British Journal of Pharmacology (1997) 122, 275–285

Differential effects of the neuroprotectant lubeluzole on bovine and mouse chromaffin cell calcium channel subtypes

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1 The effects of lubeluzole (a neuroprotective benzothiazole derivative) and its (−) enantiomer R91154 on whole-cell currents through Ca^{2+} channels, with 10 mM Ba^{2+} as charge carrier (I_{Ba}), have been studied in bovine and mouse voltage-clamped adrenal chromaffin cells. Currents generated by applying 50 ms depolarizing test pulses to 0 mV, from a holding potential of −80 mV, at 10 s intervals had an average magnitude of 1 nA.

2 Lubeluzole and R91154 blocked the peak I_{Ba} of bovine chromaffin cells in a time and concentration-dependent manner; their IC_{50}s were 1.94 μM for lubeluzole and 2.54 μM for R91154. In a current-voltage protocol, lubeluzole (3 μM) inhibited peak I_{Ba} at all test potentials. However, no obvious shifts of the *I*-V curve were detected.

3 After 10 min exposure to 3 μM lubeluzole, the late current (measured at the end of the pulse) was inhibited more than the peak current. Upon wash out of the drug, the inactivation reversed first and then the peak current recovered.

4 Blockade of peak current was greater at more depolarizing holding potentials (i.e. 35% at −110 mV and 87% at −50 mV, after 10 min superfusion with lubeluzole). Inactivation of the current was pronounced at −110 mV, decreased at −80 mV and did not occur at −50 mV.

5 Intracellular dialysis of bovine voltage-clamped chromaffin cells with 3 μM lubeluzole caused neither blockade nor inactivation of I_{Ba}. The external application of 3 μM lubeluzole to those dialysed cells produced inhibition as well as inactivation of I_{Ba}.

6 The effects of lubeluzole (3 μM) on I_{Ba} in mouse chromaffin cells were similar to those in bovine chromaffin cells. At −80 mV holding potential, a pronounced inactivation of the current led to greater blockade of the late I_{Ba} (66%) as compared with peak I_{Ba} (46% after 10 min superfusion with lubeluzole).

7 In mouse chromaffin cells approximately half of the whole-cell I_{Ba} was sensitive to 3 μM nifedipine (L-type Ca^{2+} channels) and the other half to 3 μM ω-conotoxin MVIIC (non-L-type Ca^{2+} channels). In ω-conotoxin MVIIC-treated cells, 3 μM lubeluzole caused little blockade and inactivation of I_{Ba}. However in nifedipine-treated cells, lubeluzole caused a pronounced blockade and inactivation of I_{Ba} that reversed upon wash out of the compound.

8 The results are compatible with the idea that lubeluzole preferentially blocks non-L-types of voltage-dependent Ca^{2+} channels expressed by bovine and mouse chromaffin cells. The higher concentrations of the compound also block L-type Ca^{2+} channels. The mechanism of inhibition involves the access of lubeluzole to the open channel from the outside of the cell and promotion of its inactivation. The differential blockade of Ca^{2+} channel subtypes might contribute to the neuroprotective actions of lubeluzole (which exhibit stereoselectivity). However, in view of the lack of stereoselectivity in blocking Ca^{2+} channels, this effect cannot be the only explanation for the protective activity of lubeluzole in stroke.

Keywords: Lubeluzole; R91154; calcium channel; chromaffin cell

Introduction

Lubeluzole (Figure 1) is the (+)**S**-isomer of a benzothiazole derivative that stereospecifically improves the neurological outcome and reduces infarct volume of photochemically-induced thrombotic cerebral infarcts in rats (De Ryck *et al*., 1996; De Ryck, 1997). In this model lubeluzole also prevents in a stereospecific manner, the increase of extracellular glutamate concentrations (Scheller *et al*., 1995) and normalizes neuronal excitability in the peri-infarct region (Buchkremer-Ratzmann & Witte, 1995). The compound affords protection against the glutamate-induced nitric oxide related toxicity in hippocampal neurones (Lesage *et al*., 1996), as well as against the toxicity of veratridine in cardiomyocytes (M. Borgers; personal communication) and hippocampal neurones (Pauwels *et al*., 1991). These neuroprotecting effects have been recently corroborated in a clinical trial in patients suffering acute ischaemic stroke (Diener *et al*., 1996).

Na^+ channel modulation could contribute to the neuroprotective properties of lubeluzole in the peri-infarct zone (Ashton *et al*., 1997). However, some of the effects of lubeluzole on glutamate release could also be due to blockade of the subtypes of high-threshold voltage-dependent Ca^{2+} channels described in neurones, that control the access of Ca^{2+} to the secretory machinery (Olivera *et al*., 1994). Therefore, we decided to study the effects of lubeluzole and its (−)enantiomer R91154 on the whole-cell currents through neuronal Ca^{2+} channels. The bovine adrenal medulla chromaffin cell served as a good model to conduct this study, because it expresses L-, N, P- and Q-subtypes of neuronal Ca^{2+} channels (Gandía *et al*., 1993; Albillos *et al*., 1993; 1996) the role of which in controlling catecholamine release is well defined (López *et al*., 1994; Lomax *et al*., 1997). In addition, the effects of lubeluzole on mouse adrenal chromaffin cells were investigated; this cell type

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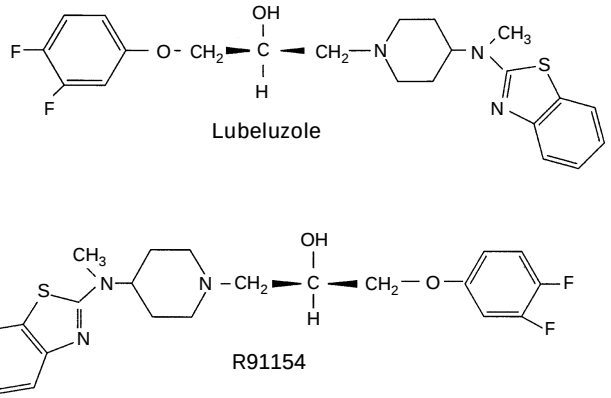

Figure 1 Molecular structure of lubeluzole ((+)-(**S**)-4-(2-benzothiazolyl-methyl-amino)-1-piperidineethanol) and of its (−)**R**-isomer R91154.

expresses different proportions of Ca^{2+} channels from the bovine cells, thus serving to detect more adequately any preference of lubeluzole for a given channel type.

Methods

Preparation and culture of bovine chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated following standard methods (Livet, 1984) with some modifications (Moro *et al*., 1990). After isolation, cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 iu ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were plated on 1 cm diameter glass coverslips at a density of 5×10^4 cells per coverslip. Cells were used 1–4 days after plating.

Isolation and culture of mouse adrenal medulla chromaffin cells

To prepare each cell batch we used 3–5 adult mice, killed by cervical dislocation. The abdomen was opened and the adrenal glands exposed. The adrenal glands were quickly removed and decapsulated and their adrenal medullae isolated under a stereoscope. They were placed in Ca^{2+}- and Mg^{2+}-free Locke buffer of the following composition (in mM): NaCl 154, KCl 3.6, $NaHCO_3$ 5.6, glucose 5.6 and HEPES 10 (pH 7.2) at room temperature. Tissues were collected under sterile conditions. Medullae digestion was achieved by incubating the pieces in 6 ml of a solution containing 8 mg collagenase, 18 mg bovine serum albumin, in Ca^{2+} and Mg^{2+}-free Locke buffer, for 40 min, at 37°C; gentle agitation was applied at 10–20 min intervals by using a plastic Pasteur pipette. The collagenase was washed out of the cells with large volumes of Ca^{2+}- and Mg^{2+}-free Locke buffer. The cell suspension was then filtered through a 80 μm nylon mesh and centrifuged at 120 × *g* for 10 min. After washing 2 times, the cells were resuspended in 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum containing 50 iu ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were plated on circular glass coverslips. After 30 min, 1 ml of DMEM was added to each well. Cells were then incubated at 37°C in a water saturated, 5% CO_2 atmosphere; they were used within 1–3 days after plating.

