Slow Onset of and Recovery from Ca Blocking Action of Benidipine in Rat Aorta and Portal Vein

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Abstract—Effects of benidipine, a newly developed 1,4-dihydropyridine derivative, on the high K⁺-induced contraction in aorta and the spontaneous contraction in portal vein of the rat were examined. When either tissue was treated with a low concentration (0.1–10 nM) of benidipine for 90, 180 min or a longer period, the evoked or spontaneous contraction was reduced in a time- and concentration-dependent manner. The time required for 50% inhibition by 1 nM benidipine was approximately 60 and 120 min in the portal vein and aorta, respectively. Although the exact IC₅₀ of benidipine was not determined because of the unexpectedly slow onset of the effect, it might have been overestimated previously and is apparently lower than 1 nM. After the withdrawal of 1 nM benidipine, its inhibitory action was not removed significantly even by washing for 5 hr. A partial or full recovery from the inhibition was observed in tissues pretreated with 0.3 nM benidipine. The persistent inhibition after withdrawal of 1 nM benidipine was not removed by Bay K 8644 treatment. The results strongly suggest that the slow recovery from the benidipine-induced inhibition is, at least in part, responsible for the long-lasting antihypertensive effects of benidipine.

Benidipine (KW-3049; 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid methyl 1-(phenylmethyl)-3-piperidinyl ester hydrochloride) is a new 1,4-dihydropyridine derivative, which exhibits potent and long-lasting antihypertensive and antianginal effects (1, 2). A clinical study (3) suggested that only once-daily administration of benidipine is sufficient for maintaining its antihypertensive action.

In vitro studies suggested that similar to other dihydropyridine Ca-antagonists, benidipine has a potent and selective inhibitory action on Ca-channels in cardiac (4, 5) and vascular (6, 7) tissues, and it is more potent in vascular tissues than in cardiac muscle (7). The mechanism of the long-lasting antihypertensive effect of benidipine has, however, not been clarified sufficiently. Moreover, there is no information about the effect of benidipine in venous tissues, while dihydropyridine-sensitive voltage-dependent Ca channels are well-developed in veins including the portal vein (8), which exhibits spontaneous rhythmic activities depending upon extracellular calcium concentration (9).

The present study was undertaken to examine the inhibitory effects of benidipine on the mechanical responses in both aorta and portal vein isolated from the rat. In particular, effects of low concentrations of benidipine and the recovery from the blockade after withdrawal were studies for an extended period of time to obtain information about the mechanisms of its long-lasting antihypertensive effects.

Materials and Methods

Preparation and measurement of mechanical response: The thoracic aorta and the portal vein were isolated from male Wistar

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rats weighing 300–350 g and cut into helical strips (3 mm in width and 8 mm in length) and longitudinal ones (2 mm in width and 6 mm in length), respectively. The preparation was mounted horizontally in an organ bath (4 ml) and continuously perfused at a rate of 6 ml min⁻¹ with Krebs’ solution of the following composition: 112.0 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 25.0 mM NaHCO₃, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄ and 14.0 mM glucose. The solution in the bath was maintained at 37±0.5°C and bubbled with 95% O₂ and 5% CO₂ (pH 7.4). Isometric tension was measured by a force displacement transducer, which was domestically made (10), and recorded on a pen-recorder (Watanabe Sokki 6202).

High K⁺-induced contractions in aorta: A resting tension of 500 mg was applied to the aortic strip at the start of the experiments. After a preincubation period of 30 min, an 80 mM potassium solution that was prepared by replacing NaCl Krebs’ solution with equimolar KCl, was applied by perfusion for 5 min every 30 min. The experiments were carried out in the presence of 1 nM atropine, 1 nM phenotolamine and 1 nM propranolol. All drugs including benidipine and Bay K 8644 were dissolved to the bathing solutions at the final concentrations and applied by continuous perfusion.

