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Potent inhibition of a recombinant low voltage-activated Ca^{2+} channel by SB-209712

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Abstract

T-type Ca^{2+} currents were recorded in 2 mM Ca^{2+} from HEK293 cells stably expressing the low voltage-activated Ca^{2+} channel sub-unit α_{1I}. These currents were inhibited by the known Ca^{2+} channel antagonist mibefradil with an IC_{50} close to 1 μM. SB-209712 (1,6,bis{1-[4-(3-phenylpropyl)piperidinyl]}hexane), a compound originally developed as a high voltage-activated Ca^{2+} channel blocker, proved to be a more potent T-type channel antagonist, exhibiting an IC_{50} in the region of 500 nM. The antagonism produced by SB-209712 was reversed following drug removal and the observed antagonism exhibited little or no voltage-dependence with respect to either holding or test potential. These data indicate that SB-209712 is amongst the most potent known non-peptide T-type channel antagonists and thus may have some use in understanding the role of these channels in cellular function.

Keywords: Ca^{2+} channel; Recombinant; HEK293 cell; Pharmacology

1. Introduction

Low voltage-activated Ca^{2+} channels (or T-type Ca^{2+} channels) are strongly inferred in the generation of rhythmical firing patterns in excitable cells. These effects have been well characterised in the CNS (e.g. Bal and McCormick, 1993; Llinas and Yarom, 1981) and have led to numerous proposals implicating the activity of these channels in the pathophysiology of epilepsy (Huguenard, 1996; Huguenard and Prince, 1994; Talley et al., 2000; Tsakiridou et al., 1995). This view is supported by the antagonism of T-type channels by certain anticonvulsant drugs for example ethosuximide, trimethadione and sipatrigine (Coulter et al., 1989; Huguenard and Prince, 1994; Macdonald and Kelly, 1995; McNaughton et al., 2000).

Until quite recently, investigation of the role of T-type channels in physiological processes was limited by two main factors. The first was a lack of potent and selective T-type channel antagonists. The second was a lack of information about T-type channels at the molecular level. The latter of these two barriers to our understanding of low voltage-activated Ca^{2+} channels has for the most part been removed by the cloning of three genes, each of which encodes a biophysically distinct T-type channel (Cribbs et al., 1998; Lee et al., 1999; Perez-Reyes et al., 1998; Randall and Benham, 1999). In contrast, only limited progress has been made to date in the quest to identify compounds that are both potent and selective T-type channel antagonists, although it is certain that the cloning of T-type channel family members will no doubt facilitate progress towards this goal.

At present the 63 amino acid Kurtoxin (Chuang et al., 1998) which comes from the scorpion *Parabuthus transvaalicus*, and the small organic molecule mibefradil (Clozel et al., 1997; Mishra and Hermsmeyer, 1994) are amongst the most potent T-type channel antagonists known. Both of these compounds exhibit state-dependent inhibition of recombinant and native T-type Ca^{2+} channels. The

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IC_{50} of mibefradil is around 1 μM, whereas Kurtoxin is a much more potent antagonist with an IC_{50} that is voltage-dependent but is approximately 15 nM for currents activated by small depolarisiations from rest (Chuang et al., 1998).

Although useful T-type channel blockers, both mibefradil and Kurtoxin also form antagonistic interactions with other ion channels. For example Kurtoxin, although without actions on high voltage-activated Ca^{2+} channels, significantly alters Na^+ channel gating (Chuang et al., 1998). In addition, its potential as a future therapeutic agent is likely to be severely limited by its peptide nature. In contrast, mibefradil is orally active and was briefly used in the pharmacotherapy of cardiovascular conditions in man. However, mibefradil is far from selective and may best be regarded as a broad spectrum ion channel antagonist with activity at high voltage-activated Ca^{2+} channels (Mehrke et al., 1994; Randall and Tsien, 1997; Viana et al., 1997), Na^+ channels (Randall, 1995), a broad range of K^+ channels (Gomora et al., 1999; Hardingham and Randall, 1996; Liu et al., 1999), Cl^- channels (Nilius et al., 1997) and store operated Ca^{2+} channels (Hahn et al., 1995).

