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Effects of the neuroprotectant lubeluzole on the cytotoxic actions of veratridine, barium, ouabain and 6-hydroxydopamine in chromaffin cells

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1 Incubation of bovine adrenal chromaffin cells with veratridine (10–100 μM) during 24 h, caused a concentration-dependent release of the cytosolic lactate dehydrogenase (LDH) into the bathing medium, an indicator of cell death. Lubeluzole or its R(−) enantiomer, R91154, did not enhance LDH release. Both lubeluzole and R91154 (0.3–10 μM) decreased the veratridine-induced LDH release.

2 Penfluridol did not increase LDH release at concentrations 0.003–1 μM; 3–10 μM increased LDH release to 50–60%, after 24 h exposure. Penfluridol (0.03–0.3 μM) did not protect against the cytotoxic effects of veratridine; at 1 μM, 15% protection was produced. Higher concentrations (3–10 μM) enhanced the cytotoxic effects of veratridine.

3 Ba^{2+} ions caused a concentration-dependent increase of LDH release. This cytotoxic effect was partially prevented by 3 μM lubeluzole and fully counteracted by 1 μM penfluridol. R91154 was less potent than lubeluzole and only protected against the lesion induced by 0.5 mM Ba^{2+}.

4 Ouabain (10 μM during 24 h) increased LDH release to about 30%. Both lubeluzole (0.3–10 μM) and the lower concentrations of penfluridol (0.003–0.3 μM) prevented the ouabain cytotoxic effects. At higher concentrations (3 μM), penfluridol increased drastically the ouabain cytotoxic effects.

5 6-Hydroxydopamine (6-OHDA) caused significant cytotoxic effects at 30 and 100 μM. Lubeluzole (3–10 μM) or penfluridol (0.03–0.3 μM) had no cytoprotective effects against 6-OHDA.

6 Lubeluzole (3 μM), R91154 (3 μM) and penfluridol (1 μM) blocked the current through Na^+ channels in voltage-clamped chromaffin cells (I_{Na}) by around 20–30%. Ca^{2+} current through Ca^{2+} channels (I_{Ca}) was inhibited 57% by lubeluzole and R91154 and 50% by penfluridol. The effects of penfluridol were not washed out, but those of lubeluzole and R91154 were readily reversible.

7 Lubeluzole (3 μM) induced reversible blockade of the oscillations of the cytosolic Ca^{2+}, $[Ca^{2+}]_i$, in fura-2-loaded cells exposed to 30 or 100 μM veratridine. Penfluridol (1 μM) inhibited those oscillations in an irreversible manner.

8 The results suggest that lubeluzole and its R-isomer caused cytoprotection against veratridine cell damage, by blocking the veratridine stimulated Na^+ and Ca^{2+} entry, as well as the $[Ca^{2+}]_i$ oscillations. The Ba^{2+} and ouabain cytotoxic effects were prevented more efficiently by penfluridol, likely by blocking the plasmalemmal Na^+/Ca^{2+} exchanger. It remains dubious whether these findings are relevant to the reported neuroprotective action of lubeluzole in stroke; the doubt rests in the stereoselective protecting effects of lubeluzole in *in vivo* stroke models, as opposed to its lack of stereoselectivity in the *in vitro* model reported here.

Keywords: Cytotoxicity; cytoprotection; lubeluzole; R91154; penfluridol; veratridine; chromaffin cell; 6-hydroxydopamine; ouabain

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 prevents also in a stereoselective 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 (Ashton *et al*., 1997). These neuroprotecting effects have been recently corroborated in a clinical trial in patients suffering acute ischemic stroke (Diener *et al*., 1996).

Na^+ channel modulation could contribute to the neuroprotective properties of lubeluzole in the peri-infarct zone (Osikowska-Evers *et al*., 1995; Ashton *et al*., 1997). However, some of the reported 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). The present study was planned to define the effects of lubeluzole and its (−)R-enantiomer R91154 as cytoprotecting agents against the cytotoxic effects of veratridine, barium, ouabain and 6-hydroxydopamine (6-OHDA)

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in bovine adrenal medulla chromaffin cells. These cells served as a good model to conduct this study because they express L-, N-, P- and Q-subtypes of neuronal Ca^{2+} channels (Gandía *et al*., 1993; Albillos *et al*., 1993; 1996; García *et al*., 1997) as well as voltage-dependent Na^+ channels (Fenwick *et al*., 1982). Na^+ as well as Ca^{2+} channels are known to be involved in the cytotoxic effects of veratridine in bovine chromaffin cells (Maroto *et al*., 1994; 1996). For comparative purposes, penfluridol, a highly lipophilic compound that has recently been shown to block Ca^{2+} entry (Villarroya *et al*., 1997) and secretion (Lara *et al*., 1997) in bovine chromaffin cells was also used. Attempts to correlate the veratridine cytotoxic effects as well as the cytoprotecting actions of lubeluzole, with cell Na^+ and Ca^{2+} homeostasis were also made.

Methods

Preparation of cells

Bovine adrenal medullary chromaffin cells were isolated as previously described (Livett, 1984) with some modifications (Moro *et al*., 1990; 1991). To reduce the number of endothelial cells in the culture, that would alter the LDH measurements, the cells were pre-plated during 30 min and proliferation inhibitors were used during the maintenance of the culture in the DMEM medium. Then chromaffin cells were re-plated on plastic culture wells (24-well Costar plates) coated with 0.01 mg ml^{-1} of poly-L-lysine, at a density of 5×10^5, containing 1 ml DMEM supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cultures were maintained for 2–5 days at 37°C in a water-saturated atmosphere with 5% CO_2. After 24 h, the medium was replaced by 1 ml serum-free fresh medium and subsequently changed every 2 days. Trypan blue exclusion yielded cell viability values greater than 95%.

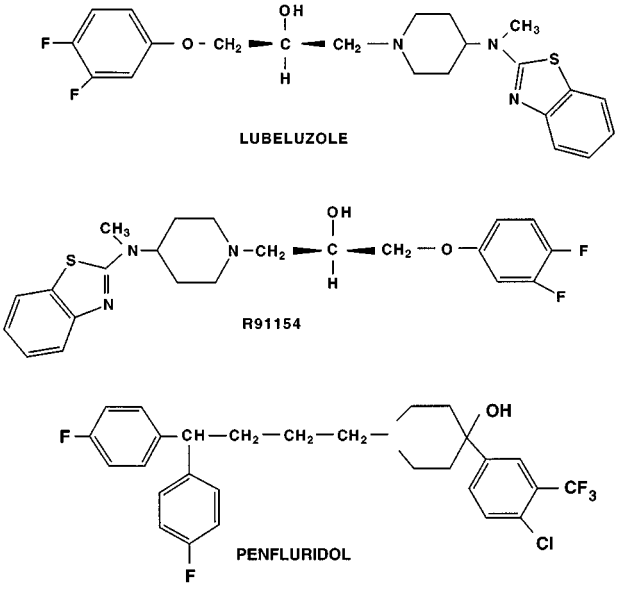

Figure 1 Molecular structure of lubeluzole [(+)(S)-4-(2-benzothiazolyl-methyl-amino)-1-piperidineethanol], its (−) R-isomer R91154, and of penfluridol.

