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# Inhibitory action of Bepridil (CERM-1978) on calcium binding to cardiac sarcolemma of guinea pig

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Bepridil (CERM-1978), 1-(3-isobutoxy-2-benzylphenylamino)propyl pyrrolidine hydrochloride, is a new antianginal and antiarrhythmic agent without beta-adrenergic receptor blocking action. This drug exerts a negative inotropic effect in dog heart [1–3]. This effect has also been demonstrated in isolated perfused guinea pig hearts, and the mechanism for depression of contraction is due to uncoupling of contraction from excitation [4]. Vogel *et al.* [4] have also demonstrated that Bepridil exerts two actions. One is to depress and block the myocardial slow channels through which calcium ions pass during the action potential, leading to the development of tension. This action of Bepridil is similar to that of Verapamil, but Bepridil is less potent. The other action of Bepridil on the cardiac cells is less clear, but it is consistent with the depression of calcium release from the sarcoplasmic reticulum.

It is well known that the sarcolemma is responsible for the regulation of the influx of calcium during the process of excitation in the intact heart. Thus, to examine the possible mechanism for the negative inotropic action of Bepridil, we have determined its effect on the calcium binding sites of isolated cardiac sarcolemma. The effect of Bepridil was compared with that of another calcium-antagonistic agent, namely, Verapamil. It was found that Bepridil, like Verapamil, depressed the calcium binding to a low affinity site on the sarcolemma.

Cardiac sarcolemma from guinea pig was isolated according to the method of Pang and Weglicki [5]. In brief, this method used gentle homogenization, extraction with 0.6 M KCl, and differential and sucrose density gradient centrifugation. The membrane fractions were collected at the sucrose range of 38–42 percent (w/w), and the $(Na^+,K^+)$-ATPase activity averaged $25.2 \pm 0.7$ $\mu$moles $\cdot$ mg$^{-1}$ $\cdot$ hr$^{-1}$.

Calcium binding to the cardiac sarcolemma was measured in the presence of 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, $1 \times 10^{-6} - 2 \times 10^{-3}$ M $CaCl_2$, 0.2 $\mu$Ci/ml $^{45}CaCl_2$, 20 mM Tris, pH 7.4, and sarcolemma (0.5 mg/ml). Bepridil was dissolved in 50% ethanol and added in a total volume of 0.01 ml to give final concentrations of $10^{-6}$ M and $10^{-5}$ M. Addition of a similar concentration of ethanol by itself had no significant effect on calcium binding. Sarcolemma was incubated at room temperature for 10 min and then was centrifuged for 10 min in an Eppendorf high speed centrifuge. The pellet was dissolved in 0.1 N NaOH at 60° for 1 hr and counted in a liquid scintillation system. To correct for non-specific binding, 10 mM EGTA [ethyleneglycolbis-(amino-ethylene) tetra-acetate] was added to the incubation medium, in place of the non-radioactive $CaCl_2$. Radioactivity measured in this blank was subtracted from the experimental values. The data were fitted to curves calculated on the assumption that the binding of calcium to the sarcolemma had taken place in two classes of sites according to the formula: $\Sigma(n_i[Ca]/K_i)/(1 + [Ca]/K_i)$, where $i = 1$ or 2; $K$ = dissociation constant; and $n$ = capacity of binding sites [6]. Statistical analysis with a computer showed that the data were not compatible with either one or three classes of binding sites. All data are expressed as means ± S.E.M. of six experiments.

As shown in Fig. 1, calcium bound passively to the cardiac sarcolemma in the presence of physiological concentrations of NaCl (150 mM), KCl (2.7 mM), and $MgCl_2$ (1 mM). Sarcolemma contained two classes of calcium binding sites. The high affinity sites had a dissociation constant of $1.5 \pm 0.2 \times 10^{-5}$ M and a capacity of $0.45 \pm 0.06$ nmole/mg. The low affinity sites had a dissociation constant of $3.3 \pm 0.3 \times 10^{-3}$ M and a capacity of $54.0 \pm 3.2$ nmoles/mg.

Table 1 shows that Bepridil, at concentrations of $10^{-6}$ M and $10^{-5}$ M, decreased the capacity of the low affinity sites from $54.0 \pm 3.2$ nmoles/mg to $34.3 \pm 1.2$ nmoles/mg and $32.0 \pm 2.1$ nmoles/mg respectively. There was also a slight change in the dissociation constant of the low affinity sites, but the difference was not significant. The capacity and the dissociation constant of the high affinity sites were not changed. Figure 2 shows the more pronounced inhibition of Bepridil at high calcium concentrations ($2 \times 10^{-3} - 10^{-1}$ M), thus further emphasizing its action on the low affinity sites and also suggesting that the action of Bepridil is already saturated at $10^{-6}$ M.

