The effects of (–)-daurisoline on Ca^{2+} influx in presynaptic nerve terminals

Y. Ming Lu & G. Qing Liu

Department of Pharmacology, China Pharmaceutical University, Nanjing, 210009, China

- 1 The effects of (–)-daurisoline on $^{45}\text{Ca}^{2+}$ uptake and [^3H]- γ -aminobutyric acid ([^3H]-GABA) release from synaptosomes of rat cerebral cortex and on contractile activity of rat aorta were examined.
- 2 Application of (–)-daurisoline (1–100 μM) produced concentration-related inhibition of high K^+ -stimulated $^{45}\text{Ca}^{2+}$ uptake and [^3H]-GABA release ($\text{IC}_{50} = 7.7 \pm 0.9 \mu\text{M}$ and $10.0 \pm 1.5 \mu\text{M}$, respectively) in synaptosomes but verapamil was only weakly active.
- 3 Neither (–)-daurisoline (100 μM) nor verapamil (100 μM) modified $^{45}\text{Ca}^{2+}$ uptake in control medium (5 mM K^+ , resting uptake) and [^3H]-GABA release in Ca -free medium (45 mM K^+ basal release).
- 4 High K^+ and noradrenaline-evoked contractions of rat aorta were inhibited by both (–)-daurisoline and verapamil.
- 5 In conclusion, (–)-daurisoline, which differed from verapamil in its mode of blocking Ca^{2+} influx may be a potent Ca^{2+} antagonist of Ca^{2+} channels in neurones.

Introduction

(–)-Daurisoline (Figure 1) is a tetrahydroisoquinoline (TIQ) isolated from the roots of *Menispermum dauricum*. TIQs, which possess a diversity of pharmacological activities, have been shown to have Ca^{2+} channel blocking properties. Recently, some TIQs, such as tetrandrine (Han *et al.*, 1987) and dauricine (Dongke *et al.*, 1987) were used clinically for the treatment of hypertension, supraventricular tachycardia and congestive heart failure. Like other Ca^{2+} channel antagonists, these TIQs block L-type Ca^{2+} channels in cardiovascular muscle (King *et al.*, 1980). We considered that (–)-daurisoline might have a similar effect on Ca^{2+} influx to tetrandrine and dauricine due to their chemical similarity and we therefore examined its Ca^{2+} antagonistic effects.

Pinched off nerve terminal endings (synaptosomes) retain the functional capacity to take up $^{45}\text{Ca}^{2+}$ during depolarization (Nachshen & Blaustein, 1980) and release neurotransmitter in response to the influx of Ca^{2+} (Drapeau & Blaustein, 1983; Suszkiw *et al.*, 1985). Although the time course of radioflux measurements (seconds) does not compare well with that of synaptic transmission as it is measured electrophysiologically (milliseconds) several features correlated well between the two systems, such as their sensitivity to block by divalent inorganic cations (Manalis *et al.*, 1984; Suszkiw *et al.*, 1985) and their similar lack of sensitivity to organic Ca channel blockers (Gotgiff & Magazanile, 1977; Suszkiw *et al.*, 1986; Atcheson & O'Leary, 1987). Because of these similarities, and because the $^{45}\text{Ca}^{2+}$ uptake and transmitter release process by synaptosomes have been well studied, we have taken advantage of this system as a model to test the effects of (–)-daurisoline on depolarization-induced Ca^{2+} influx and [^3H]- γ -aminobutyric acid ([^3H]-GABA) release.

The stimulated 'fast' phase of uptake, which occurs during the first 5 s of depolarization (Nachshen, 1985; Suszkiw *et al.*, 1986), is presumed to be mediated by a voltage-dependent Ca^{2+} channel and has been associated with release of a

number of transmitters from synaptosomes (Floor, 1983; Leslie *et al.*, 1985). The goal of the present study was to investigate the effects of (–)-daurisoline on 'fast' phase $^{45}\text{Ca}^{2+}$ influx and transmitter release in synaptosomes. In addition, the effects of (–)-daurisoline on contractile activity of rat aorta stimulated by noradrenaline (NA) and high K^+ were tested to ascertain any selectivity for Ca^{2+} channel blockade between neurones and smooth muscle.

