"Calcium Entry Blockers" as Cerebral Protecting Agents: Comparative Activity in Tests of Hypoxia and Hyperexcitability

Albert WAUQUIER, David ASHTON, Gilbert CLINCKE and
Johannes FRANSEN

Department of Neuropharmacology, Janssen Pharmaceutica,
B-2340 Beerse, Belgium

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Abstract—The group of drugs described as "Ca²⁺-entry blockers" is chemically and pharmacologically heterogenous. It is believed now that these drugs are useful in the treatment of ischemic disease. In an effort to define which drugs could offer the best therapeutic alternative, a comparative pharmacological study was made. Nine drugs (D-600, diltiazem, flunarizine, nicardipine, nifedipine, nimodipine, nitrendipine, verapamil, tiapamil) were tested in experimental screening models of brain hypoxia, ischemia, cellular intoxication and against bicuculline-induced seizures. Three types of activity were found. Verapamil, D-600, tiapamil and diltiazem were almost inactive, possibly due to their poor brain penetration. The dihydropyridines had a broad spectrum of activity, but considerable differences between these compounds exist. They all were active against hypoxia and less active against ischemia. Out of this subgroup, only nicardipine protected against metabolic intoxication and nifedipine and nicardipine could block seizure components at very high doses. Flunarizine was the only compound with a dose-related effect in all the tests. These results suggested that this combination of screening tests could be used to find compounds with an interesting activity in the field of cerebral protection.

Experimental and clinical data on intra-operative use of barbiturates suggest that these compounds have protective effects in cerebral focal ischemia (1-3). However, the originally reported beneficial effects against global ischemia (4) have not always been confirmed (e.g. 5). Furthermore, the cardio-respiratory depressant effects of these drugs limit possible benefits.

Recent hypotheses suggest that intracellular accumulation of calcium is a common denominator of ischemic cellular damage (6, 7). Therefore, prevention of excessive calcium entry may protect the brain (8). Histological evidence demonstrates that hypoxia is associated with intracellular accumulation of calcium which can be prevented by certain calcium entry blockers (9).

In *in vitro* pharmacological tests, "Ca²⁺-entry blockers" have been subdivided into 3 different types. Dihydropyridines (nimodipine,

nifedipine) block voltage-dependent Ca²⁺ channels in striated muscles, at lower doses than those altering smooth muscle tone. Compounds like flunarizine are specific anti-vasoconstrictors, and they show little or no negative inotropic effects (10). Thirdly, compounds like verapamil and D-600 prevent Ca²⁺-entry into both smooth and striated muscle. Although, nitrendipine binding sites are present in the brain, there is thus far no correlation between inhibition of ³H-nitrendipine binding and any pharmacological test (11-13). Further, little is known about the penetrability of the blood brain barrier to "Ca²⁺-entry blockers", definitive values only being available for flunarizine (14).

The development of new methods of preventing cerebral damage after ischemia or hypoxia is a major research interest of this laboratory. At present, the "Ca²⁺-entry blockers" represent the most viable phar-

macological possibility for treating ischemic disease. We are therefore interested in defining what type of "Ca²⁺-entry blocker" represents the best therapeutic alternative, which necessitates comparative pharmacological studies. These types of studies are impossible in large complicated "clinically-relevant" models such as cardiac-arrest in dogs (15). Therefore, as a first step in this study, we present here comparative studies of "Ca²⁺-entry blockers" in a series of experimental screening models of brain hypoxia, ischemia and cellular metabolic intoxication. Further seizures and hypoxic damage are both associated with an excessive influx of calcium. Therefore, the "Ca²⁺-entry blockers" were also tested against convulsive seizures induced by bicuculline.

Materials and Methods

Tests

Brain hypoxia: nitrogen exposure: The experiments were performed on male Wistar rats (200±10 g), deprived of food overnight. They were placed for 1 min in a container with a pure nitrogen atmosphere. Thereafter, they were removed from the container and placed in an observation cage. Survival was scored after removal from the closed container (16).

Complete ischemia: The experiments were performed on male Swiss mice (25 g), deprived of food overnight. They were decapitated caudal to the intersection of the medulla and the spinal cord. The time between decapitation and the last gasp (duration of gasping) was recorded.

Metabolic poisoning: histotoxic dysoxia test: The experiments were performed on male Wistar rats (200 g), deprived of food overnight. They were given a rapid i.v. injection of 5 mg/kg of potassium cyanide (KCN) and scored for mortality 2 hr after the injection (16).

