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EFFECTS OF D-600 AND ITS OPTICAL ISOMERS ON FORCE OF CONTRACTION IN CAT PAPILLARY MUSCLES AND GUINEA-PIG AURICLES

C. LUDWIG & H. NAWRATH
Pharmakologisches Institut der Universität Mainz, 6500 Mainz, Germany

1 (−)-D-600 and (+)-D-600 exerted concentration-dependent negative inotropic effects in papillary muscles from cats, the potency of (−)-D-600 being about 100 times greater than that of (+)-D-600. The action was more pronounced at high than at low frequencies of stimulation. Time to peak force, relaxation time and therefore also total duration of contraction were not significantly affected by the drugs.

2 The negative inotropic effects of both drugs were not reversible after washing in drug-free solution for 60 min at all concentrations tested.

3 The same negative inotropic effects were seen in guinea-pig left auricles with the racemic mixture of both isomers.

4 Uptake measurements of D-600-[nitrile-^{14}C] in isolated left auricles of guinea-pigs showed the drug to be accumulated in the myocardial tissue; tissue-medium ratios from 1.25 to 6.0 were found at exposure times from 1 to 100 minutes. Preparations which were loaded first with D-600-[nitrile-^{14}C] for 100 min and then washed in drug-free solution for different times lost up to about 80% of the initial radioactivity, whereas the depression of force of contraction was not reversible.

5 The results show that there are marked quantitative differences between the actions of (−)-D-600 and (+)-D-600 on isometric force of contraction in cat papillary muscles. Although the time course of the uptake of D-600-[nitrile-^{14}C] and the development of the negative inotropy in guinea-pig left auricles were reasonably well related, the magnitude of the negative inotropy does not seem to be dependent on the total tissue concentration once the negative inotropic effect has been fully developed.

Introduction

A growing body of evidence suggests that there is a close relationship between the slow inward calcium current (I_{Ca}) during the action potential and twitch tension in mammalian ventricular muscle (Beeler & Reuter, 1970; Bassingthwaighte & Reuter, 1972; Trautwein, McDonald & Tripathi, 1975). The organic compounds verapamil and its methoxyderivative D-600 were said to diminish force of contraction in guinea-pig papillary muscles (Fleckenstein, Tritthart, Fleckenstein, Herbst & Grün, 1969) and to reduce specifically I_{Ca} in cat papillary muscles (Kohlhardt, Bauer, Krause & Fleckenstein, 1972). These organic compounds are often used to identify transmembrane calcium movements; verapamil has been used to obtain further information about the mechanism of physiological and pharmacological effects on myocardial contractility (McCans, Lindenmayer, Munson, Evans & Schwartz, 1974).

Apart from the fact that the statement of a specific inhibition of I_{Ca} by verapamil or D-600 seems to require modification (Kass & Tsien, 1975), before these substances are used as tools for studying myocardial contractility their actions on force of contraction in the heart should be characterized more precisely to include concentration-response relationships, the time course of the negative inotropy, and a quantification of the reversibility or irreversibility of the effects.

In the present paper, the action of D-600 and its optical isomers on force of contraction either in cat papillary muscles or in guinea-pig auricles is described, including the effects on isometric twitch tension, time to peak force and relaxation time. There seems to be a correlation between the time course of the uptake of D-600-[nitrile-^{14}C] and the development of the negative inotropy, whereas no such correlation could be found between the recovery of force of contraction and release of D-600-[nitrile-^{14}C] when the preparations were first exposed to the drug and then washed in drug-free solution.

A preliminary account of this work has been published (Ludwig & Nawrath, 1976).

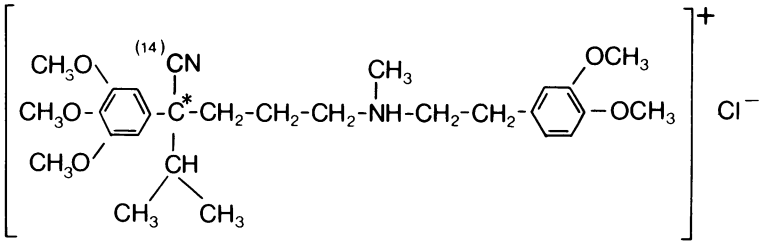

Figure 1 Structural formula of D-600-hydrochloride (α-isopropyl-α-[(*N*-methyl-*N*-homoveratryl)-γ-aminopropyl]-3,4,5-trimethoxyphenylacetonitrile-hydrochloride). The optically active C-atom is marked by an asterisk. The radioactive molecule is ^{14}C-labelled at the nitrile group.