Methods

Preparation of synaptosomes

Synaptosomes were prepared from rat cerebral cortex which was removed, pooled, and homogenized in 10 volumes of ice-cold 0.32 M sucrose with a Teflon coated pestle followed by centrifugation at 4°C for 10 min at 1000g. Crude synaptosomes were obtained by centrifugation at 17000g for 60 min at 4°C and resuspension of the pellet in 10 volumes 0.32 M sucrose. Synaptosomes were separated by sucrose gradient centrifugation at 55000g for 45 min at 4°C. Synaptosomes in the 0.8 M sucrose fraction from the sucrose gradient were equilibrated with physiological saline by the addition of 2.5 volumes of ice-cold standard HEPES buffer (composition, mM: NaCl 145, KCl 5, MgCl_2 1.2, NaH_2PO_4 2.4, and HEPES 20, adjusted to pH 7.4 at 25°C with Tris). The diluted synaptosomes were centrifuged at 10000g for 10 min at 4°C and the pellet was resuspended in cold HEPES buffer.

Measurement of $^{45}\text{Ca}^{2+}$ influx

The synaptosome suspensions (300 μl ; containing approximately 0.25 mg protein) were preincubated for 10 min at 30°C (with or without drug) before initiating test uptake of $^{45}\text{Ca}^{2+}$. Aliquots of the warmed synaptosome suspensions were added to 100 μl incubation solutions containing 1 μCi of $^{45}\text{Ca}^{2+}$. Uptake into synaptosomes of $^{45}\text{Ca}^{2+}$ was measured in either standard HEPES buffer or stimulating buffer, containing 75 mM K^+ (final concentration), substituted for equimolar concentration of sodium. After a test incubation lasting 20 s, $^{45}\text{Ca}^{2+}$ uptake was stopped by addition of 4.5 ml of ice-cold HEPES buffer. The diluted suspensions were immediately filtered by suction through Whatman glass fibre filters (GF/C 2.4 cm diameter). The filters were rapidly washed twice with 4.5 ml ice-cold HEPES. The filters were then suspended in vials containing 5 ml of scintillation liquid (toluene containing

Figure 1 The chemical structure of (–)-daurisoline.

Table 1 Effects of drugs on K-stimulated ^{45}Ca uptake in synaptosomes

Drugs (μM)	Ca uptake (nmol mg^{-1} protein)		ΔK	% inhibition
	Standard buffer	Stimulating buffer		
Control	0.83 ± 0.09	1.78 ± 0.85	0.95 ± 0.07	
Daurisoline (5)	$0.80 \pm 0.07^*$	$1.37 \pm 0.63^{**}$	0.57 ± 0.04	40.0
Verapamil (50)	$0.81 \pm 0.10^*$	$1.58 \pm 0.75^{**}$	0.77 ± 0.08	18.9

Synaptosomes were preincubated with or without drug in standard buffer and then incubated with or without drug with ^{45}Ca , in standard buffer or stimulating buffer. Stimulated ^{45}Ca uptake ΔK , was then calculated as the difference between ^{45}Ca uptake from stimulating buffer and standard buffer.

* $P > 0.1$ (NS). ** $P < 0.001$, two-tailed *t*-test.

0.5% PPO, 0.01% POPOP) and the $^{45}\text{Ca}^{2+}$ content of the material trapped by the filters was determined by liquid scintillation spectrometry. The protein concentration was determined by the method of Lowry *et al.* (1951). The extra uptake of $^{45}\text{Ca}^{2+}$ (K^+ -stimulated uptake = ΔK) was calculated as the difference between the uptake from standard HEPES buffer and the uptake from the stimulating buffer. In each experiment $^{45}\text{Ca}^{2+}$ uptake determinations were made on 6 replicates, as indicated in the table and figure legends.