Measurements of whole-cell currents through Ca^{2+} channels

Membrane currents were measured by means of the whole-cell configuration of the patch-clamp technique (Hamill *et al*.,

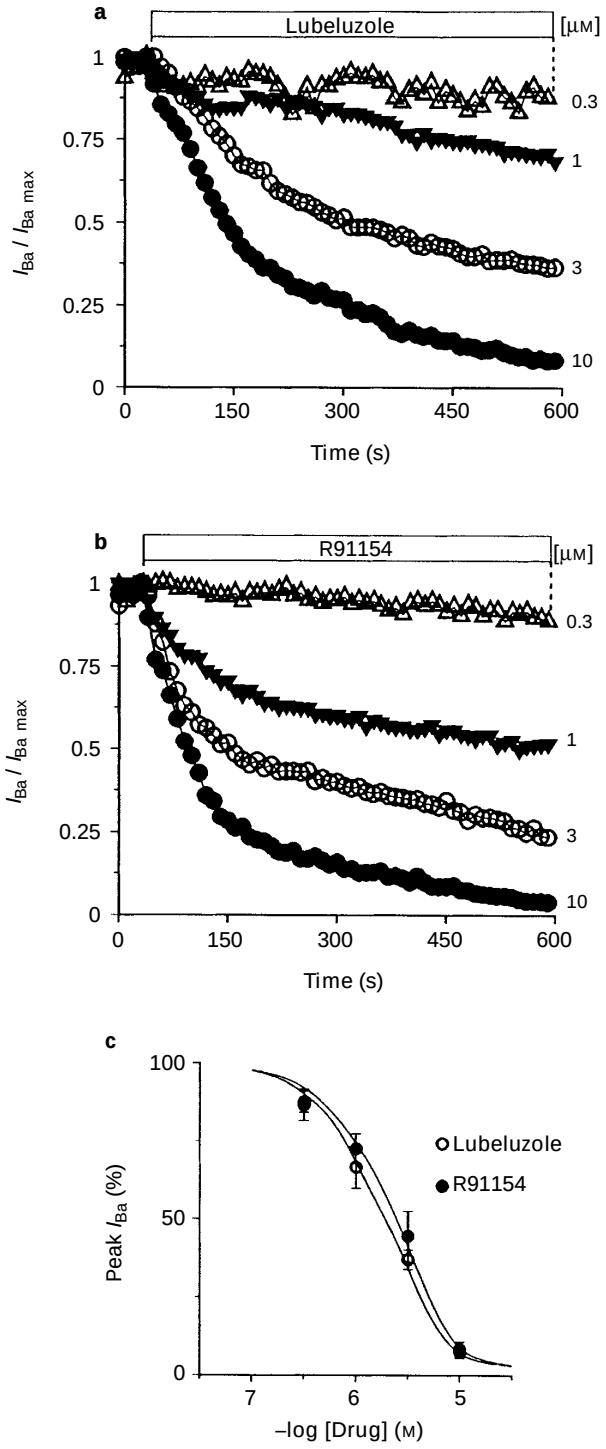

Figure 2 Time course of the inhibition by lubeluzole (a) or R91154 (b) of the whole-cell inward Ba^{2+} current (I_{Ba}). After the cytosol of a bovine voltage-clamped chromaffin cell had been penetrated, 50 ms depolarizing test pulses to 0 mV from a holding potential of −80 mV, were applied at 10 s intervals (abscissae, time in seconds). Data points represent the maximum peak current initially obtained, with 10 mM Ba^{2+} as charge carrier (see Methods). This initial current (I_{Ba} max) ranged from 500 to 1,500 pA in different cells. Once I_{Ba} stabilized, cells were continuously superfused for a 10 min period (top horizontal bars) with the concentrations of drugs shown at the right of each curve. A separate cell was used for each individual drug concentration. (c) Averaged results of the % of current remaining (ordinate scale) after 10 min superfusion with each drug concentration (abscissa scale). IC_{50} was 1.94 μM for lubeluzole and 2.54 μM for R91154. Results are means of 8–12 cells for each drug concentration; vertical lines show s.e.mean.

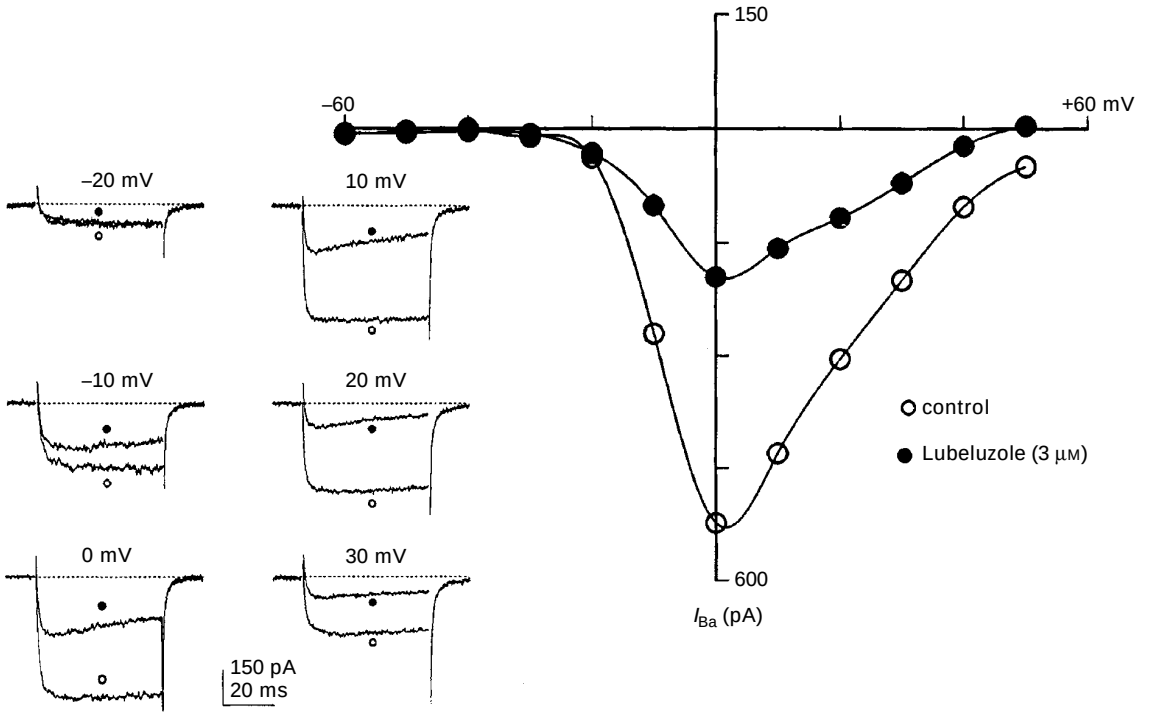

Figure 3 Voltage-current curves obtained before and 5 min after superfusing a bovine chromaffin cell with 3 μM lubeluzole. A patch-clamped cell (holding potential -80 mV) was stimulated with 50 ms test depolarizing pulses at the indicated voltages (abscissa scale); the currents generated are plotted on the ordinate scale. The insets show original current traces obtained at different test potentials. Observe (1st) that no obvious shifts of the *I*-V curve were caused by lubeluzole; and (2nd) that a clear current inactivation was produced in the presence of the drug (traces labelled with solid circles in the insets).

