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High and concentration-proportional accumulation of [^{3}H]-nitrendipine by intact cardiac tissue

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1 The binding of [^{3}H]-nitrendipine to intact, electrically driven isolated left atria of guinea-pigs was investigated over the concentration range 10^{-10} M to 3×10^{-5} M.

2 A high affinity binding site saturable in the nM range as found in ventricular homogenates could not be detected. Instead the accumulation of nitrendipine in intact atria was found to be proportional to the concentration from 10^{-10} M to 10^{-6} M; beyond 10^{-6} M the binding started to become saturated.

3 Nitrendipine was highly accumulated in atrial tissue. The cell:medium ratios amounted to about 120 in the range from 10^{-10} M to 10^{-6} M.

4 The concentration-response curve for the negative inotropic action of nitrendipine yielded an ED_{50} of 3×10^{-7} M, thus lying within the range of concentration-proportional accumulation.

5 The reduction of the contractile force proceeded faster ($t_{\frac{1}{2}} < 10$ min) than the uptake process ($t_{\frac{1}{2}} \sim 40$ min) suggesting that it is the binding of nitrendipine into a superficial compartment, which interferes with the excitation-contraction coupling.

6 The results suggest that the high concentration of nitrendipine present in hydrophobic cellular compartments such as the plasmalemma might be involved in its pharmacological action.

Introduction

Several studies have been concerned with the binding of radiolabelled dihydropyridines to membrane preparations derived from various organs. As a common finding, high affinity, specific binding sites for dihydropyridines were detected. In the case of cardiac membranes the K_D values of different dihydropyridines were found to lie in the nanomolar range (e.g. Holck *et al.*, 1982; Janis *et al.*, 1982). For nitrendipine, the K_D was usually found to be $1-3 \times 10^{-10}$ M when determined in membrane preparations from cardiac tissues (Bellemann *et al.*, 1981; Ehlert *et al.*, 1982; Janis *et al.*, 1982; Murphy & Snyder, 1982; Sarmiento *et al.*, 1983; Chatelain *et al.*, 1984; Gould *et al.*, 1984; Schwartz & Velly, 1985). When the action on the force of contraction is investigated in isolated heart preparations, no effects of nitrendipine can be detected in this concentration range. Nitrendipine starts to depress considerably the force of contraction only at a concentration of 10^{-8} M and displays an ED_{50} of about 3×10^{-7} M (Rodenkirchen *et al.*, 1979; McBride *et al.*, 1984; Vaghy *et al.*, 1984a,b). This discrepancy has been noticed earlier (e.g. DePover *et al.*, 1982; Sarmiento *et al.*, 1983; McBride *et al.*, 1984) and has led to the hypothesis that a low affinity binding site also present in cardiac tissue might be involved in the negative inotropic response induced by dihydropyridines (Marsh *et al.*, 1983; Vaghy *et al.*, 1984a,b; see also Lee & Tsien, 1983; Bean, 1984).

In experiments using cultured embryonic chicken heart cells, both the binding of dihydropyridines and their mechanical effects were directly compared. Again the discrepancy between high affinity binding and the pharmacological effect became evident (Marsh *et al.*, 1983). Also in isolated rat cardiac myocytes a high affinity binding could be demonstrated (Green *et al.*, 1985).

However, the fact that the results of binding studies performed with isolated cells are not representative of binding in intact tissue should be taken into account. First, isolated cells differ morphologically and functionally from an intact tissue: the isolation procedure includes a disruption of the myocardial syncytium, an alteration of the cellular coating, a transient exposure to unphysiologically low Ca^{2+}-concentrations; the cells can no longer contract isometrically and undergo degenerative changes in culture. Second, the reported data do not provide true equilibrium values of binding: the cells were exposed to more or less intensive

washing procedures in order to remove non-specifically bound radioligand and to improve the ratio of specific vs non-specific binding. Green *et al.* (1985) demonstrated that non-specific binding of 0.4 nM [^{3}H]-nitrendipine still amounted to about 50% of the total binding, although after termination of the incubation the cells had been immersed for 15 min in ice-cold buffer and had been subjected to three washes with buffer during filtration before counting the cell-bound radioactivity. The high non-specific uptake of [^{3}H]-nitrendipine is not surprising, since dihydropyridines are hydrophobic compounds and should thus be expected to accumulate in hydrophobic cellular compartments.