Spontaneous contractions in portal vein: After the load of an optimal resting tension (approximately 150 mg) with which total contraction (see below) occurred most actively, the tissue was preincubated for 60 min. Thereafter, the control spontaneous contractile activity was recorded in the normal solution. Taking both peak amplitude and frequency into account, the spontaneous activity was evaluated by three parameters, “frequency”, “mean of peak amplitude of contraction” and “total contraction”, which were defined as number of twitch contractions, mean of peak amplitude and a sum of peak amplitude, respectively, from a recording for 8 min. The time of measurement is expressed as the middle point of the recording for 8 min. These parameters were calculated by a personal computer (M243, Sord) after digitizing the data on a recording paper using a digitizer (Logitec).

Drugs used: Benidipine was synthesized by Kyowa Hakko. Bay K 8644 was a gift from Bayer. These drugs were dissolved in ethanol and diluted with Krebs’ or high K⁺ solution to final concentrations. Ethanol (0.003%) alone had no effect on either spontaneous or high K⁺-induced contractions. The sources of other drugs were as follows: atropine sulfate and propranolol (Wako) and phenotolamine mesylate (Ciba-Geigy).

Statistical analysis: The data are given as the mean ± S.E.M. and analyzed statistically by Student’s t-test. The number of observations are shown in parentheses in the figures. P levels (P<0.05, 0.01 and 0.001) vs. control group are represented by * ** and *** in figures, respectively.

Results

High K⁺-induced contraction in aorta: When the 80 mM K⁺ solution was applied to the aorta for 5 min every 30 min, the amplitude of evoked contraction slightly increased for the initial 120 min, but thereafter maintained a steady level over 6 hr (Fig. 1, open circles). In the continuous presence of 1 nM benidipine, the amplitude decreased with very slow time-course and reached a steady level, about 15% of the control, after 5 hr (Fig. 1A, closed circles). The time required to reduce the contraction by 50% of the control was approximately 120 min. When 1 nM benidipine was withdrawn after the application for 90 (Fig. 1A, triangles) or 180 min (squares), the inhibitory action was maintained over 3 hr at the last level before withdrawal. No significant recovery of contractile responses was observed during the washout of 1 nM benidipine within the observation period in the present study. The time-course of benidipine-induced inhibition was dose-dependent. A treatment with 0.3 and 1 nM benidipine for 180 min reduced the contraction by 35.5% (Fig. 1B) and 63.5% (Fig. 1A) of the control (at 180 min), respectively. The maximum inhibition by 0.3 nM benidipine appeared to be 50–60%, but was not determined exactly, because occasionally the effect did not reach to a steady level even after 6 hr. The effect of 0.3 nM benidipine appeared to be slightly decreased by a washout of over 2 hr (Fig. 1B).

Spontaneous contractions in portal vein:
After an initial stabilizing period for 60 min, the mean values of peak amplitude of spontaneous twitch contraction and the number of twitches occurring per min were 54.9±9.2.

**Fig. 1.** Time-course of the inhibitory effects of benidipine on 80 mM K⁺-induced contraction of rat aorta. Closed and open symbols indicate the mean values in the presence or absence of benidipine. Vertical bars denote the S.E.M. Benidipine of 1 nM (A) or 0.3 nM (B) in concentration was applied for 90 min (triangles), 180 min (square) or 450 min (circle); and thereafter, it was removed from the perfusing solution as shown by the corresponding open symbols, except for the case of the 450 min application. The numbers of experiments are shown in parentheses. The contraction just before the application of benidipine was taken as 100%. Statistical significance vs. control was indicated by a star(s); *P<0.05, **P<0.01, ***P<0.001.

**Fig. 2.** Typical records showing effects of benidipine on the spontaneous contraction in rat portal vein. Benidipine was applied for 180 min. A, control; B, 0.3 nM benidipine; C, 1 nM benidipine. Left-hand panels (0 min): before benidipine, middle (180 min): 180 min after the application, right (360 min): 180 min after the removal of benidipine.
mg and 6.1±1.0 (n=9), respectively. Even in control experiments, either peak amplitude or frequency or both decreased with time (Fig. 2A). Therefore, the evaluation of spontaneous activity by the total contraction (see Materials and Methods) is quite suitable. The total contraction in the control gradually decreased and reached to steady levels after about 3 hr and then sometimes increased slightly (Fig. 2A and Fig. 4, open circles).

