The effects of chronic treatment with the dihydropyridine, Bay K 8644, on hyperexcitability due to ethanol withdrawal, *in vivo* and *in vitro*


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1 The effects of chronic treatment with the dihydropyridine, Bay K 8644, were studied on the ethanol withdrawal syndrome, *in vivo* and *in vitro*.
2 Addition of racemic Bay K 8644 to the drinking mixture, throughout the chronic ethanol treatment, decreased the behavioural excitability seen during ethanol withdrawal *in vivo*.
3 All the signs of hyperexcitability in field potentials in the isolated hippocampal slice, caused by ethanol withdrawal, were decreased by the chronic administration of Bay K 8644.
4 These effects resembled those previously reported for chronic administration of calcium channel antagonists; racemic Bay K 8644 has both calcium channel activating and antagonist properties.
5 Measurement of brain levels of Bay K 8644 at the end of the chronic treatment showed that the compound reached micromolar concentrations during the treatment, but none could be detected in the tissues at the time of the above measurements.
6 It is possible that the results might be explained by predominance of the calcium channel antagonist properties of this compound, owing to the high central concentrations achieved during the treatment. Tolerance to the calcium channel activating properties of Bay K 8644 may also have occurred during the chronic treatment.

**Keywords:** Ethanol; withdrawal syndrome; calcium channels; dihydropyridine; hippocampus

**Introduction**

Ethanol has acute actions on many systems, but the basis of the dependence that develops on chronic treatment is not certain. There has been much interest recently in the actions of ethanol on calcium-mediated control mechanisms in neurones. Calcium spikes in cultured neurones were decreased by ethanol (Stokes & Harris, 1982). Ethanol decreased the fast phase of depolarization-induced calcium flux into neurones and tolerance developed to this effect on chronic treatment (Harris & Hood, 1980; Friedman et al., 1980).

Chronic ethanol treatment has been found to increase the number of dihydropyridine-sensitive high affinity binding sites in the CNS (Dolin et al., 1987). These sites are thought to correspond to voltage-sensitive calcium channels, of the high-voltage activated subtype. They do not appear to play a large part in normal neuronal activity, although effects have been reported in neurones under experimental conditions leading to prolonged depolarization (Docherty & Brown, 1986a; Louvel et al., 1986; Jones & Heinemann, 1987; Takahashi et al., 1989; Grover & Teyler, 1990).

Dihydropyridine calcium channel antagonists were found to have a protective effect against the ethanol withdrawal syndrome *in vivo*; this effect was stereospecific (Littleton et al., 1990). We have also found that the dihydropyridine calcium channel antagonist, PN 200-110, stereospecifically prevented all the measured signs of hyperexcitability in the isolated hippocampal slice caused by withdrawal from chronic ethanol treatment *in vivo* (Whittington & Little, 1991a). The changes in the field potentials, caused by ethanol withdrawal, included decreases in thresholds for production of population spikes, following both orthodromic and antidromic stimulation, increases in paired pulse potentiation and shifts to the left of the input/output curves (Whittington & Little, 1990a; 1991a,b). They followed different time courses during the withdrawal period, suggesting different origins of the forms of hyperexcitability.

Adaptive responses to chronic ethanol administration can be modulated by concurrent chronic treatment with a dihydropyridine calcium channel antagonist. Niitrendipine, given concurrently with ethanol in chronic treatment, prevented the development of tolerance to ethanol (Wu et al., 1987; Little & Dolin, 1987; Dolin & Little, 1989). Niitrendipine, given chronically with ethanol, also prevented the ethanol withdrawal syndrome (Whittington et al., 1991) and decreased the electrophysiological manifestations of withdrawal described above (Whittington & Little, 1991b). These effects were considered to be responses to the continued presence of nitrrendipine, rather than acute actions, as the CNS concentrations at the time of testing were too low to produce acute effects in either the tolerance studies or on the withdrawal measurements (Dolin & Little, 1989; Whittington et al., 1991). The increase in the number of dihydropyridine binding sites, measured after chronic ethanol administration, was also prevented, suggesting a possible causal relationship between the changes at these sites and development of ethanol tolerance and dependence (Dolin et al., 1988a; Dolin & Little, 1989).

Long-term administration of a dihydropyridine calcium channel antagonist causes down-regulation of the high affinity binding sites (Panza et al., 1985). We have put forward the theory that the effects of concurrently administered calcium channel antagonists in preventing ethanol dependence were due to the prevention of the upregulation of voltage-sensitive calcium channels (Littleton & Little, 1989; Whittington et al., 1991; Little, 1991).

