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Inhibition by lifarizine of intracellular Ca^{2+} rises and glutamate exocytosis in depolarized rat cerebrocortical synaptosomes and cultured neurones

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1 The effects of lifarizine (RS-87476) on intracellular Ca^{2+} rises and the release of glutamate from rat cerebrocortical synaptosomes depolarized with 30 mM KCl were investigated by use of entrapped fura 2 and exogenous glutamate dehydrogenase.

2 Prior (1 min) addition of lifarizine decreased 30 mM KCl-induced total glutamate release, with 3 μM and 10 μM causing 39% and 72% averaged decreases from controls. The calcium-dependent component of glutamate release (approx. 40% of total) was similarly decreased by 47% and 74%, whereas the calcium-independent component was decreased by only 32% and 43% respectively.

3 In parallel experiments with fura-2-loaded synaptosomes, lifarizine reduced the depolarization-induced increases in intracellular $[Ca^{2+}]$, suggesting that this is the means by which the decreases in glutamate release are brought about. Lifarizine inhibited both the plateau and the spike phases of the Ca^{2+} increases suggesting that, in addition to its known sodium channel blocking properties, it may also inhibit more than one class of calcium channel in the synaptosomes.

4 Lifarizine at 1 μM and 3 μM also inhibited the rises in intracellular $[Ca^{2+}]$ in rat cultured cortical neurones depolarized with 60 mM KCl.

5 These effects of lifarizine on intracellular Ca^{2+} and glutamate exocytosis may contribute to its neuroprotective action.

Keywords: Lifarizine; intracellular Ca^{2+}; glutamate exocytosis; synaptosomes; cultured neurones; depolarization

Introduction

Lifarizine (RS-87476) is a diphenylpiperazine analogue that has shown neuroprotective efficacy in several different *in vivo* models of both focal and global cerebral ischaemia, including the rat 4-vessel occlusion (Alps *et al.*, 1990), the cat middle cerebral artery occlusion (Kucharcyzk *et al.*, 1991), the gerbil unilateral carotid artery occlusion (Brown *et al.*, 1993), and the mouse middle cerebral artery occlusion (Brown *et al.*, 1995b). Preliminary studies in man have indicated that the drug is generally well tolerated, and some evidence of benefit in stroke patients has been obtained (Brown *et al.*, 1995a). In terms of proposed mechanism, electrophysiology studies have shown that lifarizine inhibits sodium currents in a voltage-dependent manner (Sheridan *et al.*, 1991; McGivern *et al.*, 1995). This property arises predominantly from an interaction of lifarizine with the inactivated state of the sodium channel, and consistent with this it was found that the potency of lifarizine in inhibiting sodium channels, and in the affinity of [^{3}H]-lifarizine binding to sodium channels, both increased at more depolarized holding potentials (McGivern *et al.*, 1995; MacKinnon *et al.*, 1995). The concept that its voltage-dependent inhibition of neuronal sodium channels may contribute to its anti-ischaemic action has been supported by *in vitro* studies where it was shown to protect against veratridine-induced neurotoxicity, but not glutamate-induced or cyanide-induced neurotoxicity, in rat cultured cerebrocortical neurones (May *et al.*, 1995).

Depolarization of cells as the result of cerebral ischaemia will lead to the influx of Na^+ and Ca^{2+} which can lead to cell damage (see Alps, 1992) and therefore agents which prevent these events are potentially neuroprotective. In addition to its Na^+ channel blocking effects, lifarizine has also been shown to possess some L-type (Fraser & Spedding, 1991) and T-type (Brown *et al.*, 1995a) calcium channel antagonist activity. However, it is also now clear that the release of excitatory amino acids also plays a large part in the neurotoxicity associated with cerebral ischaemia, and it has been shown that antagonism of their effects is also potentially neuroprotective (e.g. Graham *et al.*, 1992). However, this also means that agents which inhibit ion influx due to depolarization have the additional potential of reducing ischaemic injury through the consequent inhibition of the release of excitatory amino acids, and hence this may form an important part of their protective mechanism. Therefore it is the purpose of the present investigation to examine whether lifarizine can affect the stimulated release of glutamate from isolated nerve terminals, and the associated increases in intracellular $[Ca^{2+}]$, and also to study its effects on the latter response in an intact cell preparation, to see if its ion channel blocking properties may lead to this potentially important functional outcome.

