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Blockade of the inotropic effect of Bay K 8644 by cytochalasin-B and phloretin

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1 The positive inotropic effect in rabbit atria and papillary muscles of Bay K 8644 is blocked by cytochalasin-B (Cyto-B) and phloretin, two compounds known to block the facilitated diffusion of glucose. These compounds do not change the concentration-response curve of calcium.

2 Cyto-B is more potent in atria than in papillary muscles, 10^{-7} M having a maximal effect in atria whereas 2×10^{-5} M was required for a maximal effect in papillary muscles. Phloretin was fully effective at 10^{-4} M, the only concentration tested.

3 The inotropic effect of Bay K 8644 was virtually abolished in atria bathed in a glucose-free medium or one containing 5 mM pyruvate. The contractile response to Bay K 8644 of papillary muscles was not changed significantly in glucose-free or in pyruvate-containing medium.

4 Cyto-B (2×10^{-5} M) caused a slight but significant increase in the K_D for the binding of nitrendipine to a crude sarcolemnal preparation from rabbit ventricles. The B_{max} was unchanged.

5 These results may best be explained by the hypothesis that there is a metabolic requirement for the inotropic effect of Bay K 8644.

Introduction

Bailey & Dresel (1971) showed that treatment of isolated atria with phloretin at a concentration that inhibited the sugar transport system, prevented the cardiac inotropic effect of ouabain. They also showed that glucose was the optimal substrate for an inotropic effect of this glycoside. We have recently confirmed and extended these results (Ogbaghebriel & Dresel, 1986; 1987). We showed that a more specific blocker of the sugar transport system, cytochalasin-B, also blocked the inotropic response to graded concentrations of ouabain and that the effects of an aglycone, acetylstrophanthidin, were also blocked by both drugs.

Both of the above reports contained limited tests for the specificity of this effect of the blocking agents, in that the inotropic effects of isoprenaline were stated to be unaffected. We wished to extend these results to inotropic drugs of a variety of mechanisms of action. We show here that the inotropic response to Bay K 8644, an agent that increases the 'open time' of cardiac calcium channels (Hess *et al.*, 1984; Kokubun & Reuter, 1984), is blocked by phloretin and cytochalasin-B.

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Methods

Rabbits (1.5–2.5 kg) of either sex were killed by a blow to the cervical spine and the hearts were quickly removed and placed in Krebs-Henseleit solution at 30°C. The left atrium was removed and split into two flat preparations. Two papillary muscles were removed from the right ventricle. All preparations were suspended in 15 ml baths. Resting tension of the atria was adjusted to 1 g, that of the papillary muscles to the tension yielding maximal contractile force (usually close to 1 g). The tissues were equilibrated for at least 1 h. Atria were driven with Grass SD5 stimulators at 2 Hz, the papillary muscles at 1 Hz, with 2 ms pulses at 1.2–1.5 times threshold. Contractions were recorded with Grass FT 03 transducers and a Grass P7 pen recorder.

The Krebs-Henseleit solution contained (mM): NaCl 118, NaH_2PO_4 1.175, $MgSO_4$ 1.18, KCl 4.0, $CaCl_2$ 1.8 and $NaHCO_3$ 26.2. Except where stated, the medium contained 11 mM glucose. It was bubbled with 5% CO_2 : 95% O_2 to yield a pH of 7.4 at a temperature of 30°C.

Cytochalasin-B (Sigma) and phloretin (Sigma) were dissolved in ethanol to make stock solutions which were kept in the refrigerator for no longer

than 2 weeks. Bay K 8644 was dissolved fresh daily in warm polyethylene glycol 400 to which 0.9% saline was added to make the final concentration of the solvent 40%; the solution was kept in the dark. The media had no observable effect on developed tension.

