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# Blockade by *sigma* site ligands of high voltage-activated $Ca^{2+}$ channels in rat and mouse cultured hippocampal pyramidal neurones

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**1** The effects of a series of structurally-dissimilar $\sigma$ site ligands were examined on high voltage-activated $Ca^{2+}$ channel activity in two preparations of cultured hippocampal pyramidal neurones.

**2** In mouse hippocampal neurones under whole-cell voltage-clamp, voltage-activated $Ca^{2+}$ channel currents carried by barium ions ($I_{Ba}$) were reduced with the rank order ($IC_{50}$ values in $\mu$M): 1S,2R-(−)-*cis*-*N*-methyl-*N*-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamine (7.8) > rimcazole (13) > haloperidol (16) > ifenprodil (18) > opipramol (32) > carbetapentane (40) = 1-benzylspiro[1,2,3,4-tetrahydronaphthalene-1,4-piperidine] (42) > caramiphen (47) > dextromethorphan (73). At the highest concentrations tested, the compounds almost abolished $I_{Ba}$ in the absence of any other pharmacological agent.

**3** The current-voltage characteristics of the whole-cell $I_{Ba}$ were unaffected by the test compounds. The drug-induced block was rapid in onset and offset, with the exceptions of carbetapentane and caramiphen where full block was achieved only after two to three voltage-activated currents and was associated with an apparent increase in the rate of inactivation of $I_{Ba}$.

**4** In rat hippocampal neurones loaded with the $Ca^{2+}$-sensitive dye Fura-2, rises in intracellular free $Ca^{2+}$ concentration evoked by transient exposure to 50 mM $K^+$-containing medium, either in the absence or in the presence of 10 $\mu$M nifedipine (to block L-type high voltage-activated $Ca^{2+}$ channels), were also reversibly attenuated by the $\sigma$ ligands. The rank order potencies for the compounds in these experimental paradigms were similar to that observed for blockade of $I_{Ba}$ in the electrophysiological studies.

**5** These results indicate that, at micromolar concentrations, the compounds tested block multiple subtypes of high voltage-activated $Ca^{2+}$ channels. These actions, which do not appear to be mediated by high-affinity $\sigma$ binding sites, may play a role in some of the functional effects previously described for the compounds.

**Keywords:** Cultured hippocampal pyramidal neurones; $\sigma$ receptors; voltage-activated $Ca^{2+}$ channels

## Introduction

In the preceding paper (Fletcher *et al.*, 1995), the *N*-methyl-D-aspartate (NMDA) antagonist actions of a series of $\sigma$ site ligands were described. We suggested that the appreciable NMDA antagonist activity of many of the compounds tested might underlie, at least in part, their antiepileptiform and neuroprotective effects which, *in vitro*, are observed at micromolar concentrations. Nevertheless a number of compounds, notably caramiphen and carbetapentane, proved to be relatively weak NMDA antagonists and their potency in this regard appeared insufficient to be able to account for their therapeutically-useful actions. Calcium influx via voltage-activated $Ca^{2+}$ channels also plays a role in neurodegenerative phenomena and epileptogenesis (Heinemann & Hamon, 1986; Siesjö, 1992), raising the possibility that blockade of $Ca^{2+}$ channels may be an additional mechanism of action of $\sigma$ site ligands. Indeed, several lines of evidence have pointed to a possible association of $\sigma$ binding sites with voltage-activated $Ca^{2+}$ channels (reviewed by Walker *et al.*, 1990). Radioligand binding studies, for example, have suggested that at least one $\sigma$ binding site may be associated with neuronal $Ca^{2+}$ channels (Rothman *et al.*, 1991; Cagnotto *et al.*, 1994) and a variety of $Ca^{2+}$ channel blocking agents (including flunarizine) are effective competitors for high-affinity [$^3$H]-dextromethorphan (DXM) binding (Klein & Musacchio, 1989). In turn, DXM is able to block neuronal high voltage-activated (HVA) $Ca^{2+}$ channels, albeit at concentrations in excess of those associated with the displacement of tritiated ligands from high-affinity $\sigma$ binding sites (Netzer *et al.*, 1993). Finally, it is of interest that blockade of neuronal voltage-activated $Ca^{2+}$ channels has been implicated in the mechanism of action of a variety of antipsychotic agents (see Discussion), many of which possess high affinity for multiple $\sigma$ binding sites (Deutsch *et al.*, 1988).

Accordingly, in the present study we have evaluated the potency of a series of structurally-diverse $\sigma$ site ligands as blockers of HVA $Ca^{2+}$ channels in two preparations of cultured hippocampal pyramidal neurones.

## Methods

Methods for the preparation of rat and mouse cultured hippocampal pyramidal neurones, as well as a description of the general experimental procedures, were provided in the preceding paper (Fletcher *et al.*, 1995).

### *Electrophysiological studies*

Mouse hippocampal pyramidal neurones were used for whole-cell recordings of barium currents ($I_{Ba}$) by conventional patch-clamp techniques. Cultures were used 7-14 days after plating. Prior to recording, neurones were rinsed with an extracellular solution containing (mM): NaCl 140, $CaCl_2$ 1.3, KCl 5.4, N-[2-hydroxyethyl]piperazine-*N'*-2-[ethanesulphonic acid] (HEPES) 25 and glucose to a final osmolarity of 330 mOsm. Tetrodotoxin

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(TTX) at 100 nM was added and the pH of the solution was adjusted to 7.4 with 1 M NaOH. Patch electrodes were filled with a fluoride-free intracellular solution containing (mM): CsCl 110, tetraethylammonium 10, ethylene glycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) 10 and HEPES 10 to which was added, fresh on the day of the experiment, a support system consisting of 2 mM Mg-ATP, 50 mM phosphocreatine, 50 iu creatine phosphokinase and 2 mM GTP. The pH and osmolarity of the solution were then adjusted to 7.4 and 330 mOsm, respectively. The intracellular solution was kept on ice and was not used for longer than 5 h due to the instability of its components. Recordings were performed at room temperature (20–24°C).

After the whole-cell configuration was attained, the extracellular solution was changed to one containing (mM): NaCl 140, CsCl 5, $BaCl_2$ 5, $MgCl_2$ 1, HEPES 25 and glucose to a final osmolarity of 330 mOsm. In order to achieve the best possible voltage-clamp, a higher concentration of TTX (300–600 nM) was added to block voltage-activated $Na^+$ currents, whilst voltage-activated $K^+$ currents were minimized by the replacement of extracellular KCl with CsCl. Test compounds were dissolved in the extracellular solution to the final concentration (see Results) and pH adjusted to 7.4. The series resistance and capacitance were minimized using the patch-clamp amplifier (Axopatch 1B, Axon Instruments Inc., Foster City, CA, U.S.A.), although full compensation was not possible due to the morphology of the neurones employed; in order to limit this error, neurones lacking extensive processes were used as frequently as possible for recordings. Leak currents were estimated on superfusion of the neurones with a solution containing 100 μM cadmium (or, in earlier experiments, on estimation of leak from a series of hyperpolarizing currents).

$I_{Ba}$ was activated at 20 s intervals by a 200–250 ms voltage step from a holding potential (V(h)) of −80 mV to a test potential (V(t)) of −10 mV (unless otherwise indicated in the Results). In neurones employed in the present experiments, the latter voltage step activates both dihydropyridine-sensitive (L-type) and dihydropyridine-resistant, ω-conotoxin GVIA-(ω-CgTx) sensitive (N-type) HVA $Ca^{2+}$ channels, with a small component representing activation of other subtypes of HVA $Ca^{2+}$ channels (Church *et al.*, 1994a; see also Ozawa *et al.*, 1989). Currents carried by low voltage-activated (T-type) $Ca^{2+}$ channels were seen only occasionally in the neurones employed (e.g. shoulder on *I*-V plot in Figure 1b(ii)), as reported elsewhere (Ozawa *et al.*, 1989) and we did not attempt to study the effects of σ ligands on this $Ca^{2+}$ channel subtype.