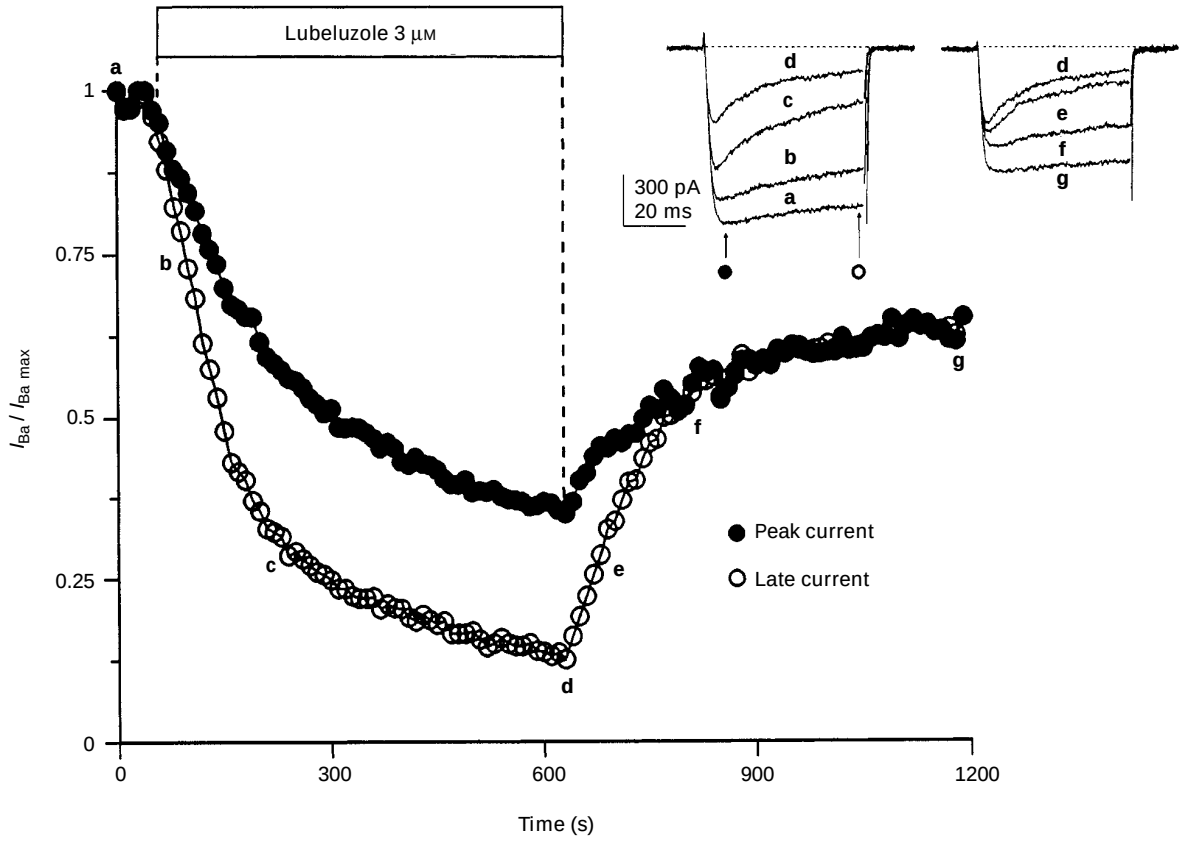

Figure 4 Effects of lubeluzole on the peak and the late Ba^{2+} currents (I_{Ba}), of bovine chromaffin cells. Peak I_{Ba} was measured at the maximum current peak in each trace (solid circle at arrow in the bottom of current traces shown in the inset at the top right part of the figure); the late current was measured at the end of the depolarizing test pulse (open circle at arrow at the inset current trace). In this experiment, a voltage-clamped cell was stimulated with 50 ms test pulses to 0 mV, from a holding potential of -80 mV, given at 10 s intervals. After the cell had been penetrated and the initial current stabilized, it was superfused with lubeluzole as shown by the top horizontal bar. Then, another 10 min superfusion with a drug-free extracellular solution followed. Insets show original I_{Ba} traces obtained in the presence of lubeluzole (blockade) or after washing out the compound, as shown by the letters.

1981). Coverslips containing the cells were placed in an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused at room temperature (22–25°C) with a control Tyrode solution containing (in mM): NaCl 137, $MgCl_2$ 1, $CaCl_2$ 2, HEPES/NaOH 10, pH 7.4. For current recordings, 2 or 10 mM Ba^{2+} (instead of 2 mM Ca^{2+}) was used as the charge carrier, and 5 μM tetrodotoxin (TTX) was added to suppress Na^+ currents. Cells were internally dialysed with a solution containing (in mM): NaCl 10, CsCl 100, TEA.Cl 20, Mg.ATP 5, EGTA 14, HEPES/CsOH 20, Na.GTP 0.3, pH 7.2.

Whole-cell recordings were made with fire-polished glass electrodes (resistance 2–5 MΩ) mounted on the headstage of a DAGAN 8900 patch-clamp amplifier, allowing cancellation of capacitative transients and compensation of series resistance. A Labmaster data acquisition and analysis board and an IBM-compatible computer with pCLAMP software (Axon Instruments Inc., CA, U.S.A.) were used to acquire and analyse the data.

Cells were clamped at −80 mV holding potential (HP). Step depolarizations to 0 mV from this HP lasted 50 ms and were applied at 0.1 Hz to minimize the 'run-down' of Ca^{2+} currents (Fenwick *et al*., 1982). Cells with pronounced rundowns were discarded. Leak and capacitative currents were subtracted by using currents elicited by small hyperpolarizing pulses.

External solutions were exchanged by a fast superfusion device consisting of a modified multi-barrelled pipette, the common outlet of which was positioned 50–100 μm from the cell. Control and test solutions were changed with miniature solenoid valves operated manually (The Lee Company, Westbrook, CT, U.S.A.). The flow rate (0.5–1 ml min^{-1}) was regulated by gravity to achieve a complete replacement of cell surroundings in less than 1 s.

Materials and solutions

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Mannheim); bovine serum albumin fraction V, cytosine arabinoside, fluorodeoxyuridine

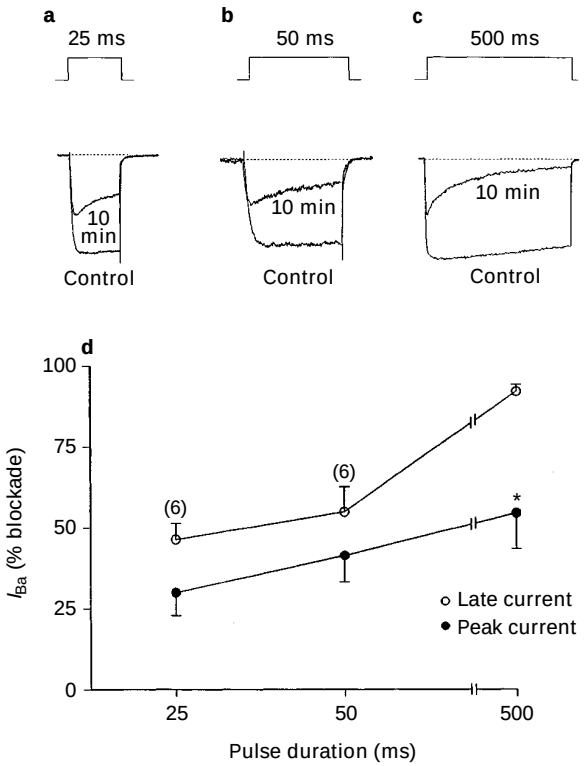

Figure 5 Inhibition of I_{Ba} by lubeluzole increased with the length of the depolarizing pulses. (a), (b) and (c) Original I_{Ba} traces taken from three separate voltage-clamped bovine chromaffin cells. Test depolarizing pulses to 0 mV, from a holding potential of −80 mV were applied at 60 s intervals; the duration of the pulses was 25 ms (a), 50 ms (b) or 500 ms (c). (d) The relationship between the length of the depolarizing stimuli (abscissa) and the degree of inhibition of the peak current or the late current, after 10 min of superfusion with 3 μM lubeluzole. Data are means of 6 cells (25 and 50 ms) and 4 cells (500 ms); vertical lines show s.e.mean. $^*P<0.01$ with respect to the late current at the same pulse duration.