Methods

Synaptosomal preparation

Cerebral cortices were rapidly dissected from the brains of 6-7 week old male Wistar rats that had been killed by cervical dislocation. The myelin layer was scraped away and the cortices rapidly placed in ice-cold 320 mM sucrose. The cortices were then homogenized and the P_2 synaptosomal fraction was prepared as previously described (Nicholls, 1978; Dunkley *et al.*, 1988). Protein concentration was measured as described by Bradford (1976); about 20 mg protein was obtained from each

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brain. Synaptosomal pellets were kept on ice and then, as required, were resuspended and incubated in HEPES-buffered medium (HBM) composition, mM: NaCl 120, KCl 5, HEPES 20, $NaHCO_3$ 5, $MgCl_2$ 1, $NaHPO_4$ 1.2, glucose 10; pH 7.4. All pellets were used within 6 h of preparation; previous experiments (unpublished) showed that glutamate release, which is one of the most sensitive parameters of viability of this preparation, remained unchanged in synaptosomes that were left as pellets for 6 h or less. The *n* values given relate to the number of separate synaptosomal preparations.

Glutamate release

Synaptosomes (0.5 mg protein in 1 ml of HBM) were pre-incubated at 37°C in a thermostatted, stirred, cuvette in a Perkin-Elmer LS-5B luminescence spectrofluorimeter. Then 1 mM $NADP^+$, 1.3 mM $CaCl_2$ (or 50 μM EGTA when measuring calcium-independent release), and 50 units of glutamate dehydrogenase were added and NADPH production was followed at 340 nm excitation and 420 nm emission (Nicholls *et al.*, 1987). Traces were standardized by the addition of 1 nmol of glutamate at the end of each assay. Data points were obtained at 2 s intervals and exported to Lotus 1-2-3 where they were standardized and averaged. Values given in the text are means ± s.e.mean; traces shown in the figures are the averaged traces and the bars shown are the s.e.mean values. The calcium-dependent traces were obtained by subtracting the data obtained from the calcium-independent release experiments (i.e. plus EGTA in the medium) from that obtained in the total release experiments (i.e. plus calcium in the medium). This methodology is fully described in Cousin *et al.* (1993).

Cytoplasmic free Ca^{2+}

The cytoplasmic (i.e. intrasynaptosomal) free calcium concentration ($[Ca^{2+}]_c$) was measured with fura-2 using the Perkin-Elmer fluorometer and the ratios of the results at 505 nm emission from the excitation wavelengths at 340 nm and 380 nm as described previously (Kauppinen *et al.*, 1988). The K_d of hydrolysed fura-2 for Ca^{2+} was taken to be 224 nM (Grynkiewicz *et al.*, 1985). For loading the fura-2, synaptosomes were pre-incubated in HBM containing 5 μM acetoxymethyl ester of fura-2 and 16 μM bovine serum albumin (essentially fatty acid free) for 35 min at 37°C, before being centrifuged for 15 s in a microfuge and then resuspended in fresh HBM.

Experiments with rat cortical neurones

These were only concerned with intracellular $[Ca^{2+}]$ measurement and the methodology was essentially similar to that described above for the synaptosomes with the following differences. Primary cultures of neurones were established from cortical cells of embryonic rats (18th day of gestation) as fully described by Pauwels *et al.* (1989) and May *et al.* (1995) on 22 mm coverslips coated with poly-D-lysine (M_R 30,000–70,000; 5–10 μg per coverslip for 60 min), and were then maintained in serum-free media (to discourage glial cell growth) for 7–9 days until use (by which time they have put out extensive neurites). Cells were loaded with fura-2 by incubation in HBM with 10 μM of the acetoxymethyl-fura-2 and 0.025% (w/v) Pluronic F127 for 60 min at 37°C. Any excess ester was removed by replacing the medium, and the coverslips were then transferred to a microscope perfusion chamber for single-cell ratio-fluorescence measurements using a PTI system, and carried out at 30°C in HBM as above except that 2 mM $CaCl_2$ and 0.8 mM $MgCl_2$ were present. Drugs were introduced into the chamber via the perfusion system (which involved a 15 s delay between addition (or washout) of a compound and contact with the cells).