Control and drug-containing solutions were perfused over the entire neurone under voltage-clamp using a multibarrel array and $I_{Ba}$ activated until steady-state block was achieved. For construction of concentration-inhibition plots, the concentration of test compound was sequentially increased to achieve full block before the start of wash-out in order to minimize the possible error contributed by run-down of $I_{Ba}$. Cells which displayed less than a 50% recovery of the control $I_{Ba}$ were excluded from analysis. Drug effect was expressed as a percentage reduction of the control response at a steady state. Final $IC_{50}$ values (the concentration of test compound resulting in a 50% inhibition of the control response) and percentage reductions of control responses are expressed as mean ± s.e.mean, with *n* the number of neurones tested. To derive $IC_{50}$ values, data points were fitted to the logistic equation described in the companion paper (Fletcher *et al.*, 1995).

### *Fluorescent dye studies*

Cultured hippocampal pyramidal neurones of the rat were used 8–21 days after plating. After loading with Fura-2, coverslips were placed in a chamber at 20–24°C and continuously superfused at 1.5 ml $min^{-1}$ with a solution containing (mM): NaCl 136.5, KCl 3, $NaH_2PO_4$ 1.5, $MgSO_4$ 1.5, D-glucose 10, $CaCl_2$ 2 and HEPES 10. Tetrodotoxin 0.5 μM was added and the pH adjusted to 7.35–7.40 with 10 M NaOH. High-$[K^+]_o$ solutions (50 mM, by substitution for NaCl) were administered in 1 ml aliquots to the inflow of the perfusion chamber and allowed to remain in contact with the neurones for 20 s before wash-out. Test compounds were applied by superfusion.

Cytoplasmic free calcium concentrations ($[Ca^{2+}]_i$) were measured by the dual excitation fluorescence ratio method, employing Fura-2. During exposure to high-$[K^+]_o$, a ratio was acquired every 1.5 s; lower rates (e.g. one ratio every 30 s) were employed between high-$[K^+]_o$ applications to minimize photobleaching, ultraviolet-mediated cytotoxicity and in the case of nifedipine-containing solutions, drug breakdown. Statistical results are reported as mean ± s.e.mean, where *n* refers to the total number of neurones from which observations were made under each experimental condition. The effects of each concentration of each test compound were examined on at least three different neuronal cultures. $IC_{50}$ values were obtained by

**Table 1** Potency of σ site ligands as antagonists of whole-cell $I_{Ba}$ in mouse hippocampal neurones and of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ in rat hippocampal neurones

| | *Antagonism of* $I_{Ba}$ | | | *Antagonism of high-$[K^+]_o$-evoked rise in $[Ca^{2+}]_i$* | |
|---|---|---|---|---|---|
| *Compound* | $IC_{50}$ (μM) | % reduction of control $I_{Ba}$ | | $IC_{50}$ (μM) (-nifedipine) | $IC_{50}$ (μM) (+nifedipine) |
| (−)-44 | 7.8 ± 1.7 (6) | 100 μM | 95 ± 2 (5) | NT | NT |
| Rimcazole | 13.1 ± 2.3 (7) | 100 μM | 94 ± 5 (5) | 3.9 ± 0.2 | 5.5 ± 0.7 |
| Haloperidol | 15.6 ± 1.1 (9)[a] | 50 μM | 85 ± 3 (8) | 7.8 ± 0.5[a] | 14.8 ± 0.7[a] |
| Ifenprodil | 17.8 ± 2.0 (8)[b] | 100 μM | 92 ± 3 (4) | 16.9 ± 0.3[b] | 12.6 ± 3.7[a] |
| Opipramol | 32.2 ± 3.6 (7) | 100 μM | 84 ± 7 (4) | 11.3 ± 1.1 | 15.6 ± 4.2 |
| Carbetapentane | 40.5 ± 3.1 (6) | 300 μM | 95 ± 2 (3) | 18.6 ± 1.7 | 30.9 ± 3.0 |
| L 687,384 | 42.4 ± 7.2 (6) | 100 μM | 81 ± 3 (7) | 20.5 ± 3.9 | 20.8 ± 0.9 |
| Caramiphen | 47.3 ± 2.6 (5) | 300 μM | 98 ± 2 (5) | 21.0 ± 1.4 | 31.7 ± 3.2 |
| DXM | 72.6 ± 10.2 (6) | 400 μM | 88 ± 2 (6) | 27.2 ± 3.5 | 44.9 ± 4.1 |
| (−)-29 | NE | 30 μM | 49 ± 5 (6) | NT | NT |
| (+)-29 | NE | 30 μM | 44 ± 3 (5) | NT | NT |
| (+)-Pentazocine | NE | 100 μM | 64 ± 6 (5) | NT | NT |
| DTG | NE | 100 μM | 40 ± 4 (6) | NT | NT |
| (+)-3-PPP | NE | 100 μM | 25 ± 2 (2) | NT | NT |
| Ketamine | NE | 100 μM | 11 ± 5 (4) | NT | NT |

Table indicates the mean ± s.e.mean of the $IC_{50}$ values (μM) for the test compounds, indicated to the left, as antagonists of the steady-state component of $I_{Ba}$ (first column) and as antagonists of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$, assessed in both the absence (fourth column) and the presence (fifth column) of 10 μM nifedipine. The percentage reductions (mean ± s.e.mean) of control $I_{Ba}$ by given concentrations of σ site ligands (second column) are shown in column 3. Number of neurones tested is given in parentheses. NE, not estimated; NT, not tested. [a]Values taken from Fletcher *et al.* (1994). [b]Values taken from Church *et al.* (1994b).