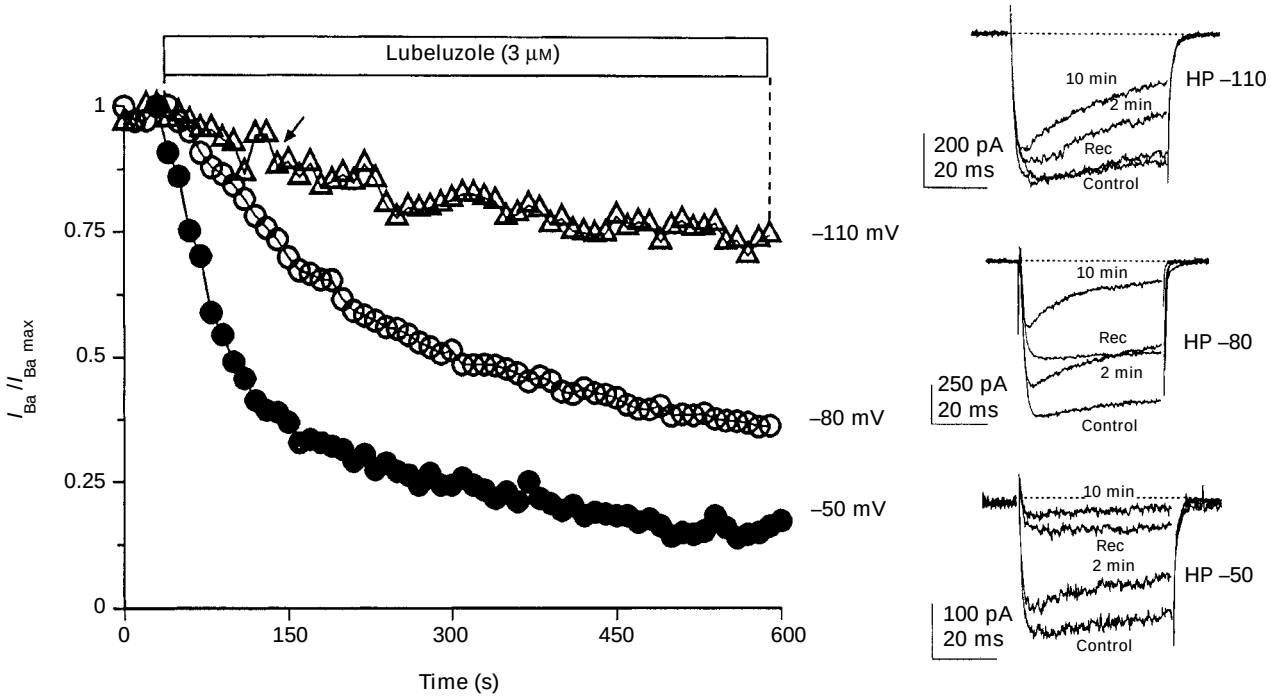

Figure 6 Blockade by lubeluzole of I_{Ba} at different holding potentials. The time course of I_{Ba} inhibition by lubeluzole (3 μM) for three different cells is shown. Three separate bovine chromaffin cells were voltage-clamped at holding potentials of −110 mV (open triangles), −80 mV (open circles), or −50 mV (solid circles). From these different potentials, test depolarizing pulses to 0 mV were applied at 10 s intervals. Once the initial I_{Ba} stabilized, lubeluzole (3 μM) was applied for a 10 min period, as shown by the top horizontal bar. In each case, I_{Ba} was normalized as a fraction of the initial current (ordinates). Insets show original current traces before (control), during and after wash out of lubeluzole.

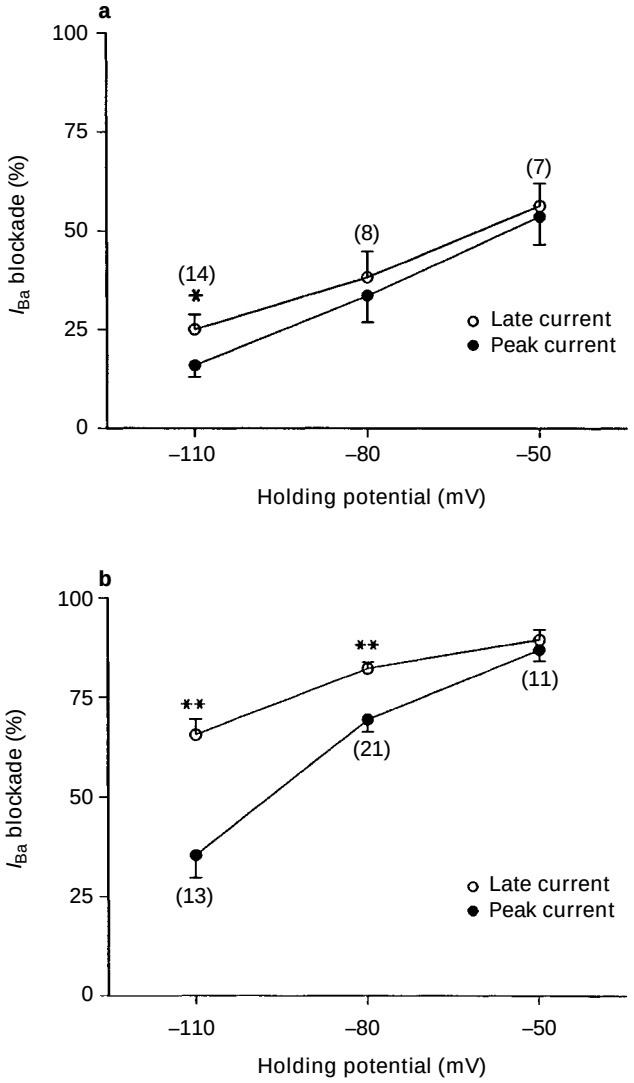

Figure 7 Blockade by lubeluzole of I_{Ba}, as a function of the holding potential of bovine voltage-clamped chromaffin cells. These averaged data were obtained from experiments with protocols the same as those described in Figure 6 legend, either in the presence of 1 μM (a) or 3 μM lubeluzole (b). Peak and late currents before and after 10 min of lubeluzole exposure were measured as shown in Figure 4. Data are means of the number of cells shown in parentheses, vertical lines show s.e.mean. $*P<0.01$ and $**P<0.001$ with respect to peak current at the same holding potential.

and EGTA (Sigma); DMEM, foetal calf serum, penicillin and streptomycin (GIBCO); tetrodotoxin (Calbiochem). Lubeluzole ((+)-(**S**)-4-(2-benzothiazolyl-methylamino)-[(3,4-difluorophenoxy)-methyl]-1-piperidineethanol) and its (−)**R**-isomer R91154 were kind gifts from the Janssen Research Foundation (Beerse, Belgium). All other chemicals were reagent grade.

Lubeluzole and R91154 were dissolved in dimethylsulphoxide (DMSO, Merck) at 10^{-2} M and diluted in saline solutions to the desired concentrations. At the highest concentrations used (no more than 0.1%), DMSO had no significant effects on I_{Ba}.

Statistical analysis

Data are expressed as means ± s.e.mean. IC_{50} values were estimated through non-linear regression analysis, by ISI software for a PC computer. Differences between non-paired groups were compared by Student's *t* test; a value of *P* equal or smaller than 0.05 was taken as the limit of statistical significance.

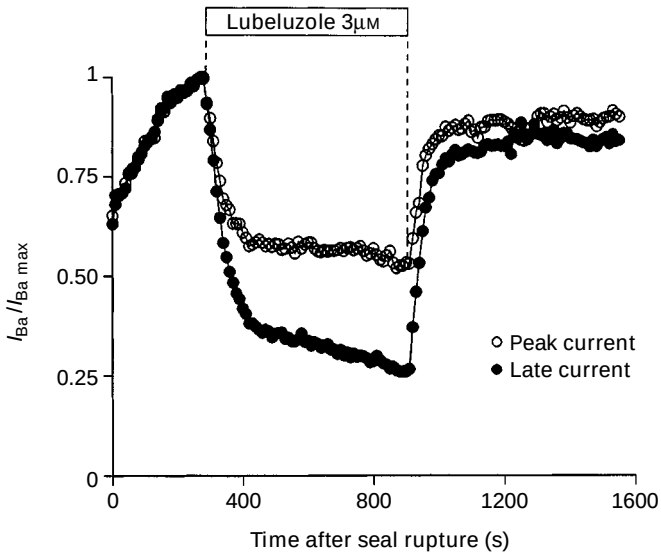

Figure 8 Effects of lubeluzole on I_{Ba} when given intra- or extracellularly. The bovine chromaffin cell of this experiment was voltage-clamped at −80 mV; Ba^{2+} currents were elicited at 10 s intervals through the application of 50 ms test pulses to 0 mV. The patch pipette intracellular solution contained 3 μM lubeluzole. Currents were recorded immediately after rupturing the seal (time 0). External lubeluzole was applied during the time period shown by the top horizontal bar. Time courses for both peak current and late current are shown.

Results

Time and concentration-dependent blockade of I_{Ba} *by lubeluzole and R91154*

In the experiments of Figure 2 each individual voltage-clamped cell tested was stimulated intermittently with 50 ms depolarizing pulses to 0 mV, applied at 10 s intervals from a holding potential of −80 mV. Peak inward I_{Ba} current elicited with each test pulse with 10 mM Ba^{2+} as charge carrier, varied between 500 and 1500 pA between the different cells tested. In 110 cells the averaged initial current (I_{Ba} max) amounted to 963 ± 40 pA. This current suffered no appreciable decline during a 10 min testing period; if a tendency to decline was observed, the cell was discarded. Once the initial current stabilized, each cell was superfused with a single concentration of lubeluzole or R91154 for a 10 min period. This protocol was selected in the light of information provided by initial experiments showing a slowly developing inhibition of I_{Ba}. This behaviour of the compounds precluded doing cumulative concentration-response curves in the same cell. To diminish variations between data the current was normalized to 1, once I_{Ba} had stabilized at the beginning of the recording from each individual cell ($I_{Ba}/I_{Ba\ max}$).

Figure 2 shows the time courses of I_{Ba} inhibition by four concentrations of lubeluzole (a) and R91154 (b). The higher the concentration the greater the blockade, which always developed slowly. In fact, even at 10 μM it took about 60 s to reach 50% blockade. This sharply contrasts with inhibition of I_{Ba} subcomponents by 1,4-dihydropyridines or by ω-conotoxin GVIA, that occurs abruptly only 10 s after cell superfusion with the compounds (Gandía *et al*., 1993; Albillos *et al*., 1993; 1996). However, the data agree with the slow blockade of I_{Ba} induced by the wide-spectrum channel blocker ω-conotoxin MVIIC, in high $[Ba^{2+}]_o$ (10 mM) (Albillos *et al*., 1996).