Methods

The hearts of young cats of either sex (body weight: 1–2 kg) were removed following sodium pentobarbitone (35 mg/kg) anaesthesia. The right ventricles were opened in a dissection chamber containing warm, oxygenated Tyrode solution. Papillary muscles were isolated by tying both ends with fine silk sutures and then dissecting them from the ventricular wall. The preparations were attached to a muscle holder assembled with a bipolar platinum stimulating electrode and mounted vertically in a muscle chamber containing 5 ml Tyrode solution. The upper, tendinous end of the preparations was connected to an inductive force displacement transducer (built in our workshop by H. Fleck) by means of a stainless steel wire. The muscles were driven electrically by rectangular pulses (Grass stimulator S 4; intensity 10–20% above threshold) of 2 ms duration at 0.1 to 1.0 Hz. Tension was recorded under isometric conditions at the apex of the preload active tension curve and monitored on a Hellige paper recorder.

The hearts of guinea-pigs (body weight: 200–300 g) were obtained from animals killed by a blow on the head. The left auricles were removed from the rest of the heart, mounted as described for the papillary muscles from cats, and driven at 2 Hz.

The Tyrode solution used was prepared with distilled deionized water and had the following composition (mM): NaCl 136.9, KCl 5.4, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $CaCl_2$ 1.8 and glucose 5.5. The Tyrode solution in the muscle chambers was continuously gassed with 95% O_2 and 5% CO_2; the temperature was maintained constant at 35°C. The experimental set up permitted a rapid exchange (1–2 s) of one solution for another.

Drugs

(−)-D-600, (+)-D-600, (±)-D-600, (−)-verapamil and (+)-verapamil were supplied by Knoll AG, Ludwigshafen. The abbreviated form D-600 always refers to the racemic mixture of the drug: (±)-D-600. In a series of experiments, D-600-[nitrile-^{14}C] (purity 99·7%; Knoll AG, Ludwigshafen) was used. The structural formula of D-600 is given in Figure 1. The asymmetric C-atom is indicated by an asterisk; verapamil is the corresponding compound but with only two methoxy groups on the left hand benzene ring. The absolute configuration of (−)-verapamil has been described as L-, and of (+)-verapamil as D-form of the optically active isomers (Ramuz, 1975). The purity of the optically active isomers is 100% (Dengel & Oberdorf, Knoll AG, personal communication). The pH of the test solutions was not changed in the presence of these drugs and ranged from 7.3 to 7.4. All drugs were freshly prepared in distilled deionized water and 0.1 ml of the corresponding stock solutions were injected into the muscle chamber containing 5 ml Tyrode solution.

Experimental procedure

Papillary muscles were first allowed to stabilize for 60 min at 0.5 Hz. Then the preparations were driven for 5 min at each of a number of different frequencies (0.1, 0.25, 0.5, 0.75, 1.0 Hz). After this period, the driving frequency was set at 0.5 Hz and the muscles were exposed to drugs when the twitch height was steady (within 5 to 10 minutes). The drug effect was monitored for 30 min and force-frequency relationships were obtained as described for the control period. Finally, the muscles were washed 3 times and tension was monitored for a further 60 minutes. At the end of each experiment, the preparations were measured in length with a microscope, then blotted with filter paper for 90 s under constant pressure (280 g) and weighed. The cross sectional area of the papillary muscles was determined according to Koch-Weser (1963). The mean cross sectional area ± s.d. of 107 preparations was 0.55 ± 0.32 mm^2. Individual isometric contraction curves were displayed at higher paper speed