The amplitude of contraction was reduced by benidipine in a concentration-dependent manner (Fig. 2B and C, also see Fig. 3). The frequency appeared to be decreased slightly, especially when 1 nM or higher concentration of benidipine was applied. In experiments shown in Fig. 2, benidipine was applied for 180 min and thereafter withdrawn. The activity was not recovered after 180 min from the withdrawal of benidipine (at 360 min in Fig. 2B and C). Figure 3 illustrates the summarized results about the effects of benidipine on total contraction. The inhibition increased in a dose- and time-dependent manner. The rate of time-dependent inhibition was accelerated by the increase in the concentration (Fig. 3). In the presence of 1 nM benidipine, the time required to reduce the total contraction to 50% of the control at the same time was approximately 60 min. When the period of treatment was prolonged to 180 min, the inhibition increased further more particularly at 0.3 nM (see triangles in Fig. 4A and B). Figure 4 illustrates the effect of 1 nM benidipine on the mean value of peak amplitude (A) and frequency (B) of spontaneous activity in portal vein. Although both parameters decreased in the presence of benidipine, the decrease in frequency was not statistically significant. When 0.3 nM benidipine was applied for 180 min, the frequency was not affected at all while the mean amplitude was significantly decreased (not shown). Therefore, the decrease in the total contraction mainly resulted from the decrease in the peak amplitude of contraction rather than the
frequency, especially when the concentration of benidipine was lower than 1 nM.

Figure 5 shows the recovery time-course after the withdrawal of benidipine following the treatment for 90 (A) or 180 min (B). The inhibition by 0.3 nM benidipine applied for 90 min was fully removed by washing for 4 hr. However, the inhibition by 1 nM benidipine was not removed by washing for this period of time. In addition to this long-lasting effect, it is noted that further significant reduction of spontaneous activities was observed after withdrawal of benidipine (Figs. 2 and 5). This effect was especially apparent when the inhibition in the presence of benidipine was rather small (triangles in Fig. 5A).

Effects of Bay K 8644: The inhibitory action of benidipine on mechanical responses in the aorta and portal vein was hardly removed by withdrawal of the drug from the bathing solution. Therefore, it was examined whether a Ca-agonist, Bay K 8644, could antagonize the inhibition by benidipine (11) or accelerate the recovery from the blockade.

After the suppression of spontaneous activities by 3 nM benidipine for 180 min, an addition of 0.1 μM Bay K 8644 to the solution containing benidipine recovered the activities (Fig. 6A). These activities were, however, abolished again after the simultaneous withdrawal of these drugs. Even when Bay K 8644 was applied 180 min after the withdrawal of benidipine, the recovery of the activities was observed only in the presence of Bay K 8644. A prolonged application of 0.1 μM Bay K 8644 for 180 min after the withdrawal of 3 nM benidipine did not accelerate the recovery of the activity from the inhibition (not shown). A similar effect of Bay K 8644 on the benidipine-induced inhibition was observed in the aorta (Fig. 6B). Bay K 8644 could not significantly accelerate the recovery from the benidipine inhibition in either the aorta or the portal vein.

Effects of nifedipine: For comparison, the effects of nifedipine at a relatively high concentration on the high K+-induced contraction in the aorta and the spontaneous contraction in the portal vein were examined. When 0.1 μM nifedipine was applied to the portal vein, the spontaneous contraction was abolished within 5 min (not shown). Nifedipine was withdrawn after treatment for 90 (triangles in Fig. 7A) or 180 min (squares). The spontaneous activity reappeared within 10 min and recovered to the control level within 60–150 min after the withdrawal. As shown in Fig. 7A, the recovery time course varied widely from preparation to preparation, but was not significantly affected by the period of treat-

