Biochemical Pharmacology, Vol. 35, No. 2, pp. 217–220, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

THE BINDING OF THE CALCIUM CHANNEL BLOCKER, BEPRIDIL, TO CALMODULIN

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(*Received* 13 *March* 1985; *accepted* 15 *July* 1985)

Abstract—Bepridil had the highest relative potency for inhibition of myosin light chain kinase (MLCK) activated by Ca^{2+}-calmodulin of all the calcium channel blockers we examined. Kinetic analysis indicated that the primary effect of bepridil was mediated through a competitive inhibition of the enzyme activation by interaction with calmodulin and the apparent K_i value of this agent was 2.2 μM. We then examined the binding of bepridil to calmodulin, using the equilibrium column binding technique. [^{3}H]bepridil bound to the calcium–calmodulin complex, but not to calmodulin in the presence of 2 mM EGTA. Scatchard analysis of the binding of bepridil to calmodulin demonstrated that the dissociation constant was 6.2 μM and the calculated number of specific binding sites was about 5 sites per molecule of calmodulin. The concentrations of unlabeled bepridil, W-7, prenylamine, verapamil and diltiazem producing 50% inhibition (IC_{50}) of the binding of [^{3}H]bepridil to calmodulin were 4 μM, 28 μM, 45 μM, 130 μM and 700 μM, respectively. However, nifedipine and nicardipine did not displace [^{3}H]labeled bepridil from calmodulin. There was a good correlation between the displacement of [^{3}H]bepridil from calmodulin and the inhibitory effect on MLCK by these calcium channel blockers and W-7. These results suggest that bepridil binds to calmodulin in the presence of calcium and potently inhibits the phosphorylation of myosin light chain.

Several drugs with similar pharmacological effects and various chemical formulae have been classified as calcium antagonists by virtue of their ability to inhibit the slow calcium channel [1]. Cosnier *et al.* [2] and Michelin *et al.* [3] showed that bepridil is an antianginal, antiarrhythmic agent with Ca^{2+}-antagonistic properties similar to those of verapamil in that it blocks slow channels in the myocardium [4]. Kerr and Sperelakis [5] found that the effects of bepridil and verapamil on slow action potential duration differed considerably and suggested that these drugs may function via different mechanisms. Recently, it was reported that bepridil interacts with calmodulin, an intracellular calcium receptor, regulates many calcium dependent processes, and inhibits the activity of calmodulin dependent enzymes [6–8].

To clarify the relationship between bepridil and calmodulin, we examined the binding of bepridil to calmodulin, then the effects of other calmodulin antagonists and Ca^{2+} channel blockers on this binding, using [^{3}H]labeled bepridil. We also compared the inhibitory effect of bepridil on the calmodulin-induced activation of MLCK,† a process which seems to be essential for induction of smooth muscle contraction [9], with effects of other calcium channel blockers on this enzyme.

MATERIALS AND METHODS

Materials. Bepridil, prenylamine, verapamil, diltiazem and W-7 were kindly provided by Nippon Organon, Hoechst Japan, Eisai Co. Ltd., Tanabe Seiyaku Co. Ltd. and Seikagaku Kogyo, respectively. [^{3}H]labeled bepridil (40 Ci/mmol) and adenoside 5′ [γ-^{32}P]triphosphate were from N.V. Organon International B.V., Oss-Holland and Amersham International Ltd., respectively. Trypsin (bovine pancreas) and trypsin inhibitor (soybean) were purchased from Sigma Chemical Company. Sephadex G-50 (fine) gel was obtained from Pharmacia Fine Chemicals. All other chemicals were of reagent grade or better.

Calmodulin was isolated from bovine brain and purified by the procedure described by Yazawa *et al.* [10]. Myosin light chain kinase (MLCK) was purified from chicken gizzard by the method of Adelstein and Klee [11]. Myosin light chain of chicken gizzard, used as substrate for the kinase assay, was prepared using the procedures of Perrie and Perry [12] and was separated from calmodulin by DEAE-cellulose chromatography [13].