Here we describe the antagonism of recombinant T-type channels by SB-209712. This compound is one of a series of phenyl (alkyl/alkoxy) 1-aminoalkyl substituted piperidines and pyrrolidines that have previously been shown to antagonize Ca^{2+} channels in cultured sensory and sympathetic neurones (Brown and Cooper, 1995). The IC_{50} of this compound for α_{1I} sub-unit-mediated T-type channels is around 500 nM, thus making it the most potent small molecule T-type channel antagonist described to date.

2. Methods

HEK293 cells stably expressing the rat α_{1I} (Ca_V 3.3) Ca^{2+} channel sub-units were prepared as described previously (Lee et al., 1999). These cells were maintained in culture for up to 25 passages and prepared for electrophysiology as described previously (McNaughton and Randall, 1997).

All experiments were carried out using whole-cell voltage-clamp techniques and were performed at room temperature using fire-polished thin-walled glass pipettes of ~ 2–4 MΩ resistance. Series resistances were always < 10 MΩ and were additionally compensated by 90–98% using the appropriate controls on the patch clamp amplifier (Axopatch 200B, Axon Instruments).

In brief, gigaseals were formed in an extracellular solution consisting of (mM): NaCl, 130; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 30; HEPES-NaOH, 20; pH 7.3. To record isolated Ca^{2+} currents, this solution was exchanged for one of the following composition (mM): tetraethylammonium chloride (TEACl), 140; CsCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 10; pH 7.35. The pipette contained a solution of the following composition (mM): CsCl, 140; $MgCl_2$, 4; HEPES, 10; EGTA, 10; pH 7.3.

All experiments were performed under control of the pClamp7 software suite (Axon Instruments, Foster City, CA). This program was also used extensively in data analysis. The current output of the amplifier was filtered at $f_c = 2$ kHz and sampled at 10–25 times f_c. Leak subtraction was performed in software using a standard P/4 method; leak-subtracted and raw data were stored on computer disk. The voltage clamp protocols employed are described within the body of the text. Compounds were applied locally via one of a movable bank of glass capillaries. This permitted complete solution changes to be completed in around 30 ms, as described previously (McNaughton et al., 1999). During analysis measurements of current amplitude and exponential fits were performed between manually placed cursors. Throughout data are presented as mean ± 1 S.E.M.

3. Results

From a holding potential of −80 mV, depolarisation of HEK293 cells expressing the T-type Ca^{2+} channel encoded by the rat α_{1I} sub-unit elicited large, robust inward currents, as described previously (McNaughton et al., 2000; Lee et al., 1999). No such currents were observed in untransfected wild-type cells. Standard current voltage relationships revealed that these currents were classically low voltage-activated in nature with significant current induction being observed at a test potential of −60 mV (see also below). These α_{1I} sub-unit-mediated currents could be completely and reversibly eliminated by the previously described non-selective T-type Ca^{2+} channel antagonist mibefradil. Fig. 1A shows a plot of peak current versus time from a typical recording in which 10 μM mibefradil was used to antagonize the Ca^{2+} current in a typical α_{1I} expressing HEK293 cell. Sample T-type current traces from this experiment are shown in Fig. 1B.

Fig. 1C illustrates pooled normalized time course data for the antagonism of α_{1I} sub-unit-mediated T-type currents by 1, 3 and 10 μM mibefradil. It is clear from the data that both the extent and development kinetics of antagonism of T-type channels are dose-dependent as would be predicted by a simple "binding to antagonize" mechanism. In line with this, the kinetics of recovery from block following mibefradil removal were independent of antagonist concentration and were well fit by a single exponential. The mean value of this current recovery time constant was 205 ± 35 s. In Fig. 1D these same data are represented in a plot of the reciprocal of the blocking time constant against mibefradil concentration. A best straight