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**Fig. 5.** The time-course of the recovery from the inhibitory effects of benidipine in rat portal vein. The drug-free solution was perfused after the application of 0.3 (triangles) or 1 nM (squares) benidipine for 90 min (A) or 180 min (B). Open and closed symbols show the absence and the presence of benidipine, respectively.
ment with 0.1 μM nifedipine. Although not examined systematically, the recovery time course appeared to be roughly dependent upon the concentration of nifedipine in the range between 10 nM and 1 μM. Figure 7B illustrates the effects of 0.1 μM Bay K 8644 applied for 90 min in the presence or after the withdrawal of nifedipine. In the presence of 0.1 μM nifedipine, the spontaneous activity recovered and, moreover, enhanced by 0.1 μM Bay K 8644 to about 200% of the initial level (triangles). This confirmed the preceding observation shown by Mikkelsen (11). Even after Bay K 8644 was withdrawn together with nifedipine, the total contraction was maintained at a level higher than the control (see Fig. 7A circles) and gradually declined towards the control level. When 0.1 μM Bay K 8644 was applied after the withdrawal of nifedipine, a marked enhancement of total contraction to about 400% was observed (squares). After Bay K 8644 was removed, the total contraction returned to the control level.

Reduction in the contractile response of the aorta to 80 mM K+ by 0.1 μM nifedipine occurred much faster than that by 1 nM benidipine, while the maximum effects were similar (Fig. 7C, cf. Fig. 1A). The recovery of the response after withdrawal of nifedipine was also relatively fast (Fig. 7C, circles). When 0.1 μM Bay K 8644 was applied for 10 min in the presence of 0.1 μM nifedipine and was withdrawn concomitantly (triangles), the recovery of the contractile response was not much different from that without the application of Bay K 8644 (circles). The treatment with Bay K 8644 for 10 min after the withdrawal of nifedipine markedly enhanced the recovery (squares). It is now clear that the inhibitory effects of 0.1 μM nifedipine can be removed relatively rapidly in both preparations. After the concomitant withdrawal of 0.1 nM nifedipine and Bay K 8644, the effects of nifedipine never remained and those of Bay K 8644 were rather predominant, especially in the portal vein.

Discussion
The present study demonstrates that
Slow Time-Course of Effects of Benidipine

Fig. 7. Effects of nifedipine on the spontaneous contraction in portal vein (A, B) and the high K+-induced contraction in aorta (C). Open and closed symbols indicate the absence and presence of nifedipine, respectively. A: Circles show the time course of total contraction in the absence of nifedipine. Nifedipine (0.1 μM) was applied for the initial 90 (triangles) or 180 min (squares). B: Bay K 8644 (0.1 μM) was applied for 90 min as indicated by horizontal bars in the presence (triangles) or after the withdrawal of 0.1 μM nifedipine (squares). C: Bay K 8644 was applied for 10 min at the arrows in the presence (triangles) or after withdrawal of 0.1 μM nifedipine (squares).

Benidipine, even at low concentrations, has a strong and long-lasting vasodilating action. Such long-lasting vasodilation by benidipine may, at least in part, account for the ability to maintain the antihypertensive effect for a whole day by once a day administration (3).

A new and important finding in the present study is that the inhibitory action of benidipine developed quite slowly in a time range of hours, especially in the aorta. This onset time appeared to be much slower than that of nifedipine (see also ref. 12). The mechanism of the slow onset was, however, not well clarified in the present study. It is probably due to the chemical characteristics of the side chain at the third carbon of the dihydropyridine ring, which includes piperidine. The inhibition of the spontaneous activity of the portal vein by low concentrations of benidipine often increased even after the withdrawal of benidipine (Fig. 5, triangles). This appeared to be closely related with the mechanism of slow onset, while no systematical experiments were done for these problems in the present study. The IC50 values of benidipine determined by the inhibition of high K+-induced contraction in canine coronary arteries (7) and 45Ca-uptake in rat aorta (Okada, Imaizumi and Watanabe, unpublished observation) are 7.4 nM and 2 nM, respectively, when the tissues were treated with the drug for 30 min. It is, however, clear now that a short application of benidipine is not enough to determine IC50 in these preparations, since the effect of the drug at low concentrations does not reach to a steady level. Actually, a long treatment with 1 nM benidipine nearly abolished the spontaneous activity in the portal vein and decreased high K+-induced contraction in the aorta to less than 20% of the control. These results indicate that the IC50 of benidipine obtained from the steady inhi-
bition in these tissues of the rat is apparently lower than 1 nM. Therefore, benidipine may have an IC50 that is lower than the previously reported value (5, 7) and be more potent than nifedipine (IC50: a range from 1.4 to 30 nM, determined from many other studies (13)). This may explain why the antihypertensive effects of benidipine are 6.1 times more potent than those of nifedipine (1).