The dihydropyridine, Bay K 8644, has been shown to increase the opening of voltage-sensitive calcium channels (Schramm et al., 1983; Brown et al., 1984; Nowycky et al., 1985) and this effect of Bay K 8644 has been shown to be the property of the (−)-isomer (Hof et al., 1985). In the present study we have measured the effects of chronic treatment with Bay K 8644, on the ethanol withdrawal syndrome and on the changes in field potentials in the hippocampal slice following chronic ethanol treatment. Bay K 8644 was given in the drinking fluid with the ethanol, throughout the chronic treatment. It was necessary to use the racemate in this chronic...
treatment study, because insufficient of the isomers was available.

**Methods**

**Chronic drug treatment**

Male mice, C57 strain, 25–30g, were given ethanol, 24% v/v, as sole fluid, for 18 weeks. Racemic Bay K 8644 was dissolved in the ethanol and the solution then added to the drinking fluid. The dihydropyridine was given with the ethanol, at 71 μM, throughout the drinking period. Bay K 8644 was removed from the drinking fluid 24 h before the experiments (the ethanol remaining) so that the effects of chronic Bay K 8644 treatment, rather than any acute action, could be studied. Controls drank tap water only. Owing to the low aqueous solubility of Bay K 8644 it was not possible to treat animals by this method with the compound in the absence of ethanol.

The addition of Bay K 8644 did not alter the ethanol intake; the mean intakes of ethanol were 16.0 ± 1.7 g·kg⁻¹·day⁻¹ (n = 15 mice) for the ethanol alone treated group, and 15.3 ± 2.7 g·kg⁻¹·day⁻¹ (n = 16 mice) for the ethanol plus Bay K 8644 treated group. The intake of Bay K 8644 was 1.9 ± 0.3 mg·kg⁻¹·day⁻¹ (n = 16 mice) during the 18 week treatment period. Mice were taken at random from the treatment groups for preparation of the hippocampal slices.

Bottles containing Bay K 8644 were protected from light, as the compound is light-sensitive. The mice were weighed at regular intervals during the chronic treatment and there were no significant differences between the weights of any of the treatment groups. Bay K 8644 (Bayer AG) is methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl)phenylpyridine-5-carboxylate.

**The ethanol withdrawal syndrome**

The ethanol withdrawal syndrome was measured by ratings of convulsive behaviour (Goldstein & Pal, 1971; Little et al., 1990). This method measures both tremor and clonic convulsions on a continuous scale. The mice were removed from the cages containing the ethanol drinking mixture between 09 h 00 min and 09 h 30 min, 24 h after removal of Bay K 8644 from the drinking solution. Behavioural ratings were made once an hour on all mice for the next 12 h, by an observer who was unaware of the prior drug treatment. The treatment groups were tested concurrently. The numbers in each group in this experiment were 8 for those drinking ethanol alone, and 10 for those receiving ethanol plus Bay K 8644.

For comparison, control (drug-naive) C57 mice were given intraperitoneal injections of (±)-Bay K 8644, 2 mg·kg⁻¹, suspended in Tween 80, 0.5%, or the vehicle, n = 8 per treatment group. Ratings were made of convulsive behaviour on handling, as above, hourly for the next 12 h.

**Statistical analysis of withdrawal ratings**

The ratings of the withdrawal syndrome, expressed as median and interquartile range, were compared by nonparametric analysis of variance, designed for repeat measurements on the same samples and multiple comparisons with controls (Meddis, 1984). Comparisons were made on the ratings over the whole 12 h period from the start of withdrawal. During the first 2 h the withdrawal syndrome was beginning, but was not seen clearly until about 3 h.

**Electrophysiological recordings**

Hippocampal slices were prepared between 09 h 00 min and 10 h 00 min immediately on removal of the ethanol drinking solution (i.e. 24 h after removal of Bay K 8644 from the drinking solution), and perfused with standard Ringer solution. The animals were not withdrawn from the ethanol before preparation of the slices, so ethanol would have been washed out of the slices during the first part of the recording period. Hippocampal slices, 400 μm thick, were prepared as described previously, (Whittington & Little, 1990a), and maintained in Krebs solution at 30°C ± 0.5°C, flow rate 1.5 ml·min⁻¹. Extracellular recordings from the stratum pyramidale in area CA1 were started 30 min after the slices were placed in the perfusion bath, that is 45 min from removal of tissues. The times given in the results sections are all from removal of tissues, i.e. from withdrawal of ethanol.