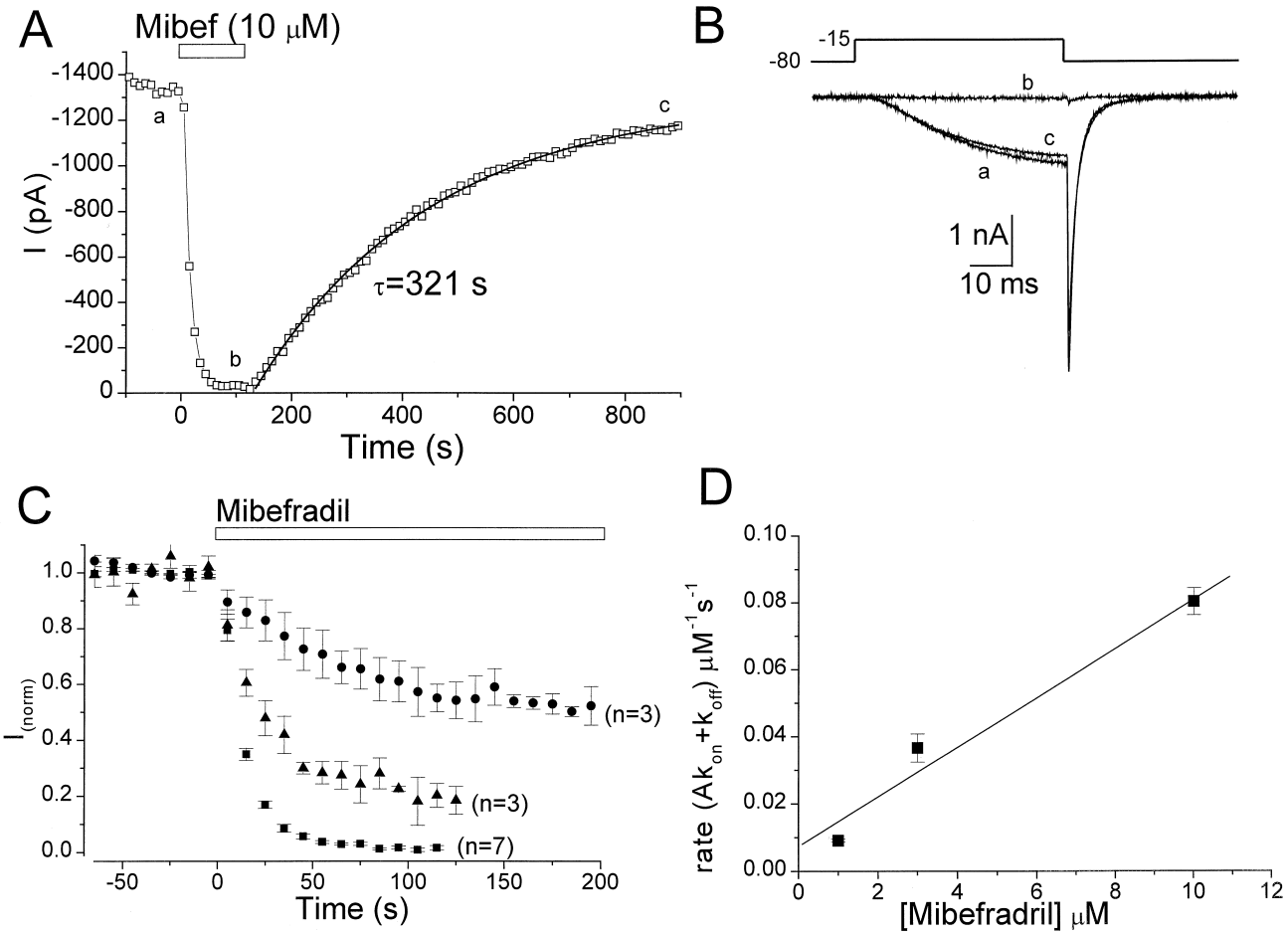

Fig. 1. The known Ca^{2+} channel antagonist mibefradil reversibly blocks α_{1I} encoded T-type Ca^{2+} channels. (A) A plot of peak Ca^{2+} current amplitude versus time from a typical experiment in which mibefradil (10 μM) was used to antagonize α_{1I} sub-unit-mediated channel function. In these experiments the test potential was −15 mV and the holding potential was −80 mV. Test pulses were applied at 0.1 Hz. The compound was applied as indicated by the open bar. A single exponential fit to the current recovery following drug removal is shown. (B) Typical traces from the experiment shown in (A), the lower case letters correspond to the timepoint accordingly indicated in (A). (C) Pooled data illustrating the timecourse of T-type channel inhibition by 1 μM (●, $n = 3$), 3 μM (▲, $n = 3$) and 10 μM (■, $n = 7$) mibefradil. Prior to averaging across cells data from each recording were averaged to the predrug current amplitude. (D) A plot of mibefradil concentration against T-type current antagonism rate and a corresponding best fit linear regression line.