The present results support the preceding suggestion that the spontaneous activity of portal vein, as well as the high K+-induced contraction in aorta, is quite useful for assay ing a Ca antagonist (11, 14). It is well-established that each twitch contraction occurs as a consequence of a short burst of spontaneous action potentials (15, 16), which are susceptible to Ca-antagonists (14). Although high K+-induced contraction is widely used as a convenient tool for this aim, the conditions are rather far from the physiological ones. The Ca current measured in large arteries is much smaller than that in portal vein (8, 17, 18). Moreover, the contractile responses of large arteries to agonists such as norepinephrine, prostaglandin F2α and angiotensin II are not highly sensitive to Ca-antagonists (13). Our results showed that the inhibitory effect of benidipine on spontaneous contractile activity in the portal vein developed faster and was more potent than that on high K+-induced contraction. This finding may be explained by the frequency- or the use-dependent block of Ca channels by 1,4-dihydropyridine derivatives, as reported in cardiac muscle (19, 20). The blockade by benidipine may be accelerated by the repetitive occurrence of spontaneous action potentials due to Ca channel activation in the portal vein. An additional but important observation concerning it is that the frequency of spontaneous activity was less sensitive to benidipine than the peak amplitude of contraction. The frequency may be directly related to pacemaker activity, while the mechanisms of pacemaking have not been revealed. At the concentration of 1 nM or less, benidipine rather selectively reduced the peak amplitude of contraction.

The inhibition of mechanical responses by benidipine was not easily removed by washing in the present study. This observation is consistent with the results from the measurement of the slow inward current (6), and the high K+-stimulated 45Ca-uptake (Okada, Imaizumi and Watanabe, unpublished observation) and contraction (7) under a short-term application of benidipine at relatively high concentrations. It should, however, be emphasized that the effect of benidipine was not irreversible but could be removed by washing for hours as shown in the Results. A study using 3H-benidipine suggests its slow dissociation rate from the tissue, while the efflux of 3H-benidipine from specific and non-specific binding sites may not be clearly distinguished in those experiments (7). It has also been reported that benidipine selectively and competitively binds to 3H-nitrendipine binding sites of rat cardiac membranes with high affinity (21). Bay K 8644, a dihydropyridine Ca agonist, displaces or inhibits the specific binding of 3H-nitrendipine in cardiac tissues (22, 23) and well-antagonizes the blockade by nifedipine in the portal vein (11). In the present study, it was additionally observed that the effect of Bay K 8644 remained predominantly after the concomitant withdrawal of nifedipine and Bay K 8644 in the portal vein. It suggests that nifedipine can be easily replaced from the binding site by Bay K 8644. Bay K 8644 reversed the inhibitory effect of benidipine when these two compounds were concomitantly present, but on the contrary, only the inhibition by benidipine remained after withdrawal of Bay K 8644 and benidipine. Benidipine possibly binds not only the effective sites of dihydropyridines but also another site which is not occupied by Bay K 8644. Therefore, it may be possible that such an additional binding site of benidipine has a key role in its long-lasting vasodilation in the aorta and portal vein as has been suggested in cardiac cells (5). The side chain at the third carbon in the dihydropyridine may again have an obligatory function for that binding.

References


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