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TWO SITE BINDING OF BEPRIDIL AND MODULATION OF ADENYLATE CYCLASE IN CARDIAC SARCOLEMMAL MEMBRANES

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A preparation of cardiac sarcolemmal membranes is described. These membranes exhibit 9–24-fold purification of $(Na^+ + K^+)$-ATPase, potassium-stimulated nitrophenolphosphatase, 5′-nucleotidase, adenylate cyclase, sialic acid content, and β-receptor number. Sarcolemmal membranes have two classes of binding sites for the calcium entry blocker, bepridil, $70 \cdot 10^{12}$ high-affinity sites/mg, K_d 25–40 nM; and $30 \cdot 10^{15}$ low-affinity sites/mg, K_d 54–70 μM. Binding of bepridil to these sites appears responsible for inhibition of isoprenaline-stimulated and activation of fluoride-stimulated adenylate cyclase. Since basal adenylate cyclase activity is not influenced, bepridil must act not at the catalytic site, but by altering the interactions between β-receptor and catalytic and regulatory components of adenylate cyclase.

Introduction

Cardiac sarcolemmal, or plasma, membranes contain protein assemblies which mediate the normal metabolic, electrophysiological and contractile functions of myocytes. Specific transport sites for sodium and potassium ions and for nutrients such as glucose [1] and amino acids [2], as well as adrenergic and peptide hormone receptors [3] have been recognised.

The present study was undertaken to develop a rat cardiac sarcolemmal membrane preparation of high purity, for use in studies of the mechanisms of action of hormones and other cardioactive molecules, including drugs, which influence plasma membrane activities.

Many cardioactive drugs influence the activity of sarcolemmal enzymes. For example cardiac glycosides inhibit the $(Na^+ + K^+)$-dependent ATPase, although whether this inhibition is necessarily associated with increased force of contraction remains a matter of debate [4]. β-Adrenergic agonists and antagonists stimulate [5] or inhibit [6] adenylate cyclase, an integral protein of the sarcolemmal membrane. The calcium entry blockers, or calcium antogonists, which are used in the treatment of angina, are a structurally heterogeneous group of compounds. Pharmacological and electrophysiological studies suggest these compounds act primarily by inhibiting the influx of calcium ions through sarcolemmal membrane channels opened during cell excitation [7]. But recent evidence points to additional intracellular sites of action for some compounds of this class. In particular, bepridil, β-[(2-methylpropoxy)methyl]-*N*-phenyl-*N*-phenylmethyl-1-pyrrolidineethanamine (ORG 5730), has been shown to concentrate in adult rat ventricular myocytes [8], and to a lesser extent in chick embryonic ventricular muscle [9]. Vogel et al. [10]

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PMSF, phenylmethylsulphonyl fluoride.

have suggested bepridil may act within cells to inhibit release of calcium from the sarcoplasmic reticulum. These observations do not preclude an action on the sarcolemmal membrane although attempts to demonstrate an influence on calcium binding to guinea pig heart sarcolemmal fragments have been less convincing for bepridil than for verapamil [11,12].

To clarify the molecular bases of drug-induced changes in membrane behaviour, a sarcolemmal membrane preparation of high purity is required which retains all activities associated with the membrane of intact tissue. A major technical difficulty in isolating functionally intact cardiac sarcolemmal fragments arises because myocardial tissue requires relatively severe homogenisation conditions. For this reason sarcolemmal preparations appear heavily contaminated with sarcoplasmic reticulum and mitochondrial membrane fragments. Disruption of myocardial tissue by proteolytic digestion may be less severe, but can result in loss of functional properties of the membrane.

Several methods for the isolation of cardiac sarcolemmal membranes have been reported, but none generally adopted. Recent reports describe the properties of sarcolemmal fractions isolated from hamster [13], guinea-pig [14,15], rabbit [16–20] and dog [21]. These preparations have been characterised by the extent to which a variety of recognised plasma membrane markers are enriched, and in general exhibit 5–10-fold purifications compared with whole tissue homogenates. Rat heart sarcolemma has been isolated from neonatal rats [22] with K^+ *p*-nitrophenolphosphatase, ($Na^+ + K^+$)-ATPase and 5′-nucleotidase activities and sialic acid content enriched 10-fold. Similar purifications have been achieved with adult rat heart [15,23]. Enrichments in the range 33–100-fold have been reported [24] for sarcolemmal fragments prepared from isolated canine myocytes. This preparation has the advantage of being free of plasma membrane fragments derived from interstitial and other non-muscle cells, but the low yield, compared with preparations from whole tissue, makes it impractical to adopt this approach with small animal hearts.

