Properties of a new calcium ion antagonist on cellular uptake and mitochondrial efflux of calcium ions

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Compound YS 035 [*NN*-bis-(3,4-dimethoxyphenethyl)-*N*-methylamine] is a new synthetic compound capable of inhibiting Ca^{2+} uptake by different cells. The inhibition of Ca^{2+} uptake by muscle cells isolated from chicken embryo is dose-dependent in the compound YS 035 concentration range 10–30 μM . The new compound also inhibits Ca^{2+} entry into rat brain synaptosomes and less effectively into baby-hamster kidney cells. Compound YS 035 partially inhibits the slow Ca^{2+} release induced by Ruthenium Red and the rapid Na^{+} -dependent efflux from heart mitochondria. The inhibition of the $\text{Na}^{+}/\text{Ca}^{2+}$ exchange appears to be of a non-competitive type with an apparent K_i of 28 μM . The new Ca^{2+} antagonist totally inhibits the Ca^{2+} efflux from liver mitochondria induced by Ruthenium Red, but it does not affect the release induced by uncoupler, respiratory inhibitor or chelator, nor the mitochondrial ATP synthesis and membrane potential. The properties shown by the new compound indicate it to be a Ca^{2+} antagonist and a useful tool for studies on the mitochondrial Ca^{2+} transport.

Ca^{2+} activates several enzyme reactions in the cytoplasm, and the regulation of its concentration is, at least in part, ascribed to mitochondria. Mitochondria in fact possess a powerful transport system and can accumulate large amounts of Ca^{2+} , thus regulating its concentration in the cytoplasm.

Ca^{2+} distribution across the mitochondrial membrane is maintained by two distinct transport systems, namely an energy-dependent uniporter to transport Ca^{2+} inside, by an electrophoretic mechanism down its electrochemical gradient, and a Ruthenium Red-insensitive electroneutral carrier, slower than the uniporter, but essential for the physiological release of Ca^{2+} from mitochondria (for reviews see Bygrave, 1978; Carafoli & Crompton, 1978; Borle, 1980; Nicholls & Åkerman, 1982).

Addition of Ruthenium Red to a mitochondrial suspension causes a slow release of Ca^{2+} as a consequence of a blockade of Ca^{2+} cycling (Stucki

& Ineichen, 1974). In cardiac mitochondria the velocity of Ruthenium Red-independent Ca^{2+} efflux is greatly enhanced by addition of Na^{+} , owing to an exchange between extra-mitochondrial Na^{+} and intramitochondrial Ca^{2+} , mediated by the electroneutral carrier (Crompton *et al.*, 1976).

The clinical use of the so-called Ca^{2+} -antagonist drugs (or Ca^{2+} -entry blockers) is now widespread, although not many reports on the ability of such drugs to inhibit the transmembrane flux of Ca^{2+} in isolated cells have appeared (Fleckenstein, 1974; Fleckenstein-Grun & Fleckenstein, 1980). Few studies also paid attention to the relationship between effects at cellular concentration and effects on mitochondria for such compounds. Ca^{2+} antagonists, moreover, do not constitute a homogeneous class of drugs, although a papaverine-like structure seems to be a common characteristic of some of them.

A new synthetic compound, *NN*-bis-(3,4-dimethoxyphenethyl)-*N*-methylamine (referred to as compound YS 035 in the present paper), chemically related to homoveratroylveratramine, was synthesized. This is a methyl derivative of a com-

Abbreviations used: BHK cells, baby-hamster kidney cells; Tes, 2-[[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid; compound YS 035, *NN*-bis-(3,4-dimethoxyphenethyl)-*N*-methylamine.

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pound described by Buth *et al.* (1939) as a papaverine analogue.

In the present paper we describe its chemical characteristics and activity on Ca^{2+} transport, tested in comparison with that of well-known Ca^{2+} antagonists such as nifedipine and compound D600 (Bossert & Vater, 1971). Ca^{2+} influx into different cells was studied together with mitochondrial Ca^{2+} influx and efflux. A significant decrease of Ca^{2+} uptake by muscle cells and brain synaptosomes was observed in the presence of the new compound.