Measurement of [^3H]-GABA release

Rat hippocampus synaptosome suspensions (1 ml containing approximately 0.8 mg protein) were incubated with 4 nM [^3H]-GABA for 15 min at room temperature (25–30°C). Uptake was stopped by the addition of 1 ml ice-cold HEPES buffer. Synaptosomes were separated by centrifugation at 35000 *g* for 5 min at 4°C. The supernatant was discarded and the pellet was used for measurement of [^3H]-GABA release. Stimulated release was studied, based on the technique of Haycock *et al.* (1977). The synaptosomes preloaded with [^3H]-GABA, were washed by addition of six consecutive 1 ml ice-cold Ca^{2+} -free wash solutions (composition, mM: NaCl 100, KCl 45, MgCl_2 1.2, NaH_2PO_4 2.4, EGTA 20 and HEPES 20, pH 7.4). [^3H]-GABA release was stabilized by the fifth wash (Cotman *et al.*, 1976). The washed synaptosomes were separated by centrifugation at 35000 *g* for 5 min at 4°C. The supernatant was discarded, and the pellet was suspended in HEPES buffer for the measurement of [^3H]-GABA release. Suspensions were preincubated for 10 min at 30°C (with or without drug) before initiating test [^3H]-GABA release. [^3H]-GABA release was measured in either 1.2 mM CaCl_2 wash (stimulated release) or Ca^{2+} -free wash buffer (basal release). The reaction was quenched rapidly with 1 ml ice-cold Ca^{2+} -free wash buffer 15 min after addition of 1.2 mM CaCl_2 to the test medium (Ca^{2+} -free wash buffer). Net Ca^{2+} -dependent release of [^3H]-GABA was calculated by subtracting the % basal release values from % stimulated release values. All values represent the mean $\pm$ s.e. mean of six determinations.

Effects of (–)-daurisoline on noradrenaline- and K^+ -induced contractions

In these experiments, inhibition of the contractions induced by noradrenaline (NA) and high K^+ -depolarization was measured as a function of the efficacy of (–)-daurisoline or verapamil. Rats were killed, exsanguinated and the aortae removed and cut into aortic rings (3 mm long). These were mounted in all-glass 20 ml muscle chambers at 37°C. The bathing fluid was Krebs solution of the following composition (mM): NaCl 118, KCl 4.8, CaCl_2 2.5, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 24, glucose 11, at 37°C pH 7.4. It was continuously gassed with 95% O_2 :5% CO_2 . Responses were recorded isometrically. First, a control maximal contraction produced with either 1 μM NA or 75 mM K^+ . All data are expressed as a percentage of this control contraction. This was followed by a contraction induced by a concentration of the agonist used for a specific (–)-daurisoline or verapamil concentration to be tested. The aorta was then challenged in the presence of (–)-daurisoline or verapamil with the agonist.

Subsequently, the agonist was washed out in the presence of the next higher concentration of (–)-daurisoline or verapamil and after 30 min, again challenged by the same agonist concentration. The process was repeated until the entire (–)-daurisoline or verapamil concentration-response curve at this agonist concentration was obtained. Only one concentration-response curve was obtained from each muscle ring. The magnitude of the control contraction did not change significantly during the time course of the experiments.

Materials

$^{45}\text{Ca}^{2+}$ (specific activity, 25 mCi g^{-1}) and [^3H]-GABA (specific activity, 35 Ci mmol^{-1}) were obtained from China Atomic Energy Institute. Verapamil and noradrenaline were obtained from Tianjing Research Institute of Pharmaceutical Industry. (–)-Daurisoline was obtained from the Department of Pharmacchemistry, China Pharmaceutical University. HEPES was obtained from U.S. Biochemical Co.

Results

Effects of (–)-daurisoline on $^{45}\text{Ca}^{2+}$ uptake in synaptosomes

Triggered $^{45}\text{Ca}^{2+}$ uptake in high K^+ buffer during the first 20 s of depolarization was approximately 2–3 fold of the basal $^{45}\text{Ca}^{2+}$ uptake in control buffer (Table 1). (–)-Daurisoline (100 μM) had little or no effect on basal uptake. The most prominent effect produced by (–)-daurisoline was concentration-dependent inhibition of stimulated $^{45}\text{Ca}^{2+}$ uptake ($\text{IC}_{50} = 7.7 \pm 0.9 \mu\text{M}$, $n = 6$). Meanwhile, for 40% inhibition of maximal $^{45}\text{Ca}^{2+}$ uptake, a verapamil concentration of 100 μM was required. These results are shown in Figure 2.

Figure 2 Effects of (–)-daurisoline on $^{45}\text{Ca}^{2+}$ uptake in synaptosomes: (▲) inhibitory effect of verapamil; (○) blocking action of (–)-daurisoline on the effects of incubating synaptosomes with high external K^+ (75 mM). Points in the curves represent the mean of 6 experiments; s.e. mean shown by vertical lines. Test incubation with $^{45}\text{Ca}^{2+}$ lasted 20 s. All buffers contained 1.2 mM CaCl_2 and 1.2 mM MgCl_2 . $^{45}\text{Ca}^{2+}$ uptake from control buffer averaged $0.81 \pm 0.09 \text{ nmol mg}^{-1}$ protein. * $P < 0.02$ (values significantly less than control); ** $P < 0.01$; *** $P < 0.001$, one-tailed *t* test.

Effect of (-)-daurisoline on [^3H]-GABA release

Addition of CaCl_2 (0.2–1.2 mM) produced a concentration-related increase in [^3H]-GABA release (Figure 3). The % maximal release was 2.98 ± 0.33 . The effect of (-)-daurisoline (1–100 μM) on K^+ -stimulated [^3H]-GABA release is seen in Figure 4. When (-)-daurisoline concentration was increased from 1 μM to 100 μM , inhibition produced by (-)-daurisoline was $15.3 \pm 1.8\%$ and $70.6 \pm 5.4\%$ respectively ($\text{IC}_{50} = 10.0 \pm 1.3$, $n = 7$). At a concentration of 10 μM , verapamil only slightly inhibited [^3H]-GABA release (% inhibition = 10.9 ± 1.8 , $n = 7$). Neither (-)-daurisoline (100 μM) nor verapamil (100 μM) decreased the basal release in Ca -free buffer significantly.

Figure 3 The influence of external Ca^{2+} on [^3H]- γ -aminobutyric acid ([^3H]-GABA) release. Points indicate [^3H]-GABA release in various Ca^{2+} concentrations. Each point represents mean for 7 determinations; s.e.mean shown by vertical lines. Test incubation with indicated Ca^{2+} concentration lasted 15 min at room temperature (25–30°C). All buffers contained 1.2 mM MgCl_2 and 45 mM K^+ . Radioefflux in a given supernatant is represented as the total radioactivity present in the pellets immediately after the centrifugation.

Figure 4 The effects of drugs on [^3H]- γ -aminobutyric acid ([^3H]-GABA) release in synaptosomes. Synaptosomes loaded with [^3H]-GABA were preincubated for 10 min at 30°C with drugs at the indicated concentrations before initiating [^3H]-GABA release: ($\blacktriangle$) (-)-daurisoline; ($\bullet$) verapamil [^3H]-GABA release from synaptosomes was measured in 1.2 mM CaCl_2 wash buffer for 15 min. Values represent the mean for 7 determinations; s.e.mean shown by vertical lines. Percentage inhibition is expressed as Net release without drug – Net release with drug/Net release without drug (%). * $P < 0.02$ (values significantly less than control); ** $P < 0.01$, one-tailed t test.

Effects of (-)-daurisoline on contractile activity of aorta

As shown in Figures 5 and 6, (-)-daurisoline produced a concentration-dependent inhibition of contraction induced by NA (1 μM) and high K^+ (75 mM) with an IC_{50} of $39.8 \pm 4.1 \mu\text{M}$ and $3.9 \pm 0.2 \mu\text{M}$ ($n = 5$). The IC_{50} of verapamil was $31.6 \pm 2.9 \mu\text{M}$ and $0.21 \pm 0.04 \mu\text{M}$ ($n = 5$), respectively.

Figure 5 Concentration-response curves for the inhibition by (-)-daurisoline ($\blacktriangle$) and verapamil ($\circ$) of noradrenaline (1 μM)-induced contractions of rat aortic rings. For details, see Methods. Each point represents a mean of five determinations; s.e.mean was less than 10% of the mean. * $P < 0.05$ (values significantly less than control), two-tailed t test.

Figure 6 Concentration-response curves for the inhibitory efficacy of (-)-daurisoline ($\blacktriangle$) and verapamil ($\circ$) against contractions produced by K^+ (75 mM). Each point represents mean of five determinations; s.e.mean was less than 10%. * $P < 0.05$, two-tailed t test.