In this paper we report characteristics of the membrane preparation, describe sarcolemmal binding sites for the calcium entry blocker bepridil, and the influence of bepridil on adenylate cyclase activities.

Materials and Methods

Bepridil and [^{3}H]bepridil (20.7 Ci/mmol, prepared by Amersham International, Amersham, Bucks, U.K.) were provided by Organon Laboratories Ltd. All other isotopes were obtained from Amersham International (Amersham, Bucks). Biochemicals including collagenase were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. All other chemicals were of analytical reagent grade. Cyclic AMP antibody was a gift from Dr. K. Siddle. Cyclic AMP binding protein was isolated from bovine adrenal glands by the method described by Brown et al. [25].

Isolation of sarcolemmal membranes. Six male rats were used in each experiment. All procedures were at 4°C unless otherwise stated. Rats were anaesthetised with ether and the excised hearts cooled in oxygenated buffer containing 124 mM NaCl/5 mM KCl/1.2 mM $MgSO_4$/20 mM sodium phosphate/10 mM glucose, pH 7.4 (solution A). Ventricular tissue was weighed, cut into 1 mm slices and incubated at 37°C for 30 min in solution A containing 25 μM $CaCl_2$ and 0.5 mg/ml collagenase. Digested tissue was washed with solution A to remove the collagenase, minced with scissors, and homogenised in 1 mM $NaHCO_3$/1 mM phenylmethylsulphonyl fluoride (PMSF)/1 mM dithiothreitol (pH 8.0) using a loose-fitting motor driven glass-teflon homogeniser (10–12 passes at 300 rpm). The homogenate was diluted with 5 volumes of 1 mM $NaHCO_3$/0.6 M KCl/50 mM sodium pyrophosphate/5 mM $MgCl_2$/1 mM PMSF/0.1 mM dithiothreitol (pH 8.0), stirred for 45 min and filtered through four layers of muslin. A crude membrane pellet was separated by centrifugation at 46 000 × *g* for 30 min, before being washed by resuspension in 1 mM $NaHCO_3$/25 mM sodium pyrophosphate/2.5 mM $MgCl_2$/0.1 mM dithiothreitol (pH 8.0) and centrifugation at 46 000 × *g* for 30 min. The pellet was suspended in 5 mM Tris/0.1 mM dithiothreitol (pH 7.4) containing 40% (w/v) sucrose. All sucrose solutions were made in the same Tris-DTT buffer. The membrane fraction was layered on 5 ml 60% (w/v) sucrose, with 10 ml of 35% (w/v) sucrose, 10 ml of

30% (w/v) sucrose and 1.5 ml Tris-DTT buffer successively layered on top. These sucrose gradients were centrifuged at 96 000 × *g* for 2 h. Material concentrating at sucrose interfaces was collected, diluted with Tris-DTT buffer, and collected by centrifugation at 46 000 × *g* for 45 min. Pellets were suspended in the Tris-DTT buffer containing 0.25 M sucrose and stored at −20°C. Interface fractions were designated A, B, C and D corresponding to material collecting at the 0–30%, 30–35%, 35–40% and 40–60% sucrose interfaces, respectively.

A sample of the initial homogenate was filtered through four layers of muslin and frozen for assays of homogenate activities. Contractile proteins solubilised by the pyrophosphate extraction procedure were precipitated by dialysis against 5 mM Tris/0.1 mM dithiothreitol (pH 8.0) collected by centrifugation at 46 000 × *g* for 30 min and resuspended in 5 mM Tris/0.3 M NaCl/0.1 mM dithiothreitol (pH 7.4). The soluble fraction derived from this dialysis is the supernatant fraction (Table I). To allow comparison of the properties of membranes prepared with and without proteolytic digestion of the tissue, some sarcolemmal preparations were prepared without the collagenase digestion step.

Membrane activities. Binding of [^{125}I]cyanopindolol to membrane fragments was measured by incubating membranes for 45 min at 30°C in 50 mM Tris/5 mM $MgCl_2$ (pH 7.4) containing 40 pM [^{125}I]cyanopindolol ($4 \cdot 10^5$ cpm/ml) before rapid filtration through Whatman GF/F or Bio-Rad Unipore 0.2 μm polycarbonate filters. Specific binding was assessed by parallel incubations in the presence of 1 μM (±)propranolol. Unless otherwise stated all enzymes were assayed at 37°C. For estimations of sarcolemmal purification the fluoride (10 mM)-stimulated adenylate cyclase activity of all fractions was determined at 30°C in the presence of 4 mM EGTA in order to complex endogenous calcium [26]. All other measurements of adenylate cyclase activity in purified membrane were carried out in the absence of EGTA and in the presence of 0.5 mM $MnCl_2$. Cyclic AMP was determined by either radioimmunoassay [27] or binding protein assay [25]. ($Na^+ + K^+$)-ATPase and K^+-stimulated nitrophenolphosphatase were determined as reported by Bers [22], with ouabain sensitivity determined in the presence of 4 mM ouabain.