Binding study. The binding of [^{3}H]bepridil to purified calmodulin was investigated using the equilibrium binding technique of Hummel and Dreyer [14] on a Sephadex G-50 fine gel filtration column, as described by Hidaka *et al.* [15]. The Sephadex G-50 fine column (0.9 × 26.5 cm) was pre-equilibrated with the buffer containing 20 mM Tris–HCl (pH 7.5), 20 mM imidazole, 3 mM magnesium acetate, various concentrations of [^{3}H]bepridil and 100 μM $CaCl_2$ or 2 mM EGTA at 25°. Purified calmodulin (180 μg) was used for each experiment. The gel filtration was carried out at 25° at a flow rate of 8.6 ml per hour and 0.86 ml fractions were collected. Samples (0.6 ml and 0.2 ml) of each fraction were analyzed for radioactivity and protein concentration, respectively. Protein was determined by the method of Lowry *et al.* [16] with purified calmodulin as a

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† Abbreviations used: W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; MLCK, myosin light chain kinase; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

standard. In this experimental procedure, standard errors of means were about ten percent.

Phosphorylation analysis. Myosin light chain kinase (MLCK) activity was assayed as described [11]. The reaction mixture (0.2 ml) contained 20 mM Tris–HCl (pH 7.5), 0.05 mM [γ-^{32}P]ATP (1 μCi/assay tube), 5 mM $MgCl_2$, 10 μM myosin light chain, 24 nM calmodulin and 0.1 mM $CaCl_2$ or 1 mM EGTA, unless otherwise noted. MLCK (specific activity; 4.5 moles/min/mg) concentration from chicken gizzard was 0.1 μg/ml. The incubation was carried out at 30° for 4 min. The reaction was terminated by addition of 1 ml of 20% trichloroacetic acid, and 0.1 ml of bovine serum albumin (1 mg/ml) was added to the reaction mixture. The sample was then centrifuged at 2000 *g* for 10 min, the pellet was resuspended in 5% trichloroacetic acid, the final pellet was dissolved in 2 ml of 1 N NaOH and the radioactivity measured in a liquid scintillation counter.

Preparation of trypsin-treated MLCK was as described [8].

RESULTS

Effects of Ca^{2+} channel blockers on the phosphorylation of myosin light chain

Figure 1 shows the effects of calcium channel blockers on calmodulin-activated myosin light chain kinase (MLCK). Nifedipine and nicardipine had little effect on the phosphorylation of chicken gizzard myosin light chain by MLCK. Verapamil and diltiazem inhibited slightly this phosphorylation and 50% inhibition (IC_{50}) was attained at the concentration of 500 μM and 1300 μM, respectively. However, prenylamine and bepridil inhibited effectively the phosphorylation with IC_{50} values of 37 μM and 14 μM, respectively (Table 1). We also examined the direct interaction of these agents with calmodulin independent MLCK, which was pretreated with trypsin and lost the portions necessary for complex formation with calmodulin. All these calcium channel blockers did not inhibit significantly the activity of the calmodulin-independent MLCK at the concentration producing 50% inhibition of calmodulin-activated MLCK (data not shown). These results suggest that among the calcium channel blockers tested, bepridil has most potent calmodulin antagonistic action.

Table 1. Effect of calcium channel blockers and W-7 on the binding of [^{3}H]bepridil to calmodulin and myosin light chain phosphorylation

Compounds	Displacement of [^{3}H] bepridil from calmodulin* IC_{50} (μM)	Calmodulin activated MLCK† IC_{50} (μM)
Bepridil	4	14
Prenylamine	45	37
Verapamil	130	500
Diltiazem	700	1300
W-7	28	40

* Sephadex G_{50} fine gel was pre-equilibrated with buffer containing 20 mM Tris–HCl (pH 7.5), 20 mM imidazole, 3 mM magnesium acetate, 0.5 μM [^{3}H] bepridil, 0.1 mM $CaCl_2$ and various concentrations of drugs.

† MLCK is activated by 24 nM calmodulin.

Figure 2 shows the kinetic analysis of bepridil-induced inhibition of calmodulin-activated MLCK, using double reciprocal plots and Dixon plots [17].