Bicuculline seizure test: The experiments were performed on male Wistar rats (200 g), deprived of food overnight. They were given an i.v. injection of 1.25 mg/kg of bicuculline. The following phenomena were observed: the presence or absence of clonic seizures, tonic backward extension of the forepaw, tonic extension of the hindpaw and mortality.

Because the different agents only blocked the tonic hindpaw extension, protection against this measurement was the criterion selected.

Drugs

Nine calcium entry blockers were tested with various routes of administration and at various time intervals as indicated in the results section. The following compounds were dissolved in distilled water: flunarizine (except for the highest dose), verapamil, D-600, tiapamil and diltiazem. The following compounds were given in suspension, i.e., water and a few drops of Tween 80: nifedipine, nimodipine and nitrendipine. Nicardipine was dissolved using 20% polypropyleneglycol. All compounds were given in a volume of 1 ml/100 g. In each test and for each compound, at least 3 doses were given in a randomized order. Maximal doses were 40 mg/kg, i.p. and 160 mg/kg, p.o., unless the appearance of toxic effects necessitated a reduction.

Statistics

In the nitrogen hypoxia test, the KCN test and the bicuculline test, ED₅₀-values (and 95% confidence limits) were calculated according to Finney (17). The number of animals tested was at least 5 per dose. In the complete ischemia test in mice, the data obtained after drug injection (n=10 per dose) were compared with those obtained in the control group (n=30). Statistical significance was assessed using Student's *t*-test, two-tailed probability.

Results

Nitrogen anoxia test

Controls: After placing the rats in the nitrogen atmosphere, agitation occurred at 20 sec and convulsions after 30 to 45 sec. None of the control rats previously tested survived the one min nitrogen exposure.

Drug effects: Table 1 shows the ED₅₀-values obtained with the various compounds. Flunarizine was the only compound active both after i.p. and p.o. administration. Verapamil, D-600, tiapamil and diltiazem were inactive after either route of administration. Nifedipine, nimodipine and nitrendipine were only active after i.p. administration, and nicardipine was only active 1 hr after p.o.

Table 1. ED50-values (limits) in mg/kg of protection against mortality following a 1-min exposure of rats in a 100% nitrogen environment

Compound	Nitrogen hypoxia		
	Route of admin.: Pretreatment time:	i.p. 1 hr	p.o. 1 hr p.o. 4 hr
Flunarizine		17.6 (7.10-43.5)	32.5 (18.8-56.0) 14.0 (8.83-22.3)
Verapamil		>40	>40
D-600		>2.5 ^a	>40 ^b
Tiapamil		>40	>160
Nifedipine		41.4 (31.7-54.1)	>160
Nimodipine		10.8 (6.24-18.6)	>160
Nitrendipine		54.5 (31.6-93.9)	≥160
Nicardipine		>160	80.0 (46.3-138.0) >160
Diltiazem		>40	≥160 >160

^aToxic at 10 mg/kg intraperitoneally (i.p.). ^bToxic at 160 mg/kg orally (p.o.).

Fig. 1. Duration of the gasping response following global ischemia in mice treated with saline or various doses of calcium entry blockers given p.o. 4 hr (upper panel) or i.p. 1 hr (lower panel) before the induction of ischemia. Student's *t*-test, two tailed probability. **P*<0.05, ***P*<0.01, ****P*<0.001.

administration.

Complete ischemia

Figure 1 shows the results obtained in controls and drug-treated mice, respectively, after i.p. (–1 hr) and p.o. (–4 hr) administration.

After i.p. administration, flunarizine and nitrendipine dose-relatedly increased the duration of gasping. D-600 and tiapamil were inactive. Verapamil, diltiazem and nicardipine increased the duration of gasping only at the highest dose tested (40 mg/kg). Nimodipine was active at 2.5 and 40 mg/kg. Nifedipine increased the duration of gasping at 10 mg/kg and decreased it at 40 mg/kg.

After p.o. drug administration, flunarizine dose-relatedly increased the duration of gasping. Nifedipine increased the duration of gasping at both 10 and 40 mg/kg, but decreased it at 2.5 mg/kg. D-600, tiapamil and diltiazem were inactive. Verapamil, nimodipine, nitrendipine and nicardipine only increased the duration of gasping at the highest dose tested (40 mg/kg).

