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UPTAKE OF BEPRIDIL INTO ISOLATED VENTRICULAR MYOCYTES

RETENTION BY ACTIN

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Abstract—Isolated rat ventricular myocytes accumulate large amounts of bepridil intracellularly. The mode of transport is uncertain but uptake is of such a magnitude as to mask possible sarcolemmal binding sites. Bepridil is tightly bound within myocytes, there being only a minimal efflux into bepridil-free medium. Purified F-actin binds bepridil in excess of 2 moles/mole actin, sufficient to account for the uptake of bepridil by myocytes incubated in 10 μM drug. At higher bepridil concentrations the amount transported into myocytes suggests that the drug may be distributed between actin-bound and cytoplasmic pools. Bepridil may alter cardiac contractility by a direct influence on the contractile filaments.

Bepridil {β-[(2-methylpropoxy)-methyl]-*N*-phenyl-*N*-phenylmethyl-1-pyrrolidineethanamine} (ORG 5730) is a new antianginal agent which, in experimental animals, exhibits both negative chronotropic and inotropic properties in the heart. It produces dilatation of peripheral and coronary vascular smooth muscle and reduces myocardial oxygen consumption [1, 2]. These properties can be explained by a calcium antagonist action [3–6]. But there is some evidence that bepridil differs from classical calcium antagonists such as verapamil, for, in contrast to verapamil, bepridil only partially inhibits the slow inward current in guinea pig myocardium [3]. In addition, Vogel *et al.* [3] provided indirect evidence that bepridil may inhibit the release of calcium from intracellular stores. There has as yet been no unequivocal demonstration that bepridil acts on intracellular sites of ventricular myocytes.

Pang and Sperelakis [7] have investigated the influence of bepridil on calcium binding to guinea pig sarcolemmal membrane fragments characterised as being enriched seven-fold in (Na^+-K^+)ATPase activity [8]. They report two populations of calcium-binding sites in their membrane preparation. Bepridil did not alter calcium binding to the high-affinity sites, but reduced binding to low-affinity sites. The response was apparently not dependent on bepridil concentration.

Our study was designed to establish whether bepridil acts specifically on myocytes of ventricular origin. We have attempted to quantitate sarcolemmal binding sites for bepridil, and have established that bepridil penetrates the sarcolemmal membrane; a prerequisite for direct intracellular action of the drug. A possible intracellular site of action has been identified.

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MATERIALS AND METHODS

Bepridil and [^{3}H]bepridil (20.7 Ci/mmole) (synthesised by Amersham International, Amersham, U.K.) were provided by Organon Laboratories Ltd. 1-Bromodecane was obtained from Aldrich Chemical Co. (Gillingham, U.K.), and 1-bromododecane from Lancaster Synthesis (Lancaster, U.K.). Sephadex G-100 was from Pharmacia (Uppsala, Sweden). Medium 199 was from Wellcome Laboratories (Beckenham, U.K.), and bovine serum albumin from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical reagent grade.

Cardiac myocytes were isolated from Wistar rats weighing 230–250 g by methods previously described [9, 10]; 65–70% of the enriched myocytes were rod-shaped. Myocytes were maintained under aerobic conditions at 37° in either Medium 199 containing 0.5 mM $CaCl_2$ and supplemented with 2% w/v bovine serum albumin or in a modified Krebs–Henseleit bicarbonate buffer [10] containing 0.5 mM $CaCl_2$ and 2% w/v bovine serum albumin. For determination of bepridil incorporation, myocytes were separated from incubation medium by spinning them in a Beckmann Microfuge through 100 μl of a 1:4 (v/v) mixture of bromodecane:bromododecane, sp. g. 1.043, layered on top of 100 μl of 25% (w/v) sucrose. These conditions were developed to ensure that rod-shaped myocytes pass through the oil phase, while round damaged cells remain at the medium–oil interface. The tubes were then frozen in dry ice:methanol and cut at the sucrose–oil interface. The sucrose phase containing only rod-shaped myocytes was counted in toluene:triton:PPO scintillant. When myocytes are incubated with ^3H_2O and [^{14}C]methyl inulin as an extracellular marker, we find that after separation from the medium, less than 5% of the myocyte pellet volume is occupied by inulin.

Actin was extracted from acetone powder prepared from fresh beef ventricular muscle as described by Spudich and Watt [11], polymerised by the

addition of $MgCl_2$ to 0.7 mM and collected by centrifugation at 80,000 *g* for 3 hr. Polymerised actin ran as a single protein band on 10% SDS–polyacrylamide gels.