Schaffer collateral/commissural fibres were stimulated using paired pulses, constant current, 50 μs duration, every 10 s throughout the 7 h recording period. The stimulating electrodes consisted of two silver insulated wires, 0.2 mm in diameter, passed through 1 mm diameter silicon tubing. Recording electrodes were made from 1.2 mm diameter electrode glass and filled with 2 M KCl; the resistances were between 5 and 15 MΩ. A 70 ms interval was used for the paired pulses because preliminary experiments showed that it gave the maximum paired pulse potentiation of the population spike in normal mouse hippocampal slices. A stimulation level of 1.25 times the threshold for eliciting a single population spike was used in the studies on paired pulse potentiation.

The thresholds for the production of population spikes, and levels of paired pulse potentiation of these responses, were measured every 15 min. Measurements were made of the thresholds for eliciting single population spikes and multiple population spikes as described previously (Whittington & Little, 1990a). Input/output curves were determined at 1, 3, 5, and 7 h from the preparation of tissues. These were made for the population spikes and for the slopes of the field e.p.s.ps (excitatory postsynaptic potentials). The field e.p.s.ps were recorded from the cell body area, so the measurements were made at some distance from the actual origin of the dendritic e.p.s.ps.

Certain precautions were taken to ensure that valid comparisons could be made between the field potentials from tissues from different treatment groups (Whittington & Little, 1990a). The order of testing and the distribution of the various treatments between the two recording chambers were carefully balanced. The positions of the stimulating and recording electrodes were determined in every tissue by using the ends of the arc of granular cells in the dentate gyrus and the apex of the CA3 region above the fimbria as common reference points between slices.

The composition of the perfusion fluid was (mm): NaCl 124, KCl 3.25, NaH₂PO₄ 1.25, NaHCO₃ 20.0, MgSO₄ 2.0, CaCl₂ 2.0, and D-glucose 10.0. The pH was 7.2 at 30°C. At the completion of every day's experiments the perfusion chamber was washed through with distilled water for at least 30 min, and washed through every 5 days with sodium hydroxide followed by citric acid.

**Statistical analysis of electrophysiological results**

The areas of the population spikes were measured by Acorn computer analysis. In every case a minimum of three responses was averaged for each stimulus. The results in the figures are expressed as mean ± s.e.mean for each of the different treatments. A minimum of five tissues, all from different animals, were studied in each treatment group. Comparisons were made between results from tissues following the different treatments by two-way analysis of variance. The times given in the results section are all from the removal of the tissues. This corresponded with the withdrawal of ethanol.

Input/output data, derived from population spike area and field e.p.s.ps. slope measurements, were fitted to a logistic equation by non-linear regression. Analysis of the resulting sigmoidal curves allowed for calculation of maximum responses, the stimulus required to produce half-maximal responses (S₅₀), and the slope of the stimulus/response relationship. Comparisons of data from these curves were
made between different tissues, following the various treatments, by Student’s nonpaired t test.

Measurement of central Bay K 8644 concentrations

Measurements were made of the concentrations of Bay K 8644 in brains removed from separate groups of animals receiving the chronic treatment, to determine how much Bay K 8644 was present during the treatment and at the time of testing. The brains were removed, following cervical dislocation, from C57 mice, that had been drinking the ethanol and Bay K 8644 solution for 18 weeks as described above. The tissues were taken at the end of the chronic treatment at 10 h 00 min, and 8 h after withdrawal of ethanol (i.e. before and 32 h after removal of Bay K 8644 from the drinking solution). The 8 h timepoint was chosen to correspond with the peak drug effect in the withdrawal testing. Six tissues were taken per treatment group. For comparison, brains were also taken from control mice (n = 6) 1 h after i.p. injection of racemic Bay K 8644, 2 mg kg⁻¹.

Whole brain Bay K 8644 concentrations were measured by gas/liquid chromatography. Electron capture detection was used to analyse samples separated with a 25 meter capillary column coated with SE30. The temperature programming for the analysis was as follows: injector 280°C, detector 320°C, oven started at 180°C for 2 min then ramped up to 290°C at 16°C min⁻¹ and left at this temperature for 10 min. Helium carrier gas at 6 p.s.i. was used to ensure the flow of sample along the column at the optimum speed for separation. The sample was flushed from the end of the capillary column into the detector with nitrogen make-up gas at 20 p.s.i.