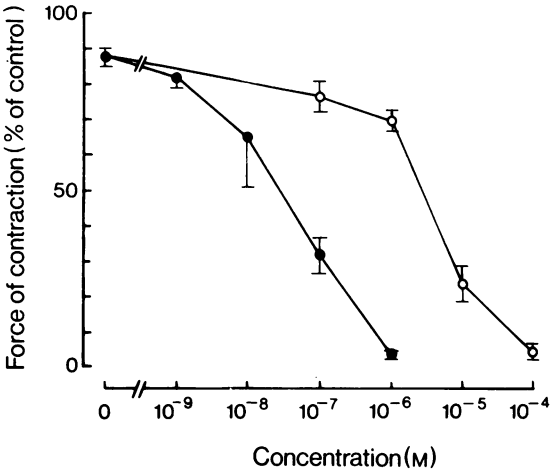

Figure 2 Concentration-response relationships of (−)-D-600 (●) and (+)-D-600 (○) in cat papillary muscles. Values are means of 6 preparations. Vertical bars show s.e. means. Both isomers exerted a concentration-dependent negative inotropic effect: ED_{50} of (−)-D-600 was approx. 3×10^{-8} M, of (+)-D-600 approx. 3×10^{-6} M.

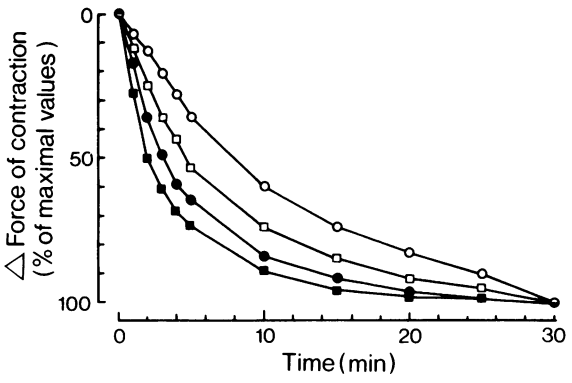

Figure 3 Time course of the negative inotropic effects of (−)-D-600 and (+)-D-600 (same preparations as in Figure 2). The drug effects were evaluated as differences (△) in force of contraction between control and test values and expressed as percentages of the maximal values reached after 30 minutes. With both isomers the effects developed faster at high than at low concentrations. Standard errors are omitted for clarity. Concentrations: (○) 10^{-8} M, (●) 10^{-6} M of (−)-D-600; (□) 10^{-6} M, (■) 10^{-4} M of (+)-D-600.

(100 mm/s) or photographed from a dual beam oscilloscope (Tektronix 502 A). These records were analyzed with regard to their height, time to peak force and relaxation time according to Reiter & Stickel (1968). Results are expressed as means ± s.e.

Tracer experiments

Experiments with D-600 or D-600-[nitrile-^{14}C] were done in guinea-pig left auricles. The muscles were first allowed to stabilize for 60 min and then exposed for different times (1–100 min) to the labelled racemic mixture of D-600. In another series of experiments, the auricles were first loaded with D-600-[nitrile-^{14}C] for 100 min and then washed in drug-free solution for different times (1–300 minutes). At the end of each experiment, the auricles were removed and after discarding the cut edges and sites of attachment they were blotted on filter paper for 90 s under constant pressure (280 g) and weighed. The preparations were then dissolved in scintillation vials by the addition of tissue solubilizer (Soluene, Packard), 0.5 ml and heating to 65°C for 3 hours. To the resulting solution 1N HCl, 1 ml and liquid scintillator (Unisolve, Zinsser), 10 ml were added. The samples were placed in a Tri-Carb liquid scintillation spectrometer (Packard, model 3380) and radioactivity was counted 3 h later.

Results

Action of the optical isomers of D-600 on force of contraction in cat papillary muscles

The inotropic effects of (−)-D-600 and (+)-D-600 in cat papillary muscles (at 0.5 Hz, 30 min after addition of the drug) are shown in Figure 2. Each concentration was tested in individual preparations. With both drugs, a concentration-dependent negative inotropic effect was observed. The concentration-response relationships suggest that there are marked quantitative differences in the action of these drugs, the potency of (−)-D-600 being about 100 times greater than that of (+)-D-600. Time to peak tension, relaxation time and therefore also total duration of contraction remained virtually unchanged with both drugs at all concentrations tested. After a 60 min washing period in drug-free solution force of contraction (F_c) remained unchanged or was even slightly smaller than 30 min after drug addition. Therefore, the negative inotropic effects of both (−)-D-600 and (+)-D-600 are thought to be completely irreversible. Figure 3 shows the time course of the action of the optical isomers of D-600, each at two concentrations. The time-dependent differences (△) between control F_c and F_c after drug addition are expressed as percentage values of maximal values measured 30 min after drug exposure. This type of