To the best of our knowledge, binding studies with [^{3}H]-nitrendipine performed on intact functioning cardiac tissues have not been reported, but recently a paper has been published on the accumulation of felodipine by rabbit hearts (Askhold & Nielsen-Kudsk, 1986). Thus, the huge body of information available about the effects of dihydropyridines and their binding to homogenized or isolated cells is in contrast to the lack of knowledge about the accumulation of dihydropyridines in intact myocardium. Therefore, we considered it necessary to study the uptake of [^{3}H]-nitrendipine in intact cardiac preparations. Our interest was focused on the extent of accumulation of nitrendipine, the dependence upon the concentration and finally the rate of uptake. The results were compared with the concentration-response curve of the inotropic effect and its time course. The experiments were conducted on guinea-pig left atria, an isolated organ preparation commonly used to study cardioactive drugs.

For comparison, [^{3}H]-nitrendipine-binding was checked in a homogenate of guinea-pig cardiac ventricles.

Methods

Left atria dissected from the hearts of guinea-pigs of either sex (weight ~ 300 g) were suspended in organ-baths containing 500 ml Krebs-Henseleit solution. The atria were positioned between stimulation electrodes made from platinum wire and driven at a frequency of 3 Hz. Twelve atria could be placed at any one time within one organ-bath. The Krebs-Henseleit solution had the following composition (in mM): NaCl 117, KCl 4.5, $CaCl_2$ 0.5, glucose 5, Tris buffer 30 and was gassed with oxygen. The pH was adjusted to 7.2 by adding equivalent amounts of HCl. The temperature was held at 32°C.

The Ca^{2+} concentration was kept rather low (0.5 mM) in order to reduce the contractile force of those atria which were exposed to low nitrendipine concentrations without a negative inotropic effect. This was necessary for methodical reasons, since high concentrations of nitrendipine depress the contractile force and thus reduce the rate of drug disposition (Lüllmann *et al.*, 1979). If all preparations are weakly beating, differences in the disposition are minimized.

After 1 h of equilibration 12 atria were transferred to two organ-baths one containing [^{3}H]-nitrendipine 10^{-10} M, the other one containing [^{3}H]-nitrendipine 10^{-10} M plus variable concentrations of unlabelled nitrendipine. Two atria were removed from each bath after 20, 60, 120, 240, 300 min. The muscle pieces were gently blotted, weighed, and dissolved in 2 ml of Soluene 350 (Packard Instr.). After addition of 10 ml of Dimilume 30 (Packard Instr.) the radioactivity was counted in a liquid scintillation counter (Packard Tricarb 460). The uptake of [^{3}H]-nitrendipine was corrected for by the amount of nitrendipine present in the extracellular space, which amounts to 0.3 ml g^{-1} of tissue under our conditions (Lüllmann & van Zwieten, 1967). The data are given as ratio nitrendipine per g cell wet weight divided by nitrendipine per ml of incubation medium (cell:medium ratio). In order to determine the concentration of [^{3}H]-nitrendipine in the incubation medium, five 100 µl aliquots were drawn from the organ-baths at the end of an experiment and were subjected to liquid scintillation counting.

In most experiments the equilibrium binding was attained within 5 h. To obtain an 'equilibrium value' which is based on all 12 atria of an uptake curve rather than on only the 300 min duplicates, an exponential function was fitted to the data applying a computer program of non-linear regression analysis according to Marquardt (1963). The exponential function was adequate for describing the time course of nitrendipine uptake (see Figure 1). The equilibrium values obtained by this procedure were used to evaluate the concentration-dependent accumulation of nitrendipine by atrial tissue.

The biological effect of nitrendipine was checked on electrically driven guinea-pig left atria, the contractile response of which was recorded isometrically. The conditions corresponded to those of the uptake experiments except that a higher Ca^{2+}-concentration was used, i.e. 1.8 mM to increase contractile force.