Exposure of cells to drugs

Cells cultured for 2–4 days were washed with serum-free DMEM and incubated with 1 ml of this medium containing veratridine or ouabain for different time periods; 6-OHDA or barium was incubated in Krebs-HEPES. To test its cytoprotective effect, a given drug was incubated in DMEM for 30 min; then the medium was removed and the cells incubated with 1 ml serum-free DMEM containing the cytoprotective drug in the absence or the presence of veratridine or ouabain. When the cells were lesioned with 6-OHDA, the incubation medium was Krebs-HEPES containing 0.3 mg ml^{-1} of ascorbic acid; the cells were exposed to 6-OHDA for 30 min although LDH assay was performed 24 h later. To test the cytoprotective action of a given drug on 6-OHDA lesion, the cells were incubated in DMEM medium containing the drug since 30 min before and 24 h after the lesion and in Krebs-HEPES with ascorbic acid, during the 30 min exposure to 6-OHDA. Control cells were treated with the solvent for each drug. At the end of the incubation period, the medium was removed and saved. Lysis of the cells attached to the bottom of the dish was achieved by adding 1 ml of DMEM containing 1% Triton X100, for LDH determination.

Lactate dehydrogenase (LDH) assay

Extracellular and intracellular LDH activities were spectrophotometrically measured by following tetrazolium reduction at an absorbance wavelength of 492 nm (Boehringer Mannheim kit). Total LDH activity was defined as the sum of intracellular and extracellular LDH activity. Released LDH was defined as the percentage of extracellular compared to total LDH activity.

Measurement of Na^{2+} and Ca^{2+} channel currents

Membrane currents were measured using the whole-cell configuration of the patch-clamp technique (Hamill *et al*., 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's solution containing (in mM): NaCl, 137; $MgCl_2$, 1; $CaCl_2$, 2; HEPES/NaOH, 10; pH 7.4. 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, USA) were used to acquire and analyse the data.

Cells were clamped at −80 mV holding potential (HP). Step depolarisations to different test potentials (TP) from this HP lasted 20–50 ms and were applied at 0.1 Hz. Leak and capacitative currents were subtracted by using currents elicited by small hyperpolarising 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, USA). The flow rate (0.2–0.5 ml min^{-1}) was

regulated by gravity to achieve a complete replacement of cell surroundings in less than 1 s.

Measurement of changes of the $[Ca^{2+}]_i$ in fura-2-loaded bovine chromaffin cells

Chromaffin cells were loaded with fura-2 by incubating them with fura-2/AM (4 μM) for 60 min at 37°C in Krebs-HEPES solution (pH 7.4) containing (in mM): NaCl, 145; KCl, 5.9; $MgCl_2$ 1.2; $CaCl_2$, 2; sodium HEPES, 10; glucose, 10. The loading incubation was terminated by washing the coverslip containing the attached cells several times with Krebs-HEPES. Then, cells were kept at 37°C in the incubator for 15–30 min. The fluorescence of fura-2 in single cells was measured with the photomultiplier-based system described by Neher (1989), which produces a spatially averaged measure of the $[Ca^{2+}]_i$. Fura-2 was excited with light alternating between 360 and 390 nm, using a Nikon 40x fluorite objective. Emitted light was transmitted through a 425 nm dichroic mirror and 500–545 nm barrier filter before being detected by the photomultiplier. $[Ca^{2+}]_i$ was calculated from the ratios of the light emitted when the dye was excited by the two alternating excitation wavelengths (Grynkiewicz *et al*., 1985).

Statistical analysis

Averaged data are means ± s.e.mean. Concentration effects were analysed by means of an analysis of variance. If significant differences were found, an appropriate multiple comparison procedure (Student-Newman-Kreuls test) was done. The level of statistical significance was taken at $P<0.05$. Analysis was performed using a SPSS software for Windows.

Materials

Veratridine, 6-hydroxydopamine (6-OHDA), and ouabain were obtained from Sigma, Madrid; Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum from GIBCO, Madrid, Spain; Fura-2 (5-Oxazolecarboxylic acid,2-(6-(bis(2-((acetoxy) methoxy)-2 oxoethyl) amino)-5-(2-(bis(2((acetyloxy)methoxy)-2-oxoethyl) amino)-5-methylphenoxy) ethoxy)-2-benzofuranyl),(acetoxy)methyl ester) was purchased from Molecular Probes: Lubeluzole, R91154 and penfluridol were from Janssen, Beerse, Belgium. Concentrated solutions of drugs were prepared in water (ouabain) and dimethylsulfoxide (veratridine, lubeluzole, R91154 and penfluridol). Appropriate dilutions were then made in DMEM or Krebs-HEPES. At the final concentrations used (less than 0.1%) DMSO had no effect on any of the parameters tested.

Results

Cell viability after treatment with veratridine, lubeluzole and R91154

The cytotoxic effects of veratridine in bovine adrenal chromaffin cells have been previously measured by using phase contrast microscopy, catecholamine content of the cells, trypan blue exclusion, and LDH release into the medium; a good correlation between these four parameters was found (Maroto *et al*., 1994). As a cell viability index we have measured here the release of LDH into the medium, after the application of a given treatment for a defined time period, as well as the LDH remaining in the cells (to express LDH released as a % of total LDH present in the cells when the experiment began). Cells were exposed to veratridine and the compounds tested dissolved in DMEM; vehicle was always used in control cells. Cells under different treatments were kept in the incubator at 37°C during 24 h. This time period was chosen because the cytotoxic effects of veratridine were delayed, as shown in a previous study on chromaffin cells (Maroto *et al*., 1994).

Incubation of the cells during 24 h at 37°C with a fresh DMEM medium released 8–15% LDH, depending on the age of the culture and the quality of the cells. Incubation with veratridine caused LDH loss in a concentration-dependent manner. Figure 2a shows that 10 μM veratridine was the threshold concentration for damaging the cells, and 100 μM caused near maximal cell death; therefore, an intermediate concentration of veratridine (30 μM) was used in most experiments. The cytotoxic effects of veratridine showed a great variability and so, in each individual multiwell plate

