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Different 1,4-dihydropyridines exhibit discriminating effects on passive calcium uptake in rat liver plasma membrane vesicles

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Abstract—The effects of a number of calcium channel effectors on Ca^{2+} uptake by rat liver plasma membrane vesicles was examined. Nifedipine, verapamil and diltiazem had to be present at 1 mM in order to produce >50% inhibition of Ca^{2+} uptake. The two structurally similar 1,4-dihydropyridines, nicardipine and nisoldipine exhibited opposite effects; nicardipine inhibited while nisoldipine stimulated Ca^{2+} uptake. The results show that low concentrations (μM) of calcium channel blockers of excitable cells have little effect on Ca^{2+} uptake by liver plasma membrane vesicles consistent with earlier findings of others that voltage-gated calcium channels are absent in hepatocytes. However, the opposite effects of higher concentrations (*ca.* 1 mM) of nicardipine and nisoldipine on Ca^{2+} uptake suggest a discriminatory action that might be useful in studying further the mechanism of passive Ca^{2+} uptake by these membrane vesicles.

The flux of Ca^{2+} across the plasma membrane is an important mechanism in the regulation of cytoplasmic $[Ca^{2+}]$ in both excitable and non-excitable cells. The mechanisms of efflux through the Ca^{2+}/Mg^{2+}-ATPase and of influx through voltage-gated channels in excitable cells are well characterized (see for example Refs 1–3). The mechanisms of these events in non-excitable cells such as liver however are less well characterized although information in recent years has begun to appear about the Ca^{2+}/Mg^{2+}-ATPase-dependent route of Ca^{2+} efflux in rat liver [4]. Liver cells exhibit a low degree of Ca^{2+}-cycling across the plasma membrane in unstimulated cells [5], and a large influx and subsequent efflux when stimulated by Ca^{2+}-mobilizing agonists [5–7]. There is some evidence to suggest that the basal influx component of the low-level cycling, and the hormone-stimulated influx are mediated by two separate routes [5–7] rather than by the one route operating at two different levels of activity. Efforts to study the influx route of Ca^{2+} uptake in hepatocytes by kinetic means, have been reported (reviewed in Ref. 6).

A preparation of rat liver plasma membrane vesicles suitable for the study of calcium transport processes has been developed and partially characterized in this laboratory [8]. These vesicles, a major population of which are rightside-out [8], show passive (ATP-independent) uptake of Ca^{2+} with properties typical of a protein-catalysed mechanism [9]. We believe, therefore that this route of uptake would reflect movement of the ion down the Ca^{2+} concentration gradient from the exterior inwards to the interior of the hepatocyte [9]. The use of specific carboxyl-group reagents has indicated the possible importance of carboxyl groups in this passive uptake [10]. Hormones which stimulate a large Ca^{2+} influx in whole liver and hepatocytes have been shown to have no effect on uptake in the isolated vesicles [9]. This is possibly related to the absence of one or more components of the appropriate second messenger system from the vesicle preparation. The passive Ca^{2+} uptake into the vesicles therefore appears to represent a basal route of Ca^{2+} influx, but this is by no means certain.

The characterization of voltage-gated Ca^{2+} channels of excitable cells has been greatly assisted by the use of a group of specific Ca^{2+} channel agonists and antagonists (see e.g. Ref. 1). Further efforts to characterize the mechanism of passive Ca^{2+} uptake in the liver vesicles would be assisted by pharmacological agents that specifically affect this mechanism. Whilst voltage-gated Ca^{2+} channels appear to be absent from hepatocytes [11] and these Ca^{2+} channel effectors have been shown to have little or no effect on liver Ca^{2+} transport at concentrations that inhibit such channels in excitable cells, there is some evidence [7, 12] to suggest that at much greater concentrations these effectors do have some effect on liver plasma membrane Ca^{2+} transport. This paper investigates the effect of a range of these voltage-gated Ca^{2+} channel effectors on the passive Ca^{2+} uptake into liver plasma membrane vesicles.