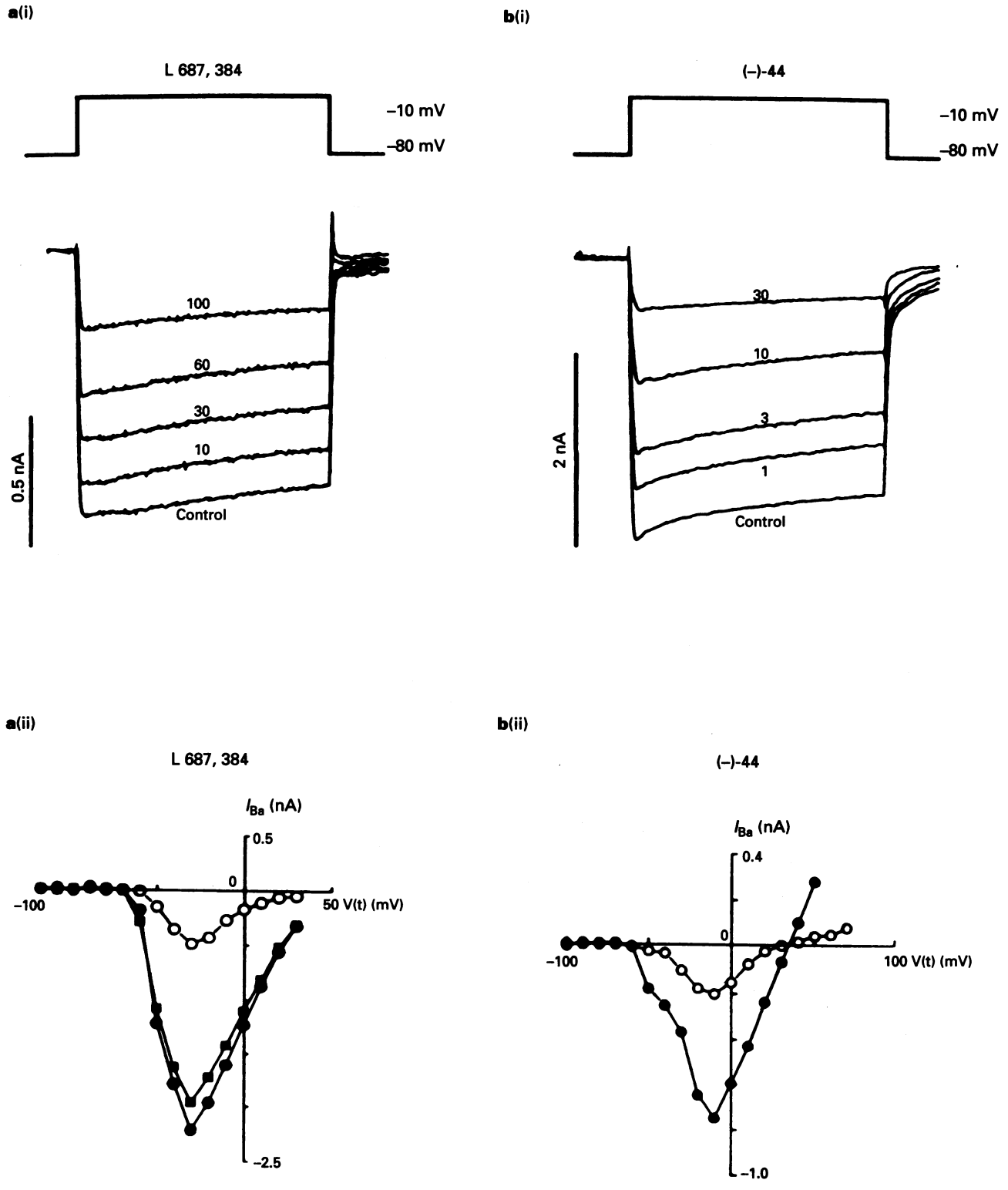



**Figure 1** Efficacy of L 687,384 and (−)-44 as antagonists of whole-cell $I_{Ba}$ in voltage-clamped mouse hippocampal neurones. Traces indicate the concentration-dependent reduction of control $I_{Ba}$ by L 687,384 (a,i) and, in a second neurone, by (−)-44 (b,i). Applied concentrations (μM) are indicated adjacent to the individual traces. $I_{Ba}$ was activated by a 250 ms voltage step from a holding potential of −80 mV to a test potential of −10 mV, as indicated above the traces. Ordinate scale, amplitude of $I_{Ba}$ (nA). In two different neurones, the effect of (a,ii) L 687,384 and (b,ii) (−)-44 was assessed on the current-voltage relationship for the whole-cell $I_{Ba}$. $I_{Ba}$ was activated from a holding potential of −90 mV to the test potential (V(t)) indicated on the abscissa scale. In (a,ii), control $I_{Ba}$ (●) was reduced by ≈80% by 100 μM L 687,384 (○) with wash (■) to near control levels. In (b,ii), 10 μM (−)-44 (○) produced an ≈60% reduction of the control $I_{Ba}$ (●). The shoulder on the control current-voltage plot apparent at hyperpolarized potentials, which corresponds to activation of T-type low-voltage-activated $Ca^{2+}$ channels (see Methods), is also attenuated by (−)-44. Abscissa scale, test potential (V(t), mV). Ordinate scale, amplitude of $I_{Ba}$ (nA).

use of the same logistic equation as in the electrophysiological studies (see above). Other experimental details are provided in the preceding paper (Fletcher *et al.*, 1995).

### *Sources and handling of compounds*

Compounds were obtained from Sigma Chemical Co., with the exceptions of ω-CgTx, rimcazole, haloperidol, ifenprodil, (+)-pentazocine, 1,3-di(2-tolyl)guanidine (DTG) and (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ((+)-3-PPP) (Research Biochemicals Inc.). 1S,2R-(−)-*cis*-N-methyl-*N*-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamine ((−)-44), 1R,2S-*cis*-(−)-N-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-(2-naphthyl)acetamide ((+)-29) and 1S,2R-*cis*-(−)-N-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-(2-naphthyl)acetamide ((−)-29) were generous gifts from Dr B.R. de Costa (National Institutes of Health, Bethesda, MD, U.S.A.). Opipramol, 1-benzylspiro[1,2,3,4-tetrahydronaphthalene-1,4-piperidine] (L 687,384) and ketamine were kindly provided by CIBA-Geigy (Summit, NJ, U.S.A.), Merck Sharp and Dohme Research Laboratories (Harlow, U.K.) and Parke-Davis Inc. (Don Mills, ON, Canada), respectively. Stock solutions of test compounds (in distilled water or saline) and nifedipine (50 mM in ethanol) were made up fresh each day from the solid and stored in glass vials. The highest concentration of ethanol in final nifedipine-containing solutions was 0.02%, which in control experiments had no effect on responses (not shown). Nifedipine-containing solutions were handled in the manner described previously (Church *et al.*, 1994a).

## Results

### *Electrophysiological studies*

The whole-cell $I_{Ba}$ evoked in the present experiments is composed mainly of current flowing through nifedipine-resistant, ω-CgTx-sensitive (N-type) $Ca^{2+}$ channels, with a smaller contribution from nifedipine-sensitive (L-type) channels and a residual (≈20%) contribution from other subtypes of voltage-activated $Ca^{2+}$ channels resistant to both dihydropyridines and ω-CgTx (Church *et al.*, 1994a). Owing to the extensive range of compounds tested, we did not attempt to investigate the actions of the compounds on individual subtypes of HVA $Ca^{2+}$ channel, although we have recently addressed this question in the cases of haloperidol (Fletcher *et al.*, 1994) and ifenprodil (Church *et al.*, 1994b). Rather, the data presented reflect the activity of the compounds as antagonists of the whole-cell $I_{Ba}$ flowing through the combination of HVA $Ca^{2+}$ channels present in the neurones employed (see also Netzer *et al.*, 1993; Biton *et al.*, 1994).

All of the σ site ligands, at micromolar concentrations, attenuated whole-cell $I_{Ba}$ in a reversible and concentration-dependent manner. The $IC_{50}$ values for the compounds tested as antagonists of $I_{Ba}$ are presented in Table 1. Hill coefficients were not significantly different from 1, with the exceptions of caramiphen (1.5±0.2; $n=5$) and opipramol (1.4±0.1; $n=7$). For those compounds observed to be weak antagonists of $I_{Ba}$ ((+)-pentazocine, DTG, (+)-3-PPP and ketamine; $IC_{50}$ values estimated at ⩾100 μM), the percentage reduction of $I_{Ba}$ observed with a single concentration of test compound (100 μM) is shown. Due to a limited supply of the stereoisomers (+)- and (−)-29, it was not possible to estimate their $IC_{50}$ values. However, at a concentration of 30 μM, (+)- and (−)-29 produced 44±3 ($n=5$) and 49±5 ($n=6$) percentage reductions of control $I_{Ba}$, respectively, suggesting $IC_{50}$ values in the region of 30 μM. The actions of L 687,384 and (−)-44, relatively potent blockers of $I_{Ba}$, are shown in Figure 1. Examples of the effects of DTG, (+)-pentazocine, ketamine and DXM, relatively weak blockers of $I_{Ba}$, are shown in Figure 2. The σ-site ligands for which $IC_{50}$ values were estimated could, at the highest concentrations tested, produce a >80% reduction of the whole-cell $I_{Ba}$ (Table 1). Since, in these neurones, dihydropyridine- and ω-Cg-Tx-sensitive $Ca^{2+}$ channels carry

