Influence of Lubeluzole on Voltage-Sensitive Ca$^{++}$ Channels in Isolated Rat Neurons

R. MARRANES, E. De PRINS and G. CLINCKE

Department of Neuropsychopharmacology, Janssen Research Foundation, B-2340 Beerse, Belgium

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ABSTRACT

Lubeluzole is neuroprotective in a photochemical stroke model, whereas the (R)-enantiomer of the same molecule is not [De Ryck, M, Keersmaekers R, Duytschaever H, Claes C, Clincke G, Janssen M and Van Reet G (1996) J Pharmacol Exp Ther 279:748–758]. We investigated the effects of lubeluzole and the (R)-enantiomer on voltage-sensitive Ca$^{++}$ channels of isolated rat dorsal root ganglion cells, using whole-cell voltage-clamp, with Ba$^{++}$ as the charge carrier. Both compounds blocked the low-voltage-activated Ba$^{++}$ current (iLVA or T current) with an IC$_{50}$ value of 1.2 µM. Lubeluzole and the (R)-enantiomer also blocked the high-voltage-activated calcium channel current (iHVA), with IC$_{50}$ values of 2.6 and 3.5 µM, respectively, and accelerated the apparent inactivation of iHVA. This acceleration was more pronounced with lubeluzole than with the (R)-enantiomer at 3 and 10 µM. Both compounds produced a clear tonic block of iLVA and iHVA, even in the absence of previous stimulation. Lubeluzole and the (R)-enantiomer induced a negative shift of the inactivation curve of iLVA and slowed down the recovery from inactivation. This resulted in a stronger inhibition of iLVA at more depolarized conditioning potentials and higher stimulation frequencies. The block of iHVA was voltage and frequency dependent. Lubeluzole and the (R)-enantiomer also blocked iHVA in isolated rat superior cervical ganglion cells and cerebellar Purkinje cells. The Ca$^{++}$ channel-blocking properties of lubeluzole may contribute to its neuroprotective effect. However, the small difference between the two enantiomers in inhibition of Ca$^{++}$ channel currents does not explain the stereospecificity of the neuroprotective properties of lubeluzole in vitro and in vivo.

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Lubeluzole, the (+)-(S)-enantiomer of a benzothiazole derivative (fig. 1), has neuroprotective properties (De Ryck, 1997). Intravenous post-treatment with lubeluzole rescued sensorimotor function and reduced infarct volume after photochemically induced neocortical infarcts in rats (De Ryck et al., 1996). The (R)-enantiomer (R091154) of lubeluzole was inactive. In the peri-infarct zone surrounding such neocortical infarcts, lubeluzole reduced the infarct-induced rise in extracellular glutamate (Scheller et al., 1995) and normalized paired-pulse, γ-aminobutyric acid-mediated inhibition (Buchkremer-Ratzmann and Witte, 1997). Again, the (R)-enantiomer was ineffective. Lubeluzole also reduced infarct volume after focal cerebral ischemia induced by middle cerebral artery occlusion (Aronowski et al., 1996) and attenuated delayed neuronal death in a model of global ischemia in rats (Haseldonckx et al., 1996). Experiments on neuronal cultures have shown that lubeluzole inhibits anoxia- and glutamate-induced nitric oxide-related neurotoxicity and that it blocks neurotoxicity induced by nitric oxide donors (Benjamins et al., 1996; Lesage et al., 1996; Maiese et al., 1997). In these in vitro paradigms of neuroprotection, the (R)-enantiomer was either less active or inactive. Therefore, the neuroprotective mechanism of action of lubeluzole may be based on stereospecific down-regulation of the NOS pathway. Lubeluzole is neither an NOS inhibitor nor an NMDA antagonist (Lesage et al., 1996). Although lubeluzole blocks the fast sodium channel and antagonizes veratridine-induced neurotoxicity (Osiowska-Evers et al., 1995; Ashton et al., 1997), these effects are not stereospecific.

In the present study, we investigated whether lubeluzole and the (R)-enantiomer act on neuronal calcium channels and, if so, whether such effects are stereospecific. Several lines of evidence suggest that intracellular Ca$^{++}$ overload, which leads to activation of proteases, nuclease, phosphatase and other degradative enzymes, can lead to free radical production and neuronal cell death (Choi, 1995; Kristian and Siesjö, 1996). Ischemic Ca$^{++}$ overload can arise by excessive release of excitatory neurotransmitters, such as glutamate, which induces a neuronal influx of Ca$^{++}$ via the NMDA receptor and some AMPA- and kainate-gated ion channels.
Influx of Ca\(^{2+}\) via inverse Na\(^+/Ca\(^{2+}\) transport after cellular Na\(^+\) overload can also contribute to Ca\(^{2+}\) overload (Urenjak and Obrenovitch, 1996). Another direct pathway for intracellular Ca\(^{2+}\) overload is Ca\(^{2+}\) influx via voltage-sensitive Ca\(^{2+}\) channels (VSCC). Ca\(^{2+}\) influx via presynaptic VSCC triggers neurotransmitter release and can thus also indirectly influence postsynaptic Ca\(^{2+}\) overload. Partial inhibition of Ca\(^{2+}\) channels might thus be neuroprotective in ischemic conditions. There are many types of Ca\(^{2+}\) channels (De Waard et al., 1996; Mori et al., 1996). They can be distinguished partly by their activation voltages. A slight depolarization to ~50 mV activates the transient LVA Ca\(^{2+}\) current (or T current). Depolarization to more positive voltages activates the HVA Ca\(^{2+}\) current, to which many types of Ca\(^{2+}\) channels can contribute. To investigate the influence of lubeluzole on Ca\(^{2+}\) channel currents, we used rat dorsal root ganglion cells, which express both LVA and HVA Ca\(^{2+}\) channels (Scroggs and Fox, 1992; Mintz et al., 1992; Diachot et al., 1995).