line through these points predicts an apparent on rate (k_{on}) of 0.00736 μM s^{-1} and an off-rate of (0.00733 s^{-1}). The ratio of these values indicates an apparent K_d of 1.0 μM, which is in good agreement both with the steady state inhibition we observed at 1 μM (Fig. 1C), and previous reports of the activity of this compound (Mehrke et al., 1994; Randall and Tsien, 1997).

Mibefradil has been shown to antagonize the activity of other ion channels including the high voltage-activated Ca^{2+} channels (Bezprozvanny and Tsien, 1995; Randall and Tsien, 1997; Viana et al., 1997). In this light, we surveyed the activity of some other small molecule antagonists of Ca^{2+} channels as T-type channel inhibitors. In these experiments α_{1I} sub-unit-mediated T-type currents were elicited by 50 ms step depolarisation from −80 to −15 mV applied every 10 s. Of the agents we have examined to date, the dihydrochloride salt of 1,6,bis{1-[4-(3-phenylpropyl)piperidinyl]}hexane (SB-209712, Fig. 2A) proved to be the most potent α_{1I} sub-unit-mediated Ca^{2+} current antagonist. An example of the timewise development of inhibition of α_{1I} sub-unit-mediated currents by this compound applied at 1 μM is shown in Fig. 2A. In this and seven other similar experiments, this concentration of SB-209712 produced an inhibition of peak current that averaged 82 ± 1% (Fig. 2B).

As with mibefradil, the extent and kinetics of SB-209712 antagonism of α_{1I} sub-unit-mediated currents followed a conventional dose-dependence. Hence, at higher concentrations, current inhibition was both more complete (94 ± 1% at 5 μM and 58 ± 2% at 0.5 μM) and developed more rapidly. The mean time constant of antagonism was 156 ± 21 s at 0.5 μM, 85 ± 8 s at 1 μM and 30 ± 2 s at 5 μM (Fig. 2C). In contrast, the rate of recovery from SB-209712-mediated T-type channel antagonism was not dependent on the concentration of compound previously applied. On average, the reversal of antagonism following