Binding of [^{3}H]-nitrendipine was measured in homogenates of cardiac ventricles of guinea-pigs. The homogenate was prepared as described by Giacomini *et al.* (1986). Ventricular myocardium from a freshly excised heart was minced with scissors and homogenized in 50 mM TrisHCl (pH 7.4) at a volume of 10 ml g^{-1} wet weight using a Potter Elvehjem glass homogenizer. The homogenate was centrifuged for 15 min at 48,000 *g*. The pellet was resuspended in 50 mM TrisHCl buffer (10 ml g^{-1} original tissue wet weight) and centrifuged again. All preparation steps were carried out at 4°C. After a total of three centrifugations, the pellet was resuspended in TrisHCl

buffer (40 ml g^{-1} wet wt.) and subjected to the binding assay. The assay was performed in triplicate in 1.5 ml medium consisting of 250 µl of the homogenate and [^{3}H]-nitrendipine at the indicated concentrations in 3 mM Mg,P_i; 50 mM TrisHCl pH 7.4. Unsaturable (non-specific) binding was determined in the presence of 10^{-5} M unlabelled nitrendipine (or 10^{-5} M nifedipine). After 2 h of incubation at 8–10°C, 1 ml aliquots were filtered under suction through glass-fibre filters (Glasfaser-Rundfilter Nr. 6, Schleicher & Schüll, Dassel, F.R.G.) followed by rinsing with 3 × 5 ml of ice-cold distilled water. Filters were placed in scintillation vials, 1 ml Soluene 350 and 1 h later 10 ml Dimilume 30 were added and the membrane-bound radioactivity was determined by liquid scintillation counting.

Since dihydropyridine derivatives are light-sensitive, all experiments were done in weak sodium-light.

[^{3}H]-nitrendipine was purchased from NEN (Dreieich, F.R.G.), the specific activity amounted to about 80 Ci $mmol^{-1}$. The purity was checked by thin layer radiochromatography. The unlabelled dihydropyridines nitrendipine and nifedipine were kindly provided by Bayer Leverkusen.

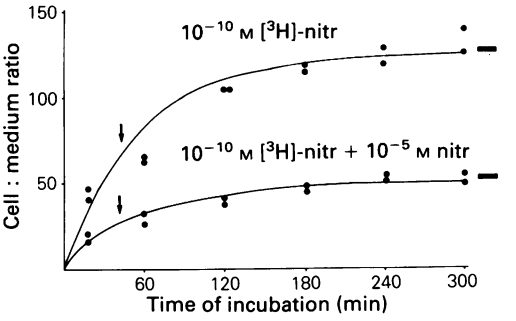

Figure 1 Accumulation of [^{3}H]-nitrendipine ([^{3}H]-nitr) by isolated electrically stimulated left atria of the guinea-pig. [^{3}H]-nitrendipine was present in both experiments in a concentration of 10^{-10} M, in one of the two experiments unlabelled nitrendipine was added to give a concentration of 10^{-5} M. The accumulation of nitrendipine by the tissue was corrected for by the amount present in the extracellular space (30% of tissue wet weight) and is expressed as cell:medium ratio (ordinate scale), the time of incubation is given in min (abscissa scale). The resulting uptake curves were calculated by means of a computer program. The extrapolated equilibrium values (bars) and half times (arrows) are marked.

Results

(1) Accumulation of nitrendipine

The cellular uptake of nitrendipine was determined in the concentration range 10^{-10} M to 3×10^{-5} M by means of [^{3}H]-nitrendipine which was always present in a concentration of 10^{-10} M. The results of a typical experiment determining the time course and the equilibrium values are illustrated in Figure 1. At the low concentration of 10^{-10} M [^{3}H]-nitrendipine the cell:medium ratio attained a value of about 130. In the presence of 10^{-5} M unlabelled nitrendipine the cell:medium ratio was approximately 50. In both cases the half-life time of accumulation amounted to about 40 min. Each individual experiment included (a) a binding curve established for the concentration of 10^{-10} M, and (b) a binding curve for various concentrations up to 3×10^{-5} M. The resulting equilibrium values (means of 2 to 3 binding curves) are shown in Figure 2. As can be seen, the cell:medium ratio remained constant up to a concentration of 10^{-6} M nitrendipine, and declined at still higher concentrations. The actual cellular concentration of nitrendipine was calculated for each concentration investigated and plotted in Figure 3. In the double logarithmic graph, a straight line was obtained up to a concentration of 10^{-6} M nitrendipine indicating that the accumulation was proportionately related to the concentration. At concentrations exceeding 10^{-6} M the experimental points deviated from the straight line indicating that binding had started to become saturated.