**Methods**

### Cell preparation

DRG neurons were isolated by use of a modification of the technique of Delree et al. (1989). Briefly, 3-month-old Wistar male rats were decapitated under isoflurane/N\(_2\)O anesthesia. DRGs were removed aseptically and freed from connective tissue. They were digested at 37°C in 1 ml of 0.5% collagenase medium for 45 min, to which 1 ml of 0.25% trypsin medium was then added, and digestion was allowed to continue for a further 30 min. The collage- nase medium contained 0.5% collagenase (Boehringer-Mannheim, Mannheim, Germany), and NaCl 120 mM, KCl 4.8 mM, KH\(_2\)PO\(_4\) 1.2 mM, MgSO\(_4\) 1.2 mM, D-glucose 3 mM, CaCl\(_2\) 0.1 mM, NaHCO\(_3\) 19.7 mM, and HEPES 40 mM at pH 7.2. The external solution contained NaCl 152 mM, KCl 3 mM, D-glucose 10 mM, HEPES 40 mM and was adjusted to pH 7.2. Solutions.

The external solution contained NaCl 152 mM, KCl 3 mM, D-glucose 10 mM, HEPES 40 mM and was adjusted to pH 7.2. The experiments were performed in a temperature-controlled water bath at 37°C in a humidified atmosphere (95% air/5% CO\(_2\)). The data were used for voltage-clamp experiments on the day of isolation and the 2 following days.

We observed that the amplitude of iLVA decreased clearly during the time in culture. In a separate series of experiments, aimed especially at investigating the effects on iLVA, this decline was retarded by storing the Petri dishes with the cells at 4°C for 24 hr from the end of the day of isolation. In this case, 4 ml of the HEPES-buffered solution used to perfuse the experimental chamber (see below) was added to the medium of each Petri dish. In the latter series of experiments, we used only medium-sized DRG cells (35–40 μm) having a large LVA Ca\(^{2+}\) current (Scroggs and Fox, 1992), which made possible accurate measurement of the effects on this current.

Several types of Ca\(^{2+}\) channels contribute to the HVA Ca\(^{2+}\) current of DRG cells (Scroggs and Fox, 1992; Mintz et al., 1992; Diachot et al., 1995). In some additional experiments, SCG cells and cerebellar Purkinje neurons were used because their iHVA consists mainly of N-type or P-type Ca\(^{2+}\) current, respectively (Boland et al., 1994; Plummer et al., 1989; Mintz et al., 1992). SCG cells from male Wistar rats (200 g) were isolated each day by use of the enzymatic dispersion technique described by Chen and Schofield (1995), except that the cells were incubated in DMEM-FCS for ≥1 hr (37°C, 5% CO\(_2\)) to achieve better attachment to the Petri dish. Cerebellar Purkinje cells were isolated from 10- to 11-day-old male Wistar rats according to Pachon et al. (1993).

### Electrophysiological recording

Whole-cell voltage-clamp (Hamill et al., 1981) was performed at room temperature (18–21°C). A Petri dish containing attached DRG cells was transferred to the stage of a Patch Clamp Tower (Luigs and Neumann, Germany) and examined with an inverted phase-contrast microscope (Diaphot TMD, Nikon). The experimental chamber was continuously perfused with a solution containing NaCl 152 mM, KCl 3 mM, D-glucose 10 mM, HEPES 10 mM, CaCl\(_2\) 2 mM and MgCl\(_2\) 1 mM at pH 7.4. Patch electrodes of borosilicate glass (Jencons, H15/10) were pulled and fire polished by means of a Zeitz DMZ puller (Augsburg, Germany). The electrode resistance ranged from 1.5 to 2.5 MΩ when measured in the bath. After the patch had been broken, the cells were allowed to equilibrate for 5 min with the contents of the electrode.

An EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany) was used, in combination with an Apple Macintosh computer and the data acquisition and analysis programs Pulse and PulseFit (HEKA) and Igor (WaveMetrics). All potentials were corrected for the liquid junction potential between the pipette solution and the bath (5 mV). The HP was ~100 mV. Series resistance was compensated by 80%. Capacitative transients were cancelled by means of the analog cancelsing circuitry of the EPC-9. Any remaining capacitive transients and the linear leak current were subtracted on-line by a P/4 procedure in the software (Armstrong and Bezanilla, 1974). In this procedure, the sum of currents elicited by four hyperpolarizing pulses starting from ~80 mV, with an amplitude one fourth of the test pulse, was added to the test pulse current. The data were low-pass filtered (667 Hz) and sampled at 2 kHz.

### Solutions

The internal pipette solution contained CsCl 100 mM, EGTA 10 mM, MgCl\(_2\) 1 mM, MgATP 3 mM, TrisGTP 0.3 mM and HEPES 40 mM and was adjusted to pH 7.2 with CsOH. The external solutions contained BaCl\(_2\) 2 mM, tetraethylammonium chloride 135 mM, tetrodotoxin 0.5 μM and HEPES 10 mM and were adjusted to pH 7.4 with tetraethylammonium hydroxide. Ba\(^{2+}\) was used in the extracellular solution instead of Ca\(^{2+}\), the current through Ca\(^{2+}\) channels was carried by Ba\(^{2+}\). This was done because Ba\(^{2+}\) suppresses residual currents through the delayed rectifier (Adams and Nonner, 1990) and inward rectifier potassium channels (Hagiwara et al., 1978) and because the high-voltage-activated Ca\(^{2+}\) channel current is then devoid of (Ca\(^{2+}\) )-dependent inactivation. Also, unlike Ca\(^{2+}\), Ba\(^{2+}\) does not permeate or barely permeates Na\(^+\) channels.

Lubeluzole and the (R)-enantiomer were prepared in 10 mM stock...
solutions in DMSO. The concentration of DMSO was always the same in the control solution as in the corresponding solutions with drug and never exceeded 0.1% (v/v).