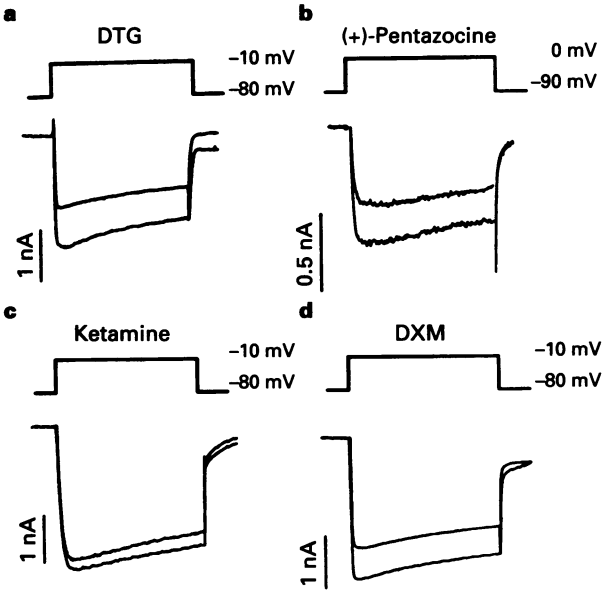
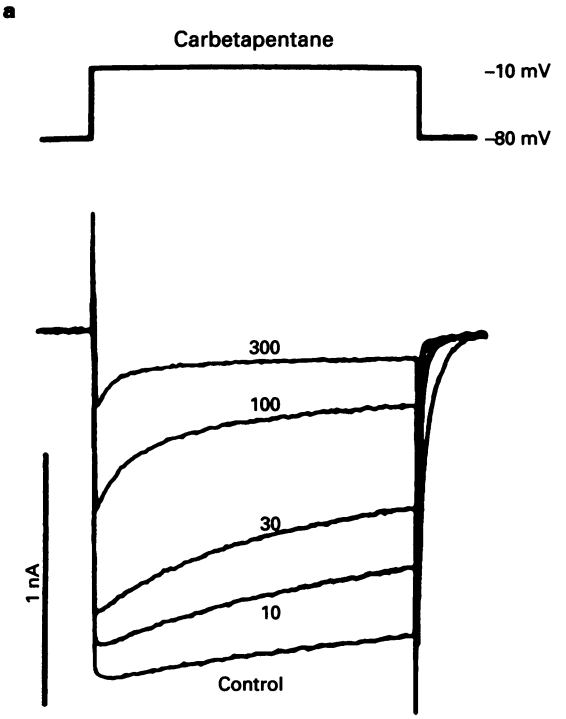
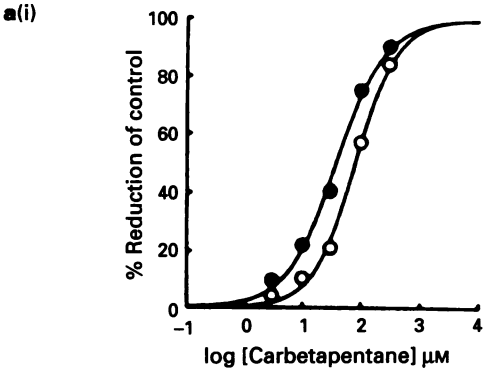


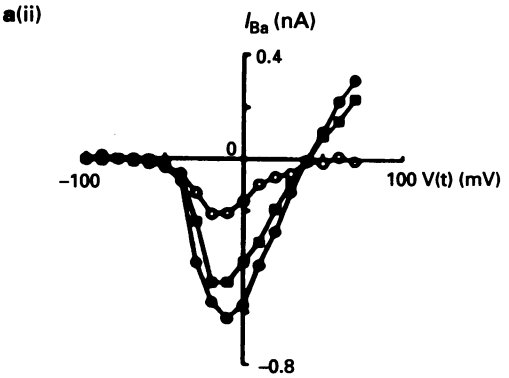

**Figure 2** Weak $I_{Ba}$ blocking actions of selected σ site ligands. Paired traces indicate antagonist actions of (a) 100 μM DTG, (b) 100 μM (+)-pentazocine, (c) 100 μM ketamine and (d) 100 μM DXM on the whole-cell $I_{Ba}$ evoked by the voltage step indicated above the individual traces. In (a) and (b) the voltage step was of 125 ms duration whilst in (c) and (d) it was 250 ms. In all cases, responses recovered to near control levels on wash (not shown). Ordinate scale, amplitude of $I_{Ba}$ (nA).

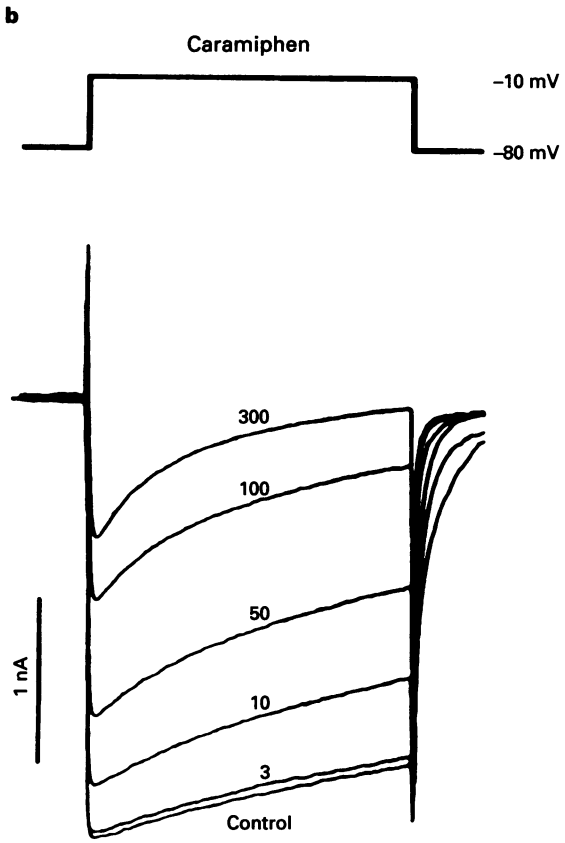
≈80% of the total whole-cell $I_{Ba}$, this finding suggests that the test compounds are able to block both L- and N-type HVA $Ca^{2+}$ channels and, possibly, additional HVA $Ca^{2+}$ channel subtypes (*e.g.* Mintz *et al.*, 1992; Wheeler *et al.*, 1994).