Histotoxic test (KCN)

Controls: KCN in control rats induces abdominal contractions and clonic seizures and is lethal within 2 min. Of the 882 rats previously tested, 53 (6%) did not die.

Drug effects: Table 2 shows the ED50-

Table 2. ED50-values (limits) in mg/kg of protection against potassium cyanide (KCN)-induced mortality

Compound	Potassium cyanide mortality		
	Route of admin.: Pretreatment time:	i.p. 1 hr	p.o. 1 hr p.o. 4 hr
Flunarizine		32.3 (18.7–55.7)	23.6 (7.34–75.9) 12.4 (3.28–47.1)
Verapamil		>40	>40
D-600		>2.5 ^a	>10 ^b
Tiapamil		>40	>40
Nifedipine		>40	>40
Nimodipine		>40	>40
Nitrendipine		>40	>40
Nicardipine		29.0 (16.8–50.0)	>40
Diltiazem		>40	>40

^a10 mg/kg—Toxic. ^b40 mg/kg—Toxic.

Table 3. ED50-values (limits) in mg/kg of protection against tonic hindpaw extension in rats following i.v. injection of 1.25 mg/kg of bicuculline

Compound	Tonic hindpaw extension		
	Route of admin.: Pretreatment time:	i.p. 1 hr	p.o. 1 hr p.o. 4 hr
Flunarizine		12.1 (7.14–21.2)	10.8 (6.24–18.6) 8.32 (4.82–14.4)
Verapamil		>40	>40
D-600		>2.5 ^a	>40
Tiapamil		>40	>40
Nifedipine		>40	82.1 (47.6–142)
Nimodipine		>40	>160
Nitrendipine		>40	37.4 (21.7–64.5)
Nicardipine		>40	>160
Diltiazem		>40	>40

^aToxic at 10 mg/kg i.p.

values obtained with the various compounds. Flunarizine was the only compound active both after i.p. and p.o. drug administration. Except for nicardipine, which is only active after i.p. administration, none of the compounds was active.

Bicuculline seizure test

Controls: A total of 84 control rats were tested. At the dose of bicuculline given, all animals showed clonic and tonic convulsions. Mortality was observed in 78 animals (7.1% survival).

Drug effects: Table 3 shows the ED50-values obtained with the different drugs. None of the compounds were active against clonic seizures or mortality. Flunarizine was the only compound active against tonic hindpaw extension both after i.p. and p.o. administration. None of the other compounds were active when given i.p. or p.o. 1 hr before the bicuculline challenge. Nifedipine and nitrendipine were active 4 hr after p.o. drug administration.

Discussion

As mentioned in the introduction, it has been demonstrated that in experimental cerebral ischemia, extracellular Ca^{2+} decreases very soon after the insult and that total brain Ca^{2+} rises during the reperfusion period. This led to the proposal that sustained Ca^{2+} -entry is the basis of neuronal toxicity in ischemia/hypoxia (6). Histological evidence obtained using the oxalate-pyroantimonate technique has unequivocally demonstrated Ca^{2+} -accumulation in ischemic-condensed neurons

in heavily injured parts of the brain (9). Some " Ca^{2+} -entry blockers" have been shown to be active in experimental cerebral ischemia models. We were presently interested to see whether " Ca^{2+} -entry blockers" were also protective in simple screening models, where the involvement of Ca^{2+} in toxicity is less obvious.

A discussion of the different tests which have been used here is necessary in order to facilitate the interpretation of the results. First, exposing rats to an environment of 100% nitrogen produces anoxia. In an unpublished study we found arterial pO_2 to be near zero at the end of the one-minute nitrogen exposure. It was previously found that protection by drugs was uniformly related to a maintenance of normocapnia (18). Drugs providing such protective action might do so in different ways. Untreated rats appear to respond to the hypoxic stimulus by a classic 'fight or flight' reaction. Massive increases in sympathetic outflow lead to an increase in peripheral vascular resistance, cardiac output and pulmonary blood flow. Effective drugs (see Table 4) may act by minimizing peripheral vasoconstriction. A maintenance of normocapnia may then be sufficient to stimulate the respiratory center (18).