The brains were homogenized in 10 vol. NaOH, 1 ml per 50 mg tissue wet weight, and residual proteins precipitated with sodium tungstate and sulphuric acid. The samples were centrifuged at 4000 r.p.m. for 10 min, then the Bay K 8644 was extracted by shaking with the same volume of toluene. The organic layer was removed and dried with anhydrous MgSO₄. Samples were evaporated to dryness and reconstituted in 100 μl toluene containing 1 μM nimodipine as internal standard.

Dihydropyridine binding

Central dihydropyridine receptor binding was measured by the method of Glossman & Ferry (1985), using [³H]-nimodipine (specific activity 130 Ci mmol⁻¹). Brains were removed 8 h after cessation of ethanol drinking (i.e. 32 h after removal of Bay K 8644 from the drinking solution), and the cerebral cortices dissected out. The tissues were homogenized in Tris buffer, 50 mmol and centrifuged (45000 g, 15 min) and washed with the buffer three times. Tissue aliquots were incubated (25°C, 45 min) with [³H]-nimodipine, 0.125 nM to 4 nm, then filtered (Whatman GF/C glass filters) and counted. All measurements were made in triplicate. Nonspecific binding was estimated with 1 μM nitrendipine. The protein content of the tissues was measured with Folin’s reagent. The results were subjected to Scatchard analysis to give the receptor density, B₅₀, in nmol mg⁻¹ protein and the dissociation constant, Kd, in nm.

Results

Behavioural studies

The ethanol withdrawal syndrome. The addition of Bay K 8644 to the drinking mixture significantly decreased the ethanol withdrawal syndrome (Figure 1). The ratings for the animals that drank ethanol alone were significantly higher than those that received ethanol plus Bay K 8644, when compared over the time period 1 h to 12 h from ethanol withdrawal (P < 0.001). The protection against the withdrawal syndrome was complete, as the values after treatment with ethanol plus Bay K 8644 were not significantly different from controls (P > 0.05).

The effects of acute Bay K 8644. When an acute injection of Bay K 8644 was given to control animals the measurements of convulsive behaviour were increased (Figure 2). This effect lasted for 2 h, then declined over the next 4 h. The difference from control values was significant over the period 1 h to 6 h from injection (P < 0.001).

Electrophysiological recordings

Thresholds for eliciting population spikes. As shown previously (Whittington & Little, 1990a), chronic ethanol treatment lowered the thresholds for eliciting both single and multiple population spikes in the CA1 area. These effects were significant over the time period 2 h to 7 h from ethanol withdrawal (P < 0.001). Chronic administration of Bay K 8644 significantly decreased the effects of chronic ethanol on both these thresholds (Figures 3 and 4). The difference between the thresholds after treatment with ethanol alone and ethanol plus Bay K 8644 was significant from 3 h to 7 h (P < 0.001). An example of the form of multiple spiking seen during ethanol withdrawal, and the absence of this form of spiking is shown in Figure 5.

Paired pulse potentiation. The paired pulse potentiation of the population spike was increased after ethanol treatment
Figure 3 Thresholds for production of single population spikes in isolated hippocampal slices prepared after chronic treatment in vivo with ethanol or ethanol plus Bay K 8644. (The dihydropyridine was removed from the drinking fluid 24 h before slice preparation.) Values after treatment with ethanol alone were significantly lower than control values or those after treatment with ethanol plus Bay K 8644, P < 0.001, over the whole test period. Values are mean with s.e.mean shown by vertical bars. Controls (□); ethanol (●); ethanol plus Bay K 8644 (▲).

(6), but, as we have demonstrated previously (Whittington & Little, 1990a; 1991a,b), this change followed a different time course from that of the changes in thresholds, being maximal at 2 h from withdrawal, and returning to control values by between 3.5 h and 4 h. The chronic Bay K 8644 treatment considerably, and significantly, reduced the effects of chronic ethanol on paired pulse potentiation. The difference between the results after ethanol alone and after ethanol plus Bay K 8644 was significant (P < 0.001) when compared over the time period 1 h to 4 h from ethanol withdrawal.

