Br. J. Pharmac. (1986), **88**, 909–914

Augmentation by calcium channel antagonists of general anaesthetic potency in mice

S.J. Dolin & H.J. Little[1]

Division of Anaesthesia, Clinical Research Centre, Watford Road, Harrow, Middlesex

1 The effects of three kinds of calcium channel antagonists on the anaesthetic potencies of ethanol, pentobarbitone and argon were examined in mice.

2 Ethanol and pentobarbitone anaesthetic potencies in mice were significantly increased by verapamil 10 mg kg^{-1}, flunarizine 40 mg kg^{-1} and nitrendipine 100 mg kg^{-1}.

3 Argon anaesthetic potency was significantly increased by nitrendipine 50 mg kg^{-1} and 100 mg kg^{-1} in a dose-related fashion.

4 Even at very high doses the calcium channel antagonists did not produce anaesthesia by themselves.

5 At the doses used the calcium channel antagonists did not affect the blood concentrations of ethanol, 2 h, or pentobarbitone, 15 min, after anaesthetic administration.

Introduction

Calcium has long been suspected of being of considerable importance in the mechanism of general anaesthesia. Suggestions of possible important sites of action have included changes in neurotransmitter release, altered post-synaptic function, membrane fluidity effects and changes in cyclic nucleotide levels. While these have all been shown to be affected by calcium concentrations, none in particular has been shown to be the crucial process for production of the state of general anaesthesia.

Some authors have suggested that anaesthetics cause reduced availability of functional intracellular calcium in synaptic terminals. Pentobarbitone (Blaustein & Ector, 1975) and ethanol (Harris & Hood, 1980) have been shown to diminish uptake of calcium into depolarized synaptosomes. Ethanol has been shown to decrease calcium entry via voltage-dependent channels (Lynch & Littleton, 1983) and also via voltage-independent channels (Stokes & Harris, 1982). On the other hand Baker & Shapira (1980) suggested that anaesthetics increased sensitivity of endogenous calcium binding proteins in the absence of any alterations in intracellular calcium levels, while Krnjević & Lisiewicz (1972) suggested that a rise in intracellular calcium activated calcium-sensitive forms of K^+ and Cl^- permeability systems resulting in reduced neuronal excitability.

There have been some recent significant advances in the pharmacology of neuronal calcium channels. High affinity binding sites have been demonstrated for a variety of calcium channel antagonists including nitrendipine, nimodipine, verapamil and diltiazem. These binding sites are thought to be a part of, or to be in close proximity to calcium channels. They have been demonstrated in a variety of tissues including brain (Murphy and Snyder, 1982; Glossman *et al.*, 1985).

We have investigated the effects of three structurally diverse calcium channel antagonists on the *in vivo* anaesthetic potencies of a variety of anaesthetic agents.

The calcium channel inhibitors we used were a dihydropyridine, nitrendipine, a phenylalkylamine, verapamil, and a diphenylalkylamine, flunarizine. We have investigated their effects on anaesthetic potencies of a barbiturate (pentobarbitone) of an alcohol (ethanol) and of an inert gas (argon).

Methods

Male T.O. mice (25–35 g) were used in all experiments. All animals were fresh from specific-pathogen-free unit, kept in controlled light cycled rooms, and fed rodent chow.

[1]Address for correspondence: Univ. Dept. of Pharmacology, South Parks Rd., Oxford OX1 3QT.

Anaesthetic potency estimations

Animals were pretreated with the calcium channel antagonists nitrendipine, 100 mg kg^{-1}, and flunarizine, 40 mg kg^{-1}, 2 h before anaesthetic administration. Verapamil, 10 mg kg^{-1}, was given 15 min before the anaesthetic drug. Concurrent control groups were used in each experiment. All were then placed in cages on heated mats with overhead illumination, and all rectal temperatures were maintained in the range 36.5°C–37.5°C by monitoring a selection of mice, and adjusting heat sources appropriately.