Ca^{2+}-stimulated ATPase (in the presence or absence of 1 mM EGTA) was measured in 50 mM imidazole/0.1 M KCl/5 mM NaN_3/3.5 mM $MgCl_2$/50 μM $CaCl_2$/3 mM ATP/0.5 mg · ml^{-1} calcium ionophore (A23187) (pH 7.4). 5'-Nucleotidase was determined in the presence of 50 mM Tris/5 mM $MgCl_2$/2.5 mM dithiothreitol/0.23 mg · ml^{-1} bovine serum albumin/1 mM [^{3}H]AMP (0.5 μC/ml) (pH 7.4). Incubations were terminated by the addition of 5 volumes of 60% (w/v) anion exchange resin slurry (BioRad AG1x2 200–400 mesh, Cl^- form), and the resin subsequently separated by centrifugation at 9000 × *g* for 1 min.

Alkaline phosphatase was measured by the method of Mircheff and Wright [28] using 5 mM *p*-nitrophenol phosphate as substrate.

Glucose-6-phosphatase was measured by a modification of Nordlie and Arion [29] as described by Bers [22].

Succinate dehydrogenase was measured using dichlorophenol indophenol [30].

Rotenone-insensitive NADPH-cytochrome *c* reductase was determined as reported by Sottocasa et al. [31].

Sialic acid was measured in sucrose-free extracts by the method of Warren [32], inorganic phosphate by the method of Kuttner and Lichtenstein [33], and protein by the method of Lowry et al. [34] using a bovine serum albumin standard.

For electron microscopy, freshly isolated membrane fractions were fixed in 2% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 for 16 h at 4°C. After washing with buffer, membranes were fixed for 1 h in 1% osmium tetroxide in phosphate buffer. The material was washed three times in 0.1 M sodium phosphate (pH 7.4) before being dehydrated in graded ethanol, embedded in araldite, and ultrathin sections cut using a Reichert OMU2 ultramicrotome. Sections were stained in 3.5% uranyl acetate followed by 0.04% lead citrate and examined using a Philips EM301 electron microscope.

Bepridil binding. Membranes were incubated at 20°C for 30 min in 50 mM Tris-HCl (pH 7.4) with bepridil at concentrations between 10 nM and 100 μM, before being filtered through Whatman GF/F

TABLE I

ENZYME AND PROTEIN DISTRIBUTION IN FRACTIONS OF RAT HEART

Fractions A, B, C and D are separated from a crude membrane fraction on sucrose gradients. For details see Methods. Enzyme activities are in the stated units ± S.D.

	Homogenate	A
Adenylate cyclase (nmol/mg/h) ($n = 9$) (*$n = 3$)	0.875 ± 0.247	20.70 ± 1.74
[^{125}I]Cyanopindolol binding (fmol/mg) ($n = 4$)	7.58 ± 1.53	88.78 ± 11.3
($Na^+ + K^+$)-ATPase, K^+-stimulated (μmol/mg/h) ($n = 7$) (*$n = 4$)	3.00 ± 1.36	28.8 ± 4.8
Ouabain sensitive	2.54 ± 0.77	30.4 ± 5.6
p-Nitrophenolphosphatase K^+-stimulated (μmol/mg/h) ($n = 9$) (*$n = 6$)	0.44 ± 0.16	9.7 ± 3.0
Ouabain sensitive	0.31 ± 0.12	4.63 ± 1.33
5′-AMPase (μmol/mg/h) ($n = 4$) (*$n = 3$)	0.42 ± 0.11	3.89 ± 1.68
Sialic acid (nmol/mg) ($n = 2$)	9.2	83:4
Alkaline phosphatase (μmol/mg/h) ($n = 3$)	0.29 ± 0.05	1.02 ± 0.52
Ca^{2+}-ATPase (μmol/mg/h) ($n = 5$) (*$n = 4$)	2.80 ± 0.83	2.59 ± 1.91
Rotenone-insensitive NADPH-cytochrome *c* reductase (nmol/mg/h) ($n = 3$)	1.42 ± 0.48	2.31 ± 0.35
Glucose-6-phosphatase (μmol/mg/h) ($n = 2$)	0.19	0.66
Succinate dehydrogenase (μmol/mg/h) ($n = 4$) (*$n = 2$)	2.02 ± 0.19	0.46 ± 0.12
cAMP phosphodiesterase (nmol/mg/h) ($n = 3$)	1.56 ± 0.06	0.27 ± 0.03
Protein recovery (mg/100 mg homogenate) (n)	100	0.359 ± 0.250 ($n = 10$)