Discussion

$^{45}\text{Ca}^{2+}$ uptake stimulated by high K^+ , was maximal within 2 min at 30°C and consisted of a 'fast' phase which occurs during the first 5 s of depolarization and a 'slow' phase which appears 20 s after prolonged depolarization (Figure 7). This result is in agreement with previous reports (Nachshen & Blaustein, 1979). The 'fast' phase of uptake is presumed to be mediated by voltage-dependent Ca^{2+} channel and is associated with release of the transmitter from synaptosomes (Floor, 1983; Leslie *et al.*, 1985). The exact nature of the 'slow' phase is as yet unclear, but is probably due in part to exchange processes. To our knowledge, it is likely that only depolarization-induced transmembrane Ca^{2+} movements are involved in synaptic effects of (-)-daurisoline in this study, since effects of

Figure 7 Synaptosomes were preincubated in standard HEPES buffer and then incubated with $^{45}\text{Ca}^{2+}$ standard HEPES buffer or stimulating buffer for the indicated test-incubation period: $^{45}\text{Ca}^{2+}$ uptake in the test incubation period with stimulating buffer (●) or standard HEPES buffer (○). The values represent the mean for 7 determinations; s.e. mean shown by vertical lines. * $P < 0.01$ (values significantly less than resting uptake), one-tailed t test.

(-)-daurisolone on $^{45}\text{Ca}^{2+}$ uptake were observed only at 'fast' phase and since the data presented in this investigation show that the resting $^{45}\text{Ca}^{2+}$ uptake and basal [^3H]-GABA release were not altered by (-)-daurisolone. (-)-Daurisolone did not chelate Ca^{2+} directly (data not shown).

In the [^3H]-GABA release experiments, only 45 mM K^+ was used to minimize the possibility of influencing intraterminal mitochondrial Ca^{2+} metabolism (Haycock *et al.*, 1977).

In a separate set of experiments, we also investigated the effect of (-)-daurisolone on contractile force of rat aorta evoked by NA and high K^+ . In vascular smooth muscle, L-type Ca^{2+} channels operated by depolarization have been reported (Nilius & Tsien, 1985). L-type Ca^{2+} channels with

highest K^+ depolarization threshold are sensitive to organic Ca^{2+} antagonists such as verapamil. Marked inhibition of Ca^{2+} conductance in vascular smooth muscle is observed with concentrations of verapamil of the order of 10 nM to 10 μM (Fleckenstein, 1977). Our results revealed that verapamil sharply inhibited the contractile force of rat aorta evoked by NA and high K^+ with an IC_{50} of $31.6 \pm 2.9 \mu\text{M}$ and $0.21 \pm 0.04 \mu\text{M}$, respectively. But the effects of verapamil on stimulated $^{45}\text{Ca}^{2+}$ uptake and [^3H]-GABA release by synaptosomes showed only 1/20 of the efficacy of (-)-daurisolone. In contrast to verapamil, daurisolone was less sensitive in inhibiting the contractile force of the rat aorta stimulated by NA and high K^+ . These results indicated that (-)-daurisolone is relatively selective as an antagonist of Ca^{2+} channels in neurones.

The mode of blocking Ca^{2+} influx of (-)-daurisolone and its specificity for a particular subpopulation of Ca channels were not explored. The electrophysiological correlate to the fast phase of tracer influx into synaptosomes is unclear, as is the actual type of Ca channel involved in neurotransmitter release (Nowicky *et al.*, 1985). Recently, Miller (1987) and Triggle (1989) have presented evidence that the N-type Ca channel is responsible for $^{45}\text{Ca}^{2+}$ uptake and transmitter release. The most prominent agent active at the N-channel is ω -conotoxin GVIA, a 27 residue peptide isolated from the toxin of molluscs of the *Conus* genus (Olivera & Cruz, 1987). ω -Conotoxin exerts potent inhibitory effects in many, but not all neuronal systems suggesting that neuronal communications may depend upon a variety of channel types differing in their pharmacological behaviour and possibly also in their cellular location. Radioligand binding studies of [^3H]-conotoxin may be useful in discerning which types of Ca^{2+} channels in neurones are affected by daurisolone. Further investigations are required to show that (-)-daurisolone is a specific antagonist of N-type Ca channels.

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