The binding of nitrendipine to microsomal preparations from rabbit hearts was studied with the method of Janis *et al.* (1984). Briefly, a homogenate was prepared by use of a Polytron (setting 7, 30 s, × 2). After preliminary centrifugations at 1000 *g* and 10,000 *g*, a microsomal pellet from a 100,000 *g* centrifugation was resuspended in a 50 mM Tris buffer at pH 7.4. Approximately 100 μg of protein was incubated in glass test tubes with [^{3}H]-nitrendipine (77.6 Ci mmol^{-1}) in 5 ml of 50 mM Tris buffer at 25°C for 90 min after which the microsomes were rapidly filtered (Whatman GF/B filters) and further washed with buffer. These were counted in 10 ml Biofluor scintillation cocktail in a Beckman scintillation counter. Non-specific binding was defined as that obtained in the presence of 1.0 μM non-radioactive nifedipine. The data were analyzed using the programme "EBDA" (Biomed. Comput. Technol. Info. Ctr, Vanderbilt Med. Ctr, Nashville, TE) on a Commodore PC/10 computer.

Statistical analysis of the results of binding studies were by Student's paired *t* test. Since the other results are given as percentage increases, we used nonparametric statistics as indicated in Results.

Results

Neither phloretin nor cytochalasin-B, at the highest concentrations used in these preparations, had any significant effect on contractile force or diastolic tension. Figure 1 shows the increase in contractile force produced by graded concentrations of Bay K 8644 in both atria and papillary muscles. When the tissues were pretreated with Cyto-B (20 μM) for 30 min before the dose-response curve was determined, the responses were greatly diminished (Figure 1). The potency of Cyto-B for blockade differed between the two tissues; the drug was maximally effective at 10^{-7} M in atria (Figure 1a) but five times that concentration was insufficient to change the response of the papillary muscles (Figure 1b). Phloretin was tested at only one concentration, 10^{-4} M and this concentration blocked completely the inotropic effect in atria (Figure 2). Identical results were obtained in papillary muscles. We did not attempt to determine whether atria and papillary muscles differed in sensitivity to blockade by phloretin.

We have shown that a full inotropic effect of digitalis is obtained only in a medium containing glucose (Bailey & Dresel, 1971; Ogbaghebriel &

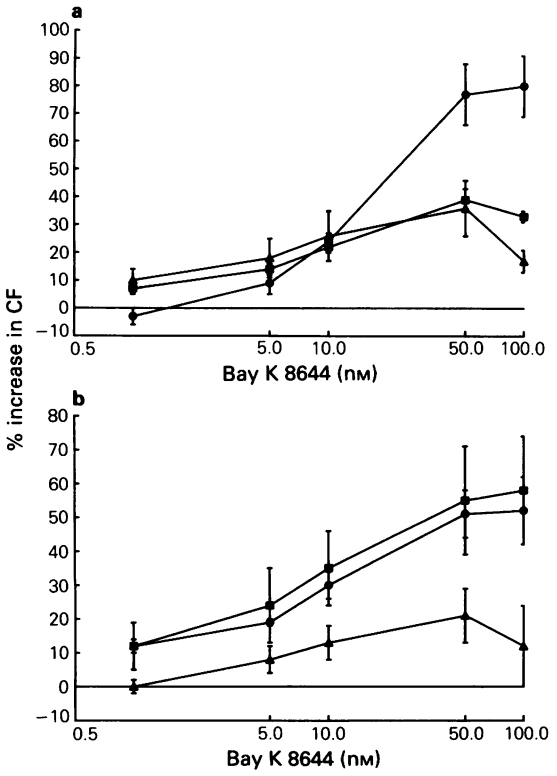

Figure 1 Effect of cytochalasin-B (Cyto-B) on the inotropic effect (CF) of Bay K 8644. (a) Left atria: (●) control (8); (■) Cyto-B 0.1 μM (5); (▲) Cyto-B 20 μM (5). (b) Papillary muscles: (●) control (7); (■) Cyto-B 0.5 μM (4); (▲) Cyto-B 20 μM (7). Means are shown with s.e. indicated by vertical lines. Numbers in parentheses are *n*. The initial strength of contraction of atria was 800 mg, that of the papillary muscles 600 mg.