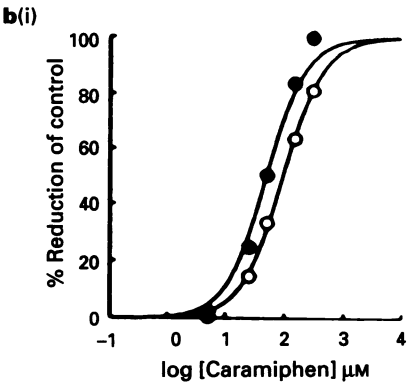
The attenuation of $I_{Ba}$ by the σ site ligands occurred without change in either the rate of onset of the whole-cell $I_{Ba}$ or the current-voltage relationship for the $I_{Ba}$ ($n\geqslant 3$ for each compound; see Figures 1 and 3,), suggesting in turn that their action occurs without influencing the kinetics of activation of HVA $Ca^{2+}$ channels. In contrast to dihydropyridines, which accelerate the inactivation of L-type HVA $Ca^{2+}$ channels (Rane *et al.*, 1987), there was no indication that the compounds tested affected the inactivation kinetics of the whole-cell $I_{Ba}$, having similar effects on both the peak and delayed components of the current (see Figures 1 and 2). Exceptions to the latter finding were seen with the non-opioid antitussives, carbetapentane and caramiphen (Figure 3). The antagonist actions of these compounds, rather than being realized completely during the first evoked current, continued to increase further over subsequent (usually up to three) voltage-activated currents and were associated with an apparent increase in the rate of inactivation of $I_{Ba}$. An analysis of the $IC_{50}$ values for reduction of the control peak and delayed (240 ms after the peak) $I_{Ba}$ indicated that carbetapentane and caramiphen have a higher affinity for the inactivated state of the whole-cell $I_{Ba}$. Thus, for carbetapentane, the $IC_{50}$ values for the peak and delayed components of the $I_{Ba}$ were 56±13 and 30±6 μM, respectively ($n=3$); for caramiphen, the respective values were 80±7 and 47±2 μM ($n=3$; see Figure 3). These data suggest the possibility that carbetapentane and caramiphen may have higher affinities for L-type than for N-type HVA $Ca^{2+}$ channels, L-type channels being less strongly inactivating than N-type $Ca^{2+}$ channels and thus making a greater contribution to the delayed component of the whole-cell $I_{Ba}$ (Tsien *et al.*, 1988). There was a similar, although less pronounced, difference in the potencies of haloperidol (Fletcher *et al.*, 1994) and DXM on the peak and delayed components of the whole-cell $I_{Ba}$ (respective $IC_{50}$ values for DXM = 94±17 and 68±10 μM ($n=5$)). Netzer and colleagues (1993) have previously reported

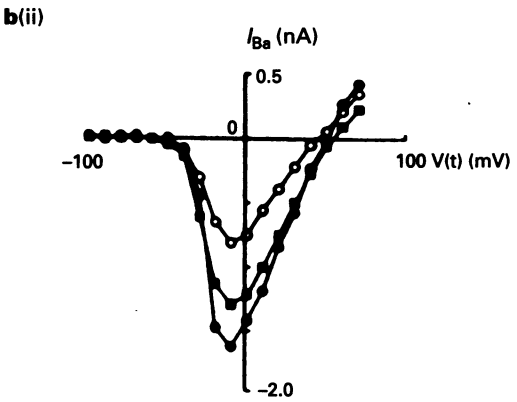

a
Carbetapentane
-10 mV
-80 mV
300
100
30
10
Control
1 nA


a(i)
% Reduction of control
100
80
60
40
20
0
-1
0
1
2
3
4
log [Carbetapentane] μM


a(ii)
$I_{Ba}$ (nA)
0.4
0
-100
100 V(t) (mV)
-0.8


b
Caramiphen
-10 mV
-80 mV
300
100
50
10
3
Control
1 nA


b(i)
% Reduction of control
100
80
60
40
20
0
-1
0
1
2
3
4
log [Caramiphen] μM


b(ii)
$I_{Ba}$ (nA)
0.5
0
-100
100 V(t) (mV)
-2.0

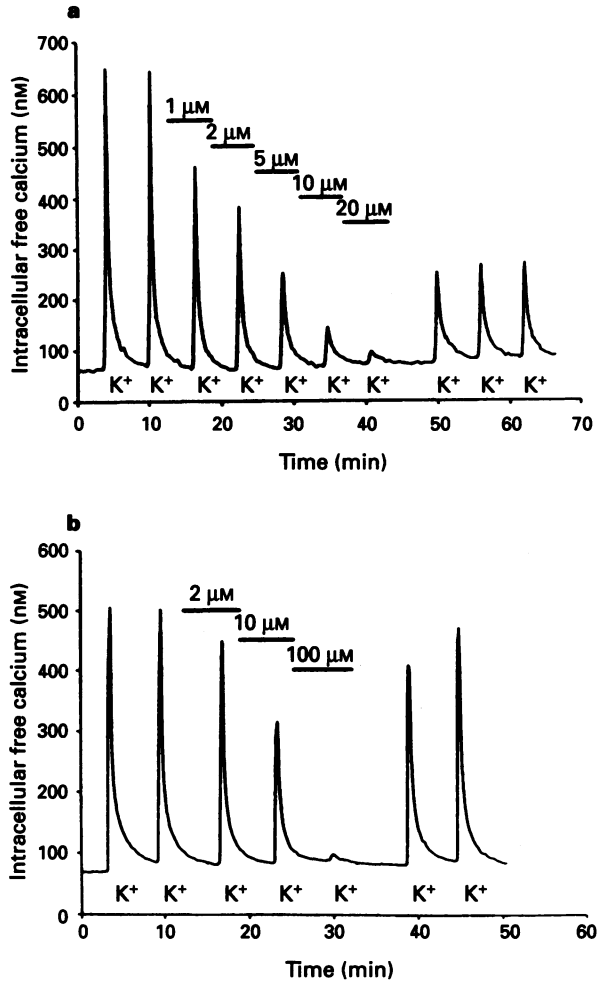



**Figure 4** The effects of rimcazole and opipramol on high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. (a) Rimcazole 1–20 μM, applied for the period indicated by the bars above the trace, attenuated high-$[K^+]_o$-($K^+$) evoked rises in $[Ca^{2+}]_i$. Recovery from the effects of concentrations of rimcazole ⩾10 μM was slow; in the experiment depicted, responses had recovered to ≈60% of control values after 2 h in wash (not shown). The trace is a mean of data obtained from 9 neurones simultaneously. (b) Opipramol 2–100 μM, applied for the periods indicated by the bars above the trace, reversibly attenuated high-$[K^+]_o$-($K^+$) evoked rises in $[Ca^{2+}]_i$. The trace is a mean of data obtained from 35 neurones simultaneously.

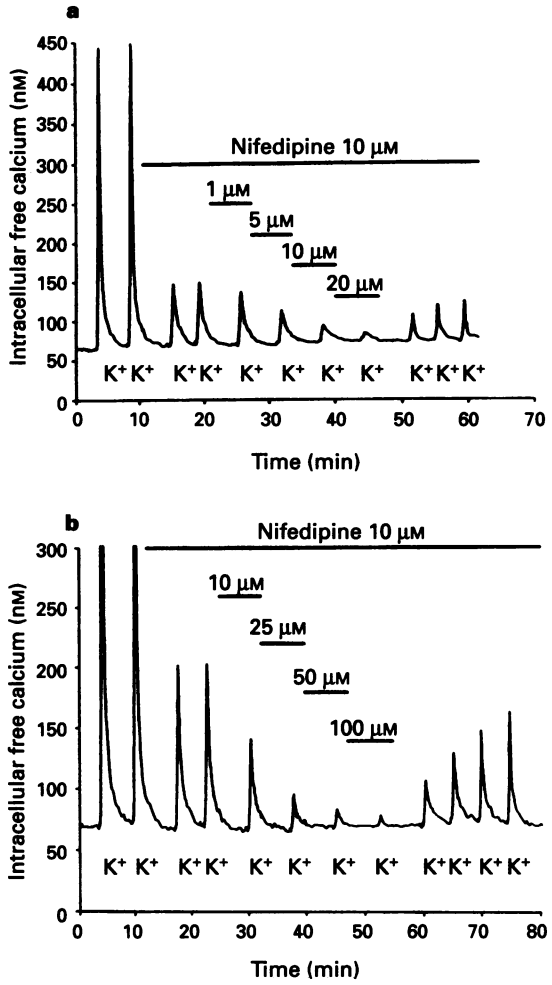



**Figure 5** Rimcazole and opipramol produce a concentration-dependent reduction of nifedipine-resistant high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. (a) Under control conditions (first and second responses) 50 mM $K^+$ ($K^+$) evoked large rises in $[Ca^{2+}]_i$ which were reduced by 79% by nifedipine 10 μM (third and fourth responses). Co-application of rimcazole 1–20 μM, for the periods indicated by the bars above the trace, produced a concentration-dependent reduction of the nifedipine-resistant residual response. The record is a mean of data obtained from 6 neurones simultaneously. (b) Under control conditions (first and second responses; peaks truncated for convenience in figure preparation) 50 mM $K^+$ ($K^+$) evoked rises in $[Ca^{2+}]_i$ which were reduced by 80% by nifedipine 10 μM (third and fourth responses). Opipramol 10–100 μM, co-applied with nifedipine 10 μM, then reversibly attenuated the nifedipine-resistant response. The record is a mean of data obtained from 5 neurones simultaneously.

that, in cultured rat cortical neurones, DXM blocks the inactivating component of $I_{Ba}$ after a conditioning potential of −100 mV (reflecting N-type $Ca^{2+}$ channel activity) with an $IC_{50}$ value of 71 μM; the $IC_{50}$ value for the component of the whole-cell $I_{Ba}$ remaining at the end of a voltage-step from −40 mV (reflecting L-type $Ca^{2+}$ channel activity) was 60 μM.