Input/output curves The input/output curves for the population spike areas showed a shift to the left after chronic ethanol treatment, measured as a decrease in the minimum stimulus intensity required to evoke half-maximal population spikes (S50). A significant decrease in the gradients after chronic ethanol treatment was also seen between 3 h and 7 h into withdrawal (see Table 1). These changes were significantly decreased by the addition of Bay K 8644 to the drinking mixture (P < 0.05). The input/output curves for the field e.p.s.p. slopes did not show any significant changes after the chronic ethanol alone, or ethanol and Bay K 8644 treatment schedules (P > 0.05, for comparison with control data) (Table 1). An example of the input/output curves obtained is shown in Figure 7, drawn from data obtained 7 h after ethanol withdrawal.

Table 1 Measurements from recordings of field potentials made from hippocampal slices prepared after chronic treatment in vivo with either ethanol or ethanol plus Bay K 8644, compared with control values

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
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<tbody>
<tr>
<td>Slopes of input/output curves for population spike areas</td>
<td></td>
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<tr>
<td>Control</td>
<td>4.7 ± 0.7</td>
<td>8.0 ± 0.5</td>
<td>7.0 ± 0.4</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6.1 ± 0.6</td>
<td>4.2 ± 0.4*</td>
<td>5.2 ± 0.3*</td>
<td>4.8 ± 0.3*</td>
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<tr>
<td>Ethanol plus Bay K 8644</td>
<td>5.4 ± 0.5</td>
<td>6.1 ± 0.6</td>
<td>8.0 ± 0.7</td>
<td>7.2 ± 0.5</td>
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<tr>
<td>S50 values for population spike areas (μA)</td>
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<tr>
<td>Control</td>
<td>36.5 ± 0.9</td>
<td>35.5 ± 0.3</td>
<td>35.7 ± 0.3</td>
<td>35.9 ± 0.3</td>
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<tr>
<td>Ethanol</td>
<td>38.0 ± 0.6</td>
<td>30.1 ± 0.8*</td>
<td>19.2 ± 0.4*</td>
<td>27.8 ± 0.4*</td>
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<tr>
<td>Ethanol plus Bay K 8644</td>
<td>37.2 ± 0.7</td>
<td>34.1 ± 0.5</td>
<td>32.4 ± 0.4</td>
<td>33.0 ± 0.3</td>
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<tr>
<td>Maximum values for population spike areas (μVs)</td>
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<tr>
<td>Control</td>
<td>10.9 ± 0.4</td>
<td>11.5 ± 0.3</td>
<td>11.8 ± 0.4</td>
<td>12.0 ± 0.5</td>
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<td>Ethanol</td>
<td>10.3 ± 0.3</td>
<td>10.4 ± 0.6</td>
<td>10.5 ± 0.3</td>
<td>10.2 ± 0.4</td>
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<tr>
<td>Ethanol plus Bay K 8644</td>
<td>10.9 ± 0.3</td>
<td>10.9 ± 0.2</td>
<td>11.2 ± 0.2</td>
<td>10.5 ± 0.2</td>
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<td>Slopes of input/output curves for field e.p.s.ps</td>
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<tr>
<td>Control</td>
<td>1.9 ± 0.8</td>
<td>3.5 ± 0.6</td>
<td>3.1 ± 0.5</td>
<td>3.7 ± 0.6</td>
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<tr>
<td>Ethanol</td>
<td>3.7 ± 0.3</td>
<td>4.0 ± 0.4</td>
<td>4.0 ± 0.7</td>
<td>4.0 ± 0.6</td>
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<tr>
<td>Ethanol plus Bay K 8644</td>
<td>3.4 ± 0.4</td>
<td>3.1 ± 0.5</td>
<td>3.2 ± 0.5</td>
<td>3.7 ± 0.4</td>
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<tr>
<td>S50 values for field e.p.s.ps (μA)</td>
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<tr>
<td>Control</td>
<td>67.6 ± 2.7</td>
<td>41.2 ± 3.2</td>
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<td>Ethanol plus Bay K 8644</td>
<td>38.2 ± 6.7</td>
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<td>45.0 ± 5.7</td>
<td>41.3 ± 3.2</td>
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<td>Maximum field e.p.s. slope (V s⁻¹)</td>
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<tr>
<td>Control</td>
<td>5.3 ± 1.9</td>
<td>3.4 ± 1.2</td>
<td>3.1 ± 0.3</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.0 ± 1.4</td>
<td>3.0 ± 0.5</td>
<td>3.2 ± 0.3</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Ethanol plus Bay K 8644</td>
<td>2.6 ± 0.7</td>
<td>3.0 ± 0.3</td>
<td>3.8 ± 0.4</td>
<td>3.3 ± 0.3</td>
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</table>

All results are expressed as mean ± s.e.mean. Times (h) are from withdrawal of ethanol.

* P < 0.05, Student's non-paired t test; comparison with control slices.
The data from the receptor binding studies (Table 2) suggested that addition of Bay K 8644 to the chronic ethanol treatment may prevent the increase in the number of cortical dihydropyridine binding sites. However, in this study, there was a high degree of variability in the measurements after ethanol administration, and the increase did not reach statistical significance ($P > 0.05$). Measurement of the $K_d$ for dihydropyridine binding showed a significant increase after chronic ethanol treatment ($P < 0.05$), but not after chronic ethanol and Bay K

**Brain concentrations of Bay K 8644**

The mean value ($\pm$ s.e.mean) for the central concentrations of Bay K 8644 at the end of the chronic treatment period was $1159 \pm 179$ ng g$^{-1}$ wet tissue weight. This corresponded to $3.26 \pm 0.5 \mu$M, assuming equal distribution throughout the brain. During the withdrawal period, $32$ h from removal of Bay K 8644 from the drinking fluid and $8$ h from withdrawal of ethanol, the concentrations were not detectable. The lowest concentration that could be detected with our assay was $53$ ng g$^{-1}$ or $150$ nm. One hour after acute administration of $2$ mg kg$^{-1}$ Bay K 8644, in naive mice, the mean central concentration was $2150 \pm 432$ ng g$^{-1}$ wet tissue weight, corresponding to $6.07 \pm 1.22 \mu$M.

**Dihydropyridine binding**

The data from the receptor binding studies (Table 2) suggested that addition of Bay K 8644 to the chronic ethanol treatment may prevent the increase in the number of cortical dihydropyridine binding sites. However, in this study, there was a high degree of variability in the measurements after ethanol administration, and the increase did not reach statistical significance ($P > 0.05$). Measurement of the $K_d$ for dihydropyridine binding showed a significant increase after chronic ethanol treatment ($P < 0.05$), but not after chronic ethanol and Bay K

**Table 2** $B_{max}$ and $K_d$ values for [3H]nimodipine binding after chronic treatment in vivo with either ethanol or ethanol plus Bay K 8644, compared with control values

<table>
<thead>
<tr>
<th></th>
<th>$B_{max}$ (fmol mg$^{-1}$)</th>
<th>$K_d$ (nM)</th>
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<tbody>
<tr>
<td>Controls</td>
<td>$174 \pm 14$ (8)</td>
<td>$1.62 \pm 0.27$</td>
</tr>
<tr>
<td>Ethanol</td>
<td>$227 \pm 32$ (7)</td>
<td>$3.01 \pm 0.98^*$</td>
</tr>
<tr>
<td>Ethanol + Bay K</td>
<td>$208 \pm 8$ (7)</td>
<td>$2.12 \pm 0.98$</td>
</tr>
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</table>

All results are expressed as mean $\pm$ s.e.mean. The values were derived from cerebrocortical tissue, taken $8$ h from the cessation of ethanol drinking.

* $P = 0.04$, Student's $t$ test for comparison with control data.
8644 treatment (P > 0.05), when comparisons were made with control values (Table 2).

Discussion

The addition of Bay K 8644 to the chronic ethanol treatment completely prevented the ethanol withdrawal syndrome in mice in vivo and significantly decreased the electrophysiological signs of withdrawal in the isolated hippocampal slices. These actions closely resembled the effects previously reported for chronic treatment with the calcium channel antagonist, nitrendipine (Whittington et al., 1991; Whittington & Little, 1991b). Bay K 8644 is normally considered to increase calcium channel opening (Brown et al., 1984; Nowycky et al., 1985), but it may have other actions (see below).

In the present study, after removal of Bay K 8644 from the drinking fluid for the last 24 h of ethanol intake, the central concentrations fell to levels below the detection of the assay, that is below about 150 nM. This is lower than would have been required to cause behavioural changes by an acute action. We conclude, therefore, that the effects seen were due to the presence of Bay K 8644 in the CNS during the ethanol chronic treatment, rather than to any acute actions at the time of testing. The concentration of any residual Bay K 8644 in the hippocampal slices during recording would also have been lower than those required to produce acute effects on the withdrawal hyperexcitability (see below). A dose of 2 mg kg⁻¹ was chosen to illustrate the acute effects, and this produced central concentrations of around 6 μM at a time when the excitatory action was maximal. This action is lost at doses below about 1 mg kg⁻¹ (our unpublished results and O'Neill & Bolger, 1988). The central concentrations measured in the present study at the acute dosing are similar to those reported by O'Neill & Bolger (1988), who found a level of approximately 2 μM in mouse brain 15 min after 2 mg kg⁻¹ i.p.

When given acutely to control mice, racemic Bay K 8644 caused a pattern of behaviour that resembled in some respects that seen during ethanol withdrawal. The ratings of convulsive behaviour on handling were increased. Bolger et al. (1985) have reported that single doses of the racemate produced signs of hyperexcitability in naive mice. We found previously that a dose of 2 mg kg⁻¹ of the racemate did not significantly affect the ethanol withdrawal syndrome when given acutely on withdrawal, but did prevent the protective action of the calcium channel antagonist, nimodipine (Littleton et al., 1990). However this was not a simple interaction, as the withdrawal syndrome was increased by the combination of nimodipine with Bay K 8644. We have also shown that the (+)-isomer of Bay K 8644 increased the ratings of convulsive behaviour during ethanol withdrawal (unpublished results).

The results from the receptor binding studies appeared to follow the same pattern as that seen in the behavioural and electrophysiological studies, but the interpretation of these results is complicated by the lack of a significant change in receptor density after the chronic treatment with ethanol alone. The reason for the large standard error in these measurements and the increase in Kᵦ values was not clear, as we have previously demonstrated consistent increases in Bₐₘₐₓ with no change in Kᵦ after such treatment (Dolin et al., 1987; Whittington et al., 1991). The effects of Bay K 8644 on the upregulation of calcium channels in cultured cells caused by growth in ethanol have been studied by two groups. Marks et al. (1989) showed that cultured PC12 cells, grown in medium containing (±)-Bay K 8644 (3 nM) showed an increase in dihydropyridine binding site number, although this change was not additive with the increase in single binding produced by ethanol. Brennan et al. (1989) grew PC12 cells in a higher concentration of (±)-Bay K 8644 (50 nM) and found the increase in dihydropyridine binding caused by addition of ethanol to the growth medium was prevented by the presence of the Bay K 8644. These results suggest that adaptations to the calcium channel activating and antagonist properties (according to the concentration used) of Bay K 8644 may be demonstrated after chronic treatment, when the interactions with ethanol are studied. Direct comparisons of the absolute Bay K 8644 concentrations used in these studies and in the present work, however, are not necessarily valid, because cultured cells are known to be more sensitive to dihydropyridines than mammalian tissues in vivo or ex vivo.

We have previously shown that when applied to hippocampal slices in the bathing medium at 500 nM or 2 μM, (–)-Bay K 8644 considerably potentiated the signs of hyperexcitability in the isolated hippocampal slice after chronic ethanol treatment, while the (+)-isomer had protective effects (Whittington & Little, 1990b). The brain concentrations of each of the isomers during the chronic treatment in the present study would have been in the same range as the concentration that increased (minus isomer) or decreased (plus isomer) the effects of ethanol withdrawal when added in vitro.

The demonstration of similar effects with a calcium channel activator and a calcium channel antagonist seem contradictory, but the results may have been due to the fact that racemic Bay K 8644 is not a pure calcium channel activator. It is well established that the effects of Bay K 8644 are stereospecific, the calcium channel activating properties of Bay K 8644 residing in the (+)-isomer, while the (-)-isomer is a calcium channel antagonist, both in vitro (Schramp et al., 1983; Hol et al., 1985; Frankowiak et al., 1985) and in vivo (O'Neill & Bolger, 1988). We have shown loss of effects and possible antagonist actions at high concentrations of Bay K 8644, as the dose-response curves in many studies showed a bell-shaped pattern. Concentrations over 100 nM of the (-)-isomer were found, by Frankowiak et al. (1985), to have less effect on cardiac tissues than lower concentrations. Biphasic dose-response curves have been demonstrated for the racemate in cardiac preparations (Dube et al., 1985). Hess et al. (1984) did not report biphasic effects on cardiac cells at concentrations of the racemate up to 10 μM, but these authors mentioned that a 'partial antagonist' action was seen when strong depolarizations were applied. Few studies of the effects of Bay K 8644 on neuronal preparations have investigated a range of concentrations, but White & Bradford (1986) demonstrated biphasic effects of Bay K 8644 on the stimulation of calcium uptake by synaptosomes. The potentiating effect of Bay K 8644 was maximal at 1–10 nM and disappeared as the concentration was raised to 1–10 μM. The stimulation of inositol phospholipid hydrolysis by Bay K 8644 was half-maximal at 0.3 μM and declined at concentrations over 10 μM (Kendall & Nahorski, 1985).

The effects of Bay K 8644 may also be influenced by the membrane potential. In patch-clamp studies on ventricular myocytes, the (–)-isomer and the racemate of Bay K 8644 have been shown to increase calcium currents evoked from negative, non-depolarizing, potentials, but to possess calcium channel antagonist properties on currents evoked from more positive holding potentials (Sanguinetti & Kass, 1984; Sanguinetti et al., 1986; Kass, 1987). Brown et al. (1984), however, did not find this pattern. It is possible that the same of the reported in vitro effects of Bay K 8644 may have been influenced by the solvent used. In many studies the drug was dissolved in ethanol and interactions between ethanol and dihydropyridines are well established (see Introduction). Even if control experiments showed no solvent effect, this does not exclude the occurrence of interactions when the two drugs are applied together.

There is some evidence that the application of Bay K 8644 may produce effects at sites other than the L-subtype of calcium channel. In hypothyralmic neurons, Akaike et al. (1989) demonstrated a blocking action on the L-type, T-subtype, of calcium channel, at Bay K 8644 concentrations above 10 μM. Docherty & Brown (1986a) found increases in the transient calcium current in hippocampal CA1 neurons at 1–5 μM Bay K 8644, but this effect was reported to be very variable. Docherty & Brown (1986b) found that 5 μM racemic Bay K 8644 caused a slow increase in membrane resistance
and suppression of outward current. It was suggested that this was due to accumulation of intracellular calcium, as a result of the agonist action of Bay K 8644, leading to inactivation of potassium channels.

Evidence of a biphasic action of Bay K 8644 was found in previous studies (Dolin et al., 1989). Bay K 8644 were given together acutely, to mice. The general anaesthetic actions of ethanol were antagonized by 1 mg kg^{-1} racemic Bay K 8644, but potentiated by 5 and 10 mg kg^{-1} of the dihydropyridine (Dolin et al., 1988b). The central concentrations produced by these doses were 1 μM for the 1 mg kg^{-1} dose and 4.6 and 11.8 μM, respectively, for the two higher doses. In the present study, no overt behavioural changes were seen during the chronic treatment.

The central concentrations of Bay K 8644 during the chronic treatment were in the low micromolar range. Calcium channel antagonist actions have been reported at concentrations of the racemate from 500 nM to 5 μM in various tissues (see above), while the calcium channel activating effects have been seen at nanomolar concentrations, and a similar pattern was seen in the in vivo interactions with ethanol (Dolin et al., 1988b). It is possible, therefore, that because the central concentrations during the chronic treatment were in this range, calcium channel antagonist actions were produced, rather than calcium channel activation. However, this does not provide a complete explanation, as the behavioural effects of single doses of the racemate, sufficient to produce these concentrations, are excitatory when the compound is given alone.

One possible explanation for the effects of the chronic treatment may be that tolerance to the excitatory effects may have occurred. O'Neill & Bolger (1988) demonstrated tolerance to the excitatory behavioural effects of racemic Bay K 8644 after four, once daily, injections of a dose that produced central concentrations of 2 μg g^{-1}. Although the incidence of convulsions was not decreased by this short course of repeated administration, their latency was increased and other signs of hyperexcitability were reduced. The brain concentrations in this study were similar to those in the present work; both were in the low micromolar range. The present authors are unaware of any reports of tolerance to the calcium channel antagonist effects of the (+)-isomer of Bay K 8644. If such tolerance occurred to the calcium channel activating actions of the (-)-isomer of Bay K 8644 during the more prolonged chronic treatment in the present study, the resultant action on the CNS would have been that of the (+), calcium channel antagonist, isomer.

One possible explanation for the results in this study, therefore, is that the protective action of chronic administration of racemic Bay K 8644 on the ethanol withdrawal syndrome in vivo and in vitro may have been due to a combination of the calcium channel antagonist properties of this compound, caused by the high concentrations in the CNS, plus tolerance to the calcium channel activating actions. Studies using lower doses of Bay K 8644, and if possible, the stereoisomers, are needed to investigate these theories. It was apparent throughout the studies that the patterns of results seen in the behavioural receptor binding and electrophysiological experiments all followed the same pattern, the only exception being the effects of the Bay K 8644 on paired pulse potentiation in the hippocampal slices.

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References


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