The DRG cell was superfused with the control or test solutions by means of a gravity-driven puffer system placed at a distance of 0.3 mm from the cell. The internal diameter of the final polyethylene tubing near the cell was 0.28 mm. This tube communicated via a small dead space with four polyethylene tubes, controlled by valves and leading to polyethylene syringes containing the different test solutions. This superfusion system changes the extracellular solution in less than a second.

Statistical analysis. All results are expressed as mean ± S.D., except for the concentration-response curves in figure 3, where mean ± S.E. is used. The difference between the effects of lubeluzole and the (R)-enantiomer on the amplitude of iLVA and iHVA (fig. 3) was evaluated for each concentration by means of the two-sided Student’s t test for independent samples. In this procedure, P values were adjusted for the number of different comparisons (five) by applying Bonferroni’s inequality. The same statistical method was used to evaluate the difference between two sequences of drug application [from lubeluzole to (R)-enantiomer and from the (R)-enantiomer to lubeluzole] in table 1 and also for the effects on the time constant of the decay of iHVA. In the experiments on the inactivation of iLVA (table 2) and on the voltage dependence of the block of iHVA, all pairwise comparisons of the three treatment groups [control (vehicle), lubeluzole and the (R)-enantiomer] were made using the Tukey-Kramer multiple comparison procedure. Values of P < .05 were considered to indicate statistical significance. Computations were carried out using the SAS system for statistical analysis.

Results

iLVA and iHVA in DRG cells. Some of the DRG cells showed both the transient iLVA (or T current) and the iHVA, whereas others showed only iHVA. The low-voltage-activated current and the high-voltage-activated current could be tested simultaneously by means of the pulse sequence shown in figure 2A. From an HP of −100 mV, first a 155-msec test pulse to −50 mV elicited mainly iLVA (if present). After a 10-msec interval at −100 mV, a 155-msec test pulse to −20 mV elicited iHVA, which was nearly maximal at this potential. The amplitude of iLVA was measured as the transient component of the current at the test pulse of −50 mV, thus as the difference between the peak inward current and the current at the end of this test pulse. The current during the test pulse to −20 mV was contaminated very little by iLVA, because iLVA was still inactivated by the preceding pulse to −50 mV.

Influence of lubeluzole and the (R)-enantiomer on iLVA and iHVA. The influence of lubeluzole on Ca2+ channel currents was investigated and compared with that of the (R)-enantiomer by means of the following protocol: the cells were stimulated every 30 sec with the pulse sequence shown in figure 2A. This was done for 5 min in the control solution, followed by 5 min in the presence of one enantiomer and thereafter by 5 min in the presence of the other enantiomer at the same concentration (fig. 2B).

Figure 2A illustrates that 3 μM lubeluzole or the (R)-enantiomer reduced both iLVA and iHVA, with the greatest effect on the transient iLVA, which was almost completely suppressed at this concentration. Both compounds decreased the peak current amplitude of iHVA (iHVApeak) and accelerated its apparent inactivation (fig. 2A). This is reflected by a larger effect on the current measured at the end of the 155-msec test pulse to −20 mV (iHVAend) than on iHVApeak (fig. 2B). Thus, the compounds have more effect on iHVA during prolonged depolarizations. Lubeluzole and the (R)-enantiomer had little or no effect on the time constant of inactivation of iLVA. The apparent inactivation of iHVA in the presence of 3 μM lubeluzole was more pronounced than that with the (R)-enantiomer. The time course of these effects is shown in figure 2B.

The amplitude of the Ca2+ channel currents decreased gradually with time, even in the control solution (fig. 2B). So as to quantify the effect of drugs in the presence of a continuous run-down of the Ba2+ currents, the time courses of iHVApeak, iHVAend and iLVA were fitted exponentially for the 5-min period in which the control solution was used, and they were extrapolated for the remainder of the experiment. The extrapolated curves give an estimation of what the current would have been if the control solution alone had been applied. Figure 2C displays the curves of ratios between the measured current and the extrapolated current at the same
time point and shows that it took >5 min for the compounds to produce their maximum effect. As late as after the first 5 min of application, the compounds produced a further gradual decline in Ca\(^{2+}\) channel currents compared with the control period, as can be seen clearly for rHVApeak in figure 2C.

To minimize the effects of variability between cells, the effects of the two enantiomers were also compared in the same cell by means of a switch from one compound to the other. The extracellular solution of lubeluzole was replaced by an extracellular solution of the (R)-enantiomer at the same concentration 5 min after the first application of 3 \(\mu\)M lubeluzole (fig. 2, B and C). The experiment showed that the ratio for iHVAend (rHVAend) clearly increased when lubeluzole was switched to the (R)-enantiomer and that this effect was reversed when 3 \(\mu\)M lubeluzole was used again 5 min later. The differences between the two enantiomers were smaller for the ratios for rLVA, rHVApeak and rHVAend. To obtain these ratios, the time courses of iLVA, iHVApeak and iHVAend in the control period were fitted exponentially and extrapolated to the end of the experiment. Division of each measured current amplitude by the value of the corresponding calculated curve obtained by fitting, at the same time point, yielded the ratios.
centrations of 0.1 and 0.3 already substantially reduced at the concentrations of 3 and 10 μM, respectively; for rHVAend: 0.9 μM (0.8) and 0.9 μM (0.8), respectively; and for rLVA, 1.2 μM (1.3) and 1.2 μM (1.1). The drug effects on rHVApeak and rHVAend were obtained after a 5-min application of the first enantiomer tested, such as time point 2 in figure 2C. Results from the second enantiomer tested in the same cell were not used for calculation of the IC50. Only one drug concentration was tested on each cell as to avoid errors in the extrapolation of the run-down of iHVA. Only a small fraction of the cells used for the measurement of the concentration-response curves of iHVA (diameter, 32.1 ± 4.1 μm, mean ± S.D., n = 78) expressed iLVA. Therefore, the concentration-response of ILVA was tested in a separate series of experiments on medium-sized DRG cells (35–40 μm), which have a larger ILVA (Scroggs and Fox, 1992).