None of the other compounds tested showed similar discrepancies, suggesting that they were not artifacts of the recording procedure.

**Figure 3** Differential activity of carbetapentane and caramiphen on the peak and delayed components of the total whole-cell $I_{Ba}$. (a) Traces indicate the block of $I_{Ba}$, evoked by the 250 ms voltage step indicated above, by carbetapentane at the concentrations indicated adjacent to the individual traces. (a,i) Plot indicates the percentage reductions of the peak (○) and delayed (240 ms after peak; ●) components of the control $I_{Ba}$ (ordinate scale) for the same neurone as in (a). In this neurone, $IC_{50}$ values were 37 and 79 μM for the delayed and peak currents, respectively. Abscissa scale, log concentration of carbetapentane (μM). (a,ii) In a second neurone, the current-voltage relationship for control $I_{Ba}$ (●) was unaltered on application of 50 μM carbetapentane (○). $I_{Ba}$ recovered to near control levels on wash (■). $I_{Ba}$ was activated from a holding potential of −90 mV to the test potential (V(t)) indicated on abscissa scale. Ordinate scale, amplitude of $I_{Ba}$ (nA). (b) Traces indicate the concentration-dependent block of $I_{Ba}$ by caramiphen. $I_{Ba}$ was activated by a 250 ms voltage step indicated above the traces. (b,i) Plot indicates the percentage reductions of the peak (○) and delayed (240 ms after peak; ●) components of the control $I_{Ba}$ (ordinate scale) for the same neurone as in (b). In this neurone, $IC_{50}$ values were 49 and 93 μM for the delayed and peak components of the $I_{Ba}$, respectively. Abscissa scale, log concentration of caramiphen (μM). (b,ii) In a second neurone, the current-voltage relationship for control $I_{Ba}$ (●) was unaltered on application of 50 μM caramiphen (○). $I_{Ba}$ recovered to near control levels on wash (■). $I_{Ba}$ was activated from a holding potential of −90 mV to the test potential (V(t)) indicated on the abscissa scale. Ordinate scale, amplitude of $I_{Ba}$ (nA).

*Fluorescent dye studies*

In our preparation of rat cultured hippocampal pyramidal neurones, rises in $[Ca^{2+}]_i$ evoked by the transient application of 50 mM $K^+$-containing medium are mediated largely by $Ca^{2+}$ flux through dihydropyridine-sensitive (presumed L-type) $Ca^{2+}$ channels, with smaller contributions from dihydropyridine-resistant, ω-CgTx-sensitive (presumed N-type) $Ca^{2+}$ channels and $Ca^{2+}$ channels insensitive to both dihydropyridines and ω-CgTx but sensitive to crude funnel-web spider venom (Church *et al.*, 1994a).

All of the σ site ligands tested produced a concentration-dependent reduction of rises in $[Ca^{2+}]_i$ evoked by 50 mM $K^+$. In all cases, the effect was fully developed during the first high-$[K^+]_o$-evoked response after the start of superfusion of the test compound (data not shown) and was not accompanied by changes in resting $[Ca^{2+}]_i$. The $IC_{50}$ values for the compounds examined in this experimental paradigm are presented in Table 1. The rank order potency for blockade of high-$[K^+]_o$-evoked responses by the σ ligands examined was similar to that found in the electrophysiological studies (Spearman rank order correlation coefficient = 0.964, $P < 0.002$), although the absolute $IC_{50}$ values were lower than those obtained for block of $I_{Ba}$ (see Table 1). At the highest concentrations tested, each of the compounds could reduce rises in $[Ca^{2+}]_i$ evoked by high-$[K^+]_o$ by >85%, a result otherwise achieved in our experimental preparation only by the combined application of nifedipine, ω-CgTx and crude funnel-web spider venom (Church *et al.*, 1994a). Thus, 50 μM rimcazole reduced the high-$[K^+]_o$-evoked response by 99±1.0% ($n = 46$). Corresponding values for the other compounds tested were: haloperidol (50 μM, 92±0.5%, $n = 152$); ifenprodil (200 μM, 98±0.3%, $n = 81$); opipramol (100 μM, 96±0.4%, $n = 78$); carbetapentane (200 μM, 97±1.6%, $n = 69$); L 687,384 (100 μM, 87±0.5%, $n = 81$); caramiphen (200 μM, 95±0.8%, $n = 84$); and DXM (200 μM, 93±2.7%, $n = 128$). These results suggested that the compounds are able to block $Ca^{2+}$ flux through multiple subtypes of HVA $Ca^{2+}$ channels. Examples of the effects of the atypical antipsychotic, rimcazole (Ferris *et al.*, 1986) and the antidepressant, opipramol (Rao *et al.*, 1990) on high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ can be seen in Figure 4.

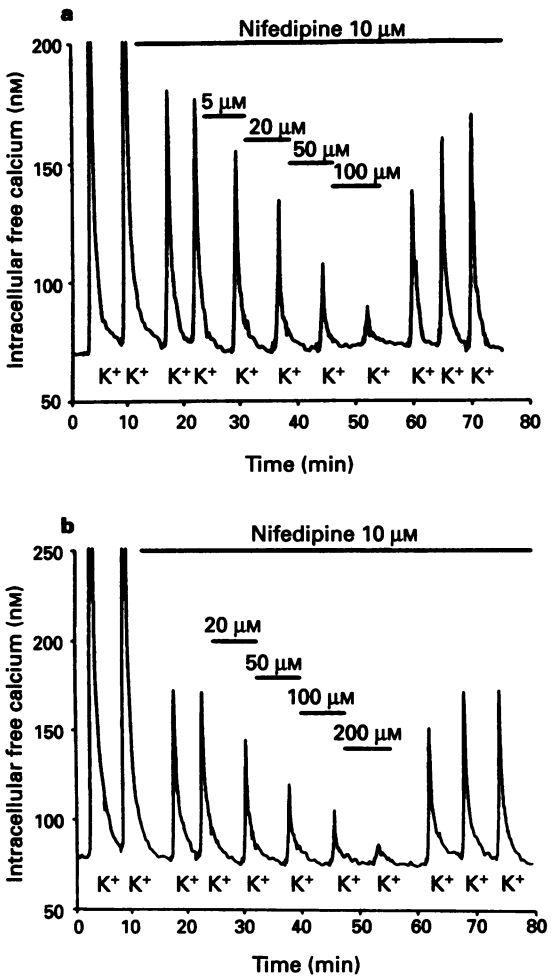



**Figure 6** The non-opioid antitussives caramiphen (a) and DXM (b) reversibly attenuate nifedipine-resistant high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. Under control conditions (first and second responses; peaks truncated for convenience in figure preparation) high-$[K^+]_o$ ($K^+$) elicited rises in $[Ca^{2+}]_i$ which were then reduced by 76% (a) and 82% (b) by nifedipine 10 μM (third and fourth responses). Caramiphen (a) and DXM (b) each then reduced the nifedipine-resistant residual response in a concentration-dependent manner. The records are means of data obtained from 13 (a) and 24 (b) neurones simultaneously.