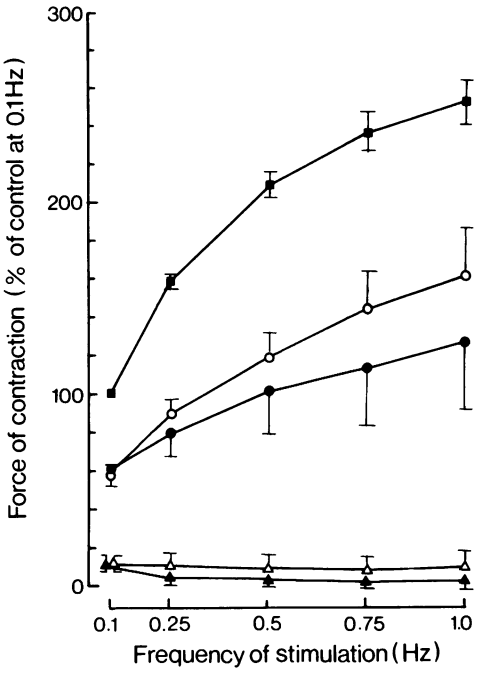

Figure 4 Force of contraction (F_c) of controls and preparations either treated with (−)-D-600 or (+)-D-600 at various frequencies of stimulation (same preparations as in Figure 2). F_c of untreated controls at 0.1 Hz is taken as 100% and all other values are expressed as percentages. F_c of untreated controls increased to about 250% at 1.0 Hz. This positive staircase phenomenon was diminished or abolished in a concentration-dependent way by (−)-D-600 and (+)-D-600: (■) control ($n=107$); (●) 10^{-8} M, (▲) 10^{-6} M of (−)-D-600; (○) 10^{-6} M, (△) 10^{-4} M of (+)-D-600.

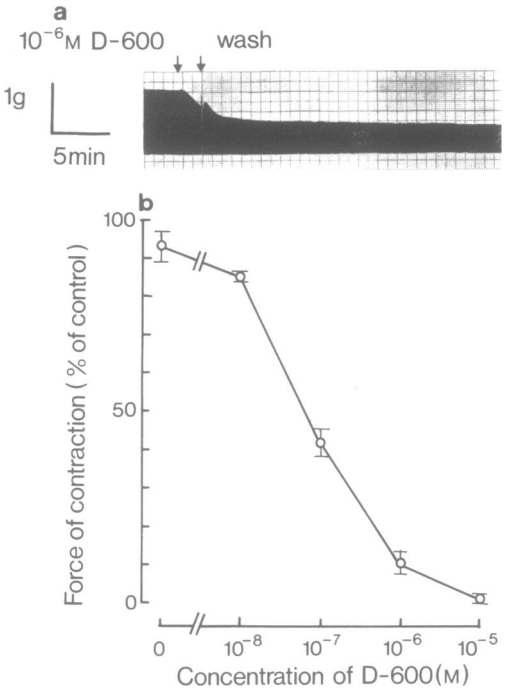

Figure 5 (a) Original tracing of a guinea-pig left auricle exposed to 10^{-6} M D-600 and washed after 90 s with drug-free solution. Note that the negative inotropic effect was still increasing although the drug in the bath fluid had been removed. (b) Concentration-response relationship of D-600 in guinea-pig left auricles. ED_{50} approx. 6×10^{-8} M.

evaluation demonstrates that the development of the negative inotropy becomes faster and the steady state drug effect is reached more rapidly with high concentrations than with low ones. This holds true for both (−)-D-600 and (+)-D-600 and, again, it is evident that both drugs show quantitative rather than qualitative differences in action.