In the latter series, two or three concentrations of a single compound were tested cumulatively in the same cell, because iLVA showed less run-down than iHVA. Drug effects were again measured 5 min after application of each new concentration. Variability between DRG cells can obscure smaller differences in effect between lubeluzole and the (R)-enantiomer, as well as for cells for which the compounds were given in the reverse order. At the concentration of 3 μM, the change in rHVAend after one enantiomer was 22.5 ± 8.0 msec (mean ± S.D.) in 3 μM lubeluzole, and after the switch to 3 μM (R)-enantiomer 5 min later, it was increased to 49.1 ± 9.1 msec (n = 6). In the cells in which the reverse sequence was applied, the change in rHVA was 49.9 ± 10.6 msec in the presence of 3 μM (R)-enantiomer and thereafter 13.1 ± 4.1 msec with 10 μM lubeluzole (n = 9). In 10 μM lubeluzole, the change was 11.6 ± 2.3 msec, and thereafter it increased to 21.0 ± 3.9 msec in the presence of 10 μM (R)-enantiomer (n = 6). In the cells with the reverse sequence, the change was 22.5 ± 2.3 msec in the presence of 10 μM (R)-enantiomer and thereafter 13.1 ± 4.1 msec with 10 μM lubeluzole (n = 7). The change in rHVA after changeover from 3 μM lubeluzole to the (R)-enantiomer (τdrug/τfree) was significantly different from the change in rHVA after the reverse sequence (P < .001). The same was true at the concentration of 10 μM (P < .001). This proves that lubeluzole accelerates the decay of iHVA more than the (R)-enantiomer at 3 and 10 μM.

The time constant was 84.9 ± 49.5 msec (n = 25) for the control solution and was thus more variable. In the presence of lubeluzole...
of 3 and 10 μM of these compounds γ was much less variable, since then iHVA decayed more rapidly; in particular, a rapidly decaying exponential function can be distinguished more reliably from the offset current a, than a very slowly decaying one, as is the case with the control solution. For the same reason, such a determination of γ becomes inaccurate at the lower concentrations 0.1 and 0.3 μM.

In two additional experiments on small DRG cells (22 μm), in the presence of 2 μM nimodipine to reduce the contribution of the L-type Ca2+ channel to iHVA (HP = −100 mV), we observed a similar reversible difference in effect on iHVAend and on the time constant of the decay of iHVA between 3 μM lubeluzole and the (R)-enantiomer.

In all cells expressing both iLVA and iHVA, lubeluzole and the (R)-enantiomer decreased rLVA to a clearly greater extent than rHVApeak at the concentrations of 1, 3 and 10 μM. At the lower concentrations of 0.1 and 0.3 μM, the initial reduction in rLVA was always larger than that of rHVApeak. The reduction in rHVApeak was more gradual. This resulted in a difference of variable sign between rLVA and rHVApeak. The reduction in rLVA was always larger than that of rHVApeak.

Part of this variability may be related to inaccuracy of the extrapolation. To estimate the extrapolation error, 29 cells remained in the control solution for 10 min; the first 5 min were used to fit the curves of iHVApeak, iHVAend and iLVA. This yielded 0.96 ± 0.06 (mean ± S.D.) for rHVApeak, 0.96 ± 0.07 for rHVAend and 0.98 ± 0.03 for rLVA at the 10th minute. Because these values are close to 1, the extrapolation technique was considered to be acceptable.

The somewhat irregular shape of the concentration-response curves of iHVApeak and iHVAend (fig. 3) at the lower concentrations (0.1, 0.3 and 1 μM) is related to the variability of the effect of the enantiomers on individual cells and to the fact that these three concentrations were not tested in the same cell. When in separate experiments these concentrations were applied cumulatively, the drug effects on iHVA clearly proved to be concentration dependent. This was seen in four cells for each enantiomer (results not shown).

**Tonic block of iLVA and iHVA.** To test the use-dependency of the block of iLVA and iHVA, the following protocol was used in a separate series of experiments. In the control solution, the cells were stimulated every 30 sec for 5 min by application of the double-pulse protocol shown in figure 4A. Thereafter, stimulation was stopped (fig. 4B). Two minutes later, 10 μM lubeluzole or the (R)-enantiomer was given. Five minutes later (i.e., 7 min after stimulation was discontinued), the stimulation was started again. Already on the first stimulation, iLVA and iHVA were greatly reduced by 10 μM lubeluzole or the (R)-enantiomer, and only small further changes in the shape of iLVA and iHVA were observed (fig. 4A). The apparent rate of inactivation of iHVA was very much increased, already from the first stimulation. In addition, no stimulation was needed in the washout period to unblock. This was seen in all medium-sized cells (n = 3 for each enantiomer). These experiments show that lubeluzole and the (R)-enantiomer exert a tonic block of iLVA and iHVA.

The decrease in iHVA after resumption of the stimulation in the washout period must be seen as a response of the amplitude of iHVA to the change in frequency of stimulation (1/30 Hz) and is not a normal run-down phenomenon.

**Voltage-dependent block of iLVA.** To investigate the influence of lubeluzole on the inactivation of iLVA, the 155-msec test pulse to −50 mV was kept constant and the voltage of the 10-sec conditioning prepulse was varied between −60 and −120 mV (fig. 5). The time interval between the test pulses was 15 sec. The curve of the amplitudes (I) of iLVA at the different conditioning potentials (V) was fitted as a function of V according to the Boltzmann equation: I = Imax/[1 + exp(V − Vh/Sl)]. This fit yielded the parameters Imax, Vh, and Sl; Imax is the maximum amplitude of iHVA in the absence of inactivation, Vh is the conditioning potential at which the inactivation is half-maximum and Sl is the slope factor.