RESULTS AND DISCUSSION

Uptake of bepridil by isolated myocytes

Myocytes were incubated at 37° with bepridil at concentrations between 0.036 and 100 μM, including [^{3}H]bepridil at 0.2–7.5 μCi/ml. Myocyte suspensions contained between 0.3×10^5 and 1.2×10^5 rod-shaped myocytes/ml. Within this range uptake of bepridil was independent of cell number. Preliminary experiments established that at 37° uptake of bepridil was essentially complete within 10 min. To ensure that myocytes did not distinguish between [^{3}H]bepridil and unlabelled bepridil, two approaches were used. In the first, incubation medium contained a fixed amount of isotope, the concentration of drug being determined by the amount of unlabelled bepridil added. In the second, labelled and unlabelled bepridil were used in constant molar proportions at all concentrations. The two approaches produced data which were not distinguishable.

Fig. 1 illustrates the relationship between bepridil concentration and uptake by myocytes. The profile suggests that uptake of bepridil may saturate at concentrations greater than 1 mM, a concentration far in excess of therapeutic doses. Uptake is independent of medium composition. Presented as a Scatchard plot, the data shows a positive correlation between the ratio bound/free bepridil and amount of bepridil bound. Thus if the drug binds to specific sarcolemmal sites, this interaction is masked by uptake into myocytes. Assuming an intramyocyte fluid content of 25 pl for an average myocyte volume of 30,000 μm^3 [12], we calculate that the intracellular bepridil concentration would be 80 μM for myocytes incubated in 1 μM bepridil, and 1.2 mM for myocytes incubated in 10 μM bepridil if the drug remained free in the cell cytoplasm.

Temperature dependence of bepridil uptake

The rate of bepridil uptake by myocytes was measured at 37°, 29° and 18°. Whilst the rate is initially slightly slower at 18° than at higher temperatures, comparable intracellular concentrations are reached at all temperatures within 20 min.

Efflux of bepridil from myocytes

Release of bepridil from intramyocyte binding sites was investigated in cells loaded for 15 min in 10 μM bepridil containing [^{3}H]bepridil. Myocytes were rescued from this medium and transferred into fresh medium containing either only unlabelled bepridil, or containing no bepridil. Cells were sampled in the ensuing efflux period at intervals up to

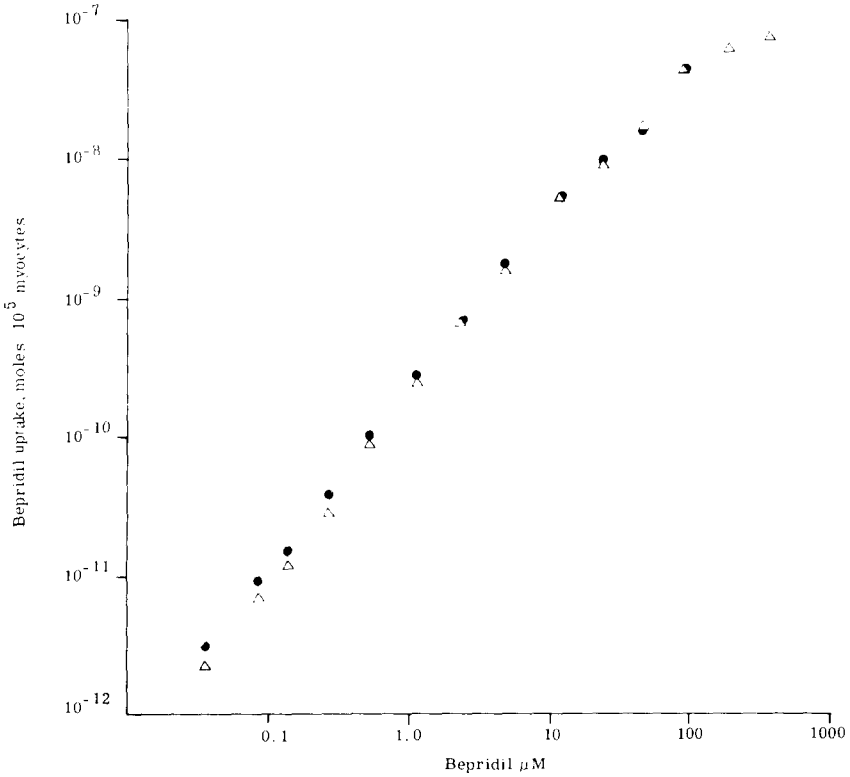

Fig. 1. Uptake of bepridil by isolated rat ventricular myocytes. Myocytes were incubated for 10 min with bepridil at the concentrations indicated. The symbols (●) and (△) indicate data from two separate myocyte preparations.