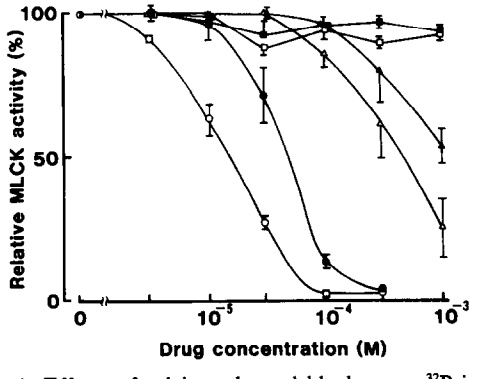

Fig. 1. Effects of calcium channel blockers on ^{32}P incorporation into myosin light chain. The reaction mixture described under Materials and Methods. The reaction initiated by adding ATP was carried out for 4 min in the presence of various concentrations of bepridil (○), prenylamine (●), verapamil (△), diltiazem (▲), nicardipine (□) and nifedipine (■). Each point represents the mean of four determination. Vertical bars indicate the standard errors.

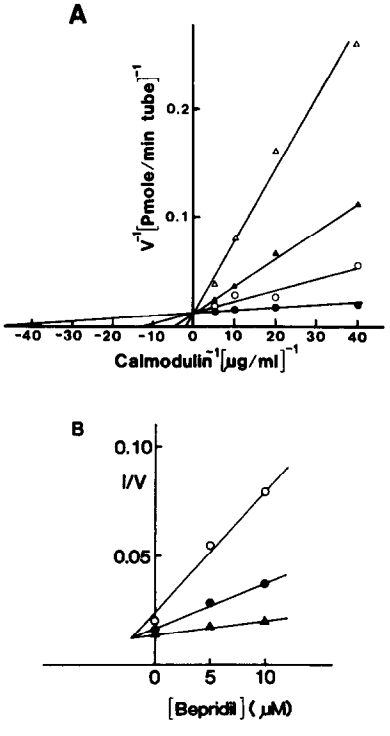

Fig. 2. Kinetic analyses of bepridil-induced inhibition of activation of smooth muscle myosin light chain kinase were carried out using double reciprocal plots (A) and Dixon plots (B). The reaction was carried out for 4 min in the presence of various concentrations of calmodulin (0.025–0.2 μg/ml). (A) The concentrations of bepridil were 0 μM (●), 5 μM (○), 10 μM (▲) and 20 μM (△). (B) The concentrations of calmodulin were 2.5 ng/ml (○), 100 ng/ml (●) and 200 ng/ml (▲).

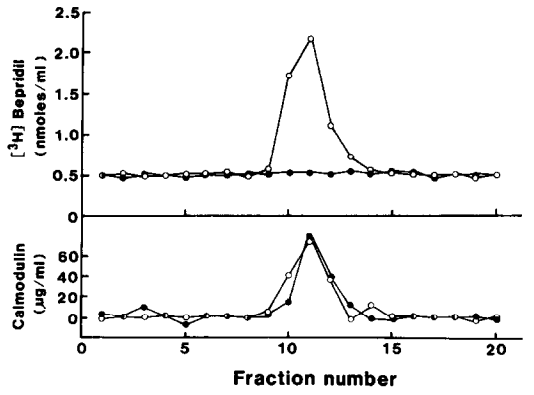

Fig. 3. Elution profile of bepridil-binding to calmodulin. Sephadex G-50 fine gel was pre-equilibrated with buffer containing 20 mM Tris–HCl (pH 7.5), 20 mM imidazole, 3 mM magnesium acetate, 0.5 μM [^{3}H]bepridil and 0.1 mM $CaCl_2$ (○) or 2 mM EGTA (●). This figure shows the typical elution profile of three independent experiments.

Bepridil inhibited this enzyme in a competitive fashion with calmodulin and the K_i value of about 2.2 μM was obtained by Dixon plot.

The binding of [^{3}H] *bepridil to calmodulin*

Figure 3 shows the elution profile for a typical binding experiment using [^{3}H] bepridil. The radioactivity peak coincided with the protein peak of the purified calmodulin, however, the radioactivity peak was not observed when calcium was replaced with 2 mM EGTA. [^{3}H]bepridil did not bind to bovine serum albumin, even in the presence of calcium (data not shown). These results suggest that bepridil specifically binds to calmodulin, the intracellular calcium receptor protein, in the presence of calcium.