The new Ca^{2+} antagonist did not affect the rate of respiration-driven mitochondrial Ca^{2+} uptake or the Ca^{2+} release from mitochondria induced by an uncoupler, a respiratory inhibitor or a chelating agent. On the other hand compound YS 035 partially inhibited the Ruthenium Red-induced Ca^{2+} efflux from rat heart mitochondria and

with HCl to pH 1–2 and, after 5 min of stirring, the solution was cooled and adjusted to pH 12–13 with NaOH. The alkaline solution was extracted with dichloromethane, and the organic phase was washed with water, dehydrated by treatment with solid Na_2SO_4 and filtered. The filtrate was evaporated to dryness, and the residue was twice crystallized with 10 ml of n-hexane. The yield was 6.7 g of chromatographically pure product, m.p. 67–69°C (Found: C, 70.23; H, 8.17; N, 3.85. Calc. for $\text{C}_{21}\text{H}_{29}\text{NO}_4$: C, 70.16; H, 8.13; N, 3.86). The ^1H -n.m.r. spectrum in C^2HCl_3 with 3-trimethylsilylpropanesulphonic acid (sodium salt) internal standard showed the following chemical shifts of protons expressed as δ values (p.p.m.): 2.35 (s, 3H, $\text{N}-\text{CH}_3$); 2.7 [s, 8H, $\text{N}(\text{CH}_2-\text{CH}_2)_2$]; 3.8 [s, 12H, $(\text{OCH}_3)_4$]; 6.7 (s, 6H, aromatic).

The new compound has M_r 359.44 and the following structure:

totally inhibited the Ca^{2+} release from rat liver mitochondria.

Both cellular uptake and mitochondrial release of Ca^{2+} produce an increase of the cytoplasmic Ca^{2+} concentration, and the inhibition of such processes exerted by compound YS 035 therefore makes the molecule an interesting regulator of the cytoplasmic Ca^{2+} concentration. Moreover, the ability of compound YS 035 to block the Ruthenium Red-induced Ca^{2+} efflux suggests the use of the new compound as a tool for studying mitochondrial Ca^{2+} -transport systems.

Materials and methods

Chemical synthesis and characteristics of compound YS 035

A 10.4 g portion of bis-(3,4-dimethoxyphenethyl)amine, prepared from 2-(3,4-dimethoxyphenyl)-*N*-(3,4-dimethoxyphenethyl)acetamide and NaBH_4 , was dissolved in 100 ml of methanol and 30 ml of 37% formaldehyde was added. [The amide was prepared from 3,4-dimethoxyphenylacetyl chloride and 3,4-dimethoxyphenethylamine (Aldrich-Europe, Beerse, Belgium).] The mixture of amine and formaldehyde was stirred and boiled for 40 min and then cooled down to 0°C, and 4 g of NaBH_4 was added in small portions. Stirring was continued for 1.5 h, and methanol was evaporated under reduced pressure.

The residue was resuspended in water, acidified

Compound YS 035 hydrochloride is soluble in ethanol and fairly soluble in dilute acids, and stable when stored at 4°C.

Other chemicals

$^{45}\text{CaCl}_2$ was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.), silicone fluid was from Hopkin and Williams (Chadwell Heath, Essex, U.K.) and all other reagents were from Sigma Chemical Co. (Poole, Dorset, U.K.). Ruthenium Red was purified before use by the method of Luft (1971).

Muscle cells from chicken embryo

Breast muscle were taken from 14–15-day chicken embryos in 0.1 g amounts, finely chopped and washed four times with Dulbecco's phosphate-buffered saline medium, pH 7.5, without Ca^{2+} and Mg^{2+} (Dulbecco & Vogt, 1954). The tissue was suspended in 0.25% trypsin (catalogue no. 1:250; Difco Laboratories, Detroit, MI, U.S.A.) in Dulbecco's medium, pH 7.5 (20 ml of trypsin solution/g of tissue), at 37°C. At 30 min intervals the supernatants containing the dispersed cells were harvested, and new trypsin solution was added to the decanted tissues. The collected supernatants were mixed with 20 ml of calf serum at 0–2°C, the cells were spun down at 2000g, and the pellets were washed with medium and finally resuspended to final values of 5×10^6 cells/ml in medium containing 120 mM-NaCl, 4.4 mM-KCl, 1.2 mM- KH_2PO_4 , 16.8 mM- Na_2HPO_4 , 2.5 mM- MgSO_4 and 5 mM-glucose, pH 7.4.