Second, in the complete ischemia test in mice, the prolongation of gasping responses is the measure of an anti-ischemic effect. Obviously, the ischemia itself cannot be prevented, but drugs might preserve the integrity of nerve cells of the respiratory

Table 4. Summary of the effects of calcium entry blockers

Test Drug	Hypoxia	Ischemia	Metabolic intoxication	Seizures
Flunarizine	++	++	++	++
Verapamil	-	+	-	-
D-600	-	-	-	-
Tiapamil	-	-	-	-
Nicardipine	++	+	++	-
Nifedipine	++	+	-	++
Nimodipine	++	+	-	-
Nitrendipine	++	++	-	++
Diltiazem	-	+	-	-

Symbols: ++ active at different doses, ED50-value exists. + at some dose active, but no ED50-value. - inactive.

center. Sustained neuronal function in the respiratory center would then lead to prolonged gasping. Maintenance of membrane stability might be involved in this form of ischemia.

Third, the lethality caused by an injection of KCN is largely due to the inhibition of cytochrome oxidase and the subsequent breakdown of cellular metabolism leading to a disruption of cellular integrity (19). The protection against KCN might not be due to an inhibition of mitochondrial energy production but rather to a protection against cellular constituents formed when the energy production is disrupted. The potent antagonistic effects of beta-adrenergic agonists against KCN seem to be due to an increased tissue perfusion which suggests that the acute lethality is due to an abrupt circulatory shock (20). Thus both cellular protective mechanisms and increased tissue perfusion without compromising the heart may be involved in the protection against KCN lethality. A similar mechanism may hold for the effects seen with flunarizine.

Finally, inactivity in the bicuculline seizure test means either no anticonvulsant activity or no brain penetration. Bicuculline has been used as the substance to provoke seizures because of its specific inhibitory effect on GABA neurotransmission. GABA is also involved in hypoxia (21), but the disturbance is rather related to a disruption of the turnover of neuronal GABA rather than to steady-state tissue concentration (22).

Table 4 presents a summary of the actions of the group of "Ca²⁺-entry blockers" in the 4 tests. The compounds blocking Ca²⁺-entry into smooth and striated muscle (verapamil, D-600, tiapamil and diltiazem) show little or no activity. The dihydropyridines showed a dose-related protection against "hypoxia" and "ischemia", but not all compounds were active with respect to "metabolic intoxication" and "seizure" antagonism. Flunarizine was active in all the tests. Thus, both dihydropyridines and flunarizine were active, although their primary site of Ca-antagonism in the peripheral system is different.

These tests cannot delineate whether drug activity was due to vascular and/or neuronal mechanisms or whether activity was due to

"Ca²⁺-entry blockade". However, some data is available which can clarify these questions to some extent. Firstly, if verapamil, tiapamil, D-600 and diltiazem were inactive because of poor brain penetrability, then vascular mechanisms of the type found in peripheral smooth muscle are insufficient to provide protection. Secondly, the active doses of the dihydropyridine compounds are higher than those required to produce negative inotropic effects on the heart *in vivo*. Thus, either poor brain penetrability requires higher doses for anti-hypoxic effects or their action is unrelated to blockade of the voltage-dependent Ca²⁺-channels of the type found in heart muscle. A third possibility is that the cerebral vasculature shows a different mechanism of reactivity to stimuli than the peripheral vasculature (23) and that this reactivity is not beneficially influenced by compounds of the verapamil type in hypoxic/ischemic conditions. Finally, it has been demonstrated that *in vivo* pretreatment of guinea-pigs with flunarizine dramatically increases the number of hippocampal slices showing recovery of synaptic activity after a hypoxic insult *in vitro* (D. Ashton and A. Wauquier, unpublished observations). This protection, achieved with a mean level of 1.22 µg flunarizine per gram hippocampal tissue, occurred in the absence of circulation and is thus a direct neuronal effect.

In summary, the present tests show differences between the three types of "Ca²⁺-entry blockers". Compounds blocking Ca²⁺-influx in both striated and smooth muscle cells were almost inactive. The dihydropyridines and flunarizine showed a broad spectrum of activity, although some differences were apparent. Little or no activity of the verapamil-like "Ca²⁺-entry blockers" and diltiazem shows that either the cerebral vasculature system reacts differently towards hypoxia than the peripheral vasculature or that active compounds achieve their effects at a receptor dissimilar to that found in peripheral vascular tissue and heart tissue. Flunarizine was active in all tests. It is the only compound shown to prevent hypoxia-induced vasoconstriction in cerebral vessels (24) and to have direct neuronal protective effects at physiological brain concentrations (D. Ashton and

A. Wauquier, unpublished observations). Such screening tests of hypoxic activity appear to be useful in delineating compounds of interest for further study, showing differences between compounds belonging to a similar pharmacological group.

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