[a] Recovery from particulate fractions.

filters and washed with ice-cold Tris-HCl buffer (pH 7.4). Bound bepridil was determined by counting the filters in toluene/triton/PPO scintillant. Because we find bepridil binds to the GF/F filters, blanks were run and subtracted for every bepridil concentration. Bepridil binding site affinity and density were determined by a computer fitted linear regression of Scatchard analysis of the data [35].

Results and Discussion

Membrane purification

Protein recovery and enzyme activities in each of the sucrose gradient fractions as well as in the initial homogenate and contractile protein and supernatant fractions are shown in Table I. Specific activities of putative sarcolemmal membrane markers were increased between 9- and 24-fold compared with the homogenate. Total recovery of most sarcolemmal enzymes was greater than 50%. Collagenase softens the tissue, facilitating subsequent homogenisation. Membrane yields, defined by both protein yield and activity of putative sarcolemmal enzymes, are reproducibly higher when collagenase digestion is included in the procedure.

Electron micrographs of the membrane fractions indicate a high degree of vesiculation in the low-density fractions, Fig. 1, similar to that reported by others [15,18,22]. Channel-forming ionophores or selective detergents have been used to unmask latent enzyme activities [19,36] but detergents are reported to reduce the activities of both 5′-nucleotidase and adenylate cyclase [15], and may adversely influence other activities dependent upon specific lipid-protein interactions. Despite the gentle homogensation procedure membrane vesicles in fraction A occasionally formed liposomal type structures (insert Fig. 1a). This vesiculation may mask enzyme activities so that recovery and purification are likely to be under-estimated.

Membrane enzyme activities

Adenylate cyclase activities, although lower than those reported for dog [19,20] and guinea-pig [14] are similar to those reported for rat [15]. Endogenous adenylate cyclase activity has been reported to be lower for the rat than for most other species [37]. In the presence of EGTA we find low basal

B	C	D	Contractile proteins	Supernatant	SL purification factor	% Recovery
13.20 ± 1.26	3.10±0.44	0.50±0.11	0.47±0.27 *	0.08 ±0.08 *	24	52
69.90 ±10.42	26.91±7.74	11.01±2.04	n.d.	n.d.	12	56
18.5 ± 9.4	6.5 ±3.3	1.9 ±1.0	1.7 ±1.2 *	1.9 ±0.4 *	10	64
19.1 ± 9.8	6.9 ±3.0	2.2 ±1.0	1.4 ±0.5	2.3 ±0.7	12	86
7.8 ± 2.7	1.63±0.70	0.56±0.26	0.41±0.17 *	0.032±0.02 *	21	66
3.72 ± 1.20	0.84±0.33	0.35±0.21	0.18±0.06 *	0.010±0.005 *	15	51
3.91 ± 1.54	1.49±0.42	0.36±0.35	0.20±0.03	0.09 ±0.06 *	9	54
63.3	5.5	6.8	5.6	0.52	9	28
1.18 ± 0.25	0.33±0.06	0.07±0.02	0.81±0.34	0.05 ±0.04	3.5	22
8.84 ± 3.4	14.14±5.7	3.75±2.46	0.96±0.68	(−26.16±5.08 *)	0.9	52 [a]
2.76 ± 0.53	4.32±1.84	0.75±0.41	0.65±0.32	1.41 ±0.26	1.6	94
0.99	0.94	0.21	0.10	0.24	3.5	96
0.99 ± 0.51	4.03±1.52	5.40±1.41	0.73 *	0.05 *	0.2	82
0.39 ± 0.02	0.40±0.02	0.58±0.05	0.20±0.01	2.80 ±0.10	0.2	96
0.500± 0.410 (n =10)	1.71±0.513 (n =10)	28.49±4.06 (n =10)	10.38±1.97 (n = 6)	46.97 ±2.31 (n = 6)		