BHK cells

BHK cells of 21/c13 strain (Macpherson & Stoker, 1962) were grown on Eagle's minimal essential medium (Eagle, 1959) containing 10% (v/v) foetal-bovine serum. At 72h intervals, after monolayer formation, the cells were suspended in Dulbecco's medium containing 0.05% trypsin and 0.25% EDTA. After being stained with 0.5% Trypan Blue and counted by using a counting chamber, the cells were diluted to a suspension containing 2×10^5 viable cells/ml. Before being tested the cells were washed twice with a medium containing 117mM-NaCl, 0.5mM-KCl, 5.5mM-glucose, 0.7mM- Na_2HPO_4 , 25mM- NaHCO_3 , 5mM- MgCl_2 and 10mM-Tris/HCl, pH 7.4, centrifuged at 5000g for 10min and resuspended at a final concentration of 5×10^6 cells/ml.

Bovine spermatozoa

Bovine sperm was collected by use of an artificial vagina and the spermatozoa were spun down by centrifugation at 3000g for 10min through 34% Percoll (density 1.05) in 0.15M-NaCl/20mM-Tris/HCl buffer pH 7.4. After two washings with the above medium the spermatozoa were resuspended at a final concentration of 2×10^9 cells/ml, measured by using a counting chamber after staining with 5% (w/v) Eosin.

Rat brain synaptosomes

Synaptosomes were prepared by the procedure of Booth & Clark (1978) and resuspended at a concentration of 1.5mg of protein/ml in medium containing 122mM-NaCl, 3.1mM-KCl, 10mM-glucose, 1.2mM- MgSO_4 , 0.4mM- KH_2PO_4 , 5mM- NaHCO_3 and 20mM-Tes, pH 7.4 (Åkerman & Nicholls, 1981).

Rat liver and heart mitochondria

Liver mitochondria were isolated from Wistar male and female rats (250g) as described by Rigoni *et al.* (1980). Heart mitochondria were obtained by the procedure of Scarpa & Graziotti (1973). Mitochondria were suspended in medium containing 200mM-sucrose, 20mM-KCl and 10mM-Tris/HCl, pH 7.4, at a concentration of 50mg of protein/ml and stored in an ice bath before use.

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

Suspensions of muscle cells or BHK cells were preincubated for 15min at 30°C in a shaking water bath, ATP (final concn. 5mM) was added and, after 1min, Ca^{2+} uptake was started by adding $^{45}\text{CaCl}_2$ (final concn. 1mM; 100 $\mu\text{Ci}/\text{ml}$). The drugs were added, where indicated, together with ATP, then at defined times 0.8ml samples of incubation mixture were withdrawn and transferred to Microfuge (Eppendorf 5412) tubes containing at the bottom 0.2ml of 12% (w/v) sucrose

and on the top 0.25ml of 50% (v/v) silicone oil (Dow Corning 550)/dinonyl phthalate mixed with 50 μl of suspension medium containing 10mM-EGTA. Centrifugation at 12000g was started after a few seconds and continued for 1.5min, the supernatant was removed and each tube was washed with water before removal of the silicone fluid. The pellet was suspended in 0.5ml of water, 0.3ml of 25% (w/v) trichloroacetic acid was added and the suspension was centrifuged for 1min. A portion of the supernatant was then removed, neutralized with Tris base and counted for radioactivity (Packard liquid-scintillation counter). Ca^{2+} uptake into synaptosomes was measured as described for the cells, without the initial addition of ATP.

The spermatozoa were preincubated for 3min at 30°C at a concentration of 2×10^8 cells/ml in 150mM-NaCl/20mM-Tris/HCl buffer, pH 7.4. The uptake was initiated by adding $^{45}\text{CaCl}_2$ (final concn. 1mM; 1 $\mu\text{Ci}/\mu\text{mol}$); at defined times 0.1ml samples were withdrawn, immediately mixed with 0.1ml of medium containing 4mM-EGTA and filtered through a Millipore HA0.45 filter. The filter was washed with 2ml of ice-cold medium containing 5mM non-radioactive Ca^{2+} , dissolved in 5ml of Instagel and counted for radioactivity.