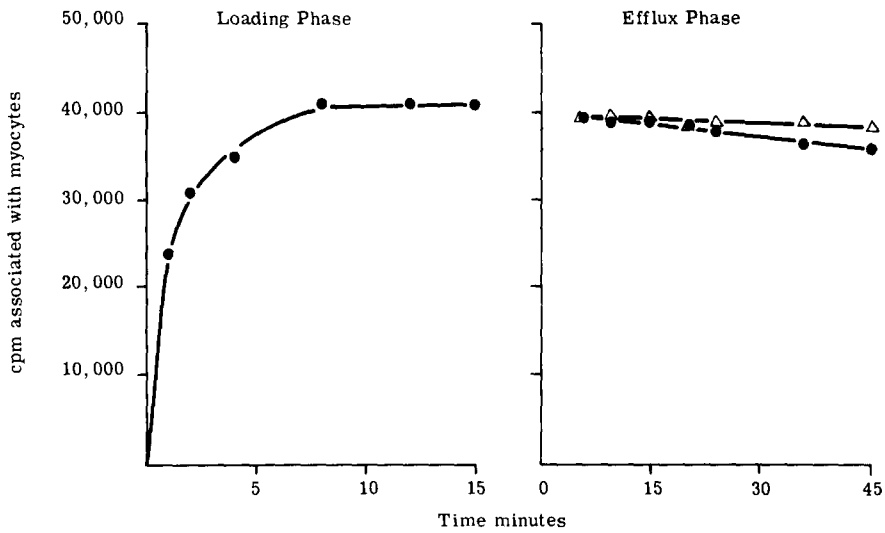

Fig. 2. Uptake and subsequent efflux of bepridil from myocytes. Myocytes were incubated with 10 μM bepridil for 15 min, collected and resuspended in fresh medium which was either (●) bepridil-free or (△) contained only unlabelled bepridil.

45 min. The data in Fig. 2 indicate that bepridil is tightly bound within myocytes, not being significantly displaced over this period either with or without bepridil in the extracellular medium.

Intracellular binding sites

Failure to elute bepridil from myocyte binding sites raises questions about the nature of intracellular site(s) which retain bepridil with sufficient avidity to prevent free exchange across the sarcolemma. Our experiments show that actin has a substantial binding capacity for bepridil. Polymerised actin was incubated with bepridil (0.3 μCi ^{3}H/μmole bepridil) at 4° for 30 min. Actin was collected by centrifugation at 80,000 *g* for 3 hr. The integrity of the binding of bepridil to actin was established by subsequently eluting actin-bound bepridil through a Sephadex G-100 column equilibrated with 0.7 mM

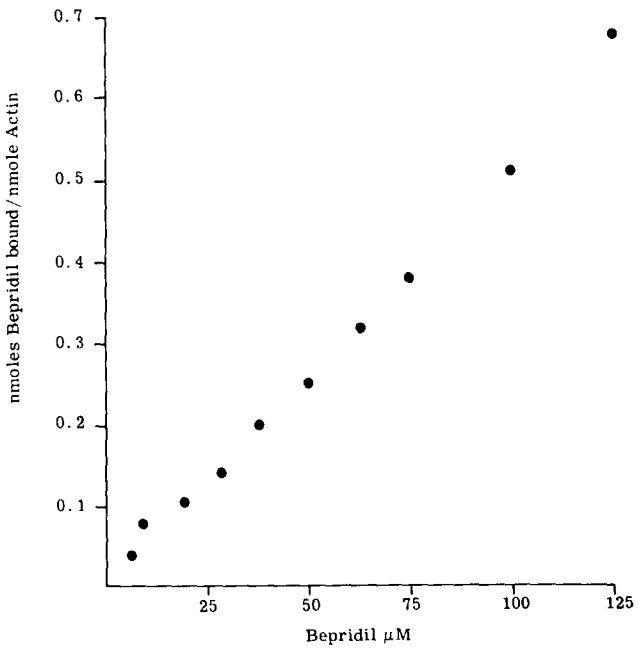

Fig. 3. Binding of bepridil to bovine cardiac actin. F-Actin, 4 mg in 4 ml, was incubated with bepridil at 4° for 30 min at the concentrations indicated. The actin was collected by centrifugation at 80,000 *g* for 3 hr. This experiment was repeated twice using different preparations of bovine cardiac actin. Results of the three experiments were not significantly different.