Since the influence of nitrendipine upon the contractile force was investigated at a Ca^{2+} concentration of 1.8 mM, binding experiments were also performed

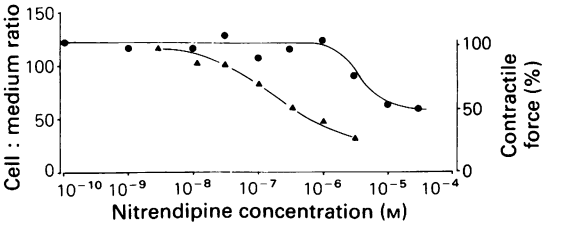

Figure 2 Cellular accumulation and negative inotropic effect of nitrendipine in guinea-pig left atria. Equilibrium values of cellular uptake of [^{3}H]-nitrendipine (left ordinate scale, (●) represent means of 2–3 uptake curves) are plotted versus the concentration of nitrendipine (abscissa scale). The concentration-response curve for the negative inotropic effect of nitrendipine is shown for comparison. [^{3}H]-nitrendipine accumulation was measured at $[Ca^{2+}] = 0.5$ mM, force of contraction was determined at $[Ca^{2+}] = 1.8$ mM; for details see text. (▲) represent means of 2–4 individual effects, the contractile force is expressed as % of control values (right ordinate scale).

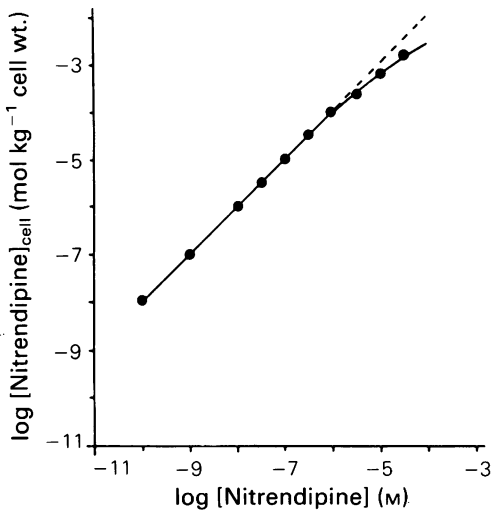

Figure 3 A double log plot showing how the cellular concentration of nitrendipine (ordinate scale) is dependent upon the concentration of nitrendipine in the Krebs-Henseleit solution (abscissa scale). The points were calculated using the cell:medium ratios shown in Figure 2 and the actual concentration of nitrendipine in the incubation medium.

at this Ca^{2+} concentration for comparison. In two binding curves obtained at 0.5 mM or 1.8 mM Ca^{2+} with nitrendipine (3×10^{-6} M) no differences were found in the cell:medium ratios at the equilibria.

Additionally, binding of [^{3}H]-nitrendipine was measured in homogenates of guinea-pig ventricular myocardium (Figure 4). Unsaturable binding of [^{3}H]-nitrendipine was determined in the presence of 10^{-5} M unlabelled nitrendipine; it amounted to 40% of the total binding at 0.1 nM [^{3}H]-nitrendipine. A similar value was found with 10^{-5} M unlabelled nifedipine. Saturable binding was calculated as the difference between total and unsaturable binding of [^{3}H]-nitrendipine. Figure 4 reveals that the saturation took place at concentrations much lower than 1 nM: half-maximum saturation was obtained with 0.2 mM [^{3}H]-nitrendipine. Thus, a high-affinity binding of nitrendipine occurred in the homogenate of guinea-pig ventricular myocardium.

(2) Reduction of the force of contraction by nitrendipine

Nitrendipine reduced the contractile force of the isolated atria in a dose-dependent manner. The time course of the effects of various concentrations of nitrendipine is shown in Figure 5. The effects were fully developed within 60 min with half-life times of less than 10 min. Taking the effects after 60 min of incubation with nitrendipine yielded the concentration response-curve depicted in Figure 2. The steep part of the concentration-response curve ranged from 3×10^{-8} M to 3×10^{-6} M with an ED_{50} of 3×10^{-7} M.

Discussion

The concentration-dependent accumulation of nitrendipine as depicted in Figures 2 and 3 showed the following remarkable features: the accumulation is high, amounting to about 120 fold; the accumulation declines only beyond a concentration of 10^{-6} M nitrendipine indicating that the binding is concentration-proportional from 10^{-10} M to 10^{-6} M and then starts to saturate; in the low concentration range (10^{-10} M–10^{-9} M) the cell:medium ratio does not decrease, thus the saturation of a high affinity binding site is not detectable; the mechanical concentration-response curve for inhibition of developed tension lies within the range of the concentration-proportional accumulation of nitrendipine.