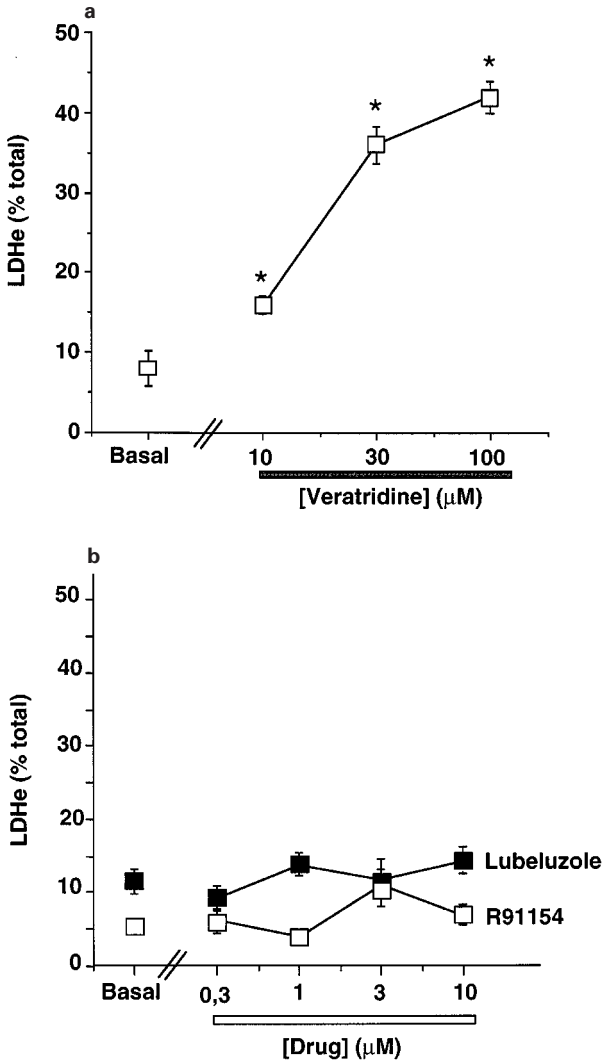

Figure 2 LDH release as an index of cell lesion. Cells (5×10^5 cells/well) were incubated at 37°C during 24 h with DMEM containing increasing concentrations of veratridine (a), lubeluzole or R91154 (b). Control cells were incubated in drug-free DMEM. LDH activity present in the medium at the end of the 24 h incubation period is expressed in the ordinates as % of total LDH present in the cells at the initiation of each experiment. Data are means ± s.e. mean of 23 wells from at least four different cell batches. *$P<0.05$ with respect to the basal release.

adequate controls were always performed (in the absence and the presence of veratridine).

Before studying the possible protecting effects of a given drug on veratridine induced cell damage, it was necessary to know whether such a compound (i.e. lubeluzole, R91154) had cytotoxic effects *per se*. Figure 2b shows that in the range of concentrations studied (0.3–10 μM) neither lubeluzole nor R91154 produced an elevation of LDH release above the levels obtained in cells exposed only to the solvent during 24 h (Basal). It was, therefore, adequate to use this concentration range to study the effects of these two compounds on veratridine-induced cytotoxicity.

Effects of lubeluzole and R91154 on veratridine-induced cytotoxicity

To study cytoprotection, cells were incubated for 30 min at 37°C in DMEM solution (1 ml) containing increasing concentrations of a given drug. Then, this medium was replaced by fresh DMEM containing the putative cytoprotectant (either lubeluzole or R91154) plus the cytotoxic agent (veratridine). Subsequently, cells were incubated in this medium during 24 h at 37°C and then analysed for LDH cell content and release as described above.

In the first protocol the concentration of veratridine was fixed (30 μM) and the concentration of cytoprotectant was varied (0.3–10 μM). Figure 3a shows that control, untreated cells lost 10±2% of LDH in 24 h. Veratridine induced 46±4% of LDH release. This loss was significantly counteracted by increasing concentrations of lubeluzole, as well as by the highest concentration of R91154 used (10 μM). The protection afforded was partial, probably because of the use of a large concentration of veratridine, as suggested by the fact that lubeluzole (3 μM) afforded full protection at lower concentrations (i.e. 10 μM) of veratridine (Figure 3c).

Cytoprotection was also studied at different levels of cytotoxicity. Cells were exposed to increasing concentrations of veratridine (10–100 μM) in the absence (control) or the presence of 0.3 and 3 μM lubeluzole or R91154. At 0.3 μM, lubeluzole showed a tendency to decrease the amounts of LDH release evoked by veratridine though this effect was not statistically significant (Figure 3b). R91154 (0.3 μM) produced a slightly greater cytoprotection, being statistically significant at the concentrations of veratridine of 30 and 100 μM. At 3 μM, both lubeluzole and R91154 afforded a statistically significant protection against the cytotoxic effects of the three concentrations of veratridine (Figure 3c); differences between lubeluzole and R91154 were found only when 100 μM veratridine was used.

The effects of penfluridol on cell viability and on the cytotoxic effects of veratridine

Penfluridol was chosen for comparative purposes, as reasoned in the Introduction and the Discussion. In contrast to lubeluzole and R91154, penfluridol increased significantly LDH release above basal levels when cells were incubated 24 h in DMEM containing concentrations of the drug over 1 μM (Figure 4a). Concentrations below 1 μM did not cause LDH release and thus, the possible cytoprotectant effects of penfluridol against veratridine could be likely seen only at the lower concentrations.

Figure 4b shows an experiment to determine the effects of increasing concentrations of penfluridol on veratridine-induced LDH release. In non-treated cells about 12±3% of total LDH was lost after a 24 h incubation period (basal). Cells exposed to veratridine (30 μM, 24 h) lost 65±3% of their LDH. This figure was unchanged in the presence of 0.03–0.3 μM penfluridol. In veratridine-lesioned cells penfluridol was incapable of affording a clear cytoprotecting action against the veratridine lesion. In fact, at 10 μM penfluridol enhanced significantly the cytotoxic effects of veratridine.