Materials and Methods

Liver plasma membrane vesicles were prepared, as described previously [8] by Percoll density-gradient centrifugation, from male Wistar rats of 280–350 g body weight. The protein concentration in the membrane preparations was determined by the method of Bradford [13] using bovine serum albumin as a protein standard.

Freshly isolated plasma membrane vesicles were diluted to a protein concentration of 0.5 mg/mL in 250 mM sucrose/5 mM HEPES (pH 7.4). Effectors of Ca^{2+} transport, dissolved in dimethylsulphoxide, were added as a 5 μL aliquot whilst vortexing, to 500 μL of diluted membranes. The solvent alone was added to control incubations. This membrane suspension was then pre-incubated for 15 min at 37°, prior to the addition of $^{45}Ca^{2+}$-labelled $CaCl_2$ to a final concentration of 100 μM. Samples (50 μL) were taken at 10, 40, 70 and 120 sec after Ca^{2+} addition, and Ca^{2+} uptake was determined by membrane filtration, as previously described [10].

Results and Discussion

We have previously established that Ca^{2+} uptake as observed in the experiments to be presented, is insensitive to washing out by EGTA and thus reflects Ca^{2+} taken up into plasma membrane vesicles (see Ref. 9). Results of a typical time course of such an experiment are shown in Fig. 1. In the experiment shown, calcium measurements were taken during a time course of 120 sec in the absence and presence of the calcium channel effector nicardipine. When the incubation is carried out at 0°, a slight uptake only occurs over the 120 sec reaction period. Extrapolation to zero time of the linear uptake observed at 0° indicates labelling of the plasma membranes to an extent of approximately 1.5 nmol of Ca^{2+} per mg of protein at the instant of Ca^{2+} addition. This is thought to represent rapid binding of Ca^{2+} to the outer surface of the membrane and thus would not represent transport of Ca^{2+} across the membranes and into the vesicles. We consider this extrapolated point to be the effective base-line representing no calcium uptake into vesicles. All Ca^{2+} uptake values