Mitochondrial Ca^{2+} transport was measured by preincubating heart mitochondria for 2min at 25°C in medium containing 90mM-KCl, 50mM-sucrose, 10mM-Tris/HCl, pH 7.4, 2mM-succinate and 1 μM -rotenone at a concentration of 5mg of protein/ml. The uptake was started by addition of 50nmol of $^{45}\text{CaCl}_2$ (100 $\mu\text{Ci}/\text{nmol}$) per mg of protein. At defined intervals 0.5ml samples of the suspension were withdrawn and rapidly transferred to tubes containing 0.2ml of medium supplemented with 2mM-EGTA to be centrifuged in a Microfuge at 12000g for 1.5min. A portion of the supernatant was counted for radioactivity. The tubes were gently washed with 1ml of ice-cold medium, and the pellet was resuspended with 0.5ml of water. After addition of 0.5ml of 20% (w/v) trichloroacetic acid and centrifuging at 12000g for 2min, the supernatant was counted for radioactivity, which was considered to be due to the intramitochondrial ^{45}Ca .

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

Rat liver mitochondria were pre-loaded with $^{45}\text{Ca}^{2+}$ by incubation in medium containing 220mM-sucrose, 10mM-Tris/HCl, pH 7.3, 10mM-KCl, 2mM-succinate, 1 μM -rotenone, 10mM-potassium acetate and 40nmol of $^{45}\text{CaCl}_2/\text{mg}$ of protein at a concentration of 4mg of mitochondrial protein/ml. After 5min, when virtually all added Ca^{2+} was accumulated, the mixture was centrifuged at 12000g for 10min. The Ca^{2+} -loaded mitochondria were resuspended in medium containing

220 mM-sucrose, 10 mM-Tris/HCl, pH 7.3, and 10 mM-KCl. Ca^{2+} efflux was induced by adding the specific compounds indicated in the Figures to the mitochondrial suspension. The intramitochondrial Ca^{2+} content was determined at defined time intervals as described above for heart mitochondria.

Other assays

Protein concentration was determined by the Lowry *et al.* (1951) method, with bovine serum albumin as a standard.

Results and discussion

It is generally supposed that Ca^{2+} antagonists exert their action by depressing the Ca^{2+} influx through plasma membranes (Fleckenstein *et al.*, 1972). The effect of compound YS 035 was therefore tested first on Ca^{2+} transport by different cells.

Fig. 1(a) shows the time course of $^{45}\text{Ca}^{2+}$ accumulation in muscle cells from chicken embryo

in the absence and in the presence of compound YS 035 and documents the significant decrease obtained when 30 μM -compound YS 035 was present in the incubation medium. The inhibitory effect was obtained at compound YS 035 concentrations higher than 1 μM , was concentration-dependent in the range 10–30 μM and was unaltered at concentrations higher than 50 μM (Fig. 1b). When we compared this with the effects of other Ca^{2+} antagonists we found that similar inhibitions are obtained with 1 μM -nifedipine and 10 μM -compound D600 (results not shown).

Compound YS 035 markedly inhibits Ca^{2+} entry into rat brain synaptosomes, whereas its effect is rather modest with BHK cells and practically nil with bovine spermatozoa (Table 1).

The effect of compound YS 035 was also studied on subcellular organelles, and Fig. 2(a) shows that the respiration-driven Ca^{2+} uptake is not significantly modified by the compound.

It is now generally accepted that mitochondria possess two different specific systems of Ca^{2+}

Fig. 1. Effect of compound YS 035 on $^{45}\text{Ca}^{2+}$ uptake by muscle cells from chicken embryo. Experimental details are given in the Materials and methods section. Means $\pm$ s.d. for three experiments are shown. (a) Time course of $^{45}\text{Ca}^{2+}$ uptake in the absence (●) and in the presence (■) of 30 μM -compound YS 035. (b) Inhibitory effect of different concentrations of compound YS 035 on the initial rate of $^{45}\text{Ca}^{2+}$ uptake.

Table 1. Initial rate of $^{45}\text{Ca}^{2+}$ uptake by rat brain synaptosomes, BHK cells and bovine spermatozoa. Experimental details are given in the Materials and methods section. The values are means $\pm$ s.d. for the numbers of experiments given in parentheses. Statistical significance by Student's *t* test is indicated; N.S., not significant.

Addition	Ca^{2+} uptake		
	By rat brain synaptosomes (ng-ions/min per mg)	By BHK cells (ng-ions/min per 10^6 cells)	By bovine spermatozoa (ng-ions/min per 10^9 cells)
None	4.89 $\pm$ 0.3 (6)	3.2 $\pm$ 0.4 (6)	11.6 $\pm$ 2.7 (4)
Compound YS 035 (30 μM)	3.11 $\pm$ 0.3 (6) $P < 0.001$	2.6 $\pm$ 0.3 (6) $P < 0.05$	11.0 $\pm$ 2.1 (4) N.S.

Fig. 2. Effect of compound YS 035 on $^{45}\text{Ca}^{2+}$ uptake and release by rat heart mitochondria. Experimental details are given in the Materials and methods section. Values are means for four experiments. (a) Time course of $^{45}\text{Ca}^{2+}$ transport in the absence (●) and in the presence (■) of 30 μM -compound YS 035. At the arrows Ruthenium Red (0.5 μM) and NaCl (25 mM) were added. (b) Inhibition by different concentrations of compound YS 035 of Ca^{2+} efflux induced by Ruthenium Red alone (△) and by NaCl in the presence of Ruthenium Red (▲).

Fig. 3. Time course of $^{45}\text{Ca}^{2+}$ efflux from rat liver mitochondria

Experimental details are given in the Materials and methods section. The following compounds were added at zero time: (a) △, Ruthenium Red (0.5 μM); ○, compound YS 035 (30 μM); ●, Ruthenium Red (0.5 μM) + compound YS 035 (30 μM); (b) □, nifedipine (10 μM); ■, Ruthenium Red (0.5 μM) + nifedipine (10 μM); ▼, compound D600 (10 μM); ▲, Ruthenium Red (0.5 μM) + compound D600 (10 μM). Values are means for four experiments.

release, respectively sensitive and insensitive to inhibition by Ruthenium Red. It is known that Ruthenium Red inhibits the mitochondrial Ca^{2+} uptake and that the addition of this dye to a mitochondrial suspension brings about a Ca^{2+} efflux from mitochondria (Moore, 1971; Rossi *et al.*, 1973; Stucki & Ineichen, 1974). The mechanism of action of Ruthenium Red has been a matter of investigation in different laboratories (Carafoli & Crompton, 1978; Nicholls & Åkerman, 1982). The Ca^{2+} efflux from mitochondria observed when

Ruthenium Red is added to inhibit the active uptake can be divided into three components: (a) a passive unspecific leakiness consequent to a damage of the membrane structure and sensitive to albumin, ADP etc. (Pozzan *et al.*, 1977; Harris, 1979; Harris *et al.*, 1979); (b) an active $\text{Na}^{+}/\text{Ca}^{2+}$ exchange particularly active in excitable tissues (Crompton *et al.*, 1976, 1977); (c) an electroneutral $\text{H}^{+}/\text{Ca}^{2+}$ exchange (Fiskum & Lehninger, 1979). In any case the Ruthenium Red-induced extrusion of Ca^{2+} from mitochondria is the result of a

blockade of Ca^{2+} cycling across the inner membrane.

Compound YS 035 drastically inhibits the slow Ca^{2+} release induced by Ruthenium Red from heart mitochondria and partially the rapid Ca^{2+} efflux due to the subsequent addition of NaCl (Fig. 2a). The dependence of the inhibition of Ca^{2+} efflux on the compound YS 035 concentration is shown in Fig. 2(b). A Dixon plot indicated that the inhibition by compound YS 035 of the Na^+ -dependent Ca^{2+} efflux is of a non-competitive type with an apparent K_i of $28\text{ }\mu\text{M}$.

It is not possible to establish at the present time whether the inhibition exerted by compound YS 035 on Ruthenium Red-induced Ca^{2+} efflux is a specific effect on the $\text{Ca}^{2+}/\text{H}^+$ antiporter or a non-specific prevention of mitochondrial membrane damage. The specific inhibition of the more physiological Na^+ -dependent carrier of Ca^{2+} seems more relevant in heart mitochondria.

The Ruthenium Red-induced Ca^{2+} efflux from liver mitochondria is completely blocked by compound YS 035 (Fig. 3a), which by itself, in the absence of Ruthenium Red, does not induce any release of Ca^{2+} . In contrast, nifedipine is able, by itself, to induce a Ca^{2+} efflux, probably as a consequence of its uncoupling effect (Fig. 3b). Compound D600, another well-known Ca^{2+} antagonist, does not induce any Ca^{2+} release when added alone, but, unlike compound YS 035, does not inhibit the Ruthenium Red-induced Ca^{2+} efflux. We have no explanations to offer for the different degree of inhibition shown by the new compound on Ruthenium Red-induced efflux of Ca^{2+} from heart and liver mitochondria.