When studying the extent of accumulation, it has to be remembered that the dihydropyridines are highly hydrophobic compounds and thus poorly water soluble. Since hydrophobic substances are known to

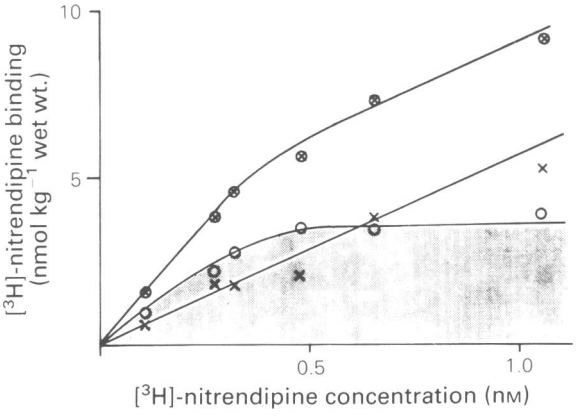

Figure 4 Binding of [^{3}H]-nitrendipine to a homogenate of guinea-pig ventricular myocardium (nmol kg^{-1} original tissue wet weight, ordinate scale) with different concentrations of [^{3}H]-nitrendipine in the medium (abscissa scale). Points represent the mean values of triplicate determinations. Non-specific binding (x) was measured in the presence of 10^{-5} M unlabelled nitrendipine. Saturable binding ((○) shaded area) was calculated as the difference between total binding (⊗) and non-specific binding.

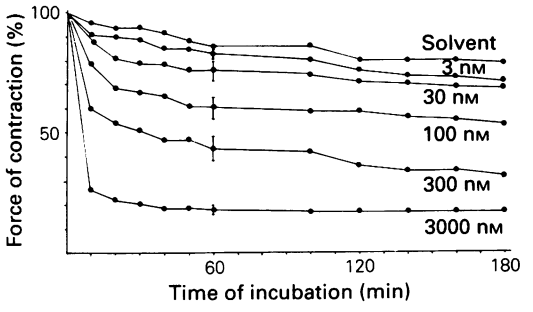

Figure 5 Force of contraction of guinea-pig isolated atria after addition of the indicated concentrations of nitrendipine. Ordinate scale: contractile force as % of the pre-drug control value; abscissa scale: time in min after addition of nitrendipine. The curves are means of 2–4 individual experiments. The upmost curve represents the decline of the force of contraction of control muscles. The force of contraction present at 60 min after addition of nitrendipine (mean ± s.e.mean) was used to construct the concentration-response curve shown in Figure 2.

accumulate in cells (Lüllmann *et al.*, 1979) mainly within their hydrophobic compartments, the high uptake of nitrendipine is not completely unexpected. Felodipine, a dihydropyridine derivative containing two chlorine atoms and therefore being still more hydrophobic, has been found to accumulate 200–500 fold in vascular tissue (Boström *et al.*, 1985) and 500 fold in rabbit heart muscle (Askholt & Nielsen-Kudsk, 1986). The cellular accumulation of nitrendipine is not evenly distributed within the cell but will be located predominantly in hydrophobic compartments such as the interior of the plasmalemma and other cellular membranes. Accordingly, the actual concentration of nitrendipine in the membranes will exceed the overall cellular concentration. By means of electron microscopic autoradiography Ashraf *et al.* (1986) found that [^{3}H]-nitrendipine was concentrated in the sarcolemma of the rat isolated perfused heart.

The decline of the cell:medium ratio beyond a nitrendipine concentration of 10^{-6} M is understandable considering the high accumulation in hydrophobic cellular compartments, since their capacity to incorporate extraneous molecules will be limited and hence the binding will eventually begin to saturate.

The question has to be raised why the high affinity binding found in membrane preparations and isolated cells was not detected in the intact atria.

One possible explanation is that the high affinity binding sites were absent under the applied physiological conditions. This notion would be in line with conclusions drawn from electrophysiological studies on the effects of nitrendipine an Ba^{2+}- (or Ca^{2+}-) currents (Lee & Tsien, 1983; Bean, 1984). Bean (1984) found that in single canine ventricular cells the potency of nitrendipine to block currents increased more than 1000 fold when the holding potential was shifted from −80 to −30 mV. The half-maximal inhibiting concentration of nitrendipine fell from 700 nM to 0.4 nM. Since the latter concentration corresponded with the reported K_D-value of high affinity binding, it was concluded that the binding sites were in a high affinity state in depolarized membranes and in a low affinity state in polarized membranes.

On first sight this conclusion may not be supported by results obtained in [^{3}H]-nitrendipine-binding studies in isolated cells. There was a high affinity binding present under normal conditions, i.e. when the cells were in the functioning state (Marsh *et al.*, 1983; Green *et al.*, 1985). Furthermore, Green *et al.* (1985) could not detect a change in the binding affinity, when the myocytes were partially depolarized by incubation in 50 mM K^+. However, it should be noted that Green *et al.*, reported that 40% of their myocytes were in a non-viable state, thus they were depolarized and prone to bind [^{3}H]-nitrendipine with high affinity. The partial depolarization of the viable cells by 50 mM K^+ would then of course be expected to double the number of high affinity binding sites (B_{max}) without changing K_D, just as was found in that study.

To summarize: electrophysiologists extrapolating from current measurements on drug-binding claim that high affinity nitrendipine binding is voltage-dependent, whereas in biochemical experiments in isolated cardiac myocytes high affinity binding of [^{3}H]-nitrendipine seemed to be independent of the membrane potential. With respect to the results of the present study, electrically stimulated guinea-pig left atria can, from a functional point of view, most likely be compared with the intact cultured embryonic chick ventricular cells (Marsh *et al.*, 1983). Accordingly, a high affinity binding of [^{3}H]-nitrendipine should be expected to be present in the guinea-pig beating atria.

The other possibility is that high affinity binding, while being present, could not be detected because of the high non-specific accumulation by the intact tissue. The density of high affinity binding sites has been found to be quite sparce (DePover *et al.*, 1982; Colvin *et al.*, 1985) and thus the capacity for specific binding of nitrendipine can only be low. The specific binding might be hidden by the high non-specific accumulation of nitrendipine. The present results do not discriminate between the two possibilities.

In any case, it is known that the pharmacological effect of nitrendipine, i.e. the reduction of the contractile force of cardiac muscles, does not correspond with the saturation of a high affinity binding site. The concentration-response curve found in the present study is in accordance with the results of other authors (Rodenkirchen *et al.*, 1979; McBride *et al.*, 1984; Vaghy *et al.*, 1984a,b). The concentration-response

curve lies in a concentration range where nitrendipine becomes bound in a concentration proportional manner. As can be seen from Figures 2 and 3, the cardiac-depressant effect of nitrendipine is paralleled by an increase in the cellular concentration of nitrendipine from about 10^{-6} M to 10^{-4} M. These are overall concentrations; information on actual concentrations within strategically important cellular compartments is not available but these concentrations will be even higher.

The analysis of the time courses of the binding process and of the negative inotropic response might, however, provide some further insight into the effect of nitrendipine. As stated above, the half-life time for the accumulation of nitrendipine by atrial tissue amounted to about 40 min, whereas the half-life time of the mechanical response was found to be less than 10 min (see Figure 5). The difference between the time courses indicates that the overall binding is unlikely to govern the pharmacological response; it rather suggests that a superficial, easily accessible cellular compartment is the site where the accumulation of nitrendipine impairs the excitation-contraction coupling. This process probably takes place at the plasmalemma which will accumulate concentrations of nitrendipine exceeding the overall cellular concentrations.

Finally, although the present study was not intended to localize the site of action of nitrendipine, the results indicate that the high concentration of nitrendipine (at least 3×10^{-5} M at the ED_{50}) present in the sarcolemma might be involved in its pharmacological effects. It seems conceivable to us that the intercalation of dihydropyridine molecules into the phospholipid matrix of the sarcolemma could alter the functional properties either of integral membrane proteins acting as Ca channels or Ca transport proteins, or of specific Ca binding sites.

Nevertheless, the high non-specific accumulation of dihydropyridines in intact functioning cardiac tissue should be taken into account, when considering the mode of action of dihydropyridine-type 'calcium-antagonists'.

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