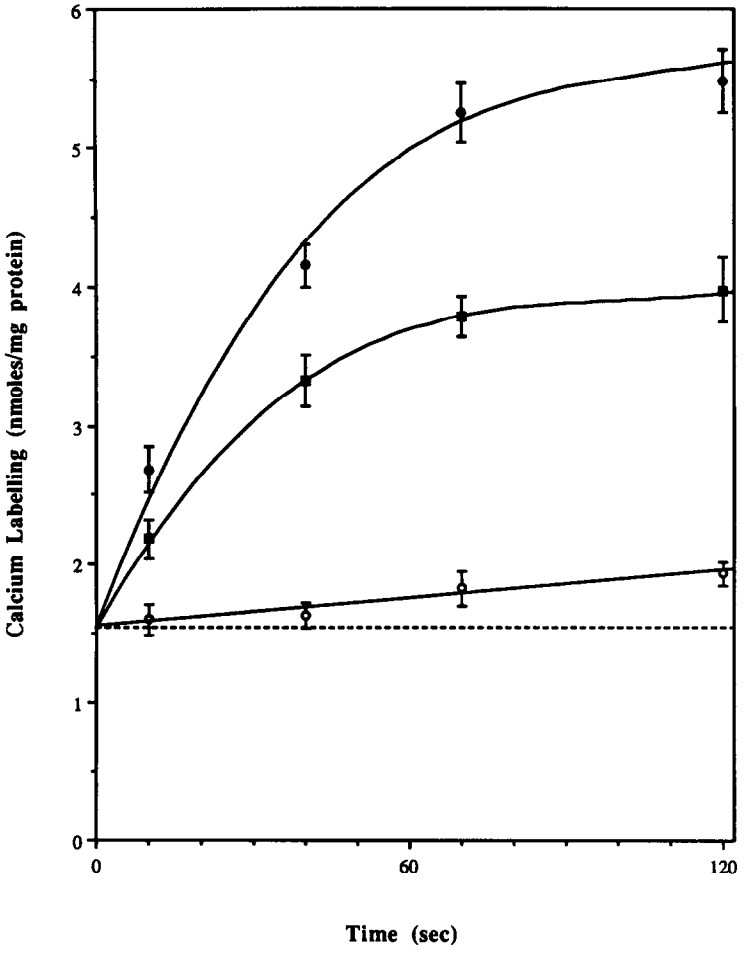

Fig. 1. Time course of Ca^{2+} uptake by isolated rat liver plasma membrane vesicles after the addition of 100 μM $^{45}Ca^{2+}$. Ca^{2+} uptake was measured as described in Materials and Methods under the following conditions: (●) incubation at 37°; (■) incubation at 37° with 300 μM nicardipine; (○) incubation at 0°. Each point is the average ± SEM of triplicate measurements from at least four membrane preparations. The dotted line is the extent of ^{45}Ca-labelling at zero time, indicated by extrapolation of the 0° time course. This is used as the zero point for measurements of Ca^{2+} uptake into vesicles.

Table 1. Calcium $^{45}Ca^{2+}$ uptake, at 120 sec after calcium addition, in the presence of various effectors

Effector	Concentration of effector		
	10 μM	100 μM	1.0 mM
Bay K8644	74 ± 1	65 ± 6	57 ± 5
Nifedipine	88 ± 8	80 ± 7	48 ± 24
Nimodipine	92 ± 8	91 ± 12	69 ± 13
Nitrendipine	97 ± 12	93 ± 6	82 ± 7
Gallopamil	94 ± 12	89 ± 14	102 ± 11
Verapamil	65 ± 3	81 ± 20	41 ± 5
Diltiazem	83 ± 4	74 ± 12	38 ± 12

Values are given as a percentage of the control uptake in the absence of any effector and taking account of the rapid binding at 0° (see text). The average control uptake was 3.9 ± 0.3 nmol Ca^{2+}/mg protein. Each value is the average ± SD of triplicate measurements from N membrane preparations where N for each of the effectors was 2 for Bay K8644 and gallopamil, 3 for verapamil, diltiazem and nifedipine, and 4 for nimodipine and nitrendipine.

are measured from it. In this experiment also it is clear that the presence of 300 μM nicardipine brings about a significant reduction in the initial rate and extent (approx. 30%) of Ca^{2+} uptake.

The effects on Ca^{2+} uptake of a range of calcium channel effectors including 1,4-dihydropyridines (nifedipine, nimodipine, nitrendipine, Bay K8644), phenylalkylamines (verapamil, gallopamil) and a benzothiazepine (diltiazem) are shown in Table 1. The >50% inhibition of Ca^{2+} uptake by nifedipine, verapamil and diltiazem at 1 mM is consistent with the results of [7] showing 20% inhibition of calcium exchange by 500 μM verapamil or nifedipine in unstimulated hepatocytes. These concentrations of effectors are very high relative to the sub-micromolar concentrations at which they affect voltage-gated calcium channels. Earlier work using perfused liver suggested the presence of voltage-gated calcium channels that were activated by depolarizing concentrations of K^+ and completely inhibited by 4 μM verapamil [14]. However, if these channels are present they must be in non-parenchymal cells as no voltage-gated calcium channels have been detected in isolated hepatocytes [11]. Also inhibition by verapamil or nifedipine of K^+- or vasopressin-stimulated Ca^{2+} uptake into isolated