rates (47.4 ± 14.6 pmol/mg membrane protein per min) which can be stimulated more than 7-fold by fluoride, and more than 12-fold in the presence of 10 μM isoprenaline plus 100 μM GTP. In the absence of EGTA and with the addition of manganese, basal activities are at least 4-fold higher with fluoride and isoprenaline stimulated activity by a further 5- to 6-fold. By contrast, dog and guinea-pig sarcolemma are reported to be stimulated only 3-fold [14,19,20]. Purification factors for other sarcolemmal enzymes are only half those for adenylate cyclase and K^+-stimulated nitrophenolphosphatase. This appears to reflect the instability of these two enzymes in the initial homogenate. We find the activity of fluoride and isoprenaline stimulated adenylate cyclase falls by 25% in the first hour, and by 90% after 6 h at 4°C, even in the presence of PMSF. Because of this rapid inactivation, samples of homogenate were immediately stored at −20°C to −70°C. These enzymes are stable to freeze-thawing cycles in purified membrane fractions stored in sucrose, but loose activity if whole homogenate fractions are stored without sucrose.

$(Na^+ + K^+)$-ATPase activity is completely inhibited by ouabain in this preparation, but the same concentration of glycoside produced only 50% inhibition of K^+-stimulated nitrophenolphosphatase. Neither lower potassium concentration, nor higher glycoside concentration altered the fraction of activity sensitive to ouabain. Although both activities are thought to be a function of the same enzyme there appear to be substrate-dependent differences in ouabain sensitivity.

There are conflicting reports about whether Ca^{2+}-stimulated ATPase is a sarcolemmal enzyme or whether its presence indicates contamination by sarcoplasmic reticulum fragments. Ca^{2+}-ATPase has been reported to co-purify with other sarcolemmal markers in membranes isolated from rabbit [18], dog [38,39], frog [40] and rat [23]. Others found no increase in Ca^{2+}-ATPase as sarcolemmal membrane fragments were purified from rat [22], dog [19,20] or mouse [41], although significant activities were still present in these latter preparations. Sarcolemmal membrane purified from dog isolated myocytes yielded the greatest enzyme purifications but no detectable Ca^{2+}-ATPase [24]. It is important to decide whether sarcolemmal Ca^{2+} transport and Ca^{2+}-dependent ATPase activ-