The average values for Imax, Vh and Sl in the control solution were −4.52 ± 1.63 nA, −83.9 ± 3.0 mV and 5.4 ± 0.6 mV (mean ± S.D., n = 18). Figure 5 and its inset show that lubeluzole reversibly decreased Imax, produced a negative shift in Vh and increased Sl (table 2). The negative shift in Vh after 1 μM lubeluzole was only partly reversed by a 5-min washout period, but further repetitive applications of lubeluzole on the same cell showed a similar reversible effect (fig. 5), confirming the effect of lubeluzole on Vh.

The observation that the effect of 1 μM lubeluzole (or the (R)-enantiomer) on Vh reversed only partly or not at all in the subsequent washout period is probably related to a spontaneous drift of Vh to more negative potentials, which was also seen in the control solution (−1.7 mV, table 2). This drift may have been due to a gradual shift in junction potential at the electrode tip caused by a gradual exchange of ions between the cell and the electrode (Marty and Neher, 1995). A second factor contributing to the fact that the reversibility of Vh was only partial could have been incomplete washout of the drug effect over the 5-min period. The effects of lubeluzole and the (R)-enantiomer on Imax, Vh and Sl were significantly larger than the spontaneous changes in these parameter values during an application of the control solution for the same duration (table 2), proving that the effects were drug induced.

The negative shift of the inactivation curve and Vh by 1 μM lubeluzole or the (R)-enantiomer predicts that these compounds should block iLVA relatively more when the conditioning potential is −85 mV than when the conditioning potential is −110 mV.

This prediction was tested in the experiment illustrated in figure 6, in which iLVA was elicited every 30 sec by a test pulse to −50 mV with the 10-sec conditioning prepulse alternating between −110 and −85 mV. This experiment showed that rLVA was indeed depressed more after a conditioning potential of −85 mV than after a conditioning potential of −110 mV (fig. 6C). The rLVA curves for the two conditioning potentials diverged from the moment of drug application, confirming additionally that there was a drug-induced negative shift in Vh. This was tested in two cells with lubeluzole and two cells with the (R)-enantiomer, with similar results (not shown). This type of experiment and the effects on the inactivation curve of iLVA thus show that the block of iLVA by lubeluzole and the (R)-enantiomer was voltage dependent.
**Frequency-dependent block of iLVA.** The frequency dependence of iLVA block was tested by application of a pair of 155-msec test pulses to \(-50\) mV every 30 sec with variation of the interval between the two test pulses. It took longer for iLVA to recover from inactivation in the presence of 1 \(\mu M\) lubeluzole (fig. 7A) than in the control solution. The effect was reversible on return to the control solution. These experiments show that the inhibition of iLVA by lubeluzole is frequency dependent and more pronounced at higher frequencies of stimulation.

The protocol and time course for these experiments are shown in figure 7B. Every 30 sec, a pair of 155-msec test pulses to \(-50\) mV was given with a constant interval of 320 msec between the two pulses, except when the recovery from inactivation was tested, in which case the interval was varied. Figure 7B shows the ratio of the amplitude of iLVA at the second pulse to iLVA at the first pulse. At the constant interval of 320 msec, 1 \(\mu M\) lubeluzole caused this ratio to decrease very rapidly, and it returned only slowly to the original value after washout of the compound. This also illustrates the time course of the drug effect on the recovery from inactivation of iLVA. A very similar effect on the recovery from inactivation of iLVA was obtained with 1 \(\mu M\) of the \((R)\)-enantiomer \((n = 4,\) results not shown).

As a consequence of the frequency dependence of the inhibition of iLVA by lubeluzole [or the \((R)\)-enantiomer], a transition from 0.03 Hz to a higher frequency of stimulation reduced iLVA relatively more in the presence of lubeluzole [or the \((R)\)-enantiomer] than in the control solution. This was true not only for long depolarizing pulses, during which iLVA inactivated nearly completely, but also for a train of shorter pulses (20 msec) at a higher frequency such as 10 Hz (not shown).

**Voltage dependence of block of iHVA.** Figure 8 shows the influence of 10 \(\mu M\) lubeluzole on the activation curve in
steady state (5 min), the former pair of 50-msec pulses to duration of these test pulses (3 msec) minimized the block by 2
a 10-sec conditioning pulse to mV was given again, first from a
more strongly at along the voltage axis. However, they did inhibit iHVAend
these compounds did not induce a shift in the iHVApeak
were obtained with the (R)-enantiomer when a larger fraction of the HVA Ca \(^{2+}\) channels are open. At 0 mV and higher test potentials, the compounds at a concentration of 10 \(\mu\)M blocked iHVAend almost completely.