Following anaesthetic administration, animals were assessed for loss of righting reflex by an observer who did not know the pretreatment given to each animal. For pentobarbitone and ethanol anaesthesia each animal was assessed by placing it on its back at 5 min intervals until awake. Failure to regain the upright posture within 60 s was taken as the criterion for anaesthesia. A minimum of four doses of each anaesthetic were studied. Ten mice were used in each group for both controls and those given calcium channel inhibitors. Each animal was used once only.

For argon anaesthesia animals were pretreated with nitrendipine 50 mg kg^{-1} or 100 mg kg^{-1}, 2 h before argon administration. The mice were placed in a rotating cage, with 4 divisions, inside a 20 litre steel pressure chamber. Oxygen was supplied to 0.5 atmospheres and argon added at a rate of 3 atmospheres per min to the predetermined pressure. An equilibration time of 15 min was chosen, based on preliminary experiments. At the end of equilibration time, loss of righting reflex was assessed by rolling the mouse onto its back in the rotating cage by remote control, and allowing it 60 s to regain the upright posture. A second pressure was then chosen and a second assessment made after a further 15 min. Each mouse was used once, at each of 2 pressures; eight mice were used per group. One half was assessed firstly at a low pressure followed by a higher pressure and the other half conversely. A minimum of 4 different pressures were used for each calcium channel antagonist. A separate control and a separate treated mouse were used for rectal temperature monitoring in every experiment. They were kept in another cage in the chamber adjacent to the rotating cage; rectal temperatures were maintained at 37°C ± 0.5°C by the alteration of ambient temperature using water-filled heating coils inside and electrical heating outside the pressure chamber.

Raw data, as numbers out of 10, (8 for argon experiments), with loss of righting reflex, at each anaesthetic dose were then analysed by probit analysis (Ross, 1980) to construct dose-response curves. Lines of best fit were compared using Chi-squared analysis, with results expressed for position, parallelism and heterogeneity.

Effects of high doses

A range of doses of calcium channel inhibitors up to 10 times the doses used in the anaesthetic experiments were administered to groups of at least 6 mice. Animals were kept on a warming mat with overhead illumination, to maintain rectal temperature close to 37°C without overheating. Rectal temperatures were monitored in all animals. Groups were observed on the bench, loss of righting reflex was assessed and any mortality noted over 24 h.

Blood concentrations

In order to determine whether changes in anaesthetic potency were due to altered metabolism of anaesthetic agents by calcium channel antagonists, blood levels of anaesthetics were measured after a standard dose. Nitrendipine 100 mg kg^{-1} was injected 2 h before anaesthetic administration; control animals receiving corresponding vehicle injection. Eight animals were used in each group. Ethanol 2 g kg^{-1} was injected and the animal temperatures kept at 37°C. Two hours later the animals were killed and blood samples taken from the carotid arteries. Two hours is on the declining part of the concentration curve and changes here would reflect altered ethanol metabolism (Goldstein, 1984). We have shown, in other studies, that the effects of nitrendipine 100 mg kg^{-1} last for 6–8 h. Pentobarbitone was administered at a dose of 40 mg kg^{-1} and blood samples were taken after 15 min. It was found that at 45 min post injection, blood levels of pentobarbitone were too low to be reliably detected, so the

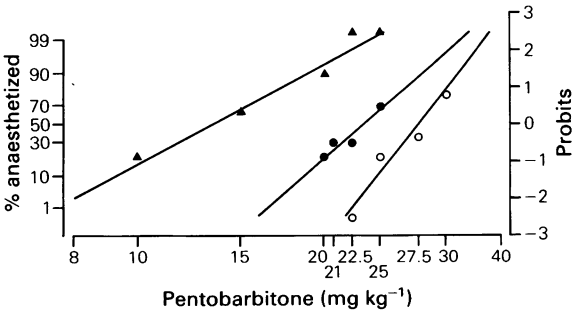

Figure 1 Dose-response curves for pentobarbitone anaesthesia at 15 min after anaesthetic administration. The lines were constructed using probit analysis. The left hand axis is percentage anaesthetized: as measured by loss of righting reflex. The right hand axis gives corresponding probit values. Concurrent controls (○) were compared with animals pretreated with nitrendipine 100 mg kg^{-1} (▲) and flunarizine 40 mg kg^{-1} (●) both at 2 h before anaesthetic administration. Each point represents $n = 10$ mice. Significance levels are shown in Table 1.