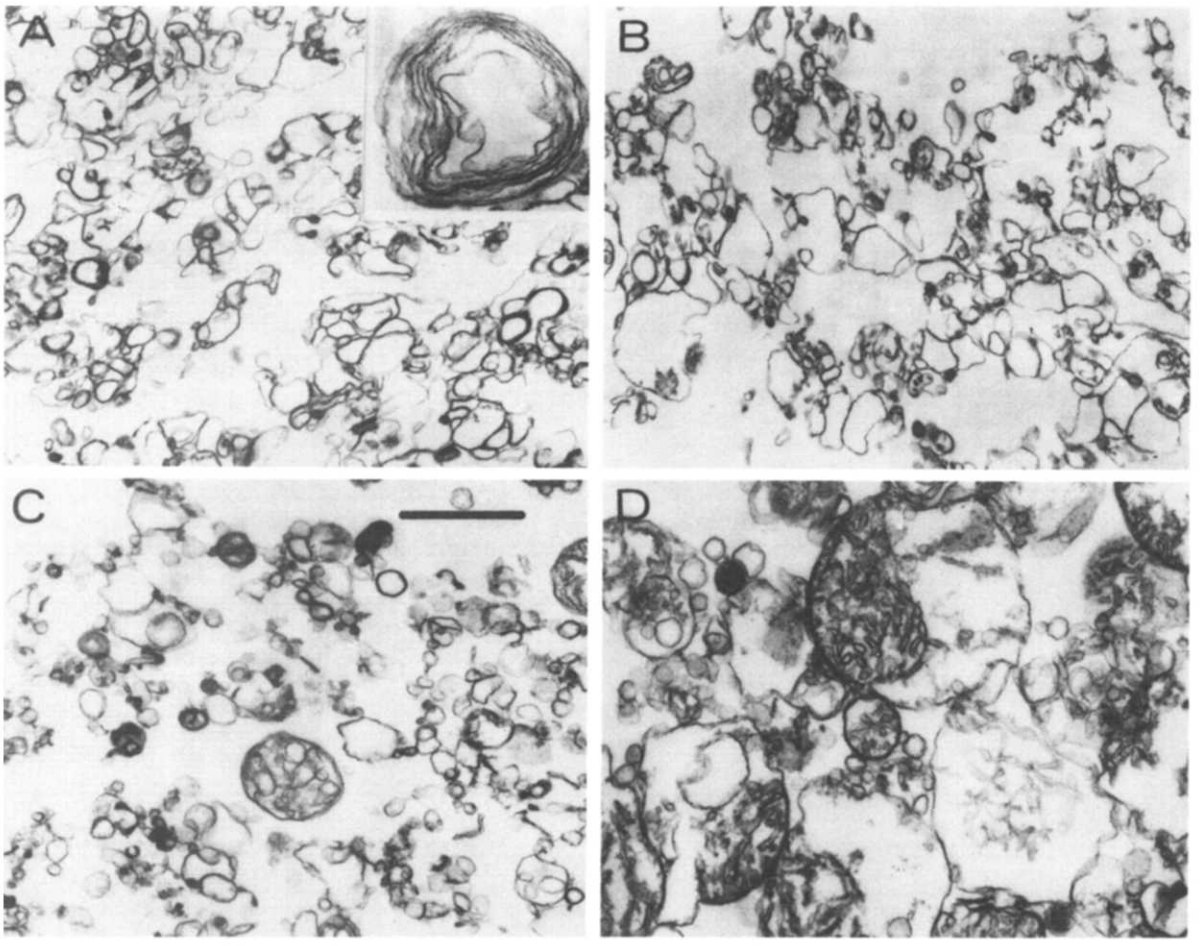

Fig. 1. Electron micrographs showing the morphology of membrane fragments in fractions A, B, C and D from sucrose gradient fractionations. Insert A shows the vesiculated structures frequently seen in fraction A. The bar represents 1 μm in A, B, C and D, but 0.5 μm in the insert to A.

ity are related, or whether Ca^{2+} transport depends solely upon Na^{+} transport, for Van Alstyne et al. [20] have demonstrated that dog sarcolemmal vesicles with low Ca^{2+}-ATPase activity have the highest reported capacity for Na^{+}-linked uptake of calcium. Our fraction A has Ca^{2+}-activated ATPase in addition to glucose-6-phosphatase and rotenone-insensitive NADPH-cytochrome *c* reductase. This enzyme combination suggests some contamination with fragments of sarcoplasmic reticulum which contains a calcium-activated ATPase [42]. The relatively low activity of succinate dehydrogenase indicates there is very little contamination with inner mitochondrial fragments. Attempts to determine the distribution of Ca^{2+}-ATPase were complicated by the consistent presence of an as yet unexplained Ca^{2+}-inhibited ATPase in the dialysed supernatant fraction, Table I.

The activities of adenylate cyclase, including fluoride-, isoprenaline- and forskolin-stimulated activities, 5′-nucleotidase and K^{+}-stimulated nitrophenolphosphatase are not significantly different for membranes prepared with or without collagenase digestion.

Bepridil binding sites

These rat heart sarcolemmal fragments have both high- and low-affinity sites binding the

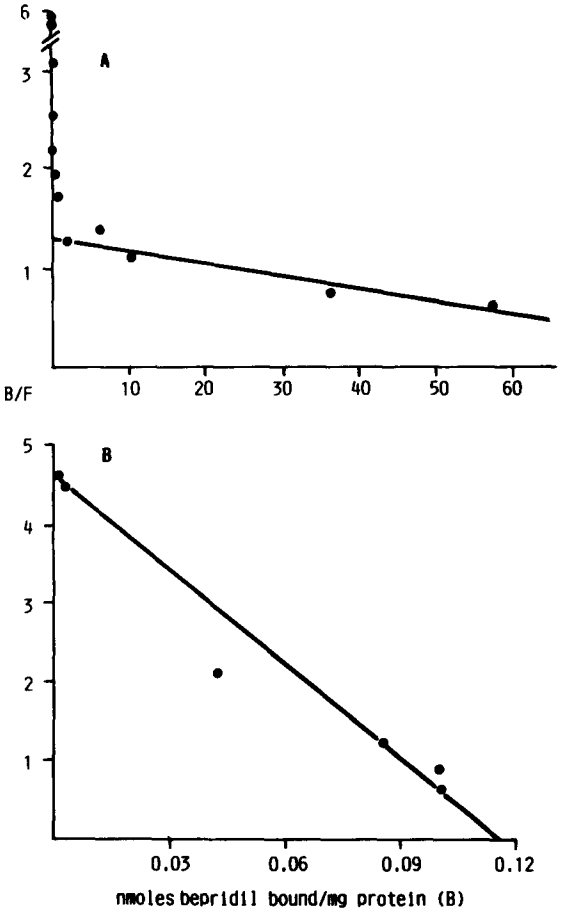

Fig. 2. Scatchard plot of bepridil binding to sarcolemmal membrane fragments. Low-affinity sites are described by the line in Fig. 3a, and high-affinity sites by the line in Fig. 3b. The units of B and F are respectively nmol bepridil bound/mg membrane protein, and μM concentration of bepridil.