The block of iHVA by lubeluzole or the (R)-enantiomer was also modulated by the voltage preceding the test pulse. The block was increased by depolarizing conditioning potentials, as shown in figure 9. In these experiments, in the control solution a 50-msec test pulse to -20 mV was given from a -100 mV HP (fig. 9A). Twenty seconds later, the HP was reset to -100 mV, and every 20 sec a short (3-msec) test pulse to -20 mV was given, to monitor iHVA in the control solution (1 min) and then for 5 min in the presence of 3 \(\mu\)M lubeluzole [or the (R)-enantiomer]. The short duration of these test pulses (3 msec) minimized the block by the compounds. When the block by lubeluzole approached steady state (5 min), the former pair of 50-msec pulses to -20 mV was given again, first from a -100 mV HP and then after a 10-sec conditioning pulse to -60 mV (fig. 9B). Then, the compound was washed out while the short 3-msec test pulses were given. Five minutes later, the pair of 50-msec pulses from -100 and -60 mV was given again (fig. 9C). This protocol was followed for the drug-treated cells and also for a group of cells that remained in the control solution. The voltage dependence of iHVApeak was expressed as the ratio of iHVApeak after the conditioning potential at -60 mV to iHVApeak after the conditioning potential at -100 mV [iHVA \(_{60}\)/iHVA \(_{100}\) mV]. In a control group, in which the cells (\(n = 7\)) remained in the control solution, this ratio decreased over time from 0.77 \(\pm\) 0.10 (mean \(\pm\) S.D.) to 0.74 \(\pm\) 0.10 after 5 min and to 0.71 \(\pm\) 0.10 after a further 5 min. In the group treated with 3 \(\mu\)M lubeluzole (\(n = 8\)), the ratio was 0.78 \(\pm\) 0.05 in the initial control solution, decreased to 0.63 \(\pm\) 0.08 after 5 min application of lubeluzole and increased again to 0.74 \(\pm\) 0.05 after 5 min of washout. In the group treated with 3 \(\mu\)M (R)-enantiomer (\(n = 9\)), this ratio was 0.67 \(\pm\) 0.10 in the initial control solution, 0.54 \(\pm\) 0.08 after 5 min application of the (R)-enantiomer and 0.61 \(\pm\) 0.11 after 5 minutes of washout. The difference between the second determination (after 5-min application of the control or drug solution) and the initial determination in the control solution was -0.03 \(\pm\) 0.03 (mean \(\pm\) S.D., \(n = 7\)) as change in ratio in the control group, -0.15 \(\pm\) 0.04 (\(n = 8\)) in the group treated with 3 \(\mu\)M lubeluzole (P < 0.001 vs. control group) and -0.13 \(\pm\) 0.05 (\(n = 6\)) in the group treated with 3 \(\mu\)M (R)-enantiomer (P < 0.001 vs. control group). The ratio was thus reduced significantly more in the presence of 3 \(\mu\)M lubeluzole or the (R)-enantiomer and this effect was reversible after a 5-min washout period. There was no significant difference between the two enantiomers for this effect. These experiments show that lubeluzole and the (R)-enantiomer blocked iHVApeak to a greater extent when the cells were in a depolarized condition, therefore more after a -60 mV than after a -100 mV conditioning potential.

**Frequency-dependent block of iHVA.** Lubeluzole and the (R)-enantiomer accelerated the apparent inactivation of iHVA. To study how long this influences the HVA Ca \(^{2+}\) channels after the return to HP and whether it could induce a frequency dependence of the block of iHVA by these compounds, iHVA was elicited twice every 30 sec, and the interval (at -100 mV) between the first iHVA (iHVA1) and the second iHVA (iHVA2) was varied (fig. 10). The effect was
studied in DRG cells not expressing iLVA (fig. 10, A–C) and in DRG cells expressing iLVA (fig. 10, D–F). For the cells expressing iLVA, the test pulses to –20 mV (to elicit iHVA) were preceded by 155-msec pulses to –50 mV to elicit and then inactivate iLVA. Figure 10, A and D, illustrates that lubeluzole blocked iHVApeak to a relatively greater extent after the shorter 100-msec interval at –100 mV (iHVA2) than after the longer 30-sec period at –100 mV (iHVA1). Similarly, iLVA elicited after the 100-msec interval (iLVA2) was blocked to a relatively greater extent by lubeluzole than was iLVA1 (fig. 10D). Similar effects were seen with the (R)-enantiomer. Figure 10, B, C, E and F, shows how long the effects of lubeluzole and the (R)-enantiomer on the apparent inactivation of iHVA lasted. They indicate that the inhibition of iHVA by lubeluzole and the (R)-enantiomer was frequency dependent and more pronounced at shorter intervals between the test pulses to –100 mV and is thus voltage dependent.

Fig. 6. Voltage dependence of the block of iLVA by lubeluzole and its time course. A, The 10-sec conditioning prepulse was alternated every 30 sec between –110 and –85 mV, whereas the 155-msec test pulse eliciting iLVA was kept constant at –50 mV. The sweeps show iLVA after the conditioning potentials indicated in the control solution and after application of 1 μM lubeluzole. The numbers between parentheses refer to the time point at which the sweeps were recorded, as shown in B and C. B, Time course of the influence of lubeluzole on iLVA after the conditioning prepulses to –110 (VC –110) and –85 mV (VC –85). C, the curves for iLVA in the control solution were fitted exponentially and extrapolated, and the ILVA values for all time points were divided by the values on the fitted curve at the same time point, yielding the ratios rLVA at the two conditioning potentials. The effect of lubeluzole on iLVA (and rLVA) is larger after a conditioning prepulse to –85 mV than after a conditioning prepulse to –110 mV and is thus voltage dependent.

Fig. 7. Influence of lubeluzole on the recovery from inactivation of iLVA. Every 30 sec, a pair of 155-msec test pulses to –50 mV was given, and the interval between the two test pulses was varied. A, The interval between the two test pulses is represented on the horizontal axis. The ratio of the amplitude of iLVA at the second test pulse to the amplitude of iLVA at the first test pulse is represented on the vertical axis. The results are expressed as mean ± S.D. The results were obtained in the control solution (n = 4), after a 5-min application of 1 μM lubeluzole (n = 4) and after a 5-min washout period in two of the four cells. Lubeluzole slowed down the recovery from inactivation of iLVA reversibly. B, Ratio of the amplitude of ILVA at the second test pulse to the amplitude at the first test pulse. Every 30 sec, a pair of 155-msec test pulses to –50 mV was given. The interval between the first and second test pulses was 320 msec except in the periods indicated by R and a horizontal bar, in which the recovery from inactivation was tested (see A). This illustrates the time course of the effect of lubeluzole and the (R)-enantiomer on the recovery from inactivation on application and washout of these compounds.
42 ± 15% and 64 ± 13% (mean ± S.D., n = 9), respectively (fig. 11, A and B). The (R)-enantiomer blocked iHVA peak and iHVA end by 34 ± 15% and 53 ± 19%, respectively (n = 6). In this cell type, too, the time constant of decay of iHVA was smaller in the presence of 3 μM lubeluzole (77.2 ± 23.9 msec, mean ± S.D.) than with 3 μM of the (R)-enantiomer (111.2 ± 41.8 msec) in the same cells (n = 15). The change in time constant after the switch from 3 μM lubeluzole to the (R)-enantiomer was significantly different from the change in time constant after the solutions were given in reverse sequence (P < .001).