earlier time was used. Blood samples (ethanol) or plasma samples (pentobarbitone) were analysed by gas-liquid chromatography. Internal standards were isopropanol for ethanol and tetraphenylethylene for pentobarbitone. A flame ionization detector was used and the areas under the curves were compared.

Drugs used

The calcium channel inhibitors used were nitrendipine (Bayer U.K. Ltd.), verapamil (Abbott Laboratories, U.K.) and flunarizine (Janssen Pharmaceutica, Belgium). The anaesthetic agents were pentobarbitone B.P. 10–30 mg kg^{-1} (May and Baker Ltd.), ethanol 1.8–3.3 g kg^{-1} (AnalaR quality, James Burrough, dissolved 20 ml per 100 ml distilled water) and argon 8–18 atmospheres (British Oxygen Company).

All drugs, where appropriate, were dissolved in water and Tween 80 (polyoxyethylene sorbitan monooleate, Sigma) one drop per 10 ml of distilled water. Nitrendipine suspensions were made up under red safe-light. Controls received corresponding volumes of water and Tween. All drugs, except argon, were administered via the intraperitoneal (i.p.) route.

Results

Dose response curves

Dose-response curves for pentobarbitone and ethanol were constructed for each 5 min interval until animals were awake. Examples of dose-response curves for pentobarbitone are shown in Figure 1. The curves are for 15 min following administration of the anaesthetic agent. Results are expressed as percentage anaesthetized on a probit scale versus log dose of anaesthetic agent. Nitrendipine and flunarizine, produced a consistent shift to the left of the dose-response curves compared with concurrent controls, which is an increase in anaesthetic potency. Verapamil (not illustrated) also produced a shift to the left for both anaesthetic agents. All dose-response curves were parallel except the nitrendipine plus pentobarbitone curves. There was no significant heterogeneity, i.e. any residual variation was consistent with binomial sampling.

The argon dose-response curves are shown in Figure 2. The curves are for two doses of nitrendipine (50 and 100 mg kg^{-1}) against concurrent controls. There is a shift to the left of the curves, i.e. an increase in anaesthetic potency. The argon curves are for a single time at the end of a 15 min equilibration period for each pressure. These curves are parallel and the data were not heterogeneous.

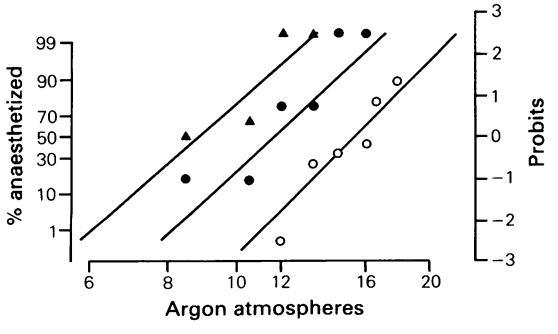

Figure 2 Dose-response curves for argon anaesthesia following equilibration for 15 min at each pressure. The lines were constructed using probit analysis. The left hand scale is percentage anaesthetized corresponding to probits on the right hand scale. Concurrent controls (○) were compared with animals given nitrendipine 100 mg kg^{-1} (▲) and nitrendipine 50 mg kg^{-1} (●) 2 h before pressurization. Each point represents $n = 8$ mice. Significance levels are shown in Table 2.