$MgCl_2$:20 mM Na phosphate (pH 7.5). In all cases more than 90% of the [^{3}H]bepridil was eluted coincident with actin.

Figure 3 illustrates the binding capacity of polymerised actin for bepridil at 4°. Similar data were obtained by incubating monomeric 'g' actin with bepridil and we found no evidence that bepridil influences actin polymerisation. The capacity of actin to bind bepridil was not saturated at 125 μM bepridil, but under these conditions the drug was not soluble at higher concentrations. As an alternative approach to saturate actin binding sites, the protein was incubated with 100 μM bepridil, collected by centrifugation, and resuspended in a fresh solution of bepridil. By successive exposures in this manner actin was progressively loaded to amounts greater than 2 nmoles bepridil/nmole actin, without evidence of saturation. Based upon the assumption that 10^5 myocytes have approximately 2.5 nmoles actin, a molar bepridil:actin binding ratio of 2:1 is sufficient to account for the bepridil uptake of myocytes incubated in 10 μM bepridil (Fig. 1). However it is not possible to establish the concentration to which free bepridil accumulates in cell cytoplasm. At higher extracellular concentrations bepridil may accumulate in both actin-bound and free pools.

Whilst this study does not establish a causal relationship between the binding of bepridil to myocyte actin and the pharmacological action of the drug, it does provide convincing evidence that rat ventricular myocytes take up substantial amounts of bepridil. Within myocytes it appears likely that bepridil binds to thin filaments. Present studies in this laboratory are designed to clarify at the molecular level whether this interaction influences contractile activity. The avidity with which purified actin binds bepridil is consistent with our failure to elute bepridil from myocytes. Despite the slight temperature dependence of bepridil uptake by myocytes, it seems unlikely that bepridil enters the cell by active transport. For, the apparent 80- to 100-fold concentration of bepridil within myocytes can be explained by the adsorption of bepridil to actin filaments within the myocyte. There is no reason to assume that bepridil accumulates to any significant free concentration in the cytoplasm.

The 'calcium antagonist' properties ascribed to bepridil could be at least partly explained if bepridil modifies myofibrillar functions or the activity of sarcolemmal or sarcoplasmic reticulum membranes.

Recent studies have shown an influence of bepridil on the handling of calcium by sarcoplasmic reticulum fragments from skeletal muscle [13]. Whether the activity of cardiac sarcoplasmic reticulum is similarly influenced remains to be established. A difficulty in approaching this question arises because the fibrous nature of myocardial tissue makes the balance between tissue disruption and preservation of membraneous tissue organelles difficult to achieve. For this reason both sarcoplasmic reticulum and sarcolemmal membrane preparations tend to be contaminated by lysosomal and by mitochondrial fragments of both outer- and inner-membrane origin. These difficulties may explain the wide variation in enrichment of sarcolemmal (Na^+–K^+)ATPase activity reported from different laboratories. Direct comparison of the data is complicated by variations in the amounts of activity reported for whole-tissue homogenates, but enrichments vary from 1.2-fold [14] to 14-fold [15] and 33-fold [16]. Sarcolemmal fragments prepared from single myocytes reportedly have an 82-fold enriched (Na^+–K^+)ATPase activity [17]. Adequate characterisation of a cardiac membrane preparation requires measurement of a broad range of activities known to be associated with the required membrane fraction, as well as those associated with membrane fragments likely to occur as contaminants of the preparation. Without characterisation in this detail questions remain about the precise nature of the reported influence of bepridil on membrane calcium-binding sites [7].

When isolated ventricular myocytes are incubated with bepridil at concentrations between 0.036 and 100 μM, the drug is concentrated approximately 100-fold within the cells. Binding is tight with little tendancy for bepridil to be released into bepridil-free medium. It has been demonstrated that most of the bepridil sequestered within cardiac myocytes is likely to be bound by actin filaments rather than remaining free in the cytoplasm. The data may indicate a direct influence of bepridil on contractile function.

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