To examine more closely the actions of the test compounds against dihydropyridine-resistant HVA $Ca^{2+}$ channels, they were each applied in the presence of 10 μM nifedipine (a maximally effective concentration in this preparation; Church *et al.*, 1994a). Application of 10 μM nifedipine alone produced a 72–82% reduction of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ (Figures 5 and 6; see Church *et al.*, 1994a,b; Fletcher *et al.*, 1994). Co-application of the σ site ligands in all cases produced a concentration-dependent reduction of nifedipine-resistant, ω-CgTx- and funnel-web spider venom-sensitive, high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. $IC_{50}$ values for the compounds examined in the presence of nifedipine are presented in Table 1. With the single exception of ifenprodil (Church *et al.*, 1994b), $IC_{50}$ values were higher in the presence than in the absence of 10 μM nifedipine and, in some cases, approached those obtained for the reduction of the whole-cell $I_{Ba}$ (see Table 1). This might be expected, given the fact that, under the voltage-clamped conditions employed in the electrophysiological studies, a greater proportion of the whole-cell $I_{Ba}$ is sensitive to ω-CgTx than nifedipine, whereas the reverse is true for high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ (see Church *et al.*, 1994a). As in the case for the effects of the test compounds observed in the absence of nifedipine (see above), the rank order potency for blockade of high-$[K^+]_o$-evoked responses by the σ site ligands examined in the presence of 10 μM nifedipine was similar to that found in the electrophysiological studies (Spearman rank order correlation coefficient = 0.952, $P < 0.005$). Examples of the effects of rimcazole and opipramol, and the non-opioid antitussives, caramiphen and DXM, on high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ in the presence of 10 μM nifedipine can be seen in Figures 5 and 6, respectively. In the cases of haloperidol, carbetapentane, caramiphen and DXM, $IC_{50}$ values obtained in the presence of 10 μM nifedipine were at least 1.5 times higher than those obtained in its absence, adding support to the possibility that the differences in potency for these compounds against the peak and delayed components of the whole-cell $I_{Ba}$ (see above) reflect greater activity at L- than at N-type HVA $Ca^{2+}$ channels.

## Discussion

Micromolar concentrations of the σ site ligands examined in the present study block $Ca^{2+}$ channel currents and high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ in cultured hippocampal pyramidal neurones. The fact that the compounds tested were able to block almost completely both the whole-cell $I_{Ba}$ and high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ in the absence of any other pharmacological agent suggests that they are able to block multiple subtypes of HVA $Ca^{2+}$ channels. This possibility was supported by the results of the microspectrofluorimetric experiments conducted in the presence of nifedipine, where the compounds were able to reduce, in a concentration-dependent manner, high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ resistant to the dihydropyridine. The present results agree well with those previously reported for DXM, which blocked both L- and N-

type HVA $Ca^{2+}$ channels in rat cultured cortical neurones with $IC_{50}$ values of 60–70 μM (Netzer *et al.*, 1993), and ketamine, which produced only a 6% reduction of steady-state $I_{Ba}$ at 100 μM in dissociated adult guinea-pig CA1 hippocampal pyramidal neurones (ffrench-Mullen & Rogawski, 1992). In agreement with ffrench-Mullen & Rogawski (1992), we have also observed that dizocilpine is a very weak antagonist of whole-cell $I_{Ba}$ (Fletcher, unpublished observations). The $Ca^{2+}$ channel blocking properties of the other compounds tested in the present experiments, with the exceptions of ifenprodil (Church *et al.*, 1994b) and haloperidol (Fletcher *et al.*, 1994), have not been previously reported, although it is of interest that the ifenprodil analogue, eliprodil (SL 82.0715) has recently been found to block the total $I_{Ba}$ carried by L- and N-type HVA $Ca^{2+}$ channels in rat cultured cortical neurones with an $IC_{50}$ of 1.5 μM (Biton 1994).

The micromolar concentrations of the compounds required to block HVA $Ca^{2+}$ channels (Table 1) are substantially higher than those nanomolar concentrations associated with competition for high-affinity σ binding sites labelled with tritiated σ ligands or non-opioid antitussives (see Fletcher *et al.*, 1995). Furthermore, the rank order potency of the compounds tested as antagonists of HVA $Ca^{2+}$ channels (Table 1) does not parallel that of their affinities for high-affinity σ binding sites. Thus rimcazole, one of the more potent $Ca^{2+}$ channel blockers tested, is a relatively weak displacer of binding to high-affinity σ sites (Ferris *et al.*, 1986; Manallack *et al.*, 1988; Beart *et al.*, 1989; Barnes *et al.*, 1992). Conversely, DTG, (+)-3-PPP, L 687,384 and (+)-pentazocine, which possess high affinities for σ sites (Manallack *et al.*, 1988; Beart *et al.*, 1989; Musacchio *et al.*, 1989; Ferris *et al.*, 1991; Middlemiss *et al.*, 1991; Barnes *et al.*, 1992; Cagnotto *et al.*, 1994) are relatively weak blockers of HVA $Ca^{2+}$ channels. Similarly opipramol, which displaces [$^3$H]-(+)-3-PPP binding with $IC_{50}$ values of 1–6 nM (Musacchio *et al.*, 1989; Ferris *et al.*, 1991), was only a moderately potent $Ca^{2+}$ channel blocker. No stereoselectivity was observed in the $Ca^{2+}$ channel blocking potencies of (+)- and (−)-29, yet they differ 160 fold in their affinities for σ sites (de Costa *et al.*, 1990). In addition, the rank order potency of the compounds tested as antagonists of HVA $Ca^{2+}$ channels does not parallel their rank order potency for inhibition of high-affinity [$^3$H]-DXM binding. Thus, the rank order potency of the compounds tested for displacement of high-affinity [$^3$H]-DXM binding ($IC_{50}$ values in nM taken from Klein & Musacchio, 1989) is opipramol (0.4) > haloperidol (1.2) > (+)-pentazocine (3) > carbetapentane = caramiphen (10) > (+)-3-PPP (25) > DXM (57) > rimcazole (120), very different from the rank order potency for $Ca^{2+}$ channel blockade (Table 1). Furthermore, the non-opioid antitussive, butamirate also has high affinity for [$^3$H]-DXM binding sites ($IC_{50}$ = 12 nM; Klein & Musacchio, 1989) but is a very weak blocker of voltage-activated $Ca^{2+}$ channels (Church, unpublished observations). In summary, as no correlation exists between the potency of the compounds tested as $Ca^{2+}$ channel blockers and their reported affinities for, or stereoselectivity at, high-affinity σ or DXM binding sites, their observed $Ca^{2+}$ channel blocking actions are unlikely to be mediated by these high-affinity sites.

The NMDA antagonist activities of the same range of compounds as those tested in the present study, with the exception of L 687,384 (examined by McLarnon *et al.*, 1994), were described in the preceding paper (Fletcher *et al.*, 1995). There is little apparent similarity between either the absolute or the rank order NMDA antagonist and $Ca^{2+}$ channel blocking potencies (as assessed under whole-cell voltage-clamp conditions) of the compounds tested, indicating that the antagonist binding sites at HVA $Ca^{2+}$ channels and at the NMDA receptor-channel complex have different pharmacological specificities. Thus, for example, whereas DXM, ketamine and (+)-pentazocine are reasonably potent antagonists of NMDA-evoked currents in mouse hippocampal neurones (respective $IC_{50}$ values 1.8, 3.5 and 7.2 μM), they are only weak blockers of HVA $Ca^{2+}$ channels (Table 1). Conversely, (−)-44, rimcazole, opipramol, carbetapentane, L 687,384 and caramiphen are more potent blockers of HVA $Ca^{2+}$ channels than they are NMDA antagonists.