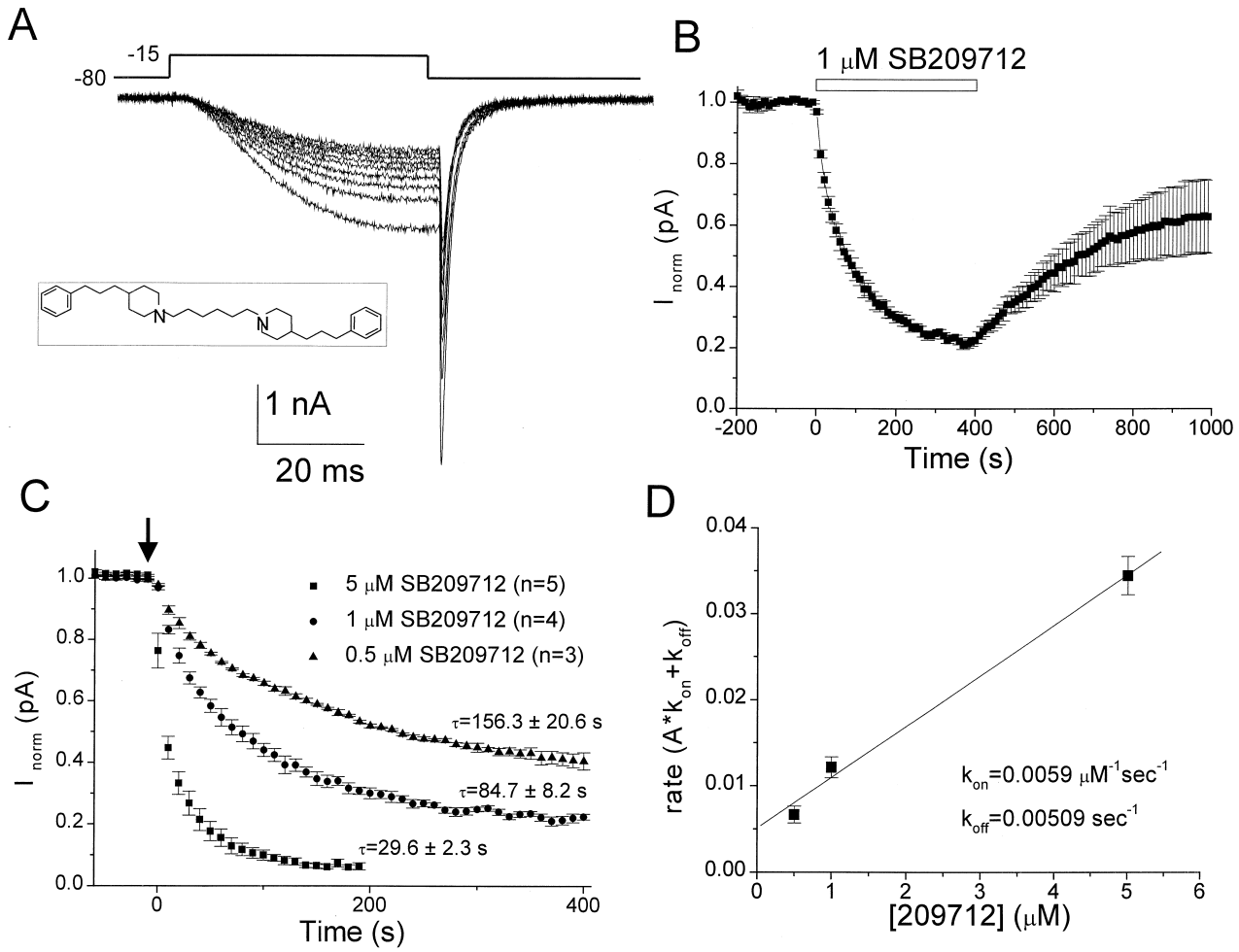

Fig. 2. T-type channel inhibition by SB-209712. (A) Consecutive traces from an experiment in which 1 μM SB-209712 (structure shown bottom left) was used to antagonize α_{1I} sub-unit-mediated T-type Ca^{2+} currents. The trace with the largest amplitude was recorded immediately prior to drug application and the remaining traces show the increasing development of antagonism with time following SB-209712 application. The test potential was −15 mV and the holding potential was −80 mV. (B) Pooled data from four cells including that in (A). The graph illustrates both the mean current antagonism rate and rate of recovery seen following SB-209712 removal. Prior to averaging across cells data from each recording were averaged to the predrug current amplitude. (C) Pooled mean current blocking trajectories for cells exposed to 0.5 μM (▲), 1 μM (●) and 5 μM (■) SB-209712. SB-209712 was applied at the time indicated by the arrow. Data were averaged as described in (B), the mean time constants of current antagonism are also shown. (D) A plot of mibefradil concentration against T-type current antagonism rate and a corresponding best fit linear regression line.

SB-209712 removal proceeded with a time constant of 264 ± 13 s. On average current recovery following drug washout only reached ~70% of predrug levels (see for example Fig. 2B). We believe that this failure to see complete recovery entirely reflects the presence of a degree of Ca^{2+} current rundown, which is a common characteristic of prolonged whole-cell recordings from α_{1I} expressing cells (RW and AR, unpublished observations).