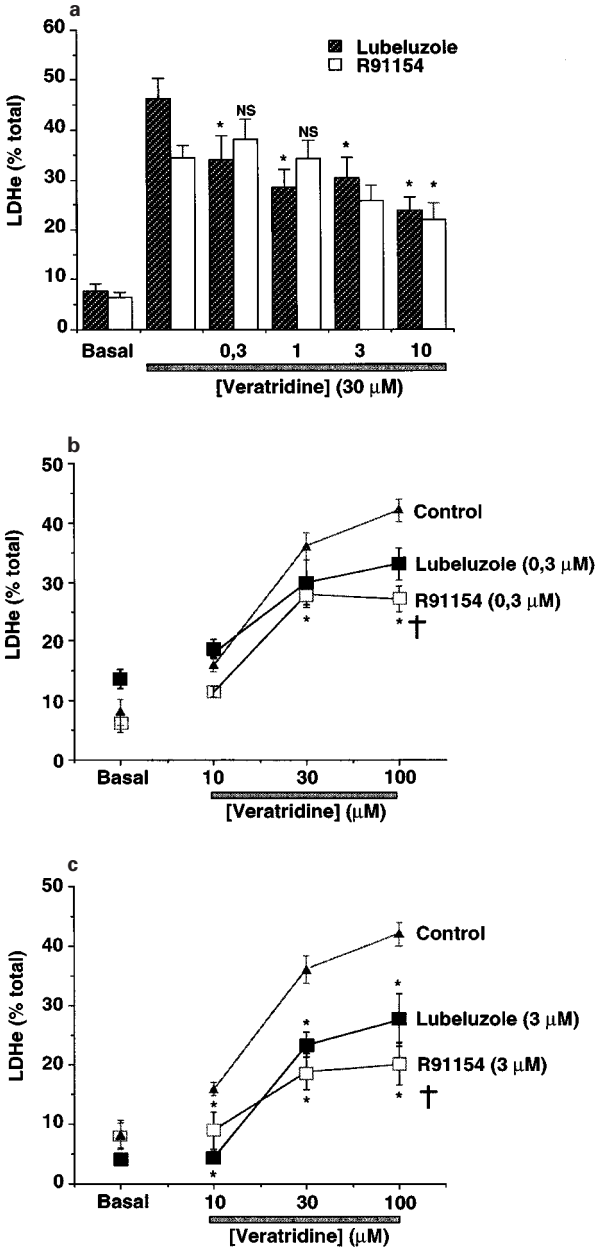

Figure 3 Protection by lubeluzole and R91154 against the cytotoxic effects of veratridine, measured as LDH release. (a) The concentration of veratridine was fixed at 30 μM and the concentrations of the cytoprotectant agents varied between 0.3 and 10 μM. Datasets were averaged from 10–18 wells from at least three different cell cultures; they are means±s.e. mean. *$P<0.05$, compared with LDH released by veratridine in the absence of drug. (b) and (c) The veratridine concentrations varied between 10 and 100 μM and the concentration of the cytoprotectants were fixed at 0.3 μM (b) or 3 μM (c). Experimental protocols and LDH release calculations are as in (a). Data are means±s.e. mean of 12–16 wells from at least three different cultures. *$P<0.05$ with respect to the corresponding value of the same veratridine concentration, but in the absence of cytoprotectant. †, $P<0.05$ compared with lubeluzole.

Cytoprotection against Ba^{2+} induced cell damage

In a previous report we demonstrated that the substitution of Ca^{2+} by Ba^{2+} could not prevent the cytotoxic effects of veratridine; moreover, Ba^{2+} itself (in the absence of veratridine) produced massive cell death after 24 h incubation with a 10 mM concentration (Abad *et al*., 1995). We corroborate here that Ba^{2+} is a powerful noxious agent in bovine chromaffin cells; in addition, we provide new information on the concentration dependence of such cytotoxic action. Figure 5 shows that the threshold [Ba^{2+}] to cause cell damage was between 0.2 and 0.5 mM. At 0.5 mM, Ba^{2+} caused about 40% LDH release while at 1 mM over 60% LDH release was produced. Further increments of [Ba^{2+}] did not cause greater cytotoxicity.

Lubeluzole (3 μM) afforded cytoprotection against the intermediate [Ba^{2+}]. Thus, at 0.5 mM Ba^{2+} lubeluzole reduced LDH release from 40% to 10%, a figure similar to the basal enzyme level found in the medium after 24 h incubation of control cells. A significant protection was also seen when 1 mM Ba^{2+} was used; lubeluzole reduced LDH release from 62 to 40%. Such reduction was smaller at 2 mM Ba^{2+} and insignificant at 5 or 10 mM Ba^{2+}. It appears, therefore, that lubeluzole loses its cytoprotecting actions at the higher [Ba^{2+}].

The cytoprotecting effects of R91154 and penfluridol against Ba^{2+}-induced cytotoxicity, are shown in Figure 5b. As in the previous experiments, 0.5 mM Ba^{2+} produced a 40% LDH loss after 24 h incubation, and over 60% at 1–2 mM Ba^{2+}. When 0.5 mM Ba^{2+} was added in the presence of 3 μM R91154, the loss of LDH was almost fully prevented; however, R91154 could not protect against the higher concentrations of Ba^{2+} (1–2 mM). In contrast, penfluridol (1 μM) afforded full protection against the cytotoxic effects of Ba^{2+} at all concentrations tested (0.5, 1 and 2 mM).

Effects of lubeluzole and penfluridol on ouabain-induced cytotoxicity

LDH release increased from about 16% in control cells, to around 30% in cells incubated during 24 h at 37°C in 1 ml DMEM containing 10 μM ouabain (Figure 6a). At 0.3 μM, lubeluzole reduced by 30% the ouabain-induced LDH increase. This reduction was similar with all the concentrations of lubeluzole tested. A more clear concentration-dependent effect of penfluridol, on the ouabain-induced LDH release, was observed (Figure 6b). At 3 μM, penfluridol enhanced the ouabain cytotoxic effects in a synergic manner (90±8% LDH release).

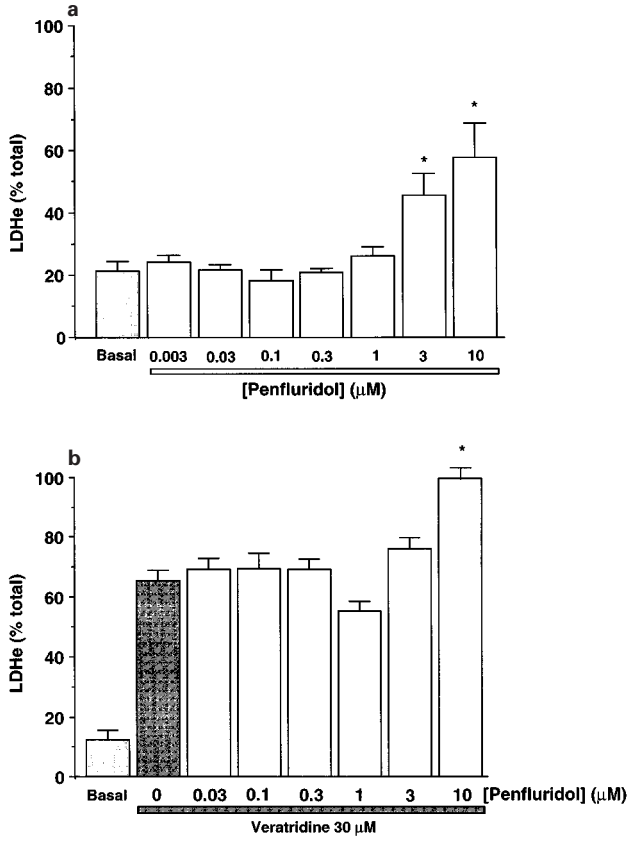

Figure 4 The cytotoxic effects of penfluridol and its actions on LDH release evoked by veratridine. (a) Shows the release of LDH in the absence (basal) and the presence of increasing concentrations of penfluridol (dissolved in DMEM), from cells incubated during 24 h at 37°C. (b) Shows LDH released in the absence of veratridine (basal) and in cells exposed to veratridine for 24 h at 37°C in the presence of the concentrations of penfluridol shown at the bottom. Data are means±s.e. mean of 12–16 wells from at least four different cultures. *$P<0.05$, compared with basal (a) or with veratridine in the absence of penfluridol (b).