Drugs and reagents

Lifarizine (RS-87476-190; 1-[2-(4-methylphenyl)-5-methyl)-1H-imidazol-4-yl-methyl]-4-diphenylmethyl-piperazine trihydrochloride) was synthesized and supplied by Syntex Research. Glutamate dehydrogenase (EC 1.4.1.3) was from Sigma (Poole, Dorset) and was dialysed before use to remove contaminating glutamate. Bovine serum albumin was also from Sigma, as were all other reagents with the exception of fura-2 acetoxymethyl ester and Pluronic F127 which were from Calbiochem (Nottingham).

Results

Effects of lifarizine on glutamate release from synaptosomes

Preliminary experiments established that the addition of lifarizine at either 1 min or 2 min before eliciting glutamate release or calcium influx revealed no significant difference in responses. We chose 30 mM KCl as the concentration for depolarization as previous work had shown that this results in a maximal and robust calcium-dependent release of glutamate which can be readily modulated by inhibitory agents (Budd & Nicholls, 1995).

Figure 1 (a) and (b) shows respectively the effects on the total and the Ca^{2+}-dependent release of glutamate stimulated

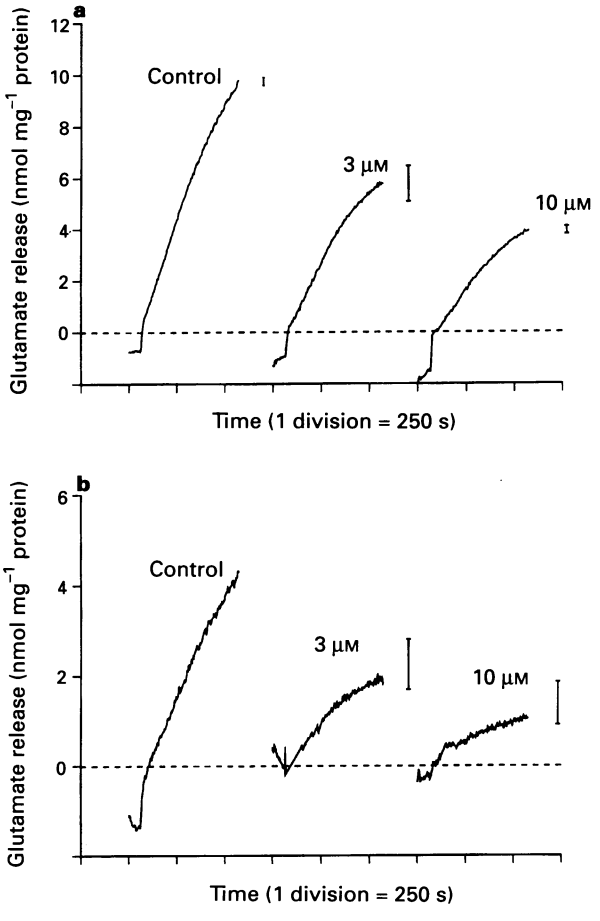

Figure 1 The effects of lifarizine on (a) the total, and (b) the Ca^{2+}-dependent release of glutamate from rat cerebrocortical synaptosomes evoked by 30 mM KCl. Lifarizine was added at the concentrations shown 1 min prior to the addition of 30 mM KCl which itself is indicated where the traces cross the dotted line. Averaged traces from experiments with three separate preparations are shown and the error bars shown are s.e.means on values (see text) measured at 6.5 min after the KCl addition.