ED_{50} values for anaesthetic potency

From the dose-response curves, ED_{50} values have been derived for all values along with 95% fiducial limits. Table 1 gives the results for pentobarbitone, ethanol

Table 1 Effects of calcium channel antagonists on anaesthetic potency

A Pentobarbitone (mg kg^{-1}, 15 min after anaesthetic administration)

Drugs	*ED_{50}*	*Fiducial limits*
Controls A	27.3	25.9–29.1
Verapamil (10 mg kg^{-1})	23.1**	21.6–25.1
Controls B	28.1	26.5–29.8
Nitrendipine (100 mg kg^{-1})	13.9**	11.7–15.8
Flunarizine (40 mg kg^{-1})	23.2**	21.9–25.0

B Ethanol (g kg^{-1}, 20 min after anaesthetic administration)

Drugs	*ED_{50}*	*Fiducial limits*
Controls A	3.3	3.1– 3.5
Verapamil (10 mg kg^{-1})	3.0*	2.7– 3.1
Controls B	3.2	3.1– 3.3
Nitrendipine (100 mg kg^{-1})	2.1***	2.0– 2.3
Flunarizine (40 mg kg^{-1})	2.7***	2.5– 2.9

C Argon (Atmospheres, 15 min equilibrium time)

Drugs	*ED_{50}*	*Fiducial limits*
Controls	15.5	14.6–16.5
Nitrendipine (50 mg kg^{-1})	11.4***	10.2–12.5
Nitrendipine (100 mg kg^{-1})	8.8***	6.7–10.2

*$P<0.05$; **$P<0.01$; ***$P<0.001$ in comparison with concurrent controls, using Chi-squared analysis for position of parallel lines following probit transformation.

and argon anaesthesia. The ED_{50} values for anaesthetic potency were all significantly lowered by the calcium channel inhibitors. The degree of potentiation by nitrendipine varied between anaesthetic agents, being greatest for pentobarbitone, less for argon, and least for ethanol.

Time courses of pentobarbitone and ethanol anaesthesia

The full time courses of changes in anaesthetic potency, as measured by percentage change in ED_{50}, for pentobarbitone and ethanol are shown in Figure 3. There was a consistent decrease in anaesthetic ED_{50} values when animals treated with the three calcium channel antagonists were compared with concurrent controls. This potentiation occurred from the onset of anaesthesia until the experiment finished owing to awakening of the animals.

High doses of calcium channel antagonists

At a dose of $1\,g\,kg^{-1}$ of nitrendipine the animals ($n = 8$) remained still, but did not lose their righting reflex. No other behavioural changes were seen, but death occurred in all cases about 6 h after drug administration.

At the dose of flunarizine $400\,mg\,kg^{-1}$ all animals ($n = 6$) experienced clonic seizures and died approximately 1 h after drug administration.

A dose of $20\,mg\,kg^{-1}$ of verapamil resulted in the death of 5 out of 10 mice within 5 min of administration without overt behavioural changes.

At lower doses of each of the calcium channel inhibitors than described above there were no overt behavioural changes and there was no mortality.

In no case did any animal lose its righting reflex prior to death.

Blood concentration of anaesthetic agents

Blood concentrations of ethanol and plasma concentrations of pentobarbitone are presented in Table 2. There were no significant differences in anaesthetic concentrations between animals pretreated with nitrendipine at $100\,mg\,kg^{-1}$ and those receiving control injections at the times examined.