A substantial number of voltage-gated ion channel antagonists exhibit state-dependent interactions. In the Ca^{2+} channel field, a classic example of such pharmacology is the selective interaction of dihydropyridines with the inactivated state of L-type channels (Bean, 1984). Mibefradil has been reported to exhibit a similar if rather less marked behaviour with respect to its inhibition of T-type channels (Mehrke et al., 1994; McDonough and Bean, 1998). To investigate if this was also the case for SB-209712-mediated inhibition of T-type channels we tested the ability of this compound to block currents elicited from three different holding potentials −90, −80 and −70 mV. Both the degree of current inhibition observed and the kinetics of block were identical when 50 ms test depolarisations to −15 mV applied at 0.1 Hz were applied to cells voltage-clamped in the steady state at each of these holding potentials (Fig. 3A). At these holding levels the degree of steady state inactivation of α_{1I} T-type channels was 89 ± 4% (−90 mV), 67 ± 5% (−80 mV) and 34 ± 5% (−70 mV) ($n = 6$). This suggests that SB-209712 does not strongly discriminate inactivated states of the α_{1I} T-type channel. In addition, current–voltage relationships col-

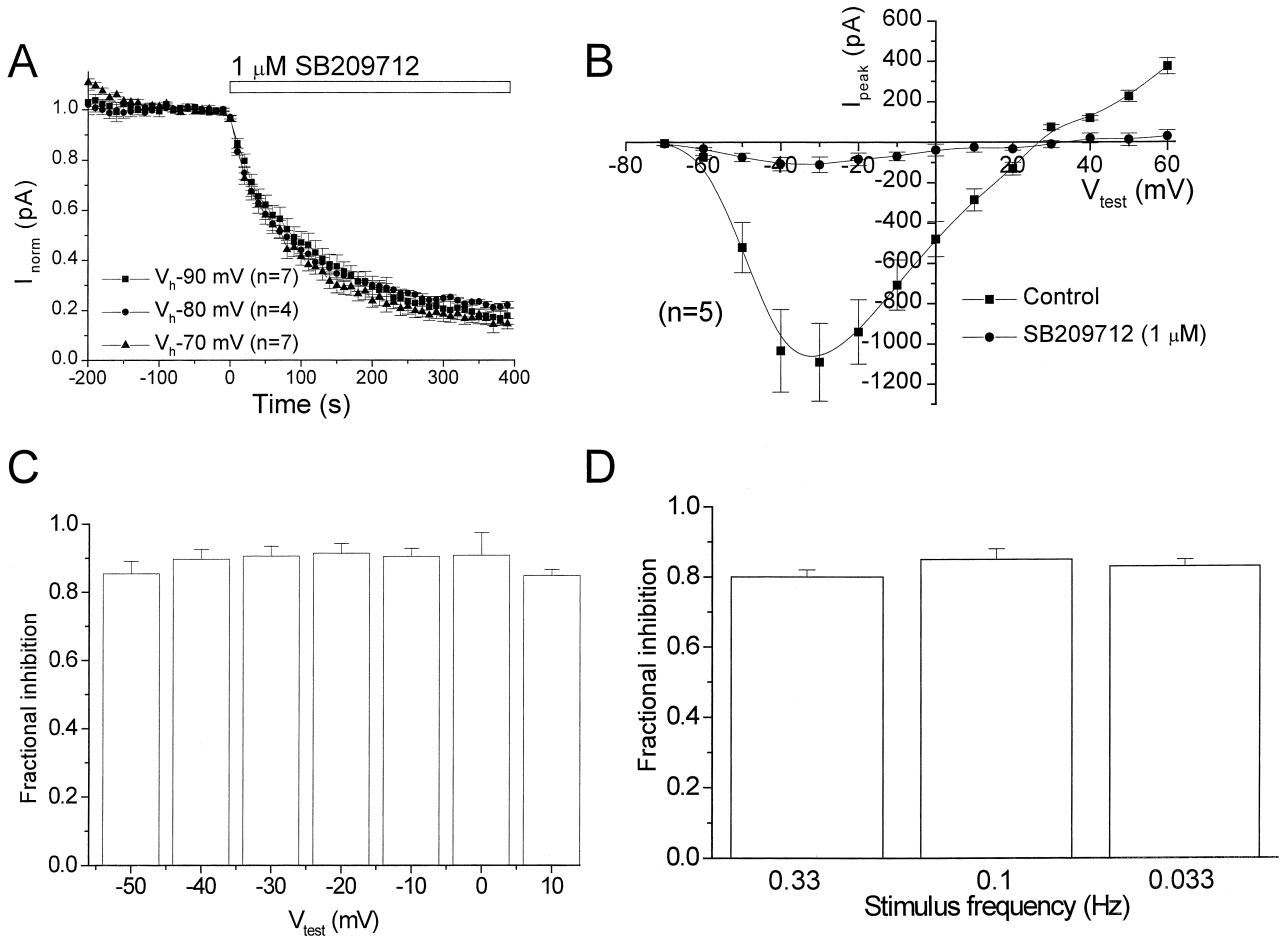

Fig. 3. Basic biophysics of SB-209712 antagonism of α_{1I} sub-unit-mediated T-type channels. (A) A graph plotting the timecourse of channel inhibition by SB-209712 for three different interstimulus holding potentials, in each case the test potential was −15 mV. Data from each recording were normalized to the mean amplitude of the five sweeps recorded prior to drug application. (B) A graph plotting pooled current voltage-relationships recorded from five cells before (■) and during (●) steady state antagonism by 1 μM SB-209712. (C) A plot of fractional SB-209712-induced antagonism against test potential for the five cells used to compile (B). (D) A plot of steady state fractional antagonism against test pulse frequency for recordings in which cells were exposed to 1 μM SB-209712.

lected under control conditions and in the presence of either 0.5 μM (not shown) or 1 μM 209712 were very similar (Fig. 3B) suggesting that antagonism by this compound is not strongly voltage-dependent with respect to activation potential (Fig. 3C). Furthermore, in experiments in which 50 ms test pulses were applied at either 0.33, 0.1 or 0.033 Hz, no evidence was found for any strong use-dependence in either the rate (not shown) or extent (Fig. 3D) of SB-209712-mediated block.

4. Discussion