To compare the effects of Bepridil and other inotropic agents on the calcium sites of the sarcolemma, the incubation of the sarcolemma was carried out in the presence of only a single concentration of $CaCl_2$ (2 mM). These results are summarized in Table 2. Bepridil ($10^{-6}$ M and $10^{-5}$ M) decreased the calcium bound to the low affinity sites from a control value of $19.6 \pm 1.4$ nmoles/mg to $16.6 \pm 0.2$ nmoles/mg and $15.4 \pm 0.7$ nmoles/mg respectively. This result was comparable to that for Verapamil ($10^{-6}$ M), which reduced the binding to $11.7 \pm 0.9$ nmoles/mg. Increasing the Verapamil concentration to $10^{-5}$ M depressed the calcium binding further. As can be

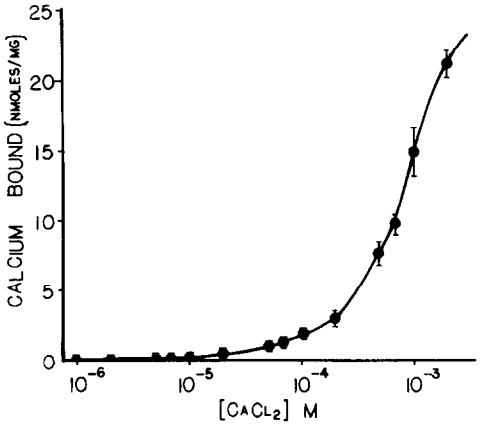


Fig. 1. Calcium binding to cardiac sarcolemma from guinea pig. Calcium binding was measured in 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$ and 20 mM Tris, pH 7.4.

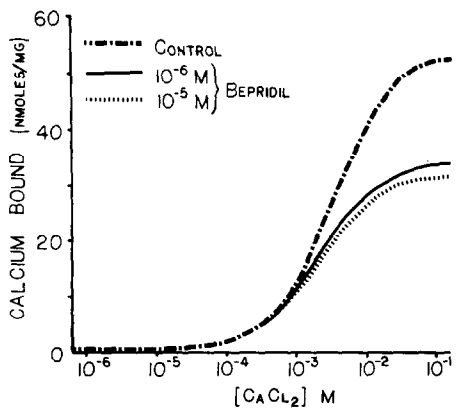


Fig. 2. Calculated calcium binding to cardiac sarcolemma in the presence and absence of Bepridil ($10^{-6}$ M and $10^{-5}$ M). Calcium binding was calculated with the formula $\Sigma (n_i[Ca]/K_i)/(1 + [Ca]/K_i)$ by the substitution of values from Table 1.

seen, $MnCl_2$ ($10^{-3}$ M) and $LaCl_3$ ($10^{-3}$ M) depressed the calcium binding to an even greater extent. Lowering the pH to 6.4 and 5.6 also lowering the calcium binding (Table 2). Decreasing the NaCl concentration from 150 to 75 mM slightly increased the amount of the calcium bound to the low affinity sites. Caffeine ($10^{-3}$ M) also produced a slight increase in the calcium binding (Table 2).

The myocardial slow channels, which are voltage-dependent and activated during excitation, are the sites through which calcium ions enter the myocardial cell during excitation–contraction coupling [8]. The amount of calcium influx controls the force of contraction. There may, in addition, be calcium release from the sarcoplasmic reticulum during contraction. The mechanism for the calcium release from the sarcoplasmic reticulum is not completely known [9, 10], and the relative importance of the calcium influx across the sarcolemma and the calcium release from the sarcoplasmic reticulum is also not known [9, 11]. Since the first step in calcium entry through the activated channels, down its electrochemical gradient, is binding to the mouth of the channel (there may be several more sequential binding sites through the channel length), and since Verapamil [12] and Bepridil [4] are known to depress and block the inward slow current (carried by both sodium and calcium) that passes through the slow channels [12], the present data suggest one possible mechanism for the blockade of calcium channels by Verapamil and Bepridil, namely, the displacement of bound calcium from the mouth of the channels.

The importance of the calcium binding sites on the sarcolemma has been emphasized by the findings that the effect of inotropic agents on the sarcolemma calcium sites (Table 2) is similar to that of the same agents on the slow inward calcium current and cardiac contraction [7]. Recent investigations from several laboratories on sarcolemmal calcium binding suggest that the calcium bound to the sarcolemma [7, 13–15], and not the calcium bound to the glycocalyx [16], is important to the regulation of calcium influx during the process of excitation–contraction coupling.

The present study shows that sarcolemma from guinea pig possesses two classes of calcium binding sites (Table 1) with dissociation constants quite similar to fractions isolated from dog [7], rat [15], rabbit [14] and neonatal heart cells [13]. It is likely that the low affinity sites ($K = 3.3 \times 10^{-3}$ M) are the ones most closely related to the slow channels, because the high affinity sites have a dissociation constant about 100-fold below the usual calcium concentration found in the interstitial fluid bathing the myocardial cells; the operating range for the slow inward calcium current observed experimentally is about $10^{-4}$ to $10^{-2}$ M calcium. The sarcolemmal calcium sites from the guinea pig are inhibited by negative inotropic agents as well as stimulated by positive inotropic agents (Table 2). This confirms the properties of the sarcolemmal calcium binding sites published previously [7] and emphasizes their possible involvement in the process of excitation–contraction coupling.