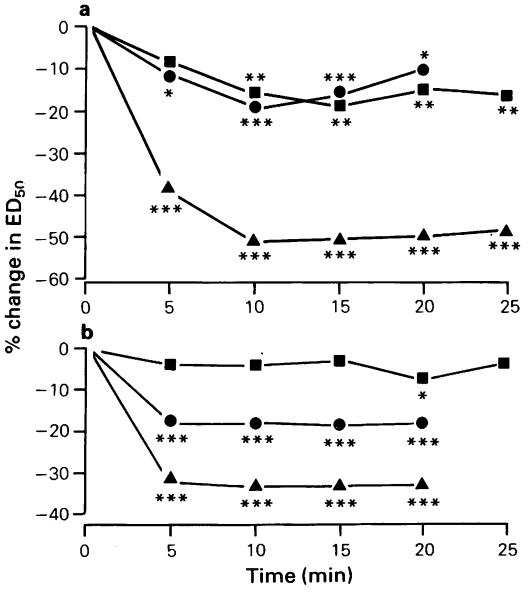

Figure 3 The time course of changes in (a) pentobarbitone and (b) ethanol anaesthetic potencies, as expressed by percentage change in ED_{50}. All results are compared with concurrent controls. The mice were pretreated with verapamil $10\,mg\,kg^{-1}$ (■) at 15 min; flunarizine $40\,mg\,kg^{-1}$ (●) or nitrendipine $100\,mg\,kg^{-1}$ (▲) at 2 h before anaesthetic administration. Each point represents $n = 10$ mice. Levels of significance are $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to concurrent controls.

Discussion

The calcium channel antagonists nitrendipine, flunarizine and verapamil, at the doses used, significantly increased the anaesthetic potencies of pentobarbitone and ethanol, as measured by ED_{50} for loss of righting reflex. In addition, nitrendipine increased the anaesthetic potency of the inert gas argon, in a dose-dependent manner.

All of the dose-response curves for anaesthesia were parallel, with the exception of nitrendipine and pentobarbitone anaesthesia. We know that the barbiturates have a multiplicity of neuronal actions which could contribute to the different pattern seen in the latter case (Goldring & Blaustein, 1980).

Table 2 Blood concentrations of anaesthetic agents following pretreatment (2 h) with nitrendipine $100\,mg\,kg^{-1}$

Drug	*Blood concentrations* (± s.e.mean)	
(a) Ethanol (2 h, $2\,g\,kg^{-1}$)		
Controls ($n = 8$)	$77 \pm 5\,mg\,100\,ml^{-1}$	
Nitrendipine ($n_1 = 8$) $100\,mg\,kg^{-1}$	$83 \pm 8\,mg\,100\,ml^{-1}$	$P > 0.1$
(b) Pentobarbitone (15 min, $40\,mg\,kg^{-1}$)		
Controls ($n = 8$)	$19.3 \pm 1.0\,\mu g\,ml^{-1}$	
Nitrendipine ($n = 8$) $100\,mg\,kg^{-1}$	$20.7 \pm 1.0\,\mu g\,ml^{-1}$	$P > 0.1$

Statistical comparison by Mann Whitney U test.

The calcium channel antagonists, even at doses up to 10 times those used to determine anaesthetic potency changes, did not themselves produce a state of anaesthesia. The increases in anaesthetic potency could not be accounted for by interference in the metabolism of pentobarbitone or ethanol.

The doses of the calcium channel inhibitors that we used were based partly on doses used by other authors to demonstrate central nervous system effects (Ashton & Wauquier, 1979; Van Reempts *et al.*, 1983) and partly on our own preliminary studies. We found that the dose of verapamil was limited by mortality at doses greater than 10 mg kg^{-1} and the dose of flunarizine to be limited by excessive hypothermia above 40 mg kg^{-1}. Owing to the different doses of the three calcium channel antagonists used it is difficult to draw conclusions about their relative efficacies on anaesthetic potency.

In general, calcium channel antagonists when given alone, have little effect on neuronal preparations. Daniell *et al.* (1983) could find no changes in calcium uptake into rat brain synaptosomes in the presence of dihydropyridine calcium channel antagonism, at concentrations up to 10 μM. Middlemiss & Spedding, (1985) observed that neuronal calcium channels were susceptible to blockade by calcium channel antagonists only when the channels were activated by Bay K 8644 (a dihydropyridine which increases opening of calcium channels). Nachshen & Blaustein (1979) noted a decrease in synaptosomal calcium uptake in the presence of verapamil and methoxyverapamil (D-600, 50–100 μM) but also noted that sodium channels were equally affected. Very few central nervous system effects have therefore been demonstrated for the calcium channel antagonists, although considerable recent interest has been generated in neuronal actions (Spedding & Middlemiss, 1985). *In vitro* studies are required to elucidate the interactions between the calcium channel antagonist, and anaesthetic agents.

