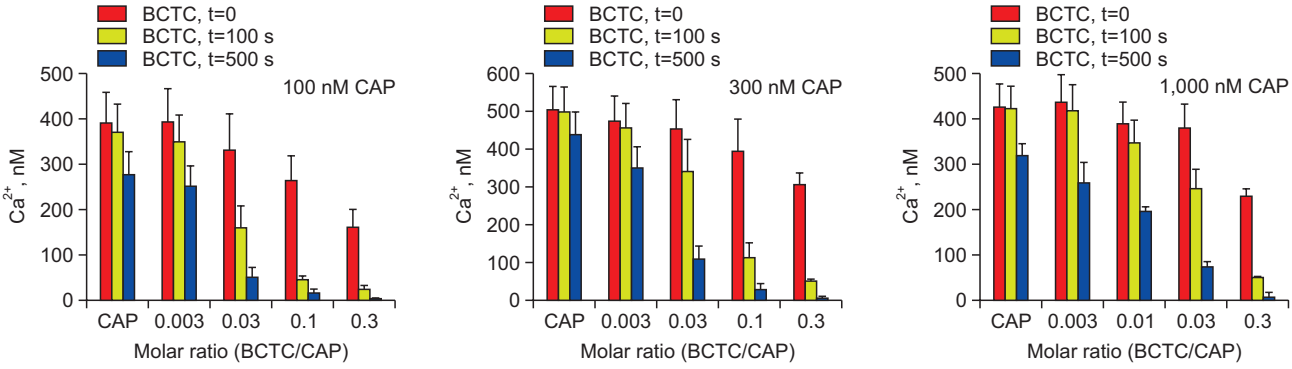

Fig. 5. Comparison of the time course of decrease in calcium signal at different capsaicin concentrations with fixed ratios of antagonist to capsaicin. Capsaicin at 100 nM, 300 nM, and 1,000 nM was added simultaneously with the indicated ratios of antagonist to capsaicin (expressed ratio values rounded to one significant figure). The initial calcium response and that after 100 s and 500 s are plotted. BCTC represents an antagonist with an apparent intermediate rate of penetration. Note the partial inhibition at T=0 at the highest ratio of antagonist to capsaicin. Values at 100 nM and 300 nM represent the mean ± SE of triplicate experiments. Values at 1,000 nM represent the mean ± SE for the data within the single experiment. Note that the time scale is shifted relative to that in Fig. 2.

ter the S-phase. This treatment combination relies on the separation in time between administration of its two components to achieve its effect, rather than on differential rates of absorption of the two components to achieve the separation in time.

Oral administration of a combination of the opiate partial agonist buprenorphine together with the opiate antagonist naloxone (the fixed dose combination is called Suboxone®), like the approach described here, entails the simultaneous administration of a combination of an agonist and an antagonist (Nutt, 2010). This drug combination is used in the treatment of opiate addiction. Here, the benefit again relies on differential absorption but with a very different objective and consequence. The buprenorphine is orally bioavailable whereas the naloxone is not absorbed and is thus ineffective through this route of administration. The combination thus gives a similar response to that obtained by orally administered buprenorphine alone. The utility of the combination lies in that it discourages drug diversion to the street and i.v. administration. Taken i.v., the antagonist in the combination is able to act, blocking the agonist effect of the buprenorphine and moreover inducing in opiate addicts the aversive symptoms of acute drug withdrawal.

Formally, another scenario in which agonists and antagonists may be used together is where the agonism and antagonism are directed at different receptors which have opposite physiological actions. For example, angiotensin II acts on two receptor subtypes, angiotensin type I (AT_1) and angiotensin type II (AT_2). AT_1 mediates vasoconstriction in response to angiotensin II, whereas AT_2 contributes to lowering of blood pressure. The combination of an AT_1 antagonist and an AT_2 agonist was shown to give an enhanced antihypertensive effect (Barber *et al*., 1999).

Duration of drug action is typically determined by receptor occupancy and desensitization. Occupancy, in turn, depends on the amount of drug, its penetration to the target site, and its loss from the target site, which is influenced both by the rate of transport away from its site of action and by its rate of metabolism (Tozer and Rowland, 2006). It is thus controlled by design of a drug with the desired pharmacokinetics together with administration of an appropriate dose for the specific drug. The approach we have described, in cases where it is applicable, provides an additional level of control, with enhanced intrinsic safety.

A further feature of the approach described here is that it provides control of duration of action largely independent of dose applied, whereas normally duration depends on dose and the rate of clearance. One obvious category of application would be for a pepper spray. Pepper sprays are used by law enforcement to temporarily incapacitate a person. Under these circumstances, the dosage to which the person is exposed is necessarily poorly controlled, and the intrinsic sensitivity of the person is not known in advance. The ability to limit the duration of action to the amount of time necessary to apply restraints would reduce the chance of severe toxicity (Busker and van Helden, 1998). It should be emphasized, however, that the current study only provides a proof of principle. Necessarily, the combination of agonist and antagonist would need to be optimized for the rates of penetration through the relevant physiological barriers, such as the respiratory epithelium.

In conclusion, we show that simultaneous addition of a fast acting agonist and a more slowly penetrating antagonist can yield a transient agonist response, the duration of which is largely independent of the agonist dose but depends on the specific antagonist and on the ratio of antagonist to agonist. While we demonstrated this behavior for TRPV1 in cultured cells, the principle should be generalizable to more physiologically relevant situations, where differential absorption will depend on the rate of tissue penetration.

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