The characteristics of bepridil binding to calmodulin are shown in Fig. 4. Scatchard analysis [18] of the data (shown in the inset) demonstrated that the dissociation constant (K_d) was 6.2 μM and the calculated number of specific binding sites was about 5 per molecule of calmodulin.

Displacement of [^{3}H]*bepridil from calmodulin by calcium channel blockers and W-7*

Table 1 is a summary of the concentration of calcium channel blockers and W-7 producing 50% inhibition (IC_{50}) of the binding of 0.5 μM [^{3}H]bepridil to calmodulin and of myosin light chain phosphorylation. Thirty μM nifedipine and 30 μM nicardipine did not displace [^{3}H] labeled bepridil from calmodulin. Unlabelled bepridil was the most potent compound with regard to displacement of [^{3}H] bepridil from calmodulin and there was a good correlation between the displacement of [^{3}H]bepridil and the inhibitory effect on MLCK by these calcium channel blockers and W-7.

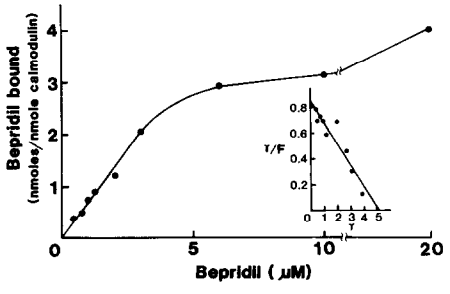

Fig. 4. Binding of bepridil to calmodulin. Equilibrium binding technique was performed in the presence of 0.1 mM $CaCl_2$ as described in Materials and Methods. Each point represents the mean value of two experiments. The inset shows the data plotted according to the Scatchard equation [18]: γ, moles of bepridil bound per mole of calmodulin; F, concentration of free bepridil.

DISCUSSION

Binding of [^{3}H]bepridil to calmodulin from bovine brain is calcium-dependent like that of other calmodulin antagonists [15, 19, 20], and a Scatchard analysis of the binding demonstrated that the dissociation constant (K_d) was 6.2 μM and the number of specific binding sites was about 5 per molecule of calmodulin. The calculated numbers of calcium dependent binding sites to calmodulin of many calmodulin antagonists including trifluoperazine (K_d = 1 μM, N = 2) [19], chlorpromazine (K_d = 3 μM, N = 3) [19], pimozide (K_d = 0.83 μM, N = 1) [20] and W-7 (K_d = 11 μM, N = 3) [15], were between 1 and 3 sites per molecule. In the case of bepridil, there may be at least two calcium dependent binding sites on calmodulin which differ from the binding sites of the above calmodulin antagonists.

Hidaka *et al.* reported that prenylamine was a calmodulin antagonist which might interact with calmodulin at sites different from those with which W-7 interacted [15]. In the present work, [^{3}H]bepridil was displaced from calmodulin by both W-7 and prenylamine. Therefore, the binding sites of bepridil to calmodulin may be responsible for both the binding sites of W-7 and prenylamine.

Since there was a good correlation between the displacement of [^{3}H]bepridil from calmodulin and the inhibition of MLCK by these calcium channel blockers and W-7, the ability of these agents to inhibit the activity of calmodulin-activated MLCK depends on antagonism of the action of calmodulin. Bepridil which bound to calmodulin in the presence of calcium inhibited the calmodulin-induced activation of MLCK more potently than did other calcium channel blockers. MLCK is a key enzyme involved in the regulation by calcium of smooth muscle contraction [9, 21]. Therefore, bepridil may affect the Ca^{2+} regulation of the vascular contractile response not only by blocking the calcium channel but also by inhibiting the function of calmodulin, an intracellular site of calcium action. Though the calmodulin antagonist action of bepridil may potentiate its calcium antagonistic action in the cardiovascular system [8], attention should also be paid to its side-effects in clinical use because of the diverse effects of calmodulin.

Acknowledgements—We thank M. Ohara of Kyushu University for comments on the manuscript. This work was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (1984).

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