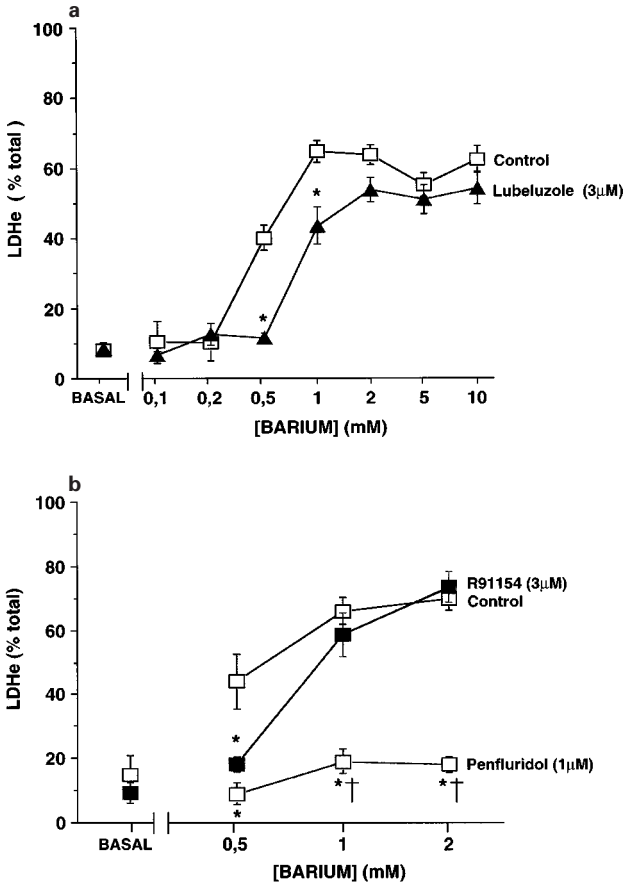

Figure 5 Cytotoxicity induced by Ba^{2+} and cytoprotection afforded by lubeluzole (a), R91154 and penfluridol (b). Cells were incubated during 24 h in normal Krebs-HEPES solution (Basal LDH release) or in solutions containing increasing concentrations of Ba^{2+} (abscissae), added to a Krebs-HEPES solution containing no Ca^{2+} (nominal 0 Ca^{2+}). Data are means±s.e.mean of 18 wells from at least four different cultures (a) or from 8 wells from two different cultures (b). *$P<0.05$, when comparing with respect to the corresponding value of cells exposed to Ba^{2+} in the absence of the drug. †, $P<0.05$ when comparing between R91154 and penfluridol.

Effects of lubeluzole and penfluridol on 6-OHDA-induced cytotoxicity

6-OHDA causes chromaffin cell damage by liberating active free radical oxygen species (Abad *et al.*, 1995). It was, therefore, interesting to test whether lubeluzole caused some cytoprotection in this model of cell lesion. Untreated cells (basal LDH release) were exposed to the solvent (see Methods), and other cells from the same plate were exposed to 6-OHDA for 30 min at increasing concentrations, either in the presence or the absence of 3 or 10 μM lubeluzole. Lubeluzole was present 30 min before, during 6-OHDA treatment (30 min) and during 24 h following 6-OHDA treatment.

At 30 μM, 6-OHDA caused about 60% cell death. Lubeluzole, at 3 and 10 μM showed a tendency to afford cytoprotection against the cell lesion produced by 30 μM 6-OHDA, although the values were not statistically different.

Penfluridol, at the two concentrations used (0.03 and 0.3 μM) afforded no cytoprotection against 6-OHDA. The use of higher concentrations was precluded because of the cytotoxic effects of penfluridol (Figure 4a).

Effects of lubeluzole, R91154 and penfluridol on Na^+ and Ca^{2+} channel currents

Whole-cell inward currents through voltage-dependent Na^+ and Ca^{2+} channels were studied in voltage-clamped chromaffin cells. Currents were elicited by 50 ms depolarising pulses to 0 mV from a holding potential of -80 mV, using an extracellular solution containing 137 mM Na^+.

Figure 7 shows original current traces of I_{Na} and I_{Ca} (2 mM Ca^{2+} in the extracellular solution, with no Cd^{2+}) obtained in three cells voltage-clamped at -80 mV. I_{Na} and I_{Ca} were elicited by 50 ms depolarising pulses to 0 mV, applied at 15 s intervals. Panel a shows the blocking effects (after 5 min superfusion) of 3 μM lubeluzole on I_{Na} (around 25%) and I_{Ca} (around 65%). Note the inactivation of I_{Ca}, already shown in a recent detailed study on the effects of lubeluzole on bovine and mouse chromaffin cell Ca^{2+} channel currents (Hernández-Guijo *et al.*, 1997). After 5 min of having washed out lubeluzole, I_{Na} and I_{Ca} recovered substantially. In the cell traces shown in panel b, R91154 (3 μM for 5 min) reduced I_{Na} by 30% and I_{Ca} by 70%. Five min after washout, I_{Na} and I_{Ca} largely recovered. Panel c shows the effects of penfluridol (1 μM), that blocked I_{Na} by only 20% and I_{Ba} by 50%.

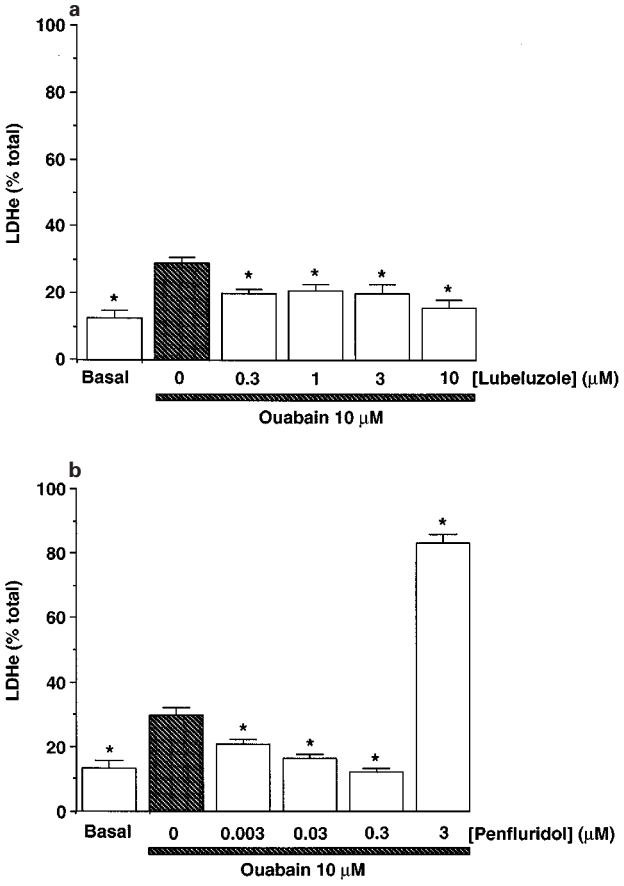

Figure 6 LDH release from chromaffin cells induced by ouabain in the absence or the presence of increasing concentrations of lubeluzole (a) or penfluridol (b). Basal LDH release represents that lost by untreated cells. Data are means ± s.e. mean of 16–20 wells from at least four different cell cultures. $*P<0.05$, when compared with LDH released by ouabain alone.