Figure 2c shows the concentration-response curves for lubeluzole and R91154 on peak I_{Ba}. Inhibition of I_{Ba} was measured in each individual cell at the end of the 10 min superfusion period with each drug concentration. The curves for both compounds overlapped and IC_{50} values were 1.94 μM

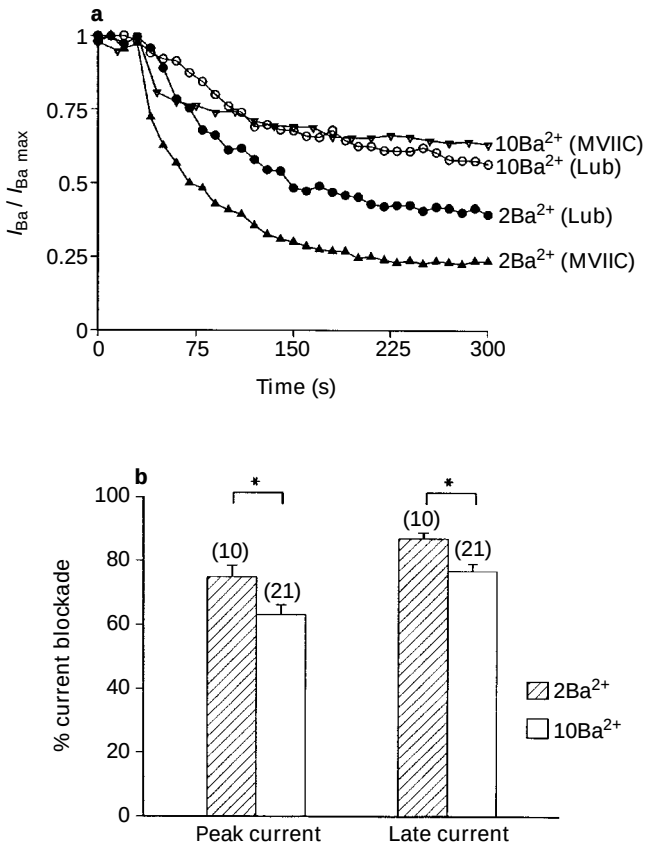

Figure 9 Blocking effects of lubeluzole on Ca^{2+} channel currents, in low and high Ba^{2+} concentrations. Bovine chromaffin cells were voltage-clamped at -80 mV, and I_{Ba} was elicited by 50 ms depolarizing pulses to 0 mV, given at 10 s intervals in the presence of 2 mM Ba^{2+} (solid circles) or 10 mM Ba^{2+} (open circles). Lubeluzole (3 μM) was superfused during the time period shown by the top horizontal bar in (a). For the sake of comparison, the time course of blockade of I_{Ba} by ω-conotoxin MVIIC in 10 mM Ba^{2+} (open triangles) and 2 mM Ba^{2+} (solid triangles), is shown (taken from Albillos *et al.*, 1996). (b) Averaged results on the fraction of peak current blocked by lubeluzole (after 10 min superfusion) in low (2 mM) and high Ba^{2+} concentration (10 mM). Data are means $\pm$ s.e.mean of the number of cells shown in parentheses. $^*P<0.05$, with respect to 2 mM Ba^{2+}.

for lubeluzole and 2.54 μM for R91154; 0.3 μM seemed to be the threshold blocking concentration and 10 μM the maximum blocking concentration. Thus, the inhibition curves were quite steep.

Effects of lubeluzole on the current-voltage relationship

A voltage-clamped bovine chromaffin cell was stimulated with depolarizing pulses of increasing strength given at 10 s intervals, before and 5 min after superfusion with 3 μM lubeluzole, with 10 mM Ba^{2+} as charge carrier. Before lubeluzole, peak I_{Ba} presented a threshold of activation at around -30 mV, peaked at 0 mV and presented a reversal potential near $+60$ mV (Figure 3). In the presence of lubeluzole no shifts of the *I*-V curve were observed. Lubeluzole inhibited I_{Ba} at all potentials tested (see original traces in the insets to Figure 3). No modifications of current activation and deactivation kinetics were observed. However, during the 50 ms depolarizing pulses, a clear inactivation of the current appeared.

Effects of lubeluzole on the peak and the late currents

The experiment shown in Figure 4 provides additional interesting features on the effects of lubeluzole on I_{Ba}. A voltage-clamped cell (HP -80 mV) was depolarized with 50 ms voltage-steps to 0 mV applied at 10 s intervals, while being superfused continuously with an external solution containing 10 mM Ba^{2+}. The introduction of lubeluzole (3 μM) into the superfusion fluid caused a gradual decline of peak I_{Ba} (65% blockade after 10 min). Initially, the amplitude of the current decreased, showing no signs of inactivation (trace b in the inset to Figure 4). However, after 30–60 s the current tended to inactivate more and more (traces c and d in the inset to Figure 4). So, when the time courses of the peak current and the late current (at the end of the trace) versus time were plotted, the blockade of the late current by lubeluzole was more pronounced than the blockade of the initial peak current.

A second interesting feature was observed on following the recovery of current upon lubeluzole wash out. Recovery of the late current seemed to be more rapid than that of the peak current (traces e, f, g in the inset to Figure 4). Because blockade of the late current was greater than that of peak current, the fraction of current recovered after washout was greater. However, at the end of the washout period both, peak and late current reached a similar amplitude of about 60% of the initial I_{Ba} (in the cell of Figure 4, initial I_{Ba} was around 1000 pA). This incomplete recovery might be due to washout of the current after such a long stimulation period (about 25 min), and/or to a persistent residual blockade of I_{Ba} by lubeluzole.

Since inactivation of the current was time-dependent and extended along the 50 ms depolarizing pulse (Figure 4, inset), it was thought that the magnitude of current inactivation in the presence of lubeluzole (3 μM) depends on the duration of the test depolarizing pulse. Figure 5 shows that I_{Ba} inactivated little when using 25 ms pulses, and that inactivation progressed with increasing pulse length (original traces in a, b and c). Figure 5d shows averaged results from several cells. Blockade of peak current rose from 30% at 25 ms pulses, to 42% at 50 ms pulses, and 55% at 500 ms pulses. Inhibition of the late current was significantly higher at all pulses tested, 46% at 25 ms, 55% at 50 ms, and 92% at 500 ms. It is worth noting that while lubeluzole blocked the peak current around half with 500 ms pulses, the late current was nearly fully inhibited.

Inhibition of I_{Ba} by lubeluzole at various holding potentials

The degree of I_{Ba} inhibition induced by lubeluzole was also tested at different holding potentials. Figure 6 shows the rate and extent of I_{Ba} blockade by lubeluzole in three different bovine chromaffin cells which had holding potentials fixed at -110 mV, -80 mV, or -50 mV after their cytosols had been penetrated. After stabilization in 10 mM Ba^{2+}, the application of 50 ms test pulses to 0 mV generated I_{Ba} of 915 ± 81 pA at a holding potential of -110 mV ($n=27$ cells), of 963 ± 40 pA at -80 mV ($n=110$ cells), and of 651 ± 59 pA at -50 mV holding potential ($n=24$ cells).

Lubeluzole (3 μM) caused a very slowly developing blockade of peak I_{Ba} at a holding potential of -110 mV (open triangles in Figure 6). The τ for the time course of blockade was 168 s, and the blockade of peak current amounted to only 35%. At -80 mV holding potential, lubeluzole caused a faster inhibition of I_{Ba}, with a time course that exhibited a τ of 129 s and a blockade of 65% after 10 min of superfusion. Finally, at a holding potential of -50 mV the blockade of I_{Ba} developed faster ($\tau=58$ s) and reached as much as 87%.