calcium entry blocker, bepridil. The number of bepridil binding sites is not different for membranes prepared with or without collagenase, but the affinity with which bepridil binds to high-affinity sites is an order of magnitude less for membranes prepared with collagenase than for those prepared without. In three different sarcolemmal preparations (collagenase-free) the number of high-affinity sites was $(69–84)\cdot 10^{12}$/mg membrane protein, with dissociation constants in the range 25–40 nM. Low affinity binding sites measured $(40–48)\cdot 10^{15}$ sites/mg membrane protein, with dissociation constants in the range 54–70 μM. Fig. 2 is a Scatchard plot of one of these sets of data. The numbers of bepridil binding sites are not similar to the numbers of high- and low-affinity calcium binding sites reported for guinea-pig isolated sarcolemmal fragments [11,12]. This suggests that bepridil may not compete directly with calcium for sarcolemmal membrane binding sites. The finding that guinea pig cardiac sarcolemmal fragments bind the calcium entry blocker, nitrendipine with a dissociation constant of 0.1 nM and 300 fmol sites/mg protein [43], has recently been confirmed for canine cardiac sarcolemmal fragments [44]. Thus nitrendipine appears to bind to less than 1% of the number of high-affinity sites available to bepridil, but with higher affinity. This difference is not surprising since bepridil and nitrendipine are structurally different and may not have a common mechanism of action.

Interaction of Bepridil with adenylate cyclase

The influence of bepridil upon sarcolemmal enzyme activities was investigated. Bepridil at concentrations of 10, 50 and 100 μM did not influence

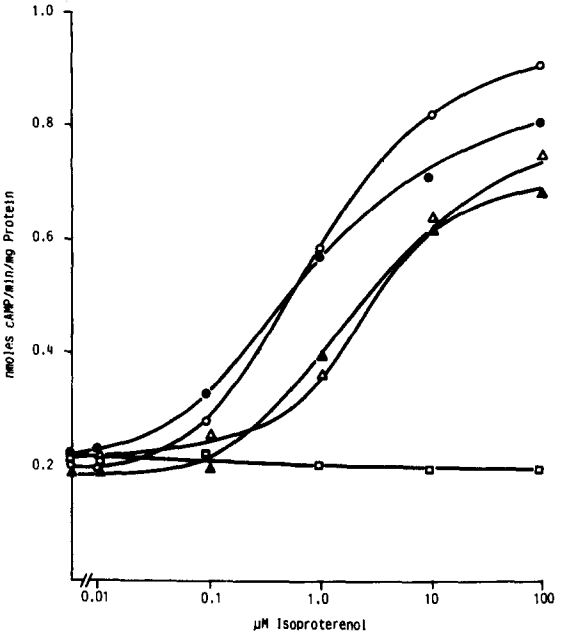

Fig. 3. Influence of bepridil on the ability of isoprenaline to stimulate adenylate cyclase activity of rat cardiac sarcolemmal membranes. Adenylate cyclase activity (nmol cAMP produced/min per mg membrane protein) measured at 30°C is presented as a function of isoprenaline concentration without bepridil (○), and in the presence of 5 μM (●), 50 μM (▲), 100 μM (△) and 500 μM bepridil (□).

5′-nucleotidase, $(Na^+ + K^+)$-ATPase, K^+-stimulated nitrophenolphosphatase or their ouabain sensitivities. In contrast the response of adenylate cyclase was complex. Neither basal nor forskolin-stimulated adenylate cyclase is influenced by bepridil between 5 and 500 μM. But, isoprenaline-stimulated adenylate cyclase is inhibited, while fluoride-stimulated adenylate cyclase is further activated at all bepridil concentrations.