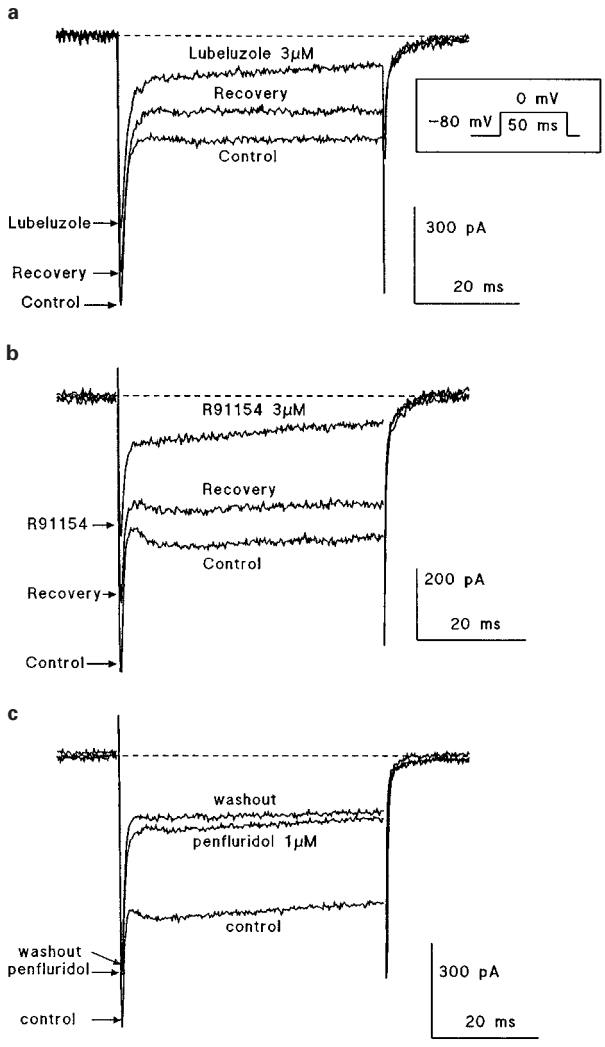

Figure 7 Na^+ currents (I_{Na}) and Ca^{2+} currents through Ca^{2+} channels (I_{Ca}) in voltage-clamped bovine chromaffin cells. The holding potential was -80 mV, and currents were evoked by 50 ms depolarising pulses applied at 15 s intervals. Control current traces were obtained immediately before the addition of drugs; the traces in the presence of the drug were obtained after 5 min superfusion with 3 μM lubeluzole (a), 3 μM R91154 (b), or 1 μM penfluridol (c). The recovery of the currents was tested 5 min after washout of each drug. 137 mM Na^+ and 2 mM Ca^{2+} were used respectively as charge carriers through Na^+ and Ca^{2+} channels.

Penfluridol did not cause inactivation of I_{Ca}. Five min washout did not lead to I_{Na} nor I_{Ca} recovery.

Figure 8 shows averaged datasets summarising the blocking effects of the three compounds on I_{Na} and I_{Ca}. Lubeluzole inhibited peak I_{Na} by $30.1 \pm 5.6\%$ and I_{Ca} by $63.1 \pm 7.7\%$. Similar effects were seen with R91154, $31.5 \pm 5.6\%$ blockade of I_{Na} and $69.4 \pm 4.9\%$ I_{Ca}. Penfluridol inhibited peak I_{Na} by $15.8 \pm 2.8\%$ and I_{Ca} by $49.1 \pm 3.3\%$. Substantial recoveries of I_{Na} and I_{Ca} were seen upon washout of lubeluzole (63% recovery) and R91154 (60% recovery), but no recovery was observed after washout of penfluridol. No significant differences between lubeluzole, R91154 and penfluridol were seen as far as their blocking effects of early I_{Ca} was concerned. However, the reduction caused by lubeluzole and R91154 of I_{Na} and the late I_{Ca} significantly differed from penfluridol (panels a and c of Figure 8).

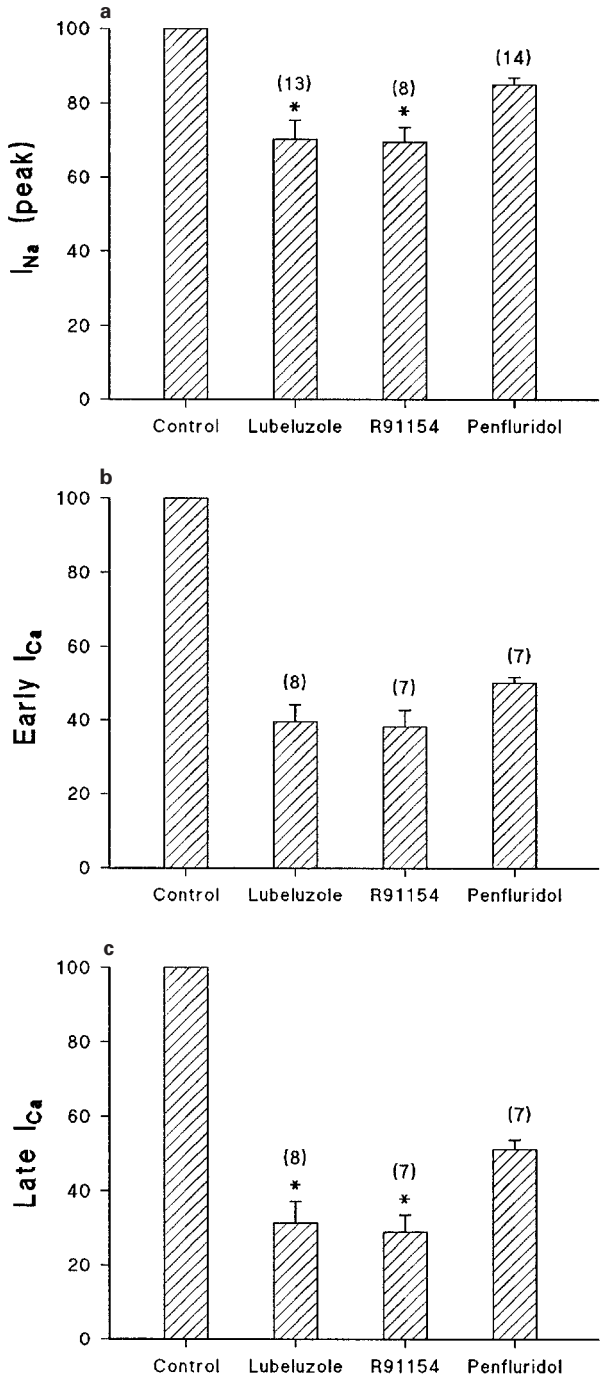

Figure 8 Quantitative inhibition of I_{Na} and I_{Ca} by lubeluzole, R91154 and penfluridol. The amplitude of peak current found after breaking into the cell with the patch electrode and stabilisation, before adding a given drug, was normalised to 100% in every individual cell studied (control current). Then the peak current obtained after 5 min exposure to the drug was expressed as % of control. (a) Shows peak I_{Na}, (b) the early peak I_{Ca} and (c) the late I_{Ca}. Data are means $\pm$ s.e.mean of the number of cells shown in parentheses on top of each column. $^*P<0.05$, with respect to penfluridol.