by 30 mM KCl, of the prior (1 min) addition of 3 μM and 10 μM lifarizine. This indicates that lifarizine at all concentrations used in the study, inhibits both total and Ca^{2+}-dependent depolarization-induced release of glutamate, which is the predominant neurotransmitter in this preparation (Cousin *et al.*, 1993), and suggests that this may be a concentration-dependent effect. The total release of glutamate evoked by 30 mM KCl reached a value of 8.48 ± 0.21 nmol mg^{-1} protein when measured at 6.5 min after the K^+-induced depolarization (Figure 1a; $n=3$). Addition of 3 or 10 μM lifarizine 1 min prior to the KCl reduced this to 5.24 ± 0.44 and 3.48 ± 0.11 nmol mg^{-1} protein respectively ($P \leq 0.01$ compared to the control in each case), representing reductions of $39 \pm 5.1\%$ and $72 \pm 1.2\%$ from the control value. The Ca^{2+}-dependent component of KCl-stimulated release was similarly decreased (Figure 1b) from a control value of 3.57 ± 0.21 nmol mg^{-1} protein ($n=3$) at 6.5 min after depolarization to values of 1.91 ± 0.28 and 0.95 ± 0.42 nmol mg^{-1} protein in the presence of 3 and 10 μM lifarizine respectively ($P \leq 0.01$ compared to the control in each case), representing reductions of $47 \pm 7.8\%$ and $74 \pm 11.7\%$ from the control value. Less inhibition of the Ca^{2+}-independent release component was observed (32% and 48% respectively for 3 μM and 10 μM lifarizine).

Effects of lifarizine on intrasynaptosomal Ca^{2+}

The plateau phase of the fura-2 signal in this preparation has been shown previously to be linked to glutamate release, whereas the spike component is not linked to glutamate release and has been shown to be primarily composed of influx through calcium channels which are insensitive to dihydropyridines (Pocock *et al.*, 1993). Figure 2 (a) and (b) thus respectively compare the effects of both post- (2 min) and pre- (2 min) addition of 3 and 10 μM lifarizine on the KCl-induced increases in cytosolic Ca^{2+} in the synaptosomes incubated in the presence of Ca^{2+}. This indicates that lifarizine decreases these depolarization-induced increases in cytosolic $[Ca^{2+}]$, whether added before or after KCl, and suggests that this may be a concentration-dependent effect. When added after KCl, 3 and 10 μM lifarizine reduced the control plateau elevation by $50.1 \pm 15.1\%$ and $52.1 \pm 10.4\%$ respectively (Figure 2a; $n=3$), thus directly indicating that lifarizine does indeed inhibit the calcium channel coupled to glutamate release. Figure 2 (b) indicates that 3 μM lifarizine did not substantially affect the spike component, but that 10 μM completely abolished it, thus indicating that lifarizine also inhibits calcium channels that are not coupled to glutamate release and that are likely to be insensitive to dihydropyridines.