As described in the Introduction, some anaesthetics decrease calcium entry into synaptic areas. It is possible that calcium channel antagonists enhance this process by further diminishing synaptic calcium entry. Our results suggest that changes in calcium movements may be involved in the production of general anaesthesia. The spectrum of anaesthetic agents whose potencies are affected by the calcium channel inhibitors suggests this may be a non-selective effect, in that it is affecting some basic mechanism underlying the production of a state of general anaesthesia. The calcium channel antagonists themselves do not produce a state of general anaesthesia. No obvious sedative effects of the doses used in the anaesthetic experiments were seen when exploratory locomotor activity was measured, (Little, Dolin and Halsey, unpublished observation) and no overt behavioural effects were seen at the doses used in the present study. This suggests that the presence of anaesthetic agents may alter the state of the calcium channel. These can exist in a variety of states: open, closed or inactive, and the calcium channel antagonists, in cardiac tissue, appear to block only channels which remain in the inactivated form for enough time to allow drug binding (Bean, 1984). It may be that while neuronal calcium channels are not normally in this state the presence of anaesthetics may allow calcium channels to remain inactivated long enough for the calcium channel inhibitors to produce further channel inhibition and thereby potentiate the effects of the anaesthetic agents. In addition, there have been shown to be at least three different types of calcium channels (Nowycky *et al.*, 1985) in neuronal tissue; anaesthetics may have selective effects on these.

Higher doses of the calcium channel antagonists were required than are needed to produce their cardiovascular effects. The dose of nitrendipine 100 mg kg^{-1} is at the top of the range used to treat hypertensive rats and on its own produced a modest (25%) fall in blood pressure in non-hypertensive rats (Knorr & Garthoff, 1984). *In vitro* evidence (see above) suggests that central nervous system calcium channels require higher doses than smooth muscle channels to demonstrate effects (Daniell *et al.*, 1983). We have excluded a pharmacokinetic interaction in the enhanced potencies of ethanol and pentobarbitone, drugs which are known to be extensively metabolised. Argon, being an inert gas, is not metabolised. There are still a number of unanswered questions, for example whether or not altered cerebral blood flow may have contributed to the changes seen. We are at present undertaking the measurement of brain levels of anaesthetic agents to determine this and intend also to examine the effects of other non-calcium channel antagonist vasodilators on anaesthetic potency. In addition, we are examining the effects on anaesthetic potency by the calcium channel activator Bay K 8644 to elucidate further the pharmacology of the calcium channel in the central nervous system.

In conclusion these results suggest that effects on calcium channels may contribute to the production of general anaesthesia. As the calcium channel antagonists produced no overt behavioural effects at the doses which increased general anaesthesia potency, and *in vitro* studies show few neuronal effects, we suggest that anaesthetic agents alter the state of the calcium channel protein (possibly via an effect on the lipid of the cell membrane), allowing the calcium channel antagonists to act.

We would like to thank Bayer UK Ltd. for supplies of nitrendipine; Janssen Pharmaceutica for flunarizine and Abbott Laboratories Ltd. for verapamil. S.J.D. is funded by W.A. and M.G. Saw Medical Research Fellowship from the University of Western Australia. We are grateful to Dr M.J. Halsey for valuable discussion.

References

ASHTON, D. & WAUQUIER, A. (1979). Behavioural analysis of the effects of 15 anticonvulsants on the amygdaloid kindled rat. *Psychopharmacology*, **65**, 7–13.

BAKER, P.F. & SHAPIRA, H.V. (1980). Anaesthetics increase light emission from aequorin at constant ionized calcium. *Nature*, **284**, 168–169.