Another clear-cut difference between the quality of blockade of I_{Ba} by lubeluzole at different holding potentials was the different degrees of current inactivation observed. The insets to Figure 6 show original current traces obtained before, at 2 and 10 min of superfusion with lubeluzole, and 5 min after its wash out (recovery). Note the scarce blockade of peak current at -110 mV and the pronounced inactivation of I_{Ba} in the presence of lubeluzole, causing a much larger blockade of the late current. At -80 mV the peak current was inhibited more and

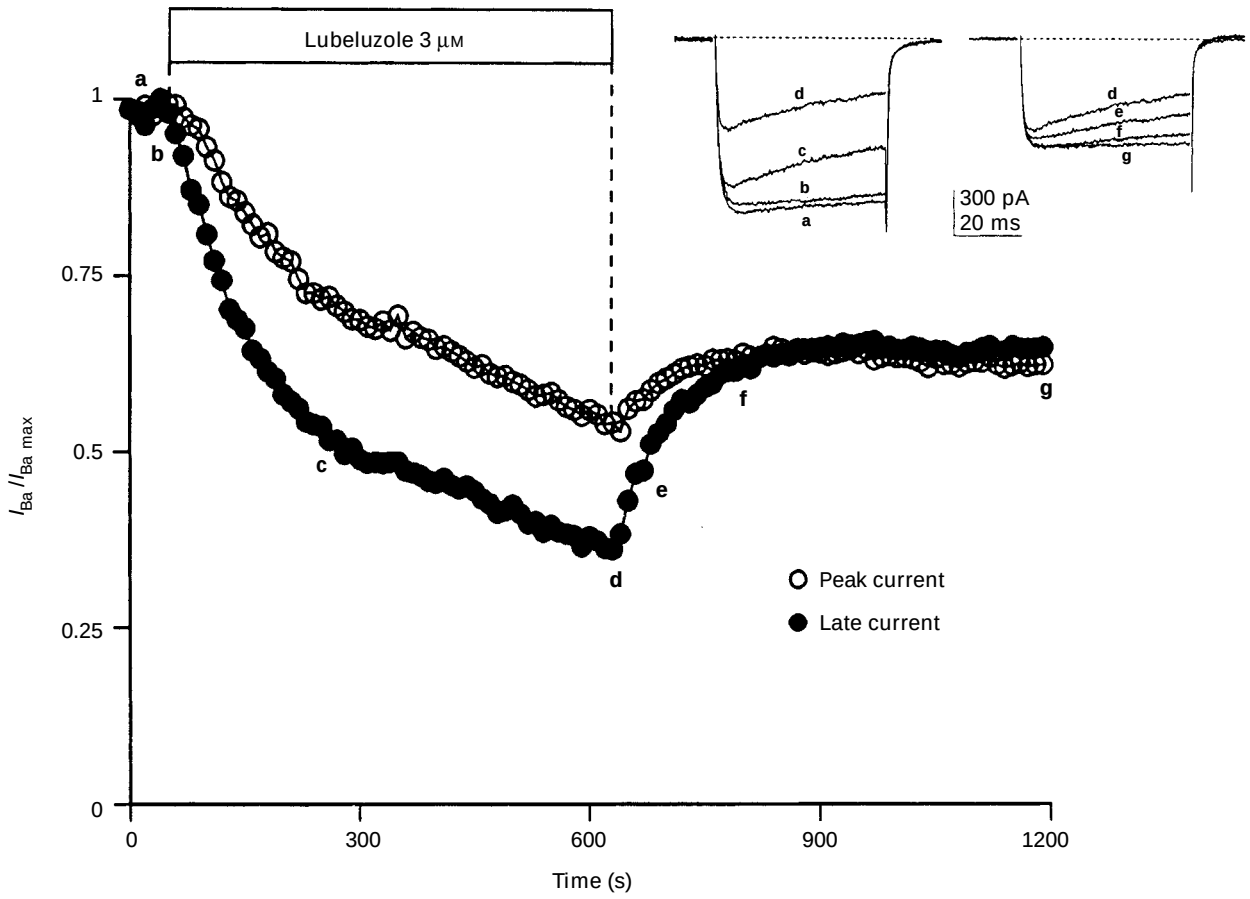

Figure 10 Effects of lubeluzole on the peak and the late Ba^{2+} currents of mouse adrenal chromaffin cells. Peak and late I_{Ba} were measured as indicated in Figure 4. In this experiment a voltage-clamped cell was stimulated with 50 ms test pulses to 0 mV, from a holding potential of −80 mV, given at 10 s intervals. Once the initial current stabilized at around 1000 pA, the cell was superfused with lubeluzole as shown on the top horizontal bar. Then, another 10 min superfusion with a drug-free extracellular solution followed. The time course of inhibition of peak current and that of the late current are shown. Insets show original I_{Ba} traces obtained in the presence of lubeluzole (blockade) or after washing out the compound, as shown by the letters.

the inactivation and blockade of the late current were concomitantly smaller. Finally, at −50 mV the fraction of peak current blocked was maximum and so, little inactivation of I_{Ba} occurred.

Figure 7 shows averaged results on the effects of 1 and 3 μM lubeluzole on peak and late currents, at the three holding potentials studied. It seems clear that as the holding potential depolarised, the blockade of I_{Ba} increased. This increase was fairly similar for peak and late currents with 1 μM lubeluzole (Figure 7a), increasing from 15% (peak) and 25% (late current) at −110 mV to 53% (peak) and 56% (late current), at −50 mV ($P<0.01$). However, with 3 μM lubeluzole (Figure 7b), inhibition of peak I_{Ba} went from around 35% at −110 mV to 87% at −50 mV ($P<0.001$). The late I_{Ba} was markedly blocked at −110 mV (about 65%) and thus, its blockade could increase only marginally at more depolarized holding potentials.

Effects of intracellularly dialysed lubeluzole on I_{Ba}

The experiment shown in Figure 8 was done to gain insight into the possible pathway by which lubeluzole blocks the Ca^{2+} channels. A bovine chromaffin cell was sealed with a patch pipette containing 3 μM lubeluzole in the intracellular solution. After the seal had been ruptured, I_{Ba} grew to reach a stable plateau. After 5 min dialysis with lubeluzole, no changes in the plateau current were observed, either in the peak or the late current. Application of external lubeluzole (3 μM) produced a typical, slowly developing blockade of I_{Ba}. Initially peak and late currents were blocked in parallel; but after 60 s the late current was inhibited substantially more, suggesting that external lubeluzole was causing gradual inactivation of the current. In 6 cells recorded with this protocol, the peak current was blocked by 55±5% and the late current by 70±3.2%. These figures are similar to those obtained in cells non dialysed with lubeluzole. After wash out of the outside drug, I_{Ba} recovered to values near to its pre-drug level (over 70%).

Effects of lubeluzole on calcium channel currents as a function of the Ba^{2+} *concentration*

These experiments were performed in an attempt to find out whether the interaction of lubeluzole with its receptor site on the Ca^{2+} channel is dependent on the concentration of the divalent cation concentration carrying the charge, as the case is for the wide-spectrum Ca^{2+} channel blocker ω-conotoxin MVIIC (Albillos *et al*., 1996).

Figure 9a shows the time course of blockade by 3 μM lubeluzole of peak I_{Ba}, in bovine chromaffin cells superfused with an extracellular solution containing 2 mM or 10 mM Ba^{2+}. Blockade developed at about the same rate in 2 mM Ba^{2+} as with 10 mM Ba^{2+}. The blockade of the peak current was greater in 2 mM Ba^{2+} compared with 10 mM Ba^{2+} (Figure 9b). It is worth mentioning that in 10 mM Ca^{2+}, the blockade of peak I_{Ca} by lubeluzole was substantially lower than in 10 mM Ba^{2+} (data not shown). For the sake of comparison, the effects of ω-conotoxin MVIIC (3 μM) on I_{Ba} are also plotted (values taken from Albillos *et al*., 1996). It is clear that the Ba^{2+} concentration affects more markedly the blocking effects of the toxin, in comparison with lubeluzole.

Effects of lubeluzole on mouse chromaffin cell Ca^{2+} channel current

In the course of a thorough study on the characteristics of its I_{Ba} currents, we observed that of the mouse adrenal medulla chromaffin cell Ca^{2+} channel population, L-type dihydropyridine-sensitive Ca^{2+} channels accounted for about 50% and non-L-type of Ca^{2+} channels the other 50% (unpublished). These proportions considerably differed from those found in bovine chromaffin cells, where L-type Ca^{2+} channels constitute only a minor fraction (15–20%) of the whole population of channels (Albillos *et al*., 1993; Gandía *et al*., 1993). It was therefore of interest to test the behaviour of lubeluzole on I_{Ba} in mouse chromaffin cells, to gain some insight on its possible preference for one or other channel subtype.