In Purkinje cells of the cerebellum, iHVA consists mainly of P current (Mintz et al., 1992). In experiments on acutely isolated rat Purkinje neurons (HP = −80 mV), 3 μM lubeluzole decreased iHVA peak and iHVA end by 44 ± 7% and 67 ± 4%, respectively (fig. 11, C and D). Similarly, the (R)-enantiomer blocked iHVA peak and iHVA end by 51 ± 6% and 73 ± 3%, respectively (n = 3). In all four cells in which the two compounds were tested in the same cell, the time constant of decay of iHVA was smaller with lubeluzole (63.5 ± 2.4) than with the (R)-enantiomer (74.9 ± 7.6).

Discussion

The results show that lubeluzole and the (R)-enantiomer block iLVA and iHVA in a concentration-, voltage- and frequency-dependent manner.

When lubeluzole or the (R)-enantiomer was applied, rHVA decreased in two phases: a first phase, which was nearly completed in 5 min, and thereafter a phase of slow progressive decrease. The latter slow phase of decline in Ca\(^{2+}\) channel currents might imply that the effect of prolonged application of lower concentrations of these compounds might be larger than the effect detected after 5 min. The small surface-to-volume ratio of the dorsal root ganglion cells prolongs the intracellular equilibration and thus also the influx into the cell and delays equilibration of lubeluzole or the (R)-enantiomer within and close to the cell membrane. This may be reflected in the time course of the block of Ca\(^{2+}\) channels by

![Fig. 8. Influence of 10 μM lubeluzole on the Ca\(^{2+}\) channel activation curve. From a −100 mV HP, 155-msec test pulses were given ranging from −80 to +50 mV. The currents were plotted as a function of the test potential. Filled symbols: peak inward current. Empty symbols: current at the end of the 155-msec test pulse. A, Medium-sized DRG cell (40 μm) with a large iLVA, activated already from −70 mV. B, Small DRG cell (25 μm) not expressing iLVA.](image)

![Fig. 9. Voltage dependence of the block of iHVA by lubeluzole. The 50-msec test pulse to −20 mV was preceded by a 10-sec prepulse to −100 mV or −60 mV. For protocol, see text. The block of iHVA peak by lubeluzole was more pronounced when the 10-sec conditioning prepulse was −60 mV than when it was −100 mV. The tail currents on repolarization to the conditioning potential are truncated in the plots.](image)
these compounds. The slow onset of block of iLVA and iHVA, elicited every 30 sec, seems not to be due to a use-dependency of the block because application of these compounds over 5 min in the absence of stimulation produced nearly the steady state block from the first stimulation after the 5-min rest period. Consequently, the block by lubeluzole or the (R)-enantiomer has a large tonic component.

The observation that lubeluzole and the (R)-enantiomer do not accelerate the inactivation of iLVA suggests that these compounds do not exert an open channel block on iLVA at the 250 mV test potential used.

These compounds not only decrease the amplitude of iLVA at its maximal availability (conditioning potential – 120 mV) but also produce a shift of the inactivation curve in the negative direction. This negative shift causes an additional decrease in iLVA at less negative membrane potentials and means that the percentage decrease in iLVA caused by these compounds is larger when the cell is more depolarized. The influence on iLVA is thus voltage dependent. In addition, these compounds also clearly slow down the recovery from inactivation of iLVA. Consequently, they inhibit iLVA more potently as the frequency of stimulation increases. The negative shift of the inactivation curve of iLVA and the slowing down of the recovery from inactivation caused by lubeluzole and its (R)-enantiomer show that they make the inactivated state of the T channel more probable, possibly by binding preferably to the inactivated state (Sanguinetti and Kass, 1984).

There also was some voltage dependence of the effect of lubeluzole and the (R)-enantiomer on iHVA, in that both
compounds inhibited iHVA more when the conditioning potential was −60 mV than when it was −100 mV. The voltage dependence was not tested at a more depolarized conditioning voltage because iHVA is elicited above −50 mV, which would result in a continuous influx of Ba$$^{2+}$$ into the cell during the preceding conditioning pulse.

Lubeluzole and the (R)-enantiomer accelerated the apparent inactivation of iHVA (decay of iHVA during a test pulse), resulting in a stronger inhibition of iHVAend than of iHVA-peak. This reduced the peak amplitude of iHVA elicited by a second test pulse when the interval between the two test pulses was short, in the presence of these compounds. After a longer interval, the peak iHVA with the second test pulse approached the peak iHVA obtained with the first test pulse. It took a few seconds (after repolarization to HP) for the amplitude to recover from the apparent inactivation of iHVA induced by these compounds. This behavior therefore induced some frequency dependence of the drug effect on iHVA, the drug effect having been greater at higher frequencies of stimulation. The acceleration of the apparent inactivation of iHVA by these compounds is probably due to an open channel block of HVA Ca$$^{2+}$$ channels because this acceleration was more pronounced at test potentials at which iHVA was more activated.

At the highest concentrations of 3 and 10 μM, lubeluzole accelerated the decay of iHVA significantly more than the (R)-enantiomer. This was the only clear difference in effect observed with these compounds. We found no difference in effect on iHVA at the lower concentrations of 0.1 and 0.3 μM, probably because then the acceleration of iHVA by these compounds was small.