BEAN, B.P. (1984). Nitrendipine block of cardiac calcium channels: high-affinity binding to the inactivated state. *Proc. natn. Acad. Sci. U.S.A.*, **81**, 6388–6392.

BLAUSTEIN, M.P. & ECTOR, A.C. (1975). Barbiturate inhibition of calcium uptake by depolarized nerve terminals *in vitro*. *Mol. Pharmac.*, **11**, 369–378.

DANIELL, L.C., BURR, E.M. & LESLIE, S.W. (1983). $^{45}Ca^{2+}$ uptake into rat whole brain synaptozomes unaltered by dihydropyridine calcium antagonists. *J. Neurochem.*, **41**, 1455–1459.

GLOSSMANN, H., FERRY, D.R., GOLL, A., STRIESSNIG, J. & SCHOBER, M. (1985). Calcium channels: basic properties as revealed by radioligand binding studies. *J. Cardiovasc. Pharmac.*, **7**, (Suppl. 6), 520–30.

GOLDRING, J.M. & BLAUSTEIN, M.P. (1980). Barbiturates; physiological effects II. In *Antiepileptic Drugs*. ed. Glaser, G.H., Perry, J.K. & Woodbury, D.M. *Advances in Neurology*, Vol. 27, pp. 523–532. N.Y.: Raven Press.

GOLDSTEIN, D. (1984). Pharmacology of Alcohol. p. 7. New York: Oxford University Press.

HARRIS, R.A. & HOOD, W.F. (1980). Inhibition of synaptozomal calcium uptake by ethanol. *J. Pharmac. exp. Ther.*, **213**, 562–568.

KNORR, A. & GARTHOFF, B. (1984). Differential influence of the calcium antagonist nitrendipine and the vasodilator hydralazine on normal and elevated blood pressure. *Archs. Int. Pharmacodyn.*, **269**, 316–322.

KRNJEVIĆ, K. & LISIEWICZ, A.J. (1972). Injection of calcium ions into spinal motoneurons. *J. Physiol.*, **225**, 363–390.

LYNCH, M.A. & LITTLETON, J.M. (1983). Possible association of alcohol tolerance with increased synaptic Ca^{2+}-sensitivity. *Nature*, **303**, 175–176.

MIDDLEMISS, D.N. & SPEDDING, M. (1985). A functional correlate for the dihydropyridine binding site in rat brain. *Nature*, **314**, 94–96.

MURPHY, K.M. & SNYDER, S.H. (1982). Calcium antagonist receptor binding sites labelled with (^{3}H) nitrendipine. *Eur. J. Pharmac.*, **77**, 201–202.

NACHSHEN, D.A. & BLAUSTEIN, M.P. (1979). The effects of some organic "calcium antagonists" on calcium influx in presynaptic nerve terminals. *Mol. Pharmac.*, **16**, 579–586.

NOWYCKY, M.C., FOX, A.P. & TSIEN, R.W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivities. *Nature*, **316**, 440–443.

ROSS, G.J.S. (1980). *Maximum Likelihood Program*. Rothamsted Experimental Station, Harpenden, Herts, England.

SPEDDING, M. & MIDDLEMISS, D.N. (1985). Central effects of calcium antagonists. *Trends Pharmac. Sci.*, **6**, 309–310.

STOKES, J.A. & HARRIS, R.A. (1982). Alcohols and synaptosomal calcium transport. *Mol. Pharmac.*, **22**, 99–104.

VAN REEMPTS, J., BORGERS, M., VAN DAEL, L., VAN EYNDHOVEN, J. & VAN DE VEN, M. (1983). Protection with flunarizine against hypoxic-ischaemic damage of the rat cerebral cortex. A quantitative morphological assessment. *Archs. Int. Pharmacodyn.*, **262**, 76–88.

(*Received January 30, 1986.*
Revised April 9, 1986.
Accepted April 30, 1986.)