Figure 4 shows force-frequency relationships of controls and preparations treated either with (−)-D-600 or (+)-D-600. Control values at 0.1 Hz were taken as 100% and all other values are given as percentages. The upper curve of untreated control muscles demonstrates the well known staircase phenomenon of increasing F_c when the driving rate is increased (Bowditch, 1871). This phenomenon was diminished in a concentration-dependent way and finally abolished with both (−)-D-600 and (+)-D-600. In other words, at low concentrations, the negative inotropic effect was dependent on the driving rate; at higher concentrations, when the staircase phenomenon was abolished, F_c was diminished to the same final value at all frequencies of stimulation tested. Figure 4 also demonstrates only quantitative differences between the action of (−)-D-600 and (+)-D-600, the factor of potency again being about 100.

Effects of D-600 on force of contraction in guinea-pig left auricles

Figure 5 shows the negative inotropic effect of D-600 in guinea-pig left auricles. F_c was depressed in a concentration-dependent way. As with the optical isomers of D-600 in cat papillary muscles, the effects of the racemic mixture of D-600 in guinea-pig left auricles were not reversible at all concentrations tested after a 60 min washing period in drug-free solution. Even when the muscles were exposed only for a short time to D-600, F_c did not return to control values. This effect is depicted in Figure 5a. The irreversibility of effects essentially also holds true for the analogous compound verapamil; Figure 6 shows the effects of

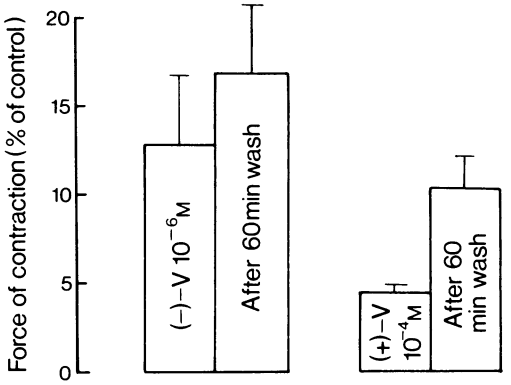

Figure 6 Influence of (−)-verapamil 10^{-6} M and (+)-verapamil 10^{-4} M on F_c in guinea-pig left auricles. Values are means of 4 preparations each. Vertical bars show s.e. mean. F_c was greatly depressed and barely recovered after a 60 min washing period in drug-free solution.

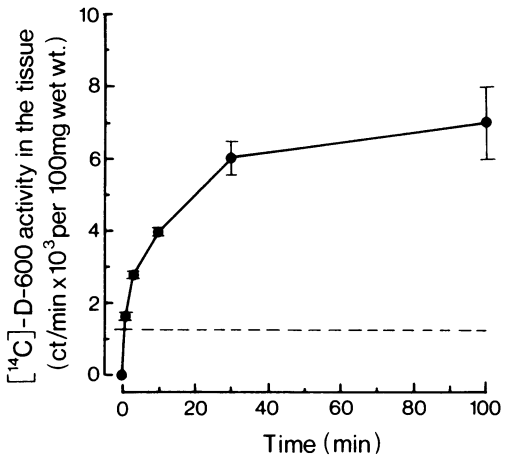

Figure 7 Time course of the uptake of 10^{-6} M D-600-[nitrile-^{14}C] in guinea-pig left auricles. Values are means of 8–16 preparations each. Vertical bars show s.e. means. The activity of 10^{-6} M D-600-[nitrile-^{14}C] per 0.1 ml is indicated by the dotted line. Tissue-medium ratios ranged from 1.25 to 6.0.

(−)-verapamil 10^{-6} M and (+)-verapamil 10^{-4} M in guinea-pig left auricles. F_c was greatly depressed at these concentrations and recovered only slightly after a 60 min washing period in drug-free solution.

Since the effects of (−)-D-600 are 100 times stronger than the effects of (+)-D-600, 99% of the action of the racemic mixture of D-600 on F_c is quantitatively explained by the presence of (−)-D-600. In the racemic mixture of D-600, 50% of either optical form is to be expected; therefore, the response to D-600 is about half in comparison to that of (−)-D-600. This is in line with the ED_{50} of about 3×10^{-8} M for (−)-D-600 in cat papillary muscles (Figure 2) and of about 6×10^{-8} M for D-600 in guinea-pig left auricles (Figure 5).