We have described the inhibitory properties of a novel T-type Ca^{2+} channel antagonist SB-209712. This compound has a submicromolar IC_{50} and exhibits reversible antagonism of the α_{1I} T-type channel. SB-209712 was originally developed as a high voltage-activated Ca^{2+} channel antagonist (Brown and Cooper, 1995). The antagonistic potency against such channels in cultured sensory neurones and superior cervical ganglion neurones was around 5–10-fold lower than that shown here for inhibition of T-type channels (Mark Harries, personal communication).

Although its activity against high voltage-activated Ca^{2+} channels clearly points to a lack of selectivity, SB-209712 remains an interesting pharmacophore because it is as potent a non-peptide T-type channel antagonist as any other compound described to date. Indeed, until quite recently, the only known pharmacological antagonists of T-type channels required concentrations approaching 0.5 mM to produce substantial block. Compounds in this category included ethosuximide, octanol and amiloride (e.g. Todorovic and Lingle, 1998). More recent work has

uncovered compounds with higher efficacy (IC_{50} 1–10 μM) against T-type channels including certain dihydropyridines (compounds better known as "selective" L-type channel blockers, (Akaike et al., 1989; Randall and Tsien, 1997; Richard et al., 1991), mibefradil, and the fluorophenylpiperazines such as U92032, U88999 and KB-2796 (Akaike et al., 1993). All of these compounds exhibit significant inhibition of at least some classes of high voltage-activated Ca^{2+} channels. Indeed, we have recently found that SB-209712 blocks the high-voltage activated recombinant human α_{1E}-sub-unit-mediated channels by ~85% at 1 μM (AR, unpublished observations). In the future, it will be important to examine in detail how SB-209712 interacts with the other two recombinant T-type channels, namely those encoded by the α_{1G} and α_{1H} sub-units, which our preliminary observations indicate are both antagonised by SB-209712. Furthermore, given the recent data on ω-Aga-IVa-mediated inhibition of different α_{1A} splice variants (Bourinet et al., 1999), careful analysis of differential actions of this SB-209712 on the emerging range of α_{1I} splice variants (Mittman et al. 1999) may eventually point to potential sites of interaction of this compound with the channel complex. Additionally, comparative analysis of inhibition of human (Mittman et al. 1999; Monteil et al., 2000) and rat α_{1I}-sub-unit mediated T-type channels could be useful for the understanding species differences, particularly with an eye to the future development of T-type channel antagonists for clinical use in man.