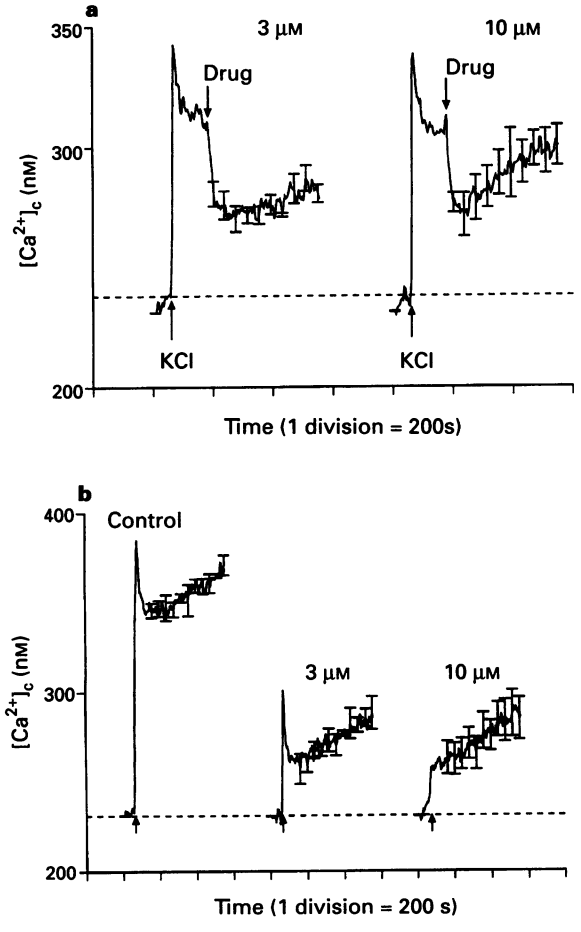

Figure 2 The effects on the rises in intracellular Ca^{2+} in rat cerebrocortical synaptosomes induced by 30 mM KCl, of the addition of lifarizine (a) 2 min after and (b) 2 min before depolarization. Lifarizine was added at the concentrations shown and KCl was added as indicated. The traces represent means from three separate experiments on different preparations and the bars shown are s.e.means.

Effects of lifarizine on intact neurones

We also wished to establish whether the above phenomena could also be demonstrated in intact whole cells, and thus used single, fura-2-loaded, rat cultured cortical neurones (see Methods). The response to a 30 s exposure to 60 mM KCl produced variable responses from cell to cell; however, for each individual cell the response was acceptably reproducible when repeated at 15 min intervals (Table 1), thus allowing the effects of drugs on the response to be quantitated. Both 1 μM and 3 μM lifarizine reduced the increases in intracellular $[Ca^{2+}]$ elicited by 60 mM KCl (Table 1). The results with 1 μM lifarizine suggest that it may be more potent in this cell system than in the synaptosomal system used above. Table 1 also shows that nitrendipine at 100 nM produced a similar degree of inhibition as 1 or 3 μM lifarizine, but that 1 μM tetrodotoxin showed a much weaker, and non-significant, response. This suggests that lifarizine may interact with dihydropyridine-sensitive channels in this preparation.

Discussion

The results of the present work clearly reveal that lifarizine is a potent inhibitor of glutamate release in rat cerebrocortical synaptosomes. Release of glutamate following Ca^{2+} and Na^+ entry as a consequence of cell depolarization is thought to play a central role in the progression of damage due to conditions such as stroke (e.g. Choi 1987, 1988), and therefore this

Table 1 Effects of lifarizine, tetrodotoxin and nitrendipine on the increases in intracellular $[Ca^{2+}]$ evoked by 60 mM KCl in rat single cultured neurones loaded with fura-2

Condition	*% of initial control response*
Control	89.1 ± 14.7 (10)
Lifarizine 1 μM	20.8 ± 11.3 (5)*
Lifarizine 3 μM	18.3 ± 11.2 (4)*
Tetrodotoxin 1 μM	66.3 ± 17.0 (4)
Nitrendipine 0.1 μM	23.8 ± 10.8 (5)*

All cells were initially depolarized with a 30 s exposure to 60 mM KCl followed by a 15 min washout period where the appropriate drug (or vehicle) was added for the last 10 min before a second 30 s exposure to 60 mM KCl. The average initial increase in intracellular $[Ca^{2+}]$ (at peak) was 527 ± 67 nM (28) (from pre-stimulus values in the 100–400 nM range). Values are means ± s.e.mean for the number of separate cells in parentheses, and $*P \leq 0.05$ versus the control condition.

property of the compound may contribute to its protective action in models of this condition (see Introduction). The ability to inhibit directly Na^+ and Ca^{2+} entry, and to inhibit indirectly depolarization and glutamate release, suggests that it may provide neuroprotection additional to that afforded by glutamate receptor antagonists. The effects described here appear to be within broadly the same efficacy concentration-range as the effects of the compound on sodium and calcium channels (MacKinnon *et al.*, 1995; McGivern *et al.*, 1995; Brown *et al.*, 1995a).