The mechanism(s) whereby the compounds tested block HVA $Ca^{2+}$ channels remain unclear and additional experiments are required to assess formally their potencies as blockers of individual (pharmacologically discriminated) subtypes of HVA $Ca^{2+}$ channels. Nevertheless, some general conclusions can be drawn. Based on the ability of the test compounds to block more than 80% of the whole-cell $I_{Ba}$ and to attenuate both nifedipine-sensitive and nifedipine-resistant high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$, it is apparent that they are able to block both dihydropyridine-sensitive and dihydropyridine-resistant HVA $Ca^{2+}$ channels. In addition, none of the compounds produced a significant shift in the current-voltage relationship of voltage-activated $Ca^{2+}$ channels, suggesting that they do not alter the voltage-dependence of channel gating, and, in contrast to dihydropyridine $Ca^{2+}$ channel antagonists (Rane *et al.*, 1987), the majority of the compounds did not affect the inactivation kinetics of the whole-cell $I_{Ba}$. Exceptions to the latter were seen with haloperidol (Fletcher *et al.*, 1994), carbetapentane, caramiphen and DXM, which blocked the delayed component of the current to a greater extent than the peak component, suggesting in turn that these compounds may have some selectivity for dihydropyridine-sensitive HVA $Ca^{2+}$ channels. The latter possibility was also suggested by their different $IC_{50}$ values for reduction of the total and nifedipine-resistant components of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. An analysis of the possible voltage-dependence of the block of $I_{Ba}$ will help to clarify the precise mechanism(s) of action of the compounds tested. In this regard, previous studies have demonstrated that whereas haloperidol (Fletcher *et al.*, 1994) and ifenprodil (Church *et al.*, 1994) block $I_{Ba}$ in a voltage-dependent fashion, the effect of DXM is not voltage-dependent (Netzer *et al.*, 1993). Finally, given the rapid onset of the block of $I_{Ba}$ by the test compounds, the frequency-dependence of the block is likely to be minimal, although this was not tested directly. Possible exceptions were seen with carbetapentane and caramiphen, whose antagonist actions were not fully achieved over the first evoked current.

What role might $Ca^{2+}$ channel blockade play in the functional effects of the compounds tested? The ability of micromolar concentrations of σ site ligands to block multiple subtypes of HVA $Ca^{2+}$ channels may, in particular, be involved in the antiepileptiform and neuroprotective actions described for some of the compounds. As noted in the preceding paper (Fletcher *et al.*, 1995), the latter effects have often been ascribed to activity at high-affinity σ binding sites despite the fact that, *in vitro*, these therapeutically-useful actions appear to be associated with micromolar concentrations of σ site ligands. As regards antiepileptiform activity, this has been described for a variety of σ ligands, including caramiphen and carbetapentane (Aram *et al.*, 1989; Tortella *et al.*, 1989; Apland & Braitman, 1990; Pontecorvo *et al.*, 1991). *In vitro*, this action is associated with concentrations of these compounds which lie within the range associated with HVA $Ca^{2+}$ channel blockade. Thus, carbetapentane and caramiphen block epileptiform bursting in rat hippocampal slices induced by $Mg^{2+}$-free medium, but not NMDA application, with $EC_{50}$ values of 37 and 23 μM, respectively (Apland & Braitman, 1990). These concentrations are lower than the $IC_{50}$ values reported in the preceding paper (Fletcher *et al.*, 1995) for inhibition of NMDA-evoked currents in cultured hippocampal pyramidal neurones under whole-cell voltage-clamp but similar to their $IC_{50}$ values for block of HVA $Ca^{2+}$ channels. Although DXM and ifenprodil are weaker blockers of HVA $Ca^{2+}$ channels than NMDA antagonists, $Ca^{2+}$ channel blockade has also been suggested to underlie, at least in part, their antiepileptiform actions (Löscher & Hönack, 1993; Netzer *et al.*, 1993; Church *et al.*, 1994b). In a similar manner, the neuroprotective actions associated with micromolar concentrations of σ ligands such as DXM, caramiphen, carbetapentane, opipramol and ifenprodil (e.g. Gotti *et al.*, 1990; Rao *et al.*, 1990; Pontecorvo

*et al.*, 1991; DeCoster *et al.*, 1995) may reflect, at least in part, blockade of multiple subtypes of HVA $Ca^{2+}$ channels, particularly given recent reports that novel broad-spectrum $Ca^{2+}$ channel blockers possess neuroprotective properties (e.g. Benham *et al.*, 1993). Carbetapentane, for example, attenuates glutamate-induced neurotoxicity in rat cultured cortical neurones with an $EC_{50}$ value of 46.3 μM (DeCoster *et al.*, 1995), similar to the $IC_{50}$ value obtained in the present study for $Ca^{2+}$ channel blockade in voltage-clamped neurones (40.5 μM; Table 1). Furthermore, the ability of micromolar concentrations of haloperidol and DTG to inhibit ischaemia-induced release of glutamate from rat hippocampal slices (Lobner & Lipton, 1990) may reflect their blockade of dihydropyridine-resistant $Ca^{2+}$ channels which participate in neurotransmitter release (see Burke *et al.*, 1993; Luebke *et al.*, 1993; Wheeler *et al.*, 1994). This possibility is supported by the fact that haloperidol, ifenprodil, rimcazole, DXM, carbetapentane and caramiphen are all able to reduce $K^+$-stimulated excitatory amino acid release from hippocampal or striatal slices with micromolar $IC_{50}$ values approximating those obtained for block of HVA $Ca^{2+}$ channels in the present experiments (Annels *et al.*, 1991; Mangano *et al.*, 1991; Ellis & Davies, 1994). Similarly, the weak $I_{Ba}$ blocking action of (+)-3-PPP parallels its low potency as an inhibitor of $K^+$-stimulated glutamate release from rat striatal slices ($IC_{50} > 100$ μM; Ellis & Davies, 1994).

In conclusion, micromolar concentrations of a series of σ site ligands block HVA $Ca^{2+}$ channels in cultured hippocampal neurones. This action, which is unlikely to be mediated by high-affinity σ binding sites, may play a role in the anticonvulsant and neuroprotective activities of some of the compounds. Indeed, the broad spectrum of activity of the compounds tested in the present experiments at HVA $Ca^{2+}$ channels resembles that of flunarizine, which is able to inhibit responses mediated by $Ca^{2+}$ flux through dihydropyridine-sensitive and -insensitive $Ca^{2+}$ channels (Tytgat *et al.*, 1991), blocks $Ca^{2+}$-dependent glutamate release (Cousin *et al.*, 1993) and possesses both anticonvulsant and neuroprotective efficacy *in vivo* (Deshpande & Wieloch, 1986; Czuczwar *et al.*, 1992). Finally, it is noteworthy that rimcazole, an atypical antipsychotic agent, was one of the more potent $Ca^{2+}$ channel blockers examined in the present experiments. This action is also part of the spectrum of activity of butyrophenones, including haloperidol (Fletcher *et al.*, 1994), and diphenylbutylpiperidine antipsychotics (*e.g.* Sah & Bean, 1994), suggesting in turn that $Ca^{2+}$ channel blockade may contribute to the clinical profile of activity of rimcazole. The results of the present study, together with those in the preceding paper (Fletcher *et al.*, 1995), emphasize the dangers of attributing unequivocally the functional effects of micromolar concentrations of σ site ligands to interactions with high-affinity σ binding sites.

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