It has been reported that iHVA of small DRG cells consists of ~30% N-type Ca$$^{2+}$$ channel current, ~53% L current and ~18% another type and that iHVA of medium-sized DRG-cells consists of ~36% N-current, ~7% L current and ~58% of another Ca$$^{2+}$$ current (Scroggs and Fox, 1992), including the P current (Mintz et al., 1992; Diochot et al., 1995). In DRG cells of embryonic mice, iHVA is composed of L-, N-, P-, Q- and possibly also R-type Ca$$^{2+}$$ channel currents (Diochot et al., 1995). The fact that 10 μM lubeluzole blocked iHVAend nearly completely at −20 mV and more positive test potentials, in small and medium-sized DRG cells, suggests that it blocks all or most high-voltage-activated Ca$$^{2+}$$ channel currents contributing to iHVA in DRG cells. However, the fact that these compounds block ILVA more potently than the total iHVA shows that these compounds display some selectivity in their inhibition of Ca$$^{2+}$$ channels.

That lubeluzole and the (R)-enantiomer also block N- and P-type HVA Ca$$^{2+}$$ channels is supported by our observation that 3 μM of these compounds clearly blocked iHVA in rat superior cervical ganglion cells, in which mainly the N-channel contributes to iHVA (Boland et al., 1994; Plummer et al., 1989), and in rat cerebellar Purkinje cells, expressing mainly the P-channel (Mintz et al., 1992).

That lubeluzole and the (R)-enantiomer also block the TTX-sensitive Na$$^{+}$$ current in isolated hippocampal cells with IC$$\text{so}_{50}$$_{2}$ values of 3.1 and 1.6 μM, respectively (Osikowska-Evers et al., 1995) and protect against veratridine-induced Na$$^{+}$$ over-

Fig. 11. Influence of 3 μM lubeluzole and the (R)-enantiomer on Ca$$^{2+}$$ channel currents in a superior cervical ganglion cell (A and B) and cerebellar Purkinje neuron (C and D). A and C, Ba$$^{2+}$$ currents elicited by the pulse sequence shown. The numbers between parentheses refer to the time point at which the sweeps were recorded (see B and D). B and D, Time course of the drug effects on the Ba$$^{2+}$$ currents measured.
load and neurotoxicity in hippocampal slices (IC_{50} = 0.54 and 0.69 \mu M, Ashton et al., 1997). Inhibition of Na^+ currents can have neuroprotective effects (Taylor and Meldrum, 1995; Urenjak and Obrenovitch, 1996). However, the effects of lubeluzole on Na^+ channels were not stereospecific, in contrast to the neuroprotective effect in the photochemical stroke model (Scheller et al., 1995; De Ryck et al., 1996; Buchkremer-Ratzmann and Witte, 1997).

Partial block of VSCC could also be neuroprotective after ischemia. It has been suggested that block of iLVA could reduce neuronal firing frequency (Akaike, 1991; Huguenard, 1996). Block of several types of HVA Ca^{2+} channels, such as the N-, P- and Q-types, can reduce neurotransmission (Gaur et al., 1994, Reuter, 1996) and glutamate release. This can lead to a reduced activation of NMDA, AMPA and kainate receptors and hereby diminish Ca^{2+} influx and intracellular Ca^{2+} overload, which is thought to play an important role in ischemic neuronal damage (Choi, 1995; Kristian and Siesjo, 1996). A temporary reduction in neurotransmission can also reduce the need for restorative ion pumping and thus be energy saving in a cerebral region at risk as a consequence of a stroke. Hence, the ability of lubeluzole to block VSCC might in principle contribute to its protective effect in stroke. Although the concentrations at which lubeluzole blocked Ca^{2+} channels in these voltage-clamp experiments were rather high compared with the effective plasma concentrations in the rat stroke model (0.23 \mu M, of which 1% is unbound) (De Ryck et al., 1996), lubeluzole could have a more potent effect on VSCC than might be expected from the IC_{50} values for inhibition of iLVA and iHVA. Indeed, its effect appeared not to be complete within 5 min of application. The IC_{50} values were also determined at a −100 mV HP and at a very low frequency of 0.033 Hz (to slow down the run-down of the Ca^{2+} channel currents). The compounds inhibited iLVA and iHVA more potently at a more depolarized conditioning potential, which may be relevant for pathological situations in which the cells are more depolarized. The observed frequency dependence of the effect of lubeluzole and the (R)-enantiomer on iLVA and iHVA indicates that the inhibition is also more potent at higher frequencies of stimulation. The fact that lubeluzole accelerated the apparent inactivation of iHVA is also interesting because it means that lubeluzole blocks iHVA especially during long depolarizations, which may be the situation in cells at risk after ischemia.

In contrast to the photochemical stroke model (Scheller et al., 1995; De Ryck et al., 1996; Buchkremer-Ratzmann and Witte, 1997), in which lubeluzole is protective and the (R)-enantiomer is virtually inactive, we found a much smaller difference in effect on VSCC between the two enantiomers, mainly in the acceleration of the decay of iHVA, and only at the highest concentrations of 3 and 10 \mu M. On the other hand, stereospecificity has been found in some in vitro models of neuroprotection (Benjamin et al., 1996; Lesage et al., 1996; Maiese et al., 1997).

In conclusion, inhibition by lubeluzole of VSCC may, in principle, contribute to the neuroprotective effect via a reduction in intracellular Ca^{2+} overload. However, the small difference in effect on VSCC between the two enantiomers in DRG cells does not explain the clear stereospecificity of the neuroprotection in the photochemical stroke model.

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Send reprint requests to: Dr. R. Marrannes, Department of Neuropsychopharmacology, Janssen Research Foundation, B-2340 Beerse, Belgium.