The inhibition of isoprenaline stimulated adenylate cyclase by bepridil is shown in Fig. 3. Both 50 μM and 100 μM bepridil shift the K_a for isoprenaline activation to higher isoprenaline concentrations, and significantly reduces maximum activation. K_a is not significantly changed in the presence of 5 μM bepridil although there is a slight reduction in the maximum activation. The ability of isoprenaline to stimulate adenylate cyclase is completely suppressed by 500 μM bepridil. The relatively high concentration of bepridil required to depress adenylate cyclase activity becomes relevant because of the extent to which bepridil concentrates within cardiac myocytes [8]. These observations are quantitatively similar to the suppression of isoprenaline-stimulated contractile activity seen in guinea-pig atria pretreated with bepridil [45]. The lack of influence on basal adenylate cyclase implies that bepridil acts at a site distinct from the catalytic site of this enzyme. Other possible mechanisms include a competitive action at the isoprenaline receptor site or an action on the guanine nucleotide-dependent regulatory binding subunit. Fluoride activation, a direct effect on the guanine nucleotide regulatory component [46], is significantly further increased by bepridil at all bepridil concentrations between 1 μM and 1 mM. Fig. 4 shows the influence of bepridil on fluoride-(25 mM), forskolin-(50 μM) and isoprenaline-(10 μM) stimulated adenylate cyclase. Forskolin activation, a direct activation of the catalytic subunit [47], is not further increased by bepridil except at 250 μM bepridil. At present we have no explanation for the reproducible increase in both fluoride and forskolin activation at 250 μM bepridil (Fig. 4).

To date definitive molecular mechanisms have not been described for the action of any drug classified as a calcium entry blocker. Among these compounds bepridil is especially lipophilic (Kelder,

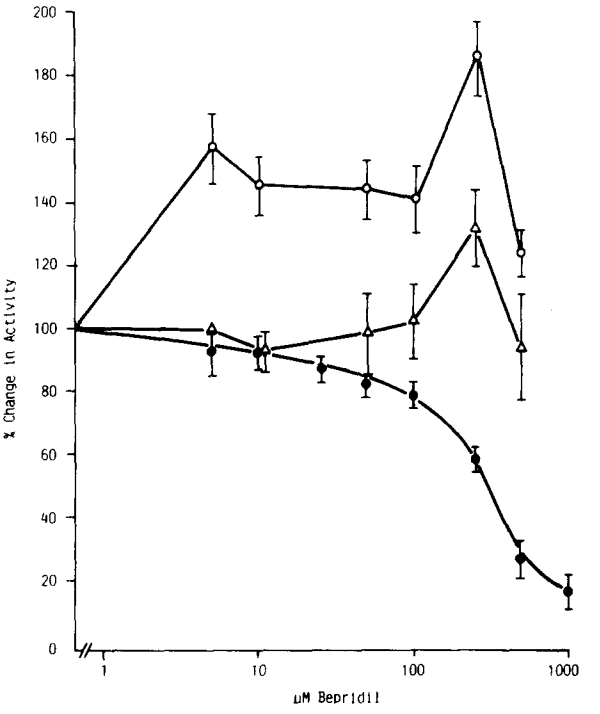

Fig. 4. Influence of bepridil on isoprenaline, fluoride and forskolin activation of adenylate cyclase activity of rat heart sarcolemmal membranes. In the absence of bepridil, adenylate cyclase activity was 0.87 nmol/min per mg in the presence of 25 mM fluoride (○), 0.93 nmol/min per mg with 10 μM isoprenaline (●) and 2.06 nmol/min per mg with 50 μM forskolin (△). Basal adenylate cyclase was 0.2 nmol/min per mg. Results are expressed as % change in activity as a function of bepridil concentration.

J., personal communication). This lipophilicity may mean that bepridil intercalates with sarcolemmal membrane lipids to change membrane fluidity, and the properties of biologically active membrane protein complexes.

On this basis our observations could be explained if bepridil intercalates into the membrane structure in such a manner as to alter interactions between the molecular components of the receptor and the catalytic and regulatory subunits of adenylate cyclase.

Alternatively the demonstrated ability of actin to bind bepridil [8] raises the possibility that bepridil interacts with cytoskeletal actin or other microtubular or microfilamentous proteins of the cell matrix [48] which remain associated with the sarcolemmal fragments. Studies with intact tissues

have provided evidence that cytoskeletal proteins have a role in controlling interactions between the β receptor and adenylate cyclase components [49,50].

The present study demonstrates the existence of two classes of bepridil binding site on sarcolemmal membranes. Since there is no convincing relationship between calcium and bepridil binding [11] these binding sites may be related to the regulation of adenylate cyclase. The dissociation constant for binding to low-affinity sites may imply that this interaction is associated with the bepridil-dependent inhibition of isoprenaline-stimulated and activation of fluoride-stimulated adenylate cyclase. The absence of influence on basal adenylate cyclase activity further implies there may be additional intracellular sites of action to explain the antianginal properties and intracellular binding [8].

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