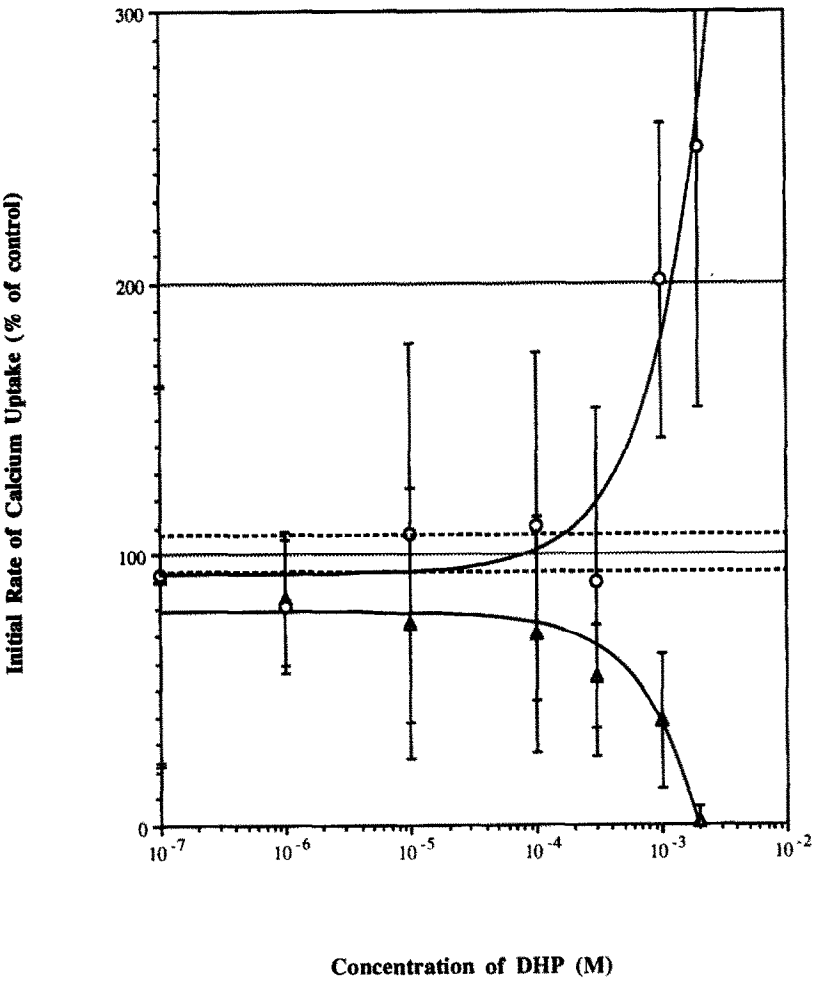

Fig. 2. Effect of nicardipine and nisoldipine on the initial rate of Ca^{2+} uptake into isolated rat liver plasma membrane vesicles. Initial rates of Ca^{2+} uptake were determined from time course experiments as shown in Fig. 1. Membranes were incubated at 37° in the presence of (○) nisoldipine, or (△) nicardipine at the indicated concentrations. The average initial rate for the 37° control was 8.1 ± 0.5 nmol/mg/min. Each point is the average of triplicate measurements from at least three membrane preparations. Error bars represent 99% confidence interval by Student's *t*-test. Dashed lines indicate 99% confidence intervals for the control uptake of 100%.

hepatocytes, requires concentrations of 50–400 μM for 50% inhibition [7, 12].

The high concentrations of calcium channel effectors required to inhibit the passive Ca^{2+} uptake in plasma membrane vesicles raises the possibility that the observed effect could be due to non-specific interactions such as disruption of membranes or interaction with other proteins. This possibility is strengthened by the fact that these compounds have been shown to have a diverse range of pharmacological effects [15, 16].

The effect of two other 1,4-dihydropyridines, nicardipine and nisoldipine was examined in more detail, with Fig. 2 showing their effect on the initial rate of Ca^{2+} uptake. Neither compound produced any significant effect on Ca^{2+} uptake at concentrations less than 300 μM. At higher concentrations however, nicardipine inhibited Ca^{2+} uptake with a dose–response curve similar to that of nifedipine, whilst nisoldipine showed the opposite effect, greatly stimulating Ca^{2+} uptake as its concentration is increased. The fact that nisoldipine is very similar in structure to the other dihydropyridines studied yet has the opposite effect on Ca^{2+} uptake, is particularly interesting and indicates a degree of specificity in the action of these two compounds. This specificity could prove useful in using them to study further the mechanism of passive Ca^{2+} uptake in these vesicles.