Fig. 4 shows that compound YS 035 does not affect the mitochondrial release of Ca^{2+} obtained after addition of a chelating agent (EGTA), an uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) or an inhibitor of respiration (antimycin A).

Compound YS 035, at concentrations lower than $100\text{ }\mu\text{M}$, produces a slight improvement of the common parameters of evaluation of mitochondrial function, such as the respiratory control index, the rate of ATP synthesis or the membrane potential (Table 2).

Nifedipine produces, on the other hand, a decrease of all parameters, and this agrees with its Ca^{2+} -releasing effect, which is presumed to be a consequence of a damage to the mitochondrial

Fig. 4. Effect of compound YS 035 on $^{45}\text{Ca}^{2+}$ efflux from rat liver mitochondria

Experimental details are given in the Materials and methods section. The Figure shows the Ca^{2+} efflux induced by EGTA (2 mM) ($\square$ and $\blacksquare$) antimycin A (0.1 nmol/mg of protein) ($\circ$ and $\bullet$) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone ($0.5\text{ }\mu\text{M}$) ($\triangle$ and $\blacktriangle$), in the absence ($\square$, $\circ$ and $\triangle$) and in the presence ($\blacksquare$, $\bullet$ and $\blacktriangle$) of $30\text{ }\mu\text{M}$ -compound YS 035. Values are means for four experiments.

Table 2. Effect of compound YS 035 on the respiratory control index, membrane potential and ATP synthesis of rat liver mitochondria

ATP synthesis was measured by bioluminescence, by the method of Lundin *et al.* (1976). The respiratory control index, the ratio of O_2 consumption in State 3 (with ADP excess) to that in State 4, was determined as reported by Chance & Williams (1955). The membrane potential was measured by monitoring the tetraphenylphosphonium distribution across the mitochondrial membrane with a specifically selective electrode (Lötscher *et al.*, 1980). Other experimental details are given in the Materials and methods section. Values are means $\pm$ S.D. for three experiments.

Addition	Respiratory control index	ATP synthesis (nmol/min per mg of protein)	Membrane potential (mV)
None	4.2 ± 0.4	1.12 ± 0.15	168 ± 9
Compound YS 035 ($10\text{ }\mu\text{M}$)	4.6 ± 0.3	0.96 ± 0.16	173 ± 10
Compound YS 035 ($30\text{ }\mu\text{M}$)	4.4 ± 0.3	1.10 ± 0.11	182 ± 10
Compound YS 035 ($100\text{ }\mu\text{M}$)	3.9 ± 0.2	1.08 ± 0.13	160 ± 12
Compound YS 035 ($300\text{ }\mu\text{M}$)	3.5 ± 0.3	0.87 ± 0.10	147 ± 8
Nifedipine ($10\text{ }\mu\text{M}$)	2.2 ± 0.2	0.65 ± 0.10	92 ± 16

membrane with a resulting collapse of the transmembrane potential (Table 2).

Both cellular uptake and mitochondrial release of Ca^{2+} contribute to increasing the cytoplasmic concentration of Ca^{2+} , and therefore compound YS 035, capable of inhibiting both processes, has the features of an ideal Ca^{2+} antagonist, i.e. a molecule acting both outside and inside the cells, through a decrease of the cytoplasmic Ca^{2+} concentration.

Compound YS 035 seems to be particularly suitable for inhibiting Ca^{2+} uptake of cells of excitable tissues, and this is another requisite of a Ca^{2+} antagonist, together with the effects of the new compound found on the isolated cardiac muscle and on the whole animal (Borzeix *et al.*, 1983).

At the moment we have no direct evidence that compound YS 035 enters into the cells, although its chemical structure and some very preliminary results seem to indicate that this is the case. A noteworthy characteristic of compound YS 035 seems to be that of stabilizing rather than damaging the mitochondrial membrane. Its property of modifying the active extrusion of Ca^{2+} from cardiac mitochondria allows us to propose the compound as: (a) a first example of that new class of Ca^{2+} -entry blockers the need for which has been expressed in a recent review (Smith, 1983), and (b) a potential tool for studying the mitochondrial systems of Ca^{2+} transport.

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