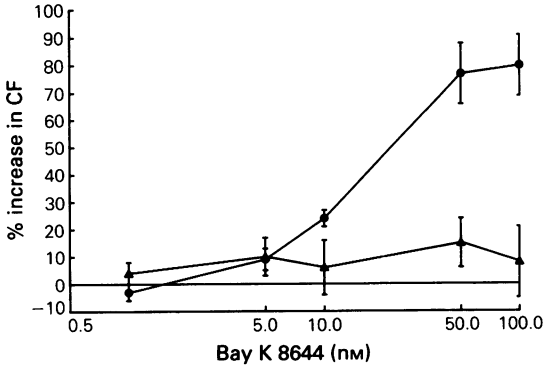

Figure 2 Effect of phloretin (10^{-4} M) on the inotropic effect (CF) of Bay K 8644 on atria: (●) control; (▲) in the presence of phloretin. $n = 8$

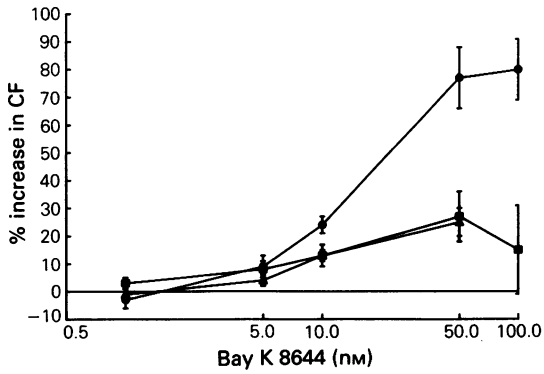

Figure 3 The effect of Bay K 8644 on atria in various media: (●) 11 mM glucose (8); (▲) zero glucose (7); (■) 5 mM Na pyruvate (8).

Dresel, 1987). Sodium pyruvate (5 mM) permitted a partial inotropic effect, but zero-glucose medium virtually abolished inotropy in atria. We therefore tested the effect of Bay K 8644 in media devoid of glucose or containing 5 mM sodium pyruvate. Figure 3 shows that the maximal effect of Bay K 8644 was only approximately 30% of control in rabbit atria in zero-glucose medium. A similar partial inotropic effect could be obtained when pyruvate medium was used. The effect of isoprenaline was unchanged in glucose-free medium (not shown).

An additional difference between atria and papillary muscles was observed in these experiments. Figure 4 shows the inotropic effect of Bay K 8644 on

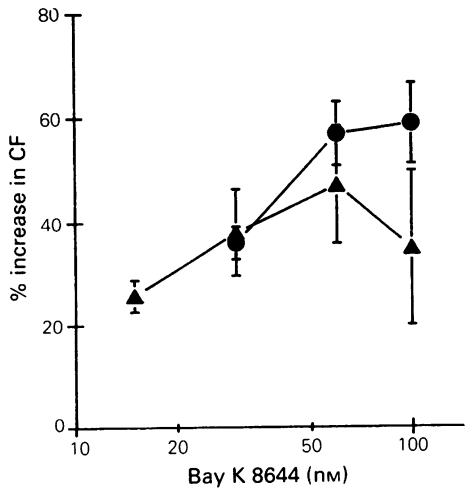

Figure 4 The effect of Bay K 8644 on papillary muscles in various media: (●) 11 mM glucose (7); (▲) zero glucose (6).

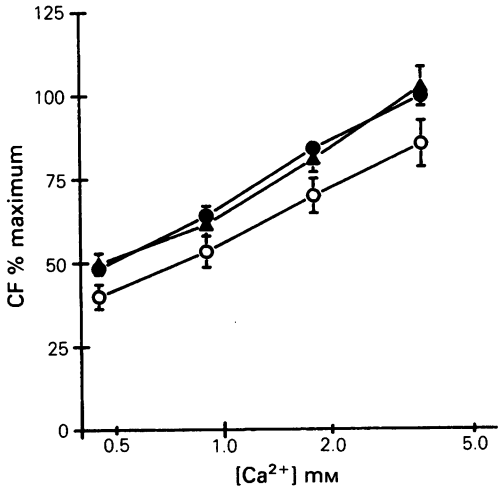

Figure 5 Lack of effect of cytochalasin-B (Cyto-B) