Blockade by ifenprodil of high voltage-activated Ca^{2+} channels in rat and mouse cultured hippocampal pyramidal neurones: comparison with N-methyl-D-aspartate receptor antagonist actions

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1 The block by ifenprodil of voltage-activated Ca^{2+} channels was investigated on increases in intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$) evoked by 50 mM K^+ (high- $[\text{K}^+]_o$) in Fura-2-loaded rat hippocampal pyramidal neurones in culture and on currents carried by Ba^{2+} ions (I_{Ba}) through Ca^{2+} channels in mouse cultured hippocampal neurones under whole-cell voltage-clamp. The effects of ifenprodil on voltage-activated Ca^{2+} channels were compared with its antagonist actions on N-methyl-D-aspartate (NMDA) evoked responses in the same neuronal preparations.

2 Rises in $[\text{Ca}^{2+}]_i$ evoked by transient exposure to high- $[\text{K}^+]_o$ in our preparation of rat cultured hippocampal pyramidal neurones are mediated predominantly by Ca^{2+} flux through nifedipine-sensitive Ca^{2+} channels, with smaller contributions from nifedipine-resistant, ω -conotoxin GVIA-sensitive Ca^{2+} channels and Ca^{2+} channels sensitive to crude funnel-web spider venom (Church *et al.*, 1994). Ifenprodil (0.1–200 μM) reversibly attenuated high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ with an IC_{50} value of $17 \pm 3 \mu\text{M}$, compared with an IC_{50} value of $0.7 \pm 0.1 \mu\text{M}$ for the reduction of rises in $[\text{Ca}^{2+}]_i$ evoked by 20 μM NMDA. Tested in the presence of nifedipine 10 μM , ifenprodil (1–50 μM) produced a concentration-dependent reduction of the dihydropyridine-resistant high- $[\text{K}^+]_o$ -evoked rise in $[\text{Ca}^{2+}]_i$ with an IC_{50} value of $13 \pm 4 \mu\text{M}$. The results suggest that ifenprodil blocks Ca^{2+} flux through multiple subtypes of high voltage-activated Ca^{2+} channels.

3 Application of the polyamine, spermine (0.25–5 mM), produced a concentration-dependent reduction of rises in $[\text{Ca}^{2+}]_i$ evoked by high- $[\text{K}^+]_o$. The antagonist effects of ifenprodil 20 μM on high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ were attenuated by spermine 0.25 mM but not by putrescine 1 or 5 mM. In contrast, spermine 0.1 mM increased rises in $[\text{Ca}^{2+}]_i$ evoked by NMDA and enhanced the ifenprodil (5 μM) block of NMDA-evoked rises in $[\text{Ca}^{2+}]_i$.

4 Similar results were obtained in mouse cultured hippocampal pyramidal neurones under whole-cell voltage-clamp. Ifenprodil attenuated both the peak and delayed whole-cell I_{Ba} with an IC_{50} value of $18 \pm 2 \mu\text{M}$, whilst it attenuated steady-state NMDA-evoked currents with an IC_{50} of $0.8 \pm 0.2 \mu\text{M}$. Block of I_{Ba} by ifenprodil 10 μM was rapid in onset, fully reversible and occurred without change in the current-voltage characteristics of I_{Ba} . The ifenprodil block of I_{Ba} was enhanced on membrane depolarization and was weakly dependent on the frequency of current activation. Spermine 0.1 mM potentiated control NMDA-evoked currents but attenuated I_{Ba} . In agreement with the microspectrofluorimetric studies, co-application of spermine produced a small enhancement of the inhibitory effect of ifenprodil 10 μM on NMDA-evoked responses whereas the reduction of I_{Ba} by ifenprodil 10 μM in the presence of spermine was less than expected if the inhibitory effects of ifenprodil and spermine on I_{Ba} were simply additive.

5 The results indicate that ifenprodil blocks high voltage-activated Ca^{2+} channels in rat and mouse cultured hippocampal pyramidal neurones. Although the Ca^{2+} channel blocking actions of ifenprodil are observed at higher concentrations than those associated with NMDA antagonist activity, Ca^{2+} channel blockade may contribute, at least in part, to the established neuroprotective and anticonvulsant properties of the compound.

Keywords: Ifenprodil; voltage-activated calcium channels; N-methyl-D-aspartate (NMDA); polyamines; cultured hippocampal pyramidal neurones

Introduction

The phenylethanolamine, ifenprodil, has been employed in the treatment of human cerebrovascular disease (Otomo & Kodama, 1976). Recent interest has centred on its activity as an antagonist of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor as the functional basis for both its neuroprotective and anticonvulsant actions (reviewed by Wong & Kemp, 1991; Carter *et al.*, 1991). Although NMDA receptor-mediated events undoubtedly play a role in the pathogenesis of neurodegenerative and convulsive phenomena, Ca^{2+} flux through voltage-activated Ca^{2+} channels is also an important contributing factor (see Heinemann &

Hamon, 1986; Siesjö & Bengtsson, 1989). It is therefore of interest that ifenprodil blocks Ca^{2+} channel-mediated responses in vascular smooth muscle, a property thought to underlie its vasorelaxant effects (Honda & Sakai, 1987; Honda *et al.*, 1988).

In this study we examined the ability of ifenprodil to block high voltage-activated (HVA) Ca^{2+} channels in rat and mouse cultured hippocampal neurones and compared this effect with its NMDA receptor antagonist actions in the same neuronal preparations. Because ifenprodil has been reported to interact with the polyamine modulatory site on the NMDA receptor-channel complex (Ransom & Stec, 1988; Schoemaker *et al.*, 1990; Carter *et al.*, 1991; Williams *et al.*, 1991) and, in turn, polyamines interact with voltage-activated

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Ca²⁺ channels (reviewed by Scott *et al.*, 1993; see also Pullan *et al.*, 1990; Schoemaker, 1992; Herman *et al.*, 1993), we also investigated the ability of polyamines to modulate the HVA Ca²⁺ channel and NMDA receptor antagonist actions of ifenprodil. We find that ifenprodil can block neuronal HVA Ca²⁺ channels, an action which is modified to a limited extent by spermine. Although the concentrations of ifenprodil required to block HVA Ca²⁺ channels are more than 10 times higher than those required to attenuate NMDA-evoked responses in the same neurones, the Ca²⁺ channel blocking properties of this compound may contribute to its established neuroprotective and anticonvulsant actions.

Methods

Fluorescent dye studies

Details of the methods employed have been described previously (Church *et al.*, 1994). In brief, hippocampal pyramidal neurones were obtained from 18-day-old foetal Wistar rats, maintained in culture for 9–14 days, and loaded with the membrane-permanent form of Fura-2 (Grynkiewicz *et al.*, 1985). Coverslips were then placed in a chamber at 20–24°C and superfused at 1.5 ml min⁻¹ with a solution containing (mM): NaCl 136.5, KCl 3, NaH₂PO₄ 1.5, MgSO₄ 1.5, D-glucose 10, CaCl₂ 2, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10, tetrodotoxin (TTX) 0.0005 (final pH 7.35–7.4). In experiments employing NMDA, Mg²⁺ was omitted and 2 µM glycine added. Both NMDA (20 µM) and 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; their effects were expressed as the percentage change of the peak response to an excitant during their administration, in relation to the peaks of the control and recovery responses obtained before and after their administration, respectively.

Cytoplasmic free calcium concentrations ([Ca²⁺]_i) were measured by the dual excitation fluorescence ratio method on an Attofluor Digital Fluorescence Microscopy System (Atto Instruments Inc.; Carl Zeiss Canada Ltd.). Fluorescence intensity measurements were obtained from multiple neurone somata simultaneously and raw intensity data at each excitation wavelength (334 and 380 nm) were corrected for background prior to calculation of the ratio. The *in situ* calibration method was used to convert fluorescence ratios into [Ca²⁺]_i. During exposure to 10 µM Br-A23187, calibration parameters (R_{max} , R_{min} and β) were obtained in the presence (2 mM) and absence (nominally zero-Ca²⁺ solution in the presence of 100 µM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA)) of Ca²⁺. The published K_D value of 135 nM (at 20°C) was employed (Grynkiewicz *et al.*, 1985).

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. Each experiment was performed on at least three different neuronal cultures. Averaged data points were fitted to a standard logistic equation (see Church *et al.*, 1994) to derive IC₅₀ values (the concentration of test compound resulting in a 50% inhibition of the control responses). Statistical analysis was performed by Student's *t* test, *P* < 0.05 being considered significant.

Electrophysiological studies

Dissociated mouse hippocampal pyramidal neurones grown in culture were used for whole-cell recordings using conventional voltage-clamp techniques, as previously described (MacDonald *et al.*, 1989; Church *et al.*, 1994). Cultures were

used at 7–14 days following plating. Recordings were performed at 20–24°C.

Prior to the investigation of excitatory amino acid-evoked responses, cultures were rinsed with an extracellular solution containing (mM): NaCl 140, CaCl₂ 1.3, KCl 5.4, HEPES 25, glucose 33, TTX 0.0001 and glycine 0.003 (unless otherwise indicated) at a final pH of 7.35–7.4. Patch electrodes constructed from thin-walled borosilicate glass were filled with a solution containing (mM): CsF 110, CsCl 10, HEPES 10 and EGTA 10, adjusted to pH 7.35–7.4 with 1 M CsOH. The membrane holding potential (V_h) was –60 mV unless otherwise indicated in the text. Control currents were evoked by 5 s applications of 60 µM NMDA, 60 µM kainate and 30 µM (RS)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA).

For recording voltage-activated Ca²⁺ channel currents, an intracellular solution was used of composition (mM): CsCl

Figure 1 Ifenprodil inhibits NMDA-evoked rises in [Ca²⁺]_i. (a) The effects of ifenprodil 0.5–100 µM, applied for the periods indicated by the bars above the trace, on NMDA- (N) evoked rises in [Ca²⁺]_i. A concentration-dependent reduction of NMDA-evoked responses is seen. The record is a mean of data obtained from 14 neurones simultaneously. (b) The effects of ifenprodil 5 µM, applied in the absence and presence of spermine 100 µM for the periods indicated by the bars above the trace, on NMDA- (N) evoked rises in [Ca²⁺]_i. The record is a mean of data obtained from 10 neurones simultaneously. In these neurones, ifenprodil 5 µM produced a 64% reduction in NMDA-evoked rises in [Ca²⁺]_i. Spermine 100 µM potentiated the NMDA-evoked responses by 12% and increased the magnitude of the block of NMDA-evoked responses by ifenprodil 5 µM to 77%.

110, TEA 10, EGTA 10 and HEPES 10, to which was added Mg-ATP 2, phosphocreatine 50, creatine phosphokinase 50 iu and GTP 2 (final pH 7.35–7.4; final osmolarity 310 mOsm) to retard run-down of the whole-cell currents (MacDonald *et al.*, 1989). The support solution was kept on ice for the duration of the experiment to prevent degradation of its components and was not used for periods longer than 5 h. After attaining the whole-cell patch configuration the standard extracellular solution (see above) was changed to one containing (mM): NaCl 140, CsCl 5, BaCl_2 5, MgCl_2 1, HEPES 25 and glucose (pH 7.35–7.4, 330 mOsm). The concentration of TTX was increased to 0.3–0.6 μM and extracellular KCl was replaced with CsCl to block, respectively, voltage-activated Na^+ and K^+ channels. The series

resistance and capacitance of the electrode were compensated for (Axopatch 1B, Axon Instruments), whilst that of the membrane was only partially achieved due to the difficulty of clamping neurones with extensive processes. Leak currents were estimated in the presence of extracellular solution with 100 μM cadmium added. Barium currents (I_{Ba}) flowing through voltage-activated Ca^{2+} channels were evoked by 250 ms voltage-steps from a V_h of -80 mV to a test potential (V_t) of -10 mV at 20 s intervals. Other protocols adopted are detailed in the text. Ifenprodil was perfused over the neurone and I_{Ba} activated until steady-state block was attained. For construction of concentration-inhibition plots the concentration of ifenprodil was sequentially increased to achieve full block before recovery was attempted in order to minimize the possible error contributed by run down of I_{Ba} . Cells which displayed less than 50% recovery of control current amplitude were excluded from analysis.

All solutions were applied to neurones from a 3-barrelled perfusion system allowing for rapid (<50 ms) application to the entire neurone under voltage-clamp. The IC_{50} values, EC_{50} values (the concentration of NMDA evoking a response 50% that of maximum) and percentage reductions of control responses are expressed as mean $\pm$ s.e. mean, with n the number of neurones tested. IC_{50} values were estimated by fitting data points to the same logistic equation as in the fluorescent dye experiments (see above).

Sources and handling of compounds

Compounds were obtained from Sigma Chemical Co., with the exceptions of ifenprodil tartrate, TTX, AMPA (Research Biochemicals Inc.) and Fura-2 AM (Molecular Probes Inc.). Stock solutions of ifenprodil (1 or 5 mM in distilled water) and nifedipine (10 mM in ethanol) were made up fresh each day from the solid, stored in glass vials and diluted in perfusion medium immediately prior to use. Ifenprodil stock solutions were briefly sonicated to ensure solubilization of the compound. The highest concentration of ethanol in final working solutions was 0.1%, which in control experiments had no effect on responses. Nifedipine-containing solutions

Figure 2 Ifenprodil inhibits rises in $[\text{Ca}^{2+}]_i$ evoked by high- $[\text{K}^+]_o$. (a) The effects of ifenprodil 1–50 μM , applied for the periods indicated by the bars above the trace, on rises in $[\text{Ca}^{2+}]_i$ evoked by 50 mM K^+ (K^+). A concentration-dependent reduction of high- $[\text{K}^+]_o$ -evoked responses is seen. The record is a mean of data obtained from 39 neurones simultaneously. (b) Ifenprodil produces a concentration-dependent reduction of nifedipine-resistant high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$. Under control conditions (first and second responses; peaks truncated for convenience in figure preparation), 50 mM K^+ (K^+) evoked large rises in $[\text{Ca}^{2+}]_i$, which were reduced by 80% by nifedipine 10 μM (third and fourth responses). Co-application of ifenprodil 1–50 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 9 neurones simultaneously.

Figure 3 The polyamine, spermine, attenuates high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ and reduces the inhibitory effect of ifenprodil. Spermine 250 μM , applied for the period indicated by the bar above the trace, reduced high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ by 33%. Ifenprodil 20 μM , co-applied with spermine, reduced high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ by 52% from control values established in the presence of spermine. Following recovery from spermine application, ifenprodil 20 μM produced a 69% depression of high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$. The record is a mean of data obtained from 8 neurones simultaneously.

were handled in the manner described previously (Church *et al.*, 1994).

Results

Fluorescent dye studies

Ifenprodil block of NMDA-evoked responses Ifenprodil (0.02–100 μM) produced a concentration-dependent reduction of rises in $[\text{Ca}^{2+}]_i$ evoked by NMDA, with an apparent IC_{50} value of $0.7 \pm 0.1 \mu\text{M}$ ($n > 75$ at each concentration

tested; Figure 1). At the highest concentration tested (100 μM) ifenprodil produced an incomplete inhibition of NMDA-evoked responses ($88 \pm 0.4\%$ reduction; $n = 177$), as previously reported in rat cultured hippocampal pyramidal neurones (Legendre & Westbrook, 1991) and in *Xenopus* oocytes expressing NR1A/NR2B heteromeric NMDA receptors (Williams, 1993). The polyamine, spermine (100 μM), potentiated NMDA-evoked responses by $13 \pm 2\%$ ($n = 95$, reviewed by Williams *et al.*, 1991; see also Rock & MacDonald, 1992; Araneda *et al.*, 1993; Benveniste & Mayer, 1993) and increased the potency of ifenprodil as an antagonist of NMDA-evoked rises in $[\text{Ca}^{2+}]_i$ (Figure 1b).

Figure 4 Ifenprodil block of amino acid-evoked currents in voltage-clamped neurones. (a) To left: dose-inhibition plot indicates the mean $\pm$ s.e. mean percentage reduction by ifenprodil of the control steady-state ($\bullet$) and peak ($\circ$) components of NMDA-evoked currents for 7 neurones at a V_h of -60 mV. The respective IC_{50} values for the ifenprodil block of the peak and steady-state components of the NMDA-evoked response were 2.8 ± 0.9 and $0.8 \pm 0.2 \mu\text{M}$. Abscissa scale: ifenprodil concentration in μM . Ordinate scale: % reduction of control NMDA-evoked response. To right: sample traces indicate block of control NMDA-evoked currents (NMDA 60 μM ; applied for the period indicated by the bar above the trace) by increasing concentrations of ifenprodil (0.1–100 μM) as indicated to right. On wash in control solution there was recovery to 75% of control (not shown). Calibration: current amplitude in nA; time in s. (b) On the same neurone currents evoked by AMPA (30 μM) and kainate (60 μM), applied for the periods indicated by the bars above the traces, were attenuated by 23% and 4%, respectively, on co-application of 100 μM ifenprodil (applied for the periods indicated by the bars below the traces). Calibration: current amplitude in nA; time in s. (c) On a different neurone the interaction of ifenprodil and spermine on NMDA-evoked responses is highlighted. Prolonged application of NMDA (bar above trace) evoked a control current (trace (i)) that was potentiated on co-application of spermine (100 μM ; bar below trace). On a subsequent NMDA-evoked response in the same neurone (trace (ii)) ifenprodil 10 μM (applied between arrows) produced an approximately 50% block of control. Co-application of spermine (100 μM) increased the ifenprodil-induced block to more than 60%. The action of ifenprodil was reversed on wash in control solution (not shown). Calibration: current amplitude in nA; time in s.

Thus, ifenprodil $5\text{ }\mu\text{M}$ produced a $61 \pm 1\%$ reduction in NMDA-evoked rises in $[\text{Ca}^{2+}]_i$ which, on the same neurones, increased to a $72 \pm 1\%$ reduction in the presence of spermine $100\text{ }\mu\text{M}$ ($n = 95$; $P < 0.001$, Student's paired t test).

Ifenprodil block of high- $[\text{K}^+]_o$ -evoked responses We have previously shown that, under our experimental conditions, rises in $[\text{Ca}^{2+}]_i$ evoked by the transient application of 50 mM K^+ -containing medium are largely a consequence of Ca^{2+} flux through nifedipine-sensitive HVA Ca^{2+} channels, with smaller components reflecting Ca^{2+} flux through both nifedipine-resistant, ω -conotoxin GVIA- (ω -CgTx) sensitive channels and channels resistant to both nifedipine and ω -CgTx but sensitive to crude funnel-web spider venom (Church *et al.*, 1994). The contribution of endogenously-released glutamate to the observed responses appears to be minimal (see Church *et al.*, 1994). Ifenprodil (0.1 – $200\text{ }\mu\text{M}$) produced a concentration-dependent reduction of the rise in $[\text{Ca}^{2+}]_i$ evoked by high- $[\text{K}^+]_o$, with an apparent IC_{50} value of $16.9 \pm 3.0\text{ }\mu\text{M}$ ($n > 50$ at each concentration tested; Figure 2a). The effect was rapid in onset and reversible, except at concentrations $\geq 100\text{ }\mu\text{M}$ when recovery was slow and often incomplete. At the highest concentration tested ($200\text{ }\mu\text{M}$), ifenprodil reduced rises in $[\text{Ca}^{2+}]_i$ evoked by 50 mM K^+ by $98 \pm 0.3\%$ ($n = 81$), a result otherwise achieved in our experimental preparation only by omitting Ca^{2+} from the external medium or by the combined addition of nifedipine, ω -CgTx

and crude funnel-web spider venom to Ca^{2+} -containing medium (see Church *et al.*, 1994). The latter finding suggests that ifenprodil is able to block Ca^{2+} flux through both nifedipine-sensitive and nifedipine-resistant HVA Ca^{2+} channels. To examine more closely the actions of ifenprodil against dihydropyridine-resistant HVA Ca^{2+} channels, it was applied in the presence of a maximally-effective concentration of nifedipine ($10\text{ }\mu\text{M}$; see Church *et al.*, 1994). Nifedipine $10\text{ }\mu\text{M}$ produced an $80 \pm 1\%$ reduction ($n = 101$) of high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$; co-application of ifenprodil 1 – $50\text{ }\mu\text{M}$ reduced the residual nifedipine-resistant response in a concentration-dependent manner with an apparent IC_{50} of $12.6 \pm 3.7\text{ }\mu\text{M}$ ($n > 90$ at each concentration tested; Figure 2b).

Ifenprodil may act, at least in part, at a polyamine-sensitive modulatory site on the NMDA receptor-channel complex to produce functional NMDA antagonism (see Introduction). Because polyamines also modulate the activity of neuronal L- and N-type HVA Ca^{2+} channels (Pullan *et al.*, 1990; Schoemaker, 1992; Scott *et al.*, 1993), we examined the effects of spermine and putrescine on high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ in both the absence and presence of ifenprodil $20\text{ }\mu\text{M}$. Spermine alone, applied at 0.25 , 0.5 , 1 and 5 mM , produced a concentration-dependent reduction of high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$; the respective percentage inhibitions from control values were $32 \pm 1\%$ ($n = 87$), $39 \pm 2\%$ ($n = 29$), $46 \pm 2\%$ ($n = 66$), and $64 \pm 2\%$ ($n = 46$). In contrast to a recent study on NE-115 mouse neuroblastoma cells (Herman *et al.*, 1993) putrescine, applied at 1 and 5 mM , also produced reductions in high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$, although it was less effective than spermine in this regard ($1 \pm 1\%$, $n = 109$ and $27 \pm 2\%$, $n = 34$ reductions by 1 and 5 mM putrescine, respectively). Spermine, but not putrescine, attenuated the inhibitory action of ifenprodil $20\text{ }\mu\text{M}$ against high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ (Figure 3). Thus ifenprodil $20\text{ }\mu\text{M}$, applied in the absence or presence of spermine 0.25 mM , reduced high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ by $63 \pm 1\%$ and $54 \pm 2\%$, respectively ($n = 70$, $P < 0.001$, Student's paired t test). Applied in the absence or presence of putrescine, ifenprodil $20\text{ }\mu\text{M}$ produced $66 \pm 2\%$ and $64 \pm 2\%$ (putrescine 1 mM ; $n = 47$, not significant, Student's paired t test), and $65 \pm 3\%$ and $63 \pm 2\%$ (putrescine 5 mM ; $n = 18$, not significant, Student's paired t test), reductions in high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ respectively.

Electrophysiological studies

Ifenprodil block of NMDA-evoked responses Superfusion of ifenprodil (0.1 – $100\text{ }\mu\text{M}$) produced a reversible block of the steady-state currents evoked on 5 s applications of NMDA with an IC_{50} value of $0.80 \pm 0.19\text{ }\mu\text{M}$ and a Hill coefficient of 0.53 ± 0.05 ($n = 7$; Figure 4a), comparable to the values reported by Legendre & Westbrook (1991) for the high affinity component of ifenprodil block of NMDA-evoked currents in rat cultured hippocampal pyramidal neurones. The NMDA antagonist action of ifenprodil ($1\text{ }\mu\text{M}$) was not competitive, producing a non-parallel shift in the NMDA concentration-response plot with a $50 \pm 3\%$ ($n = 5$) reduction of the maximum NMDA-evoked response without a change in the EC_{50} value for NMDA (data not shown). Antagonism of NMDA-evoked responses by ifenprodil ($1\text{ }\mu\text{M}$) was unaffected by changes in V_h over the range -100 mV to $+60\text{ mV}$ ($n = 5$; not shown; see Legendre & Westbrook, 1991; Williams, 1993) and channel activation was not required for the development of block by ifenprodil. Ifenprodil inhibition of the steady-state component of the NMDA-evoked response was greater than that of the peak, as evidenced by the approximately 4 fold difference between the IC_{50} values for the two components (see Figure 4a). The IC_{50} value for the peak component was $2.8 \pm 0.9\text{ }\mu\text{M}$ with a Hill coefficient 0.54 ± 0.06 ($n = 7$). The block of the steady-state NMDA-evoked responses by ifenprodil was not significantly altered on elevating the extracellular glycine con-

Figure 5 Ifenprodil blocks HVA I_{Ba} in hippocampal neurones. (a) Concentration-inhibition plot for ifenprodil as an antagonist of I_{Ba} evoked on a voltage step from a V_h of -80 mV to a V_t of -10 mV . Data points indicate mean $\pm$ s.e. mean for 8 neurones tested. Abscissa scale: concentration of ifenprodil in μM . Ordinate scale: % reduction of control I_{Ba} . (b) Sample traces of the ifenprodil block of control I_{Ba} evoked by the voltage step indicated above trace. Concentration of ifenprodil applied is indicated to the right of traces. Complete recovery was obtained (not shown). Calibration: current amplitude in nA; time in ms.

Figure 6 Voltage-dependence of ifenprodil block of I_{Ba} . (a) Ifenprodil does not affect the current-voltage relationship for I_{Ba} . Traces indicate the amplitude of I_{Ba} evoked on a 200 ms step from a V_h of -80 mV to V_t as indicated on abscissa scale. Control currents ($\bullet$) were reduced by 10 ($\circ$) and 100 ($\square$) μ M ifenprodil with recovery ($\blacksquare$) to control levels on wash. (b) On a second neurone, ifenprodil 10 μ M ($\circ$) produced 43 and 50% reductions, respectively, of control I_{Ba} ($\bullet$) evoked on voltage steps to $+10$ mV from a V_h of -80 mV (left) and -50 mV (right) as indicated above traces. Calibration: current amplitude in nA; time in ms.

centration; ifenprodil, 10 μ M, reduced NMDA-evoked responses by $47 \pm 5\%$ and $47 \pm 6\%$ in the presence of 1 and 10 μ M glycine, respectively ($n = 6$; not significant, Student's paired t test; see Legendre & Westbrook, 1991). The differential effect of ifenprodil on the peak and steady-state components of NMDA-evoked currents (see above) was also unaffected on elevation of the extracellular glycine concentration. Ifenprodil 100 μ M attenuated kainate- and AMPA-evoked responses by, respectively, $20 \pm 7\%$ ($n = 6$) and $44 \pm 6\%$ ($n = 5$; Figure 4b), in contrast to its reported selectivity for NMDA at this concentration (Legendre & Westbrook, 1991). Superfusion with 100 μ M spermine potentiated NMDA-evoked responses by $22 \pm 6\%$ (range 0–70%; $n = 17$; Figure 4c). The effect appeared to be optimal as lower concentrations of spermine (10 or 50 μ M; $n = 4$ at each concentration) produced no additional potentiation and elevating the concentration above 100 μ M resulted in attenuation of the control NMDA-evoked currents (see Williams *et al.*, 1991; cf. Rock & MacDonald, 1992). Irrespective of the amplitude of the polyamine-evoked potentiation of NMDA-evoked currents, the percentage reduction of control NMDA responses by ifenprodil 10 μ M was increased on co-application of 100 μ M spermine. In 14 out of 17 neurones tested there was a $50 \pm 4\%$ reduction of NMDA-evoked responses by 10 μ M ifenprodil, which increased to a $64 \pm 3\%$

reduction of the new control response in the presence of 100 μ M spermine ($P < 0.02$, Student's paired t test; Figure 4c). In the remaining 3 neurones no change in the inhibitory effect of ifenprodil was observed.

Ifenprodil block of I_{Ba} Inward barium currents evoked on voltage steps from a V_h of -80 mV to a V_t of -10 mV were concentration-dependently blocked by ifenprodil (0.1–100 μ M). Ifenprodil reduced both the peak and delayed (240 ms following onset of the voltage step) components of I_{Ba} with an IC_{50} value of 17.8 ± 2.0 μ M and a Hill coefficient of 1.2 ± 0.1 ($n = 8$; Figure 5), a value similar to that seen in the fluorescent dye studies for reduction of high-[K^+] $_o$ -evoked rises in [Ca^{2+}] $_i$. The action of ifenprodil was rapid in onset and fully reversible on wash in control solution. At the highest concentrations tested (50 and 100 μ M) there was almost complete attenuation of the whole-cell I_{Ba} , which we have shown previously to be comprised of both nifedipine-sensitive and nifedipine-resistant, ω -CgTx-sensitive components (Church *et al.*, 1994). Furthermore, in agreement with the microspectrofluorimetric studies (see above), ifenprodil 10 μ M produced similar reductions of both the control I_{Ba} and that current remaining in the presence of a maximally-effective concentration (10 μ M) of nifedipine (respec-

Figure 7 Interaction of the spermine and ifenprodil block of I_{Ba} . (a) Histogram indicates mean $\pm$ s.e. mean of the % reduction of control I_{Ba} by 100 μM spermine, 10 μM ifenprodil or a combination of the two on 8 neurones. An estimate of the predicted effect (the sum of the % reductions for spermine and ifenprodil) is shown at far right. Ordinate scale: % reduction of control I_{Ba} . (b) Sample traces of the I_{Ba} evoked following a 200 ms voltage step from a V_h of -80 mV to a V_t of -10 mV . Spermine (100 μM) reduced the I_{Ba} by 27%, which on co-application of 10 μM ifenprodil increased to a 48% block. Following wash, and recovery to control levels (not shown), application of 10 μM ifenprodil alone resulted in a 41% reduction of I_{Ba} . Calibration bars: current amplitude in nA; time in ms.

tively $38 \pm 4\%$ and $36 \pm 4\%$ reductions of I_{Ba} in the 5 neurones tested).

Ifenprodil 10 μM did not alter the current-voltage relationship for I_{Ba} ($n = 4$; Figure 6a) but an increase in the percentage reduction of the control I_{Ba} was observed when V_h was decreased from -80 mV to -50 mV ($31 \pm 3\%$ and $53 \pm 3\%$ reduction, respectively, of controls in 10 neurones tested; $P < 0.05$, Student's paired t test; Figure 6b). The percentage block of I_{Ba} by ifenprodil, 10 μM , was also increased from $35 \pm 3\%$ to $46 \pm 3\%$ ($n = 10$) when the frequency of current activation was increased from 0.05 Hz to 0.2 Hz (not shown).

In agreement with previous studies (see Scott *et al.*, 1993), spermine attenuated HVA Ca^{2+} channel currents. Superfusion with 100 μM spermine produced a reversible $16 \pm 3\%$ reduction (range 8–27%, $n = 7$) of the control I_{Ba} . Superfusion of the same neurones with 10 μM ifenprodil produced a $33 \pm 3\%$ reduction (range 20–45%) of I_{Ba} which increased to a $37 \pm 5\%$ reduction (range 22–53%) during co-application of spermine 100 μM . The latter value is less than might be expected if the inhibitory effects of ifenprodil and spermine were additive (computed mean of combination, $48 \pm 4\%$; see Figure 7).

Discussion

Ifenprodil blocks high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ and HVA Ca^{2+} channel currents in cultured hippocampal pyramidal neurones. The Ca^{2+} channel blocking action of ifenprodil

described in the present study adds another element to the diverse pharmacological profile of this compound, which encompasses interactions with the NMDA receptor-channel complex and σ binding sites (see below), as well as α -adrenoceptors (Honda *et al.*, 1988; Chenard *et al.*, 1991). The concentrations of ifenprodil effective at blocking neuronal HVA Ca^{2+} channels are similar to those which attenuate responses mediated by voltage-activated Ca^{2+} channels in smooth muscle (e.g. Adeagbo & Magbagbeola, 1985; Honda & Sakai, 1987; Honda *et al.*, 1988). Although the mechanism whereby ifenprodil blocks HVA Ca^{2+} channels remains unclear, some aspects of this action (i.e. voltage- and frequency-dependence) are reminiscent of the effect of dihydropyridines such as nifedipine (see Bertolino & Llinás, 1992). In contrast to nifedipine, however, ifenprodil is able to block completely I_{Ba} and high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ in our neuronal preparations (see Church *et al.*, 1994). In this regard, ifenprodil shows similarities to the phenylalkylamine (e.g. verapamil) and piperazine (e.g. flunarizine) classes of Ca^{2+} channel blockers, which can also inhibit Ca^{2+} flux through dihydropyridine-insensitive Ca^{2+} channels (e.g. Mangano *et al.*, 1991; Tytgat *et al.*, 1991; Bowman *et al.*, 1993). Interestingly, ifenprodil has been found to inhibit KCl-stimulated $^{45}\text{Ca}^{2+}$ uptake into rat brain synaptosomes (a dihydropyridine- and ω -CgTx-resistant response; see Mintz *et al.*, 1992) at concentrations associated in the present study with dihydropyridine-insensitive HVA Ca^{2+} channel blockade (Honda *et al.*, 1989).

Polyamines have been suggested to act as modulators of neuronal L- and N-type HVA Ca^{2+} channels (reviewed by Scott *et al.*, 1993). For example, spermine allosterically inhibits ^3H -nitrendipine binding to L-type Ca^{2+} channels in rat cortical membranes with an EC_{50} value of 260 μM (Schoemaker, 1992) and inhibits the binding of ^{125}I - ω -CgTx to N-type Ca^{2+} channels in rat hippocampal synaptosomes with an IC_{50} value of 9 μM (Pullan *et al.*, 1990). In the present experiments, spermine inhibited high- $[\text{K}^+]_o$ -evoked rises in $[\text{Ca}^{2+}]_i$ and I_{Ba} , a result in accordance with previous voltage-clamp data obtained in dorsal root ganglion neurones (Scott *et al.*, 1993). However, in contrast to the differing potencies of spermine as a modulator at L- and N-type HVA Ca^{2+} channels (see above), in the present experiments ifenprodil appeared equally efficacious as a blocker of HVA Ca^{2+} channel-mediated responses recorded in either the presence or absence of nifedipine. It seems unlikely that ifenprodil modulates HVA Ca^{2+} channel activity in a manner completely analogous to polyamines, although some overlap of their site(s) of action may exist, given the ability of spermine (but not putrescine) to modulate the Ca^{2+} channel blocking action of ifenprodil.

Ifenprodil has nanomolar affinity for σ binding sites in the central nervous system, the pharmacological profile of which is similar to that of σ_2 , rather than σ_1 , sites (see Benavides *et al.*, 1992; Hashimoto & London, 1993). Although binding studies have suggested a possible association of σ receptors with voltage-activated Ca^{2+} channels (Rothman *et al.*, 1991) and a variety of other σ ligands also block neuronal HVA Ca^{2+} channels (Church & Fletcher, unpublished observations; also Mangano *et al.*, 1991; Netzer *et al.*, 1993), the affinities of these compounds for σ binding sites are much higher than the concentrations associated with their HVA Ca^{2+} channel blocking actions. The Ca^{2+} channel blocking properties of ifenprodil are therefore unlikely to be mediated via interactions with σ binding sites.

The potency and mechanism of the NMDA antagonist action of ifenprodil has been extensively investigated (reviewed by Carter *et al.*, 1991; Wong & Kemp, 1991) and it was not our intention to replicate the data here. Nevertheless, in our hands, ifenprodil attenuated NMDA-evoked responses at concentrations that are in good agreement with a previous study on rat cultured hippocampal neurones (IC_{50} for block of high affinity component = 0.75 μM ; Legendre & Westbrook, 1991). In addition, ifenprodil antagonism of NMDA-evoked

responses did not involve interactions with the agonist or strychnine-insensitive glycine binding sites and the absence of use- and voltage-dependency of the ifenprodil block argues against an open-channel blocking mechanism (see Legendre & Westbrook, 1991). Binding profiles employing [3 H]-dizocilpine indicate that ifenprodil may act as a closed-channel blocker (Reynolds & Miller, 1989); electrophysiological correlates of this mechanism could include the ifenprodil-induced modal shift in NMDA channel gating (Legendre & Westbrook, 1991) and, as observed here, its higher affinity for the steady-state (i.e. predominantly desensitized) component of the NMDA-evoked response. Indeed, the observed potentiation of the ifenprodil block by spermine highlights the sensitivity of the ifenprodil block to the state of activation of the NMDA receptor-channel complex and argues against a simple competitive interaction between these two ligands (see Reynolds & Miller, 1989; Bakker *et al.*, 1991). Finally, molecular cloning techniques have revealed the heterologous nature of NMDA receptors (Moriyoshi *et al.*, 1991; Monyer *et al.*, 1992) and studies on recombinant heteromeric NMDA receptors have indicated that they differ in their sensitivity to, and mechanism of antagonism by, ifenprodil (Williams, 1993) and polyamines (Durand *et al.*, 1992). Although it is likely that the NMDA-evoked responses observed in the present study are a composite of activation of a mixed population of NMDA receptors, it is of interest that the IC_{50} value for NMDA antagonism by ifenprodil and the absence of use- and voltage-dependency of the ifenprodil block (but not its insensitivity to glycine) is similar to the reported effects of ifenprodil at NR1A/NR2B, rather than NR1A/NR2A, receptors (see Williams, 1993).

The ability of ifenprodil to block multiple subtypes of HVA Ca^{2+} channels may play a role in its neuroprotective

and anticonvulsant actions. In particular, the ability of ifenprodil to block dihydropyridine-insensitive HVA Ca^{2+} channels may be associated with reduced excitatory transmitter release (see Bertolino & Llinás, 1992; Luebke *et al.*, 1993), as has been shown for compounds such as dextromethorphan (Annels *et al.*, 1991; Mangano *et al.*, 1991; Netzer *et al.*, 1993) and flunarizine (Tytgat *et al.*, 1991; Bowman *et al.*, 1993; Cousin *et al.*, 1993), both of which also possess neuroprotective and anticonvulsant efficacy *in vivo* (e.g. Deshpande & Wieloch, 1986; Prince & Feaser, 1988; Czuczwar *et al.*, 1992; Löscher & Hönack, 1993). In addition, ifenprodil has been reported to be only 3 fold lower in potency as an antagonist of audiogenic seizures in genetically epilepsy-prone rats than dizocilpine (De Sarro & De Sarro, 1993), despite the fact that dizocilpine is at least 80 fold more potent as an NMDA antagonist than ifenprodil when assessed under voltage-clamp conditions (see MacDonald *et al.*, 1991). The discrepancy between the NMDA antagonist potencies of ifenprodil and dizocilpine compared with their similar anticonvulsant activities might reflect, at least in part, the more pronounced Ca^{2+} channel blocking actions of ifenprodil compared to dizocilpine (IC_{50} for block of Ca^{2+} channel current by dizocilpine in dissociated guinea-pig CA1 pyramidal neurones = 63 μ M; French-Mullen & Rogawski, 1992).

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