The present work also suggests that the mechanism by which the inhibition of glutamate release is achieved is most probably predominantly due to the direct inhibition of the calcium channels that are intimately associated with release, as a clear and rapid inhibition of the plateau phase of the fura-2 signal was observed (Figure 2) (Pocock *et al.*, 1993). This plateau phase has been shown to be linked to glutamate release and to be composed of non-inactivating calcium channels that are sensitive to the toxin Aga-GI (Pocock *et al.*, 1993). However, lifarizine at 10 μM also appeared to abolish the spike phase of the fura-2 signal, indicating that it also inhibits other calcium channels which are not linked with glutamate release and which transiently open and then rapidly inactivate on depolarization, and which appear to be dihydropyridine-insensitive (Pocock *et al.*, 1993).

The results obtained in the experiments carried out in calcium-free buffer suggest that lifarizine may also inhibit Ca^{2+}-independent release of glutamate to some degree, though overall not as potently as it inhibited the Ca^{2+}-dependent component. Calcium-independent release of glutamate may also become important under ischaemic conditions where reversal of the Na^+/glutamate transporter, as well as the increase in calcium-dependent exocytosis, is thought to occur (Szatkowski & Atwell, 1994). It is also of interest to note in this regard the recent work by Romano-Silva *et al.* (1994) using tityustoxin, which stimulates Na^+ influx through tetrodotoxin-sensitive Na^+ channels, which suggests that localized Na^+ entry through these channels exerts a modulatory role on Ca^{2+}-dependent release from synaptosomes.

The experiments on the cultured neurones indicate that the inhibitory effects of lifarizine in a whole cell system appear to be similar to the findings with the synaptosomes, though an indication of greater potency was found in the former. Interestingly, a known sodium-channel blocker was not able to block the responses in this case, whereas a blocker of non-inactivating L-type calcium channels was effective, suggesting that the predominant effects of lifarizine in this system may similarly have been due to its effects on L-type calcium channels (Fraser & Spedding, 1991). Thus lifarizine may interact with a number of channels implicated in depolarization-induced effects, and inhibitory effects on T-type calcium channels have also already been described (Brown *et al.*, 1995a). However, it is important to note that basal conditions appeared to be unaffected by the drug.

Other drugs have also been found to inhibit glutamate release from neuronal preparations. The profile of lifarizine described in the present work appears to be most similar in terms of potency and effects to that of flunarizine, an agent which has been shown to inhibit both the calcium-dependent and -independent release of glutamate from synaptosomes and cultured neurones from rat brain, and when challenged by either high KCl or by the sodium channel opener, veratridine (Cousin *et al.*, 1993). As already noted in the Introduction, lifarizine has also additionally been shown to protect against veratridine-induced neurotoxicity in cultured neurones from rat brain (May *et al.*, 1995). Another compound which has been reported to be neuroprotective and to have similar use-dependent sodium channel blocking activity to lifarizine and flunarizine is BW-619C89 (Graham *et al.*, 1994). Interestingly, this compound inhibited glutamate release from slices of rat brain cerebral cortex induced by veratridine, but not that induced by KCl depolarization (Leach *et al.*, 1993). Thus the apparently rather less specific channel blocking activities of lifarizine may in fact allow it greater efficacy in inhibiting glutamate release under ischaemic conditions through its ability to interfere with more than one of the potentially deleterious openings of ion channels caused by and resulting in cell depolarization, and which lead to enhanced and toxic glutamate release. More specific blockers of both sodium (e.g. Prenen *et al.*, 1988; Yamasaki *et al.*, 1991) and calcium (e.g. Weiss *et al.*, 1990; Alps, 1992) channels, and glutamate receptors (e.g. Park *et al.*, 1988; Gill *et al.*, 1991) have individually been shown to have protective efficacy in stroke and neurotoxicity models.

The present studies demonstrate a potential functional outcome, i.e. the blockage of intracellular Ca^{2+} rises and glutamate release, for the previously described ion channel blocking properties of lifarizine which may play a mechanistic role in its neuroprotective action. An agent with blocking properties at both sodium and calcium channels may thus offer further advantages to agents which block only at one or the other, especially if, as shown, it can also result in reduced glutamate release.

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