An obvious question to ask is what is so special about the structure of nisoldipine. The structures of the five dihydropyridine compounds studied are shown in Fig. 3. They reveal no clear structural feature which distinguishes nisoldipine from the others. It is unlikely, however, that such a structure–function relationship would be immediately obvious without any knowledge about interaction sites. Much work has been done on the structure–function relationship of calcium channel effectors interacting with voltage-gated channels [17, 18] and information from the models generated therein may prove useful in the future in regard to passive Ca^{2+} uptake in liver plasma membranes.

In light of our findings presented here, it is of interest to note that nisoldipine has been shown to have special properties in other systems as well. Of the dihydropyridines, nisoldipine shows a particularly high specificity for calcium

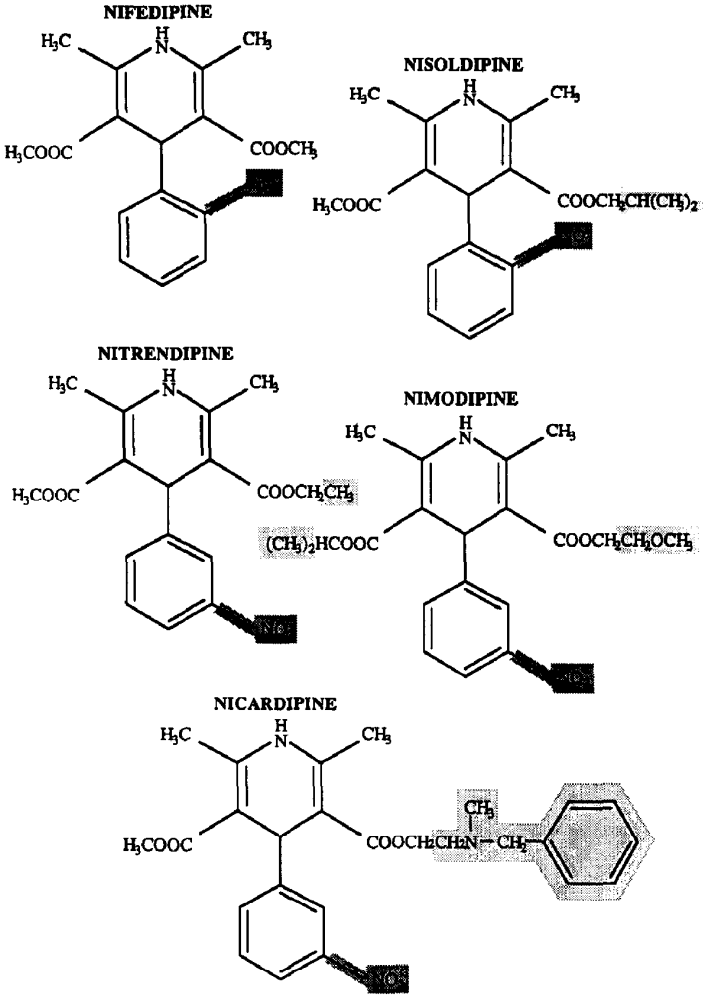

Fig. 3. Structure of 1,4-dihydropyridines used in this work. The sites of variation between structures are highlighted.

channels in vascular smooth muscle with little or no effect on myocardial tissue [19]. Also of interest is that it has also been found to increase the survival time of liver transplants [20].

Conclusion

A range of voltage-gated calcium channel agonists and antagonists was found to affect passive Ca^{2+} uptake by plasma membrane vesicles isolated from rat liver, but only at relatively high concentrations where non-specific effects may be significant. Of particular interest are a group of structurally very similar 1,4-dihydropyridines that at concentrations of 1 mM have very different effects on passive Ca^{2+} uptake. Of these, nicardipine and nifedipine inhibit by approximately 50%, nimodipine and nitrendipine inhibit by approximately 25%, and nisoldipine stimulates Ca^{2+} uptake by approximately 100%. This variation of effect by structurally similar compounds indicates a discriminatory action that may prove useful in elucidating the mechanism of passive Ca^{2+} uptake in liver plasma membrane vesicles.

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