Uptake and release of D-600-[nitrile-^{14}C] in guinea-pig left auricles

Since the effects of D-600 and its optical isomers were irreversible at all concentrations tested either in cat papillary muscles or in guinea-pig left auricles, a series of experiments was undertaken with D-600-[nitrile-^{14}C] to follow the uptake and release of the drug in the myocardial tissue. Auricles were taken in preference to papillary muscles because the specific activity of D-600-[nitrile-^{14}C] was not higher than 6.6 mCi/mM and effective doses were easier amd more precisely detected as radioactivity in tissue samples with greater weight: the mean wet weights ± s.d. amounted to 1.89 ± 1.03 mg in cat papillary muscles ($n = 107$) and to 28.9 ± 10.4 mg in guinea-pig left auricles ($n = 116$). Uptake measurements were made by immersing the preparations for 1 to 100 min in Tyrode solution containing 10^{-6} M D-600-[nitrile-^{14}C]. The time course of the uptake of the drug into the myocardial tissue is depicted in Figure 7. The concentration of the drug in the tissue was already about as high as in the Tyrode solution after 1 minute. From then on, the drug was accumulated to a value of about 6 times greater than the concentration in the Tyrode solution; equilibrium was reached after about 30 minutes. The time course of the uptake of D-600-[nitrile-^{14}C] seems to be reasonably related to the development of the negative inotropy as shown in Figure 8.

Figure 9 shows the release of the drug when the preparations were first loaded with D-600-[nitrile-^{14}C] for 100 min and then washed in drug-free solution for up to 5 hours. The accumulation of the drug was reversible after washing, indicated by the loss of radioactivity increasing with longer washing times. However, even after 5 h washout of the drug, a residual amount of radioactivity corresponding almost to a concentration of 10^{-6} M was found in the tissue. The release of D-600-[nitrile-^{14}C] does not seem to correspond to the total irreversibility of the negative inotropic effects after washing; on the other hand, the final amount of radioactivity found after long washing periods is reasonably high and might well be in line with the persistence of the negative inotropy once the effect has developed.

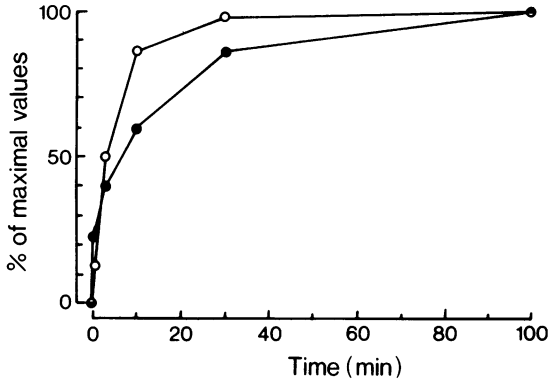

Figure 8 Time course of the uptake of 10^{-6} M D-600-[nitrile-^{14}C] and development of the negative inotropy (same preparations as in Figure 7). Evaluation and expression of results are as described in Figure 3. F_c was depressed to about 16% of control values after 100 minutes. Standard errors are omitted, since neither a statistically defined similarity would prove nor a dissimilarity would disprove a causal relationship between two completely different parameters. (○) △ Force of contraction; (●) [^{14}C]-D-600 activity in the tissue.

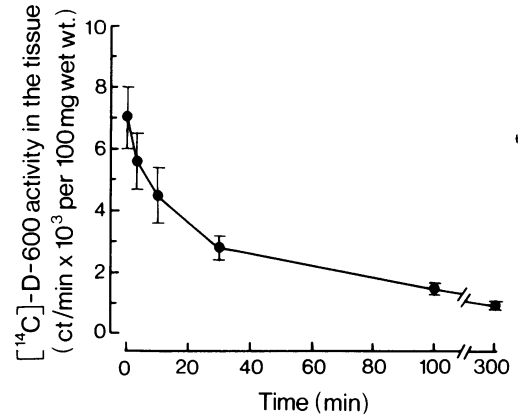

Figure 9 Release of D-600-[nitrile-^{14}C] in guinea-pig left auricles which were first loaded with D-600-[nitrile-^{14}C] for 100 min and then washed for different time periods in drug-free solution. Values are means of 8–16 preparations each. Vertical bars show s.e. means. Although considerable amounts of the drug were being washed out, a residual radioactivity corresponding to almost 10^{-6} M was still found after 5 h of washing.