Effects of penfluridol, R91154, and lubeluzole on the oscillations of cytosolic Ca^{2+} concentrations induced by veratridine

As previously shown (López *et al*., 1995), veratridine induces an oscillatory pattern of $[Ca^{2+}]_i$ in fura-2-loaded single bovine chromaffin cells. These $[Ca^{2+}]_i$ oscillations are evoked by the veratridine concentrations (30 μM) that causes 60–80% cell damage. Figure 9a shows the oscillations of $[Ca^{2+}]_i$ induced by veratridine; the oscillations reached peaks of about 0.6–0.7 μM $[Ca^{2+}]_i$, without an elevation of the basal $[Ca^{2+}]_i$ (around 0.1–0.2 μM). The oscillations were maintained for the entire 2 h 45 min-period of continued superfusion of the cell with veratridine, without ostensible variations in their frequency and amplitude.

In the cell shown in Figure 9b, veratridine induced the typical oscillatory pattern of $[Ca^{2+}]_i$. When the oscillations reached a 'steady state', the concentration (1 μM) of

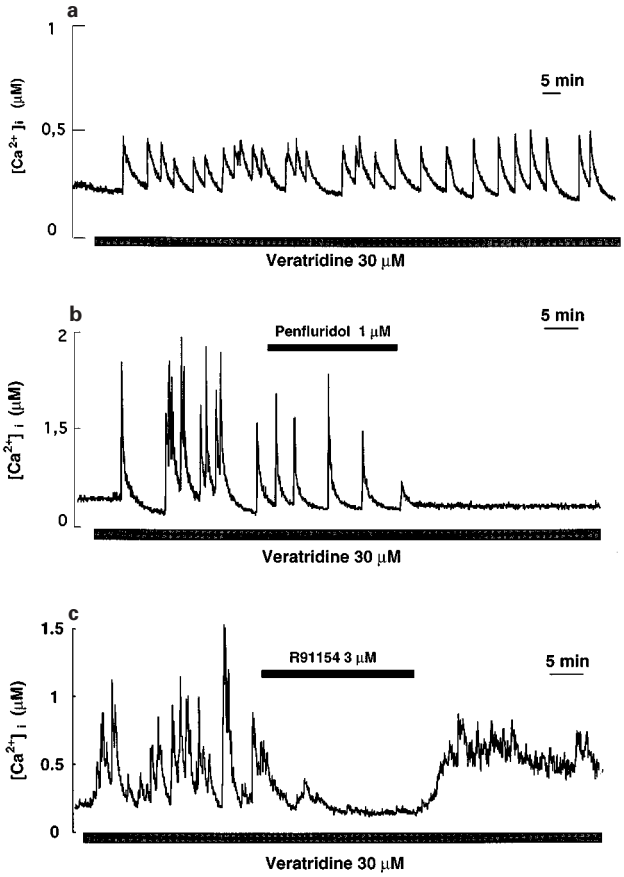

Figure 9 Penfluridol and R91154 decreased the veratridine induced oscillations of $[Ca^{2+}]_i$ in fura-2-loaded single bovine chromaffin cells. (a) Shows a cell that was superfused with veratridine (30 μM) during the entire recording period of 2 h 45 min (bottom horizontal bar). (b) Shows a second cell, with its typical oscillations of $[Ca^{2+}]_i$ induced by veratridine, and their blockade by penfluridol (top horizontal black bar). (c) Shows a third cell in which the $[Ca^{2+}]_i$ oscillations were reversibly interrupted by 3 μM R91154.

penfluridol showing cytoprotection against veratridine was added. After a delay period in which the oscillations became more infrequent, $[Ca^{2+}]_i$ remained constant at around 0.1–0.2 μM. The oscillations did not resume even after 20 min washout of penfluridol.

R91154 also reduced the veratridine-induced $[Ca^{2+}]_i$ oscillations, as the experiment of Figure 9c shows. This cell was induced to oscillate through its continued superfusion with veratridine. R91154, at the concentration (3 μM) that showed cytoprotection against veratridine, reduced the amplitude and frequency of the oscillations of $[Ca^{2+}]_i$, after a delay of 3–5 min. A quick recovery of the initial oscillatory pattern was produced when R91154 was washed out.

Figure 10 shows the results of experiments performed in three different fura-2-loaded chromaffin cells. An initial test with a short pulse of a K^+-enriched solution (70 mM K^+, 5 s) was always performed to know that the cell responded adequately with a Ca_i^{2+} spike. Upon recovery, veratridine was applied as indicated by the horizontal bar below each trace. In panel a the cell initiated large Ca^{2+} oscillations that stopped soon after addition of 3 μM lubeluzole. The oscillations resumed after washout of the drug. In panel b both, veratridine and lubeluzole were simultaneously added; no oscillations were produced but as soon as lubeluzole was removed from the superfusion medium the cell initiated small oscillations of $[Ca^{2+}]_i$ that grew up as time elapsed. Reintroduction of lubeluzole produced a delayed reduction of the amplitude of the $[Ca^{2+}]_i$ oscillations that eventually stopped, and resumed slowly after lubeluzole washout. The cell shown in panel c was initially superfused with lubeluzole (20 min) and then also with veratridine; $[Ca^{2+}]_i$ oscillations were not produced while lubeluzole was present; removal of lubeluzole led to a prompt appearance of $[Ca^{2+}]_i$ oscillations whose size grew up with time.

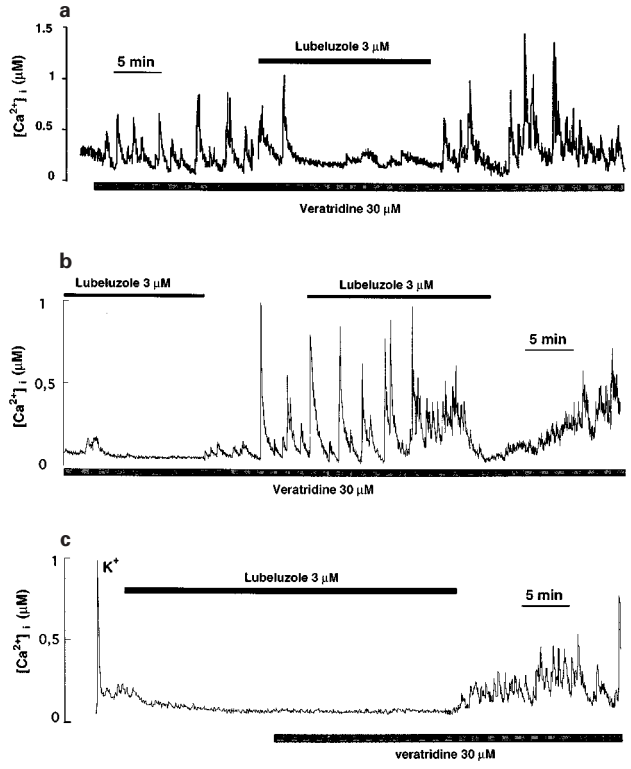

Figure 10 Lubeluzole stops the oscillations of the cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_i$, induced by veratridine (30 μM), in single fura-2-loaded chromaffin cells. After the basal levels of $[Ca^{2+}]_i$ reached an equilibrium (usually at around 0.1 μM), the addition of veratridine or lubeluzole was initiated and extended during the periods shown by the horizontal bars. Lubeluzole (3 μM) was added after veratridine (a), simultaneously with veratridine (b), or before veratridine (c). The traces are original and were taken from three different cells.