The inhibition by Bepridil of the calcium binding sites of the sarcolemma is similar to that predicted by its action on the slow action potential and cardiac contraction. Vogel *et al.* [4] have shown that Bepridil rapidly depresses ($5 \times 10^{-7}$ M) and abolishes ($10^{-5}$ M) the contractions of isolated guinea pig hearts perfused with normal Ringer's solution. The maximal upstroke velocity ($+V_{max}$) and overshoot of the normal fast action potential are only slightly affected; thus, excitation–contraction uncoupling is produced, In addition, Bepridil (above $1 \times 10^{-6}$ M) depresses $+V_{max}$, amplitude, and duration of the slow action potential within 15 min. The slow action potentials are induced by several agents (isoproterenol, tetraethylammonium) in hearts partially depolarized (to about −40 mV) by elevated $K^+$ (26 mM) Ringer's solution to voltage-inactivate the fast sodium channels. The mean $V_{max}$, which is proportional to the magnitude of the slow inward current, is depressed by 41 percent at $10^{-5}$ M Bepridil. This depression can be compared with the 37 percent decrease of the capacity of the low affinity calcium binding sites produced by Bepridil (Table 1).

The fact that caffeine ($10^{-3}$ M) exerted a slight, but statistically significant, increase in calcium binding to the low affinity sites (Table 2), suggests another possible mechanism of action for the known positive inotropic action of caffeine. A greater amount of calcium bound to the low affinity sites may allow a greater inward calcium current

Table 1. Effect of Bepridil on calcium binding to cardiac sarcolemma from guinea pig*

| | Calcium binding sites | | | |
|---|---|---|---|---|
| | High-affinity sites | | Low-affinity sites | |
| | $n_1$ | $K_1(\times 10^{-5})$ | $n_2$ | $K_2$ ($\times 10^{-3}$) |
| Control | 0.45 ± 0.02 | 1.5 ± 0.2 | 54.0 ± 3.2 | 3.3 ± 0.3 |
| Bepridil ($10^{-6}$ M) | 0.45 ± 0.01 | 1.4 ± 0.1 | 34.3 ± 1.2† | 2.1 ± 0.4 |
| ($10^{-5}$ M) | 0.42 ± 0.07 | 1.6 ± 0.3 | 32.0 ± 2.1† | 2.1 ± 0.6 |

* Key: $n_1$ and $n_2$, maximal number of binding sites (nmoles/mg); $K_1$ and $K_2$, dissociation constants (M). Conditions were the same as in Fig. 1. All values are means ± S.E.M., N = 6.
† $P < 0.01$ that the difference between the value and its control would occur by chance.

Table 2. Effect of inotropic agents on the low affinity calcium binding sites of cardiac sarcolemma from guinea pig*

| Conditions | Low affinity calcium sites (nmoles/mg) |
|---|---|
| Control (pH 7.4) | 19.6 ± 1.4 |
| Bepridil ($10^{-6}$ M) | 16.6 ± 0.2† |
| ($10^{-5}$ M) | 15.4 ± 0.7‡ |
| Verapamil ($10^{-6}$ M) | 11.7 ± 0.9‡ |
| ($10^{-5}$ M) | 8.6 ± 0.6‡ |
| $MnCl_2$ ($10^{-3}$ M) | 7.5 ± 0.8‡ |
| $LaCl_3$ ($10^{-3}$ M) | 1.9 ± 0.1‡ |
| pH 6.4§ | 13.8 ± 1.0‡ |
| pH 5.6§ | 10.4 ± 0.6‡ |
| NaCl (75 mM)‖ | 28.3 ± 3.2‡ |
| Caffeine ($10^{-3}$ M) | 24.4 ± 0.4‡ |

* Calcium binding to the sarcolemma was measured in the presence of 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 2 mM $^{45}CaCl_2$ and 20 mM Tris, pH 7.4. The low affinity sites were obtained by subtraction of the high affinity sites from the total calcium bound. The high affinity sites were calculated by the formula $(n_1\ [Ca]/K_1)/(1 + [Ca]/K_1)$ with values from Table 1 and were assumed not to be affected by the agents [7].

† $P < 0.05$.

‡ $P < 0.01$.

§ A concentration of 20 mM MES (4-morpholineethanesulfonic acid) at either pH 6.4 or 5.6 was used instead of the Tris buffer.

‖ Instead of 150 mM NaCl, 75 mM NaCl and 75 mM Tris were added.

during excitation. The other presumed mechanisms of action for caffeine include: (a) action as a phosphodiesterase inhibitor, thus elevating cAMP and leading to phosphorylation of a greater function of the slow channels [17] which would, in turn, allow a greater inward calcium current, and (b) the effect on calcium uptake and release by the sarcoplasmic reticulum [18].

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