Discussion

The results confirm the finding that D-600 and its optical isomers exert a concentration-dependent negative inotropic effect in the mammalian myocardium (Fleckenstein *et al.*, 1969; Bayer, Kaufmann & Mannhold, 1975). There were no differences found in the action of the optical isomers except in potency. This also applies to the influence of these drugs on the staircase phenomenon which was reduced at low and abolished at high concentrations. The latter finding corresponds to the data of McCans *et al.* (1974) who demonstrated that the negative inotropic effect of verapamil was also seen at all frequencies, but was more pronounced at higher frequencies of stimulation. The negative inotropic effect of D-600 can be explained by a decrease in the slow inward calcium current (Kohlhardt *et al.*, 1972) that is brought about by a decrease in the calcium conductance, and, at longer exposure times, also by an increase in the activation time of the calcium current (Nawrath, TenEick, McDonald & Trautwein, 1976).

Why the staircase phenomenon is diminished or abolished in the presence of these drugs is not known. Bayer *et al.* (1975) proposed, on the basis of computer calculations, that the cycling time of calcium in the sarcoplasmic reticulum might be prolonged by verapamil and D-600, thereby explaining the different response at different frequencies. It was claimed by the same authors that the staircase phenomenon was not only abolished but even reversed when the (−)-forms of either verapamil or D-600 were present. In the study of Bayer *et al.* (1975) F_c was measured in isotonically contracting cat papillary muscles and F_c was increased by about 20% when comparing 6 beats/min to 60 beats/minute. In our experimental set up, F_c was measured in isometrically contracting cat papillary muscles and was increased by about 150% when comparing the same frequency difference. It might well be that under conditions where the staircase phenomenon is weak, a negative staircase can be established by verapamil or D-600. This also applies to the investigation of McCans *et al.* (1974) who described a very slight inversion of the positive staircase at higher concentrations of verapamil.

Furthermore, Bayer *et al.* (1975) stated that the positive staircase phenomenon was only affected by the (−)-isomers of verapamil and D-600 and virtually unaffected by the (+)-isomers. However, this conclusion was based upon the comparison of concentrations having quantitatively different effects on F_c.

Uptake measurements of D-600-[nitrile-^{14}C] in guinea-pig left auricles showed the drug to be accumulated in the myocardial tissue. Preparations which were first loaded with D-600-[nitrile-^{14}C] for 100 min and then washed for different times in drug-free solution lost up to about 80% of the radioactivity

whereas the depression of F_c remained virtually unchanged. The time course of the uptake of D-600-[nitrile-^{14}C] was similar to the development of the negative intropy. This suggests that the negative inotropic effect is somehow related to the binding and/or accumulation of D-600 in the myocardium. Which binding sites in the myocardial cell are important in mediating the negative inotropic effect remains an open question. The fact that D-600 accumulation is dependent on exposure time indicates the difficulty in obtaining exact concentration-response relationships. It seems only possible to compare the effects of defined bath concentrations in individual preparations at defined intervals after addition of the drug. Even then, when comparing different derivatives, a similar uptake behaviour has to be assumed.

Quite a reasonable amount of D-600 remained in the myocardium after exposure to the drug. If one assumes different binding sites for D-600 more or less determining the magnitude of the negative inotropy, this tightly bound D-600 is possibly responsible for the development and/or persistence of the negative inotropy. On the other hand, the possibility has not been excluded that the negative inotropy would persist without the D-600 that was still found in the tissue after 5 h washing.

In summary, it has been shown that (−)-D-600 and (+)-D-600 exert concentration-dependent negative inotropic effects with virtually no change in time to peak force or relaxation time. The negative inotropic effect of the racemic mixture of both isomers is mostly determined by the presence of (−)-D-600. Since there are only quantitative differences between the action of (−)-D-600 and (+)-D-600, there is no obvious advantage in using either of these isomers in preference to the racemic mixture. When D-600 is used in any studies of myocardial contractility the following limitations based on this study should be kept in mind: the effect of the drug on F_c is not reversible at any concentration and the effect of the concentration investigated depends on the exposure time, since the drug is being accumulated in the myocardial tissue.

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