In this study we specifically compared the antagonistic properties of SB-209712 with those of mibefradil. We chose mibefradil because it is probably the best known of all the T-type Ca^{2+} channel antagonists. This stems mainly from its short-lived clinical use in the treatment of cardiovascular conditions in man. Under our recording conditions SB-209712 is clearly a more potent T-type channel antagonist than mibefradil. In addition, contrary to the activity of mibefradil, SB-209712-mediated antagonism of T-type channels does not appear to be dependent on the prestimulus holding potential.

A similarity between mibefradil and SB-209712 is that they both significantly antagonize the function of other ion channels. Amongst these are the high voltage-activated Ca^{2+} channels, the target against which SB-209712 was originally developed and also a major site of action of mibefradil (Mehrke et al., 1994; Randall and Tsien, 1997; Viana et al., 1997). Mibefradil also has actions on a broad range of other channels including K^+ channels, Na^+ channels and Cl^- channels (Ellar et al., 2000; Gomora et al., 1999; Hardingham and Randall, 1996; Liu et al., 1999; Nilius et al., 1997; Randall, 1995). Presently, less information is available about the actions of SB-209712 on other channels, however, some preliminary experiments indicate that it may block background K^+ channels in the two pore domain family (ADR and Helen Meadows, unpublished observations).

One other class of non-peptide compound has been reported to exhibit a submicromolar IC_{50} for T-type channel antagonism. These are the carbonitrile steriods such as (3β,5α,17 beta)-17-hydroxyestrane-3-carbonitrile. These compounds produce a stereoselective antagonism of native T-type channels in sensory neurones with a reported IC_{50} of around 300 nM (Todorovic et al., 1998). Interestingly, unlike the effects of SB-209712 on α_{1I} channels shown here, native T-type channel inhibition by these compounds is incomplete, peaking at ~45% of total low voltage-activated current. As sensory neurones express all three T-type channel α_1 sub-units this partial antagonism potentially reflects selective antagonism of one or two types of T-type Ca^{2+} channel. Instead it may just reflect a partial block of all channel types, similar to that seen in the antagonism of certain high voltage-activated Ca^{2+} channels by the spider toxin ω-Aga-IIIA (Mintz, 1994). Clearly, as with SB-209712, it would be interesting to examine the effects of these carbonitrile steroids on all three recombinant T-type Ca^{2+} channels separately.

In common with the carbonitrile steroids (Nakashima et al., 1999), SB-209712 also blocks high voltage-activated Ca^{2+} channels. For this reason it can certainly be questioned as to whether such a compound could ever form a useful lead molecule in the search for a selective T-type channel antagonist. Indeed, the view that many hydrophobic skeletons bearing one or more amines protonatable around physiological pH are more than likely to act as ion channel blockers is gaining increasing credence. As a case in point, high throughput screens against recombinant Ca^{2+} channels using diverse chemical libraries continue to isolate hydrophobic amines as relatively non-selective voltage-gated channel blockers (e.g. McNaughton et al., 1999). In a similar vein, recent work demonstrates substantial non-subtype specific antagonism of high voltage-activated Ca^{2+} channels by submicromolar levels of simple aliphatic amines. Interestingly, the work of these authors demonstrates important structure activity aspects with relationship to the length of the carbon backbone with the most potent block being produced by C12 and C13 compounds (Beedle and Zamponi, 1999).

Whether SB-209712 or related chemical entities have potential as chemical platforms for the future development of useful pharmacophores will only be clear after much additional work. However, from what we currently understand about T-type channel function, agents which modulate the activity of T-type Ca^{2+} channels with some degree of selectivity certainly exhibit potential for the pharmacotherapy of a number of disorders, the most commonly cited of which are the epilepsies. Furthermore, even if its own lack of selectivity limits its progression as a pharmaceutical lead, access to a submicromolar "tool" antagonist of T-type Ca^{2+} channels is likely to be very useful in developing and validating novel biological screens for other compounds with activity against members of this interesting ion channel family.

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