Discussion

The central finding of this study is that lubeluzole protects chromaffin cells against damage (LDH release) induced by veratridine. The cytotoxic effects of veratridine in bovine chromaffin cells (Maroto *et al.*, 1994; 1996) seem to be associated to cyclic activation and inactivation of voltage-dependent Na^+ and Ca^{2+} channels, leading to chronic oscillations of $[Ca^{2+}]_i$ (López *et al.*, 1995). Therefore, the easiest explanation for the cytoprotecting actions of lubeluzole is the interruption of such cyclic behaviour i.e. by blocking Na^+ and/or Ca^{2+} channels.

At concentrations clearly cytoprotectant (3 μM), lubeluzole was a poor blocker (30%) of Na^+ channel currents and a better inhibitor (75%) of Ca^{2+} channel currents. In addition, this concentration of lubeluzole completely cancelled the oscillations of $[Ca^{2+}]_i$ in veratridine-exposed cells. Thus, it seems that lubeluzole protects chromaffin cells by preventing Ca^{2+} entry and Ca^{2+} overload, through blockade of Ca^{2+} channels. A recent detailed study on the effects of lubeluzole on Ca^{2+} channel currents, came to the conclusion that lubeluzole is blocking preferentially N/P/Q-types of Ca^{2+} channels, that are known to control neurotransmitter release in the central and peripheral nervous system (Hernández-Guijo *et al.*, 1997).

Lubeluzole caused no protection against the cytotoxic effects of 30 μM 6-OHDA. The cytotoxic effects of 6-OHDA in chromaffin cells are related to free radical production, and unrelated to Ca^{2+} ions (Abad *et al.*, 1995). Hence, the lack of effects of lubeluzole against 6-OHDA suggest that lubeluzole does not behave as a free-radical scavenger. Also worth consideration was the cytoprotection against ouabain-induced cytotoxicity. In chromaffin cells, ouabain is known to favour the intracellular accumulation of Na^+ secondary to Na^+-K^+ ATPase inhibition; this reduces the Na^+ gradient across the cell membrane, the increase of intracellular Na^+ levels activates the Na^+-Ca^{2+} exchanger so that Na^+ can be extruded in favour of Ca^{2+} entry (Fuente *et al.*, 1996). If lubeluzole were inhibiting the Na^+/Ca^{2+} exchanger, it could prevent the cell Ca^{2+} overloading and the cytotoxic effects of ouabain.

Some other interesting findings derive from the comparison of the effects of lubeluzole with those of penfluridol. The first emerges from the cytotoxic effects of penfluridol at 3–10 μM and the lack of cytotoxic effects of lubeluzole. This could be explained on the basis of differences in lipophilicity; thus, the octanol/water partition coefficient of penfluridol is 7.6 while that of lubeluzole is 4.6 (Lara *et al.*, 1997). The prompt reversibility of the effects of lubeluzole on I_{Na}, I_{Ba} and $[Ca^{2+}]_i$ and the 'irreversible' or long-lasting actions of penfluridol are in line with this different lipophilicity. The third difference concerns the poor protection against the veratridine cytotoxic effects afforded by penfluridol and the potent protection against the ouabain cytotoxic effects, just the opposite to lubeluzole, that showed a clear concentration-dependent protecting effect against veratridine, and more tenuous effects against ouabain. Thus, it may well be that penfluridol causes cytoprotection against veratridine by blocking the Na^+/Ca^{2+} exchanger, while the targets for lubeluzole are, preferentially, the Ca^{2+} channels. Ongoing experiments on the bovine

chromaffin cell Na^+/Ca^{2+} exchanger suggest that this might be an adequate explanation (unpublished results).

Another issue concerns lubeluzole and its (−)R-enantiomer R91154. The cytoprotection against veratridine induced by R91154 was somehow similar to that of lubeluzole, as were its blocking actions on I_{Na} and I_{Ba}. Thus, a clear stereoselective action between lubeluzole and R91154 was not apparent. These findings contrast with the reported stereospecificity concerning the capabilities of lubeluzole and R91154 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 normalisation by the compound of neuronal excitability in the peri-infarct region (Buchkremer-Ratzmann & Witte, 1995; 1997), in the protection against toxicity induced by the glutamate-activated nitric oxide (NO) synthase pathway (Lesage *et al*., 1996) and against NO-donor induced neurotoxicity (Maiese *et al*., 1997). In the study by Lesage *et al*. (1996), after prolonged treatment, lubeluzole was neuroprotective with an IC_{50} of 48 nM, while R91154 was nine times less active. cGMP production was inhibited with an IC_{50} of 37 nM for lubeluzole and R91154 was seven times less active. The stereospecificity is even more pronounced for the effect of lubeluzole on the peri-infarct region (De Ryck, 1997). In the present study, a certain degree of stereoselectivity was seen against the cytotoxic effects of Ba^{2+} ions, where lubeluzole afforded cytoprotection against 0.5 and 1 mM Ba^{2+} while R91154 produced only partial protection at the 0.5 mM Ba^{2+} concentration.

Other *in vitro* studies in addition to ours, could not show such stereoselectivity. For instance, Ashton *et al*. (1997) showed that veratridine induced neurotoxicity in hippocampal slices was equipotently prevented by lubeluzole and its (−)R isomer. On the other hand, Hernández-Guijo *et al*. (1997) showed that both isomers inhibited the whole-cell Ca^{2+} channel currents in bovine chromaffin cells with equal potencies. It is, therefore, uncertain that the mechanism involved in the veratridine-induced cytotoxicity in hippocampal slices (i.e. Na^+ channel modulation) or blockade of neuronal Ca^{2+} entry can explain the *in vivo* neuroprotective effects of lubeluzole in the photochemical stroke model. For the same reason, it is also unlikely that blockade of neuronal Ca^{2+} channels and *in vitro* protection against veratridine, Ba^{2+} ions or ouabain (our present results) can explain fully such *in vivo* neuroprotective effects.

The relevance of N/P/Q-Ca^{2+} channel blockade to neuronal protection *in vivo* is uncertain. N-type (Takahasi & 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). It is therefore likely that lubeluzole might be limiting the massive release of glutamate during an ischemic episode, by blocking Ca^{2+} channels and Ca^{2+} entry into presynaptic glutamatergic nerve terminals. Partial depolarisation of ischemic neurones might facilitate the access of lubeluzole to channels, as suggested by their voltage-dependent blockade (Hernández-Guijo *et al*., 1997). At postsynaptic levels lubeluzole could also contribute to prevent neuronal Ca^{2+} overloading, a major cause of cell death (Choi, 1995). However, lubeluzole is a stereoselective modulator of the NO synthase pathway and downregulation of NO decreases glutamate release. This is the reason why De Ryck (1997) links the stereoselective neuroprotective mechanism of action of lubeluzole to its stereoselective blockade of peri-infarct glutamate *in vivo*. However, the difference in potencies between both stereoisomers is only 5–10 times and hence, Na^+ and Ca^{2+} channels cannot completely be discarded as targets to explain the neuroprotective effects of lubeluzole.

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