At a holding potential of −80 mV, by use of 50 ms depolarizing test pulses to 0 mV in 10 mM Ba^{2+}, the initial peak I_{Ba} amounted to 936±92 pA ($n=27$ cells). This initial current declined progressively in the presence of 3 μM lubeluzole. Figure 10 shows the time courses of peak and late I_{Ba} upon superfusion with 3 μM lubeluzole. On comparing Figures 4 and 10 it seems clear that the blocking effects of lubeluzole on mouse chromaffin cell Ca^{2+} channels are smaller than those seen in bovine chromaffin cells. However, qualitatively the picture is similar, since delayed inactivation of the current takes place also in the mouse chromaffin cell (inset to Figure 10) and consequently, greater blockade of the late current occurs. After 10 min superfusion with lubeluzole the peak current was blocked by 46±3.8% ($n=8$ cells) and the late current by 66±2.1% ($n=8$ cells). After wash out of the compound about 50% of the inhibited current recovered gradually; relatively more recovery was seen of the late I_{Ba}, as compared with peak I_{Ba}. In current-voltage experiments, lubeluzole depressed I_{Ba} equally at all test depolarizing potentials; as for the bovine cells, no shifts of *I*-V curves were seen in the presence of lubeluzole (not shown).

Effects of lubeluzole on the I_{Ba} remaining after treatment with nifedipine or ω-conotoxin MVIIC, of mouse chromaffin cells

The mouse chromaffin cell used for the experiment shown in Figure 11 was first superfused with 3 μM nifedipine, which abruptly depressed the peak I_{Ba} by 45%. When added together with nifedipine, lubeluzole (3 μM) caused a further substantial blockade of the remaining peak and late currents. Though nifedipine promoted the appearance of some current inactivation, lubeluzole accentuated such inactivation (see inset to Figure 11). Wash out of lubeluzole (still in the presence of nifedipine) produced a quick reversal of current inactivation. The final wash out of nifedipine caused a prompt recovery of peak and late currents to near their original values.

In Figure 12, the cell was first exposed to 3 μM ω-conotoxin MVIIC, which caused 55% blockade of I_{Ba} with no apparent inactivation (see inset). In the presence of the toxin, lubeluzole (3 μM) caused a further current blockade (19.4±0.8% of the total current; $n=4$ cells), with a slight inactivation. Wash out of lubeluzole gave rise to a mild recovery of the late, but not the peak current. Wash out of ω-conotoxin MVIIC produced about 15% recovery of current.

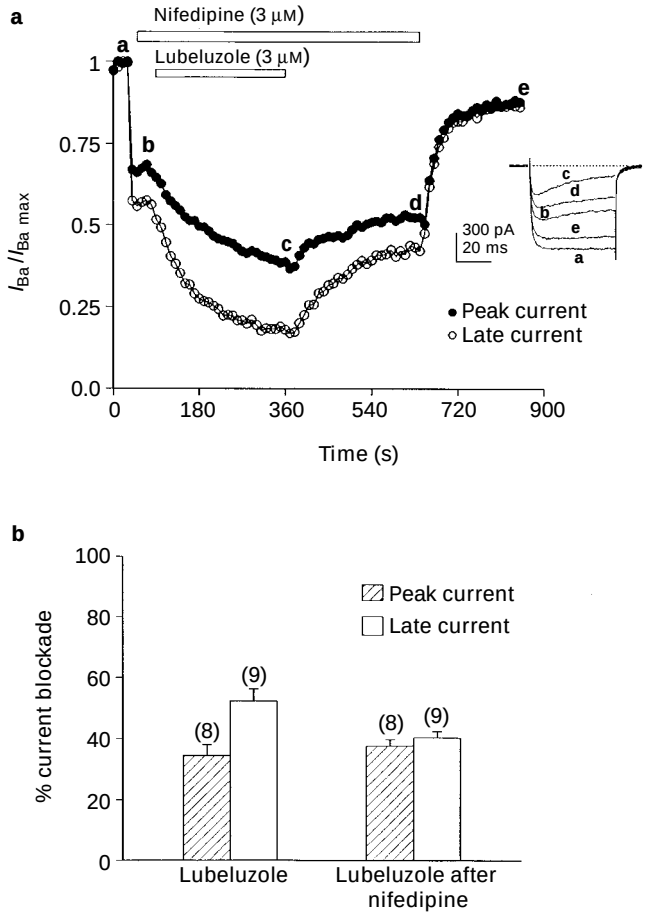

Figure 11 (a) The time course of the blockade of I_{Ba} of a mouse chromaffin cell after sequential addition of 3 μM nifedipine, and 3 μM lubeluzole (top horizontal bars). Stimulation pattern was as in Figure 10. Inset shows original current traces obtained at the times indicated by the letters. (b) Average data on the blockade of lubeluzole (3 μM) when applied alone or after pretreatment of the cells with nifedipine (3 μM). Data are means±s.e.mean of the number of cells shown in parentheses.

Discussion

In this study we have shown that lubeluzole blocks the whole cell currents through voltage-dependent Ca^{2+} channels, of bovine and mouse adrenal chromaffin cells, in a time- and concentration-dependent manner. In addition, the blocking effects of lubeluzole have the following characteristics: (i) the divalent cation concentration, and the type of cation used as a charge carrier (i.e. Ca^{2+} or Ba^{2+}) modifies the degree of current blockade; (ii) although partially, the inhibited current recovered readily upon wash out of the compound; (iii) the blockade and inactivation of the current strongly depended on the holding potential of the cell; and (iv) the compound did not block I_{Ba} when applied intracellularly. All these properties indicate a specific type of blockade by lubeluzole of Ca^{2+} channels; although the slow development of the blockade could suggest a non-specific accumulation of the drug in the plasma membrane lipid bilayer, related to the high lipophilicity of the molecule (log P=4.89), it seems unlikely to be the cause for such blockade because of the following reasons: (i) some hydrophilic peptide Ca^{2+} channel blockers (i.e. ω-conotoxin MVIIC) that block N-, P- and Q-subtypes of Ca^{2+} channels in bovine chromaffin cells also follow a slow time course (Albillos *et al*., 1996); and (ii) no effects of lubeluzole were observed when applied intracellularly.

Specificity does not apparently imply a selective action of a compound on a given Ca^{2+} channel subtype. The whole-cell current in bovine chromaffin cells is generated by a diversity of Ca^{2+} channels that have been pharmacologically separated into L-, N and P/Q subtypes (see García *et al*., 1997 for a review). Because the current could be completely blocked by 10 μM lubeluzole (Figure 2c), it seemed logical to conclude that the spectrum of Ca^{2+} channel blockade by lubeluzole is closer to that of ω-conotoxin MVIIC (N,P,Q-type Ca^{2+} channel blocker) rather than to that of ω-agatoxin IVA (P,Q-type blocker), ω-conotoxins MVIIA and GVIA (N-type blockers), or to that of 1,4-dihydropyridines (L-type blockers). Since bovine chromaffin cells express a minority of L-type Ca^{2+} channels (15–20%), it was unclear to what extent lubeluzole

could block preferentially a given subcomponent of the whole-cell current. Therefore, we had to recourse to a model expressing more L-type Ca^{2+} channels (about 50%), the mouse chromaffin cell. Here the behaviour of lubeluzole in blocking the current and causing its inactivation was similar to that seen in bovine chromaffin cells, though some selectivity was unraveled by blocking L-type or non-L-type Ca^{2+} channels with saturating concentrations of nifedipine and ω-conotoxin MVIIC. A greater blockade by lubeluzole of the current remaining in nifedipine-treated cells, as compared with toxin-treated cells, suggests a preferential action of the compound on non-L-type Ca^{2+} channels of mouse chromaffin cells. In addition, it seems that the compound blocks differentially the L-type and non-L-type of Ca^{2+} channels, since lubeluzole caused more inactivation of the latter, as compared with the former. In this sense, at appropriate concentrations, lubeluzole might be a good pharmacological tool to distinguish L- from non-L-type of channels.

Inasmuch that lubeluzole blocked more current at more depolarizing holding potentials (Figure 7), it seems pertinent to conclude that in blocking Ca^{2+} channels the compound exhibits voltage-dependence. In addition, the inactivation of the current strongly suggests that lubeluzole requires the channels to be open to gain access to its binding blocking site. This could justify the delayed appearance of current inactivation (Figures 4 and 6); after induction of depolarizing pulses in the presence of lubeluzole, the compound occupies more and more channels and then inactivation of the current develops.

A puzzling finding was the lack of stereoselectivity of lubeluzole in blocking Ca^{2+} channel currents. Thus, the (−)enantiomer of lubeluzole, R91154, behaved similarly to lubeluzole as a Ca^{2+} channel blocking agent; both exhibited equal potencies with IC_{50}s around 2 μM. This finding contrasts with the stereospecificity for these compounds, concerning their capabilities to improve the neurological outcome and to reduce the infarct volume of photochemically induced thrombotic cerebral infarcts in rats (De Ryck *et al*., 1996). Stereospecificity was also shown in the prevention by lubeluzole of the increase of extracellular glutamate concentrations (Scheller *et al*., 1995), in the normalization by the compound of neuronal excitability in the peri-infarct region (Buchkremer-Ratzmann & Witte, 1995), in the protection against NO toxicity induced by NO generators (Maiese *et al*., 1997), and in the inhibition of the glutamate-activated nitric oxide synthase pathway (Lesage *et al*., 1996). This last study was done to elucidate the mechanism of protection of lubeluzole against glutamate toxicity in primary hippocampal cell cultures. After prolonged treatment, lubeluzole was neuroprotective with an IC_{50} of 48 nM and its **R**-isomer was nine times less active. Guanosine 3′:5′-cyclic monosphosphate (cyclic GMP) production was inhibited with an IC_{50} of 37 nM for lubeluzole and R91154 was seven times less active. This stereospecificity is even more pronounced for the effect of lubeluzole on peri-infarct taurine (De Ryck, 1997).

However, other *in vitro* studies could not show such stereoselectivity. For instance, Ashton *et al*. (1996) found that veratridine induced neurotoxicity in hippocampal slices were equipotently prevented by lubeluzole and its **R**-isomer. It is therefore unlikely that the mechanism involved in the veratridine-induced cytotoxicity in hippocampal slices (i.e. Na^+ channel modulation) can explain the neuroprotective effects of lubeluzole in the photochemical stroke model. For the same

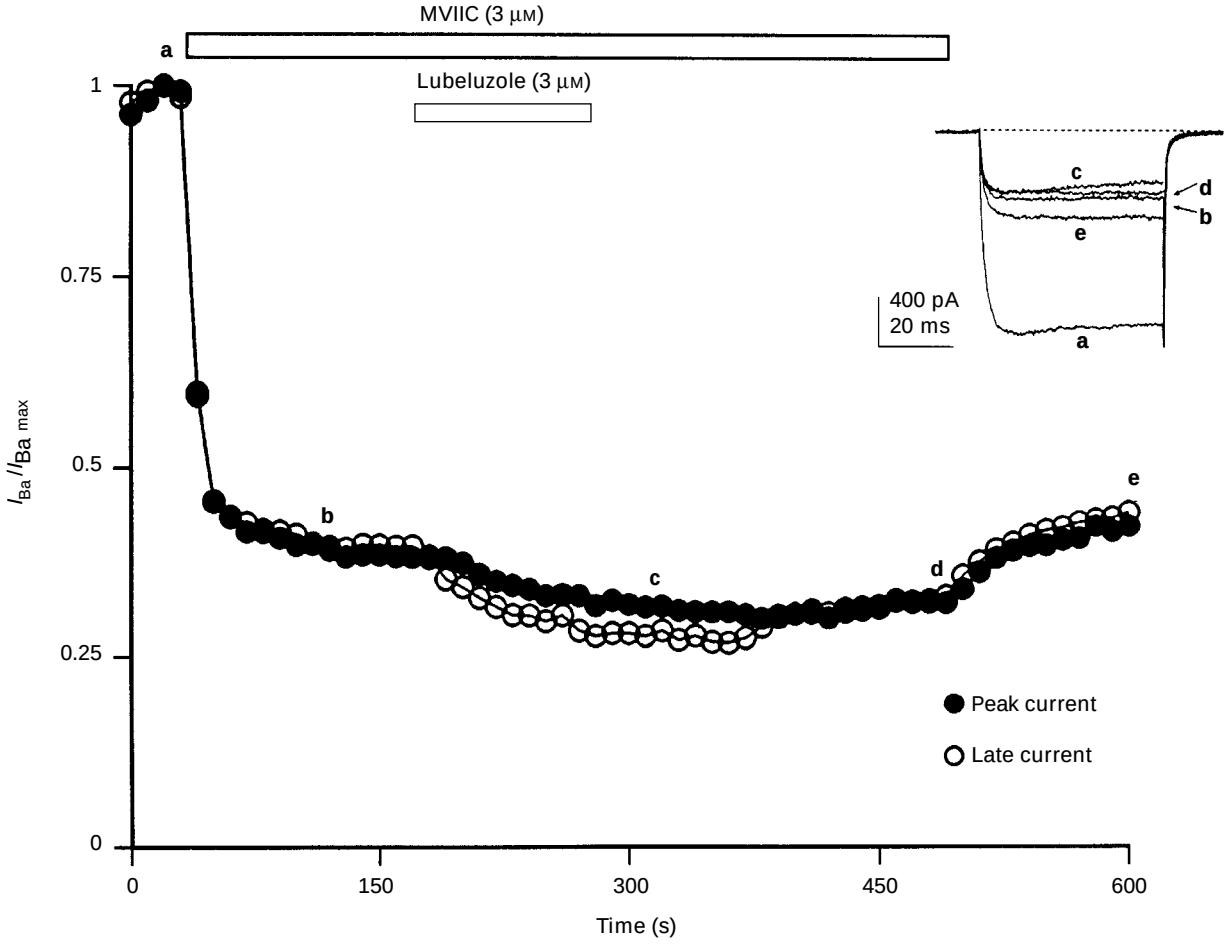

Figure 12 Time course of the blockade of mouse chromaffin cell I_{Ba} after the sequential addition of 3 μM ω-conotoxin MVIIC, followed by 3 μM lubeluzole (as shown by top horizontal bars). Stimulation pattern was as in Figure 10. Inset shows original current traces obtained at the times indicated by the letters.

reason, it is also unlikely that blockade of neuronal Ca^{2+} channels can explain those neuroprotective effects of the compound (our present results). In any case, the veratridine-induced cytotoxic effects in bovine cultured chromaffin cells could be prevented not only by Na^+ deprivation or by tetrodotoxin, but also by Ca^{2+} deprivation or Ca^{2+} channel blockers (Maroto *et al*., 1994). In the presence of veratridine, the cyclic activation of Na^+ channels causes cyclic depolarizations and oscillations of the cytosolic concentrations of Ca^{2+}, $[Ca^{2+}]_i$. These oscillations depend on activation of both Na^+ channels as well as Ca^{2+} channels (López *et al*., 1995). Because of the heterogeneity of Ca^{2+} channel subtypes expressed in bovine chromaffin cells, only the wide-spectrum Ca^{2+} channel blockers, either peptide (i.e. ω-conotoxin MVIIC) or non-peptide, i.e. R56865 (Garcézdo-Carmo *et al*., 1993) or dotarizine (Villarroya *et al*., 1995), block efficiently the veratridine-induced $[Ca^{2+}]_i$ oscillations, and protect the chromaffin cells against the cytotoxic effects of veratridine (Maroto *et al*., 1994; 1996). Lubeluzole seems to belong to this class of wide-spectrum Ca^{2+} channel blockers, capable of blocking L- as well as non-L-type of Ca^{2+} channels, and of protecting against the cytotoxic effects of veratridine in bovine chromaffin cells (unpublished results).

The relevance of wide-spectrum Ca^{2+} channel blockade to neuronal protection *in vivo* is uncertain. N-type (Takahashi & Momiyama, 1993) as well as P-subtypes of Ca^{2+} channels (Turner *et al*., 1992) have been implicated in the control of glutamate release in various brain areas (see review by García *et al*., 1997). In fact, lubeluzole attenuates the increase of extracellular glutamate concentrations in the peri-infarct area (Scheller *et al*, 1995). It is therefore likely that lubeluzole limits the massive release of glutamate during an ischaemia episode, by blocking Ca^{2+} channels and Ca^{2+} entry into presynaptic glutamatergic nerve terminals. Partial depolarization of ischaemic neurones might facilitate the access of lubeluzole to channels, as suggested by the voltage-dependent experiments (Figures 6 and 7). At the postsynaptic level, lubeluzole could also help to prevent neuronal Ca^{2+} overloading, a major cause of neuronal cell death (Choi, 1995). However, it should be kept in mind that the inhibition of glutamate release by lubeluzole is stereospecific (Scheller *et al*., 1995), while inhibition of Ca^{2+} channels is not.

This work was supported in part by the Janssen Research Foundation (Beerse, Belgium) and Janssen-Cilag (Madrid, Spain). Also by grants from DGICYT (No. PB94-0150) and Fundación Ramón Areces (Spain). We thank Mrs Carmen Molinos for the typing of this manuscript. J.M.H.G. is a fellow of CAM, Madrid, Spain.

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(Received February 26, 1997
Revised May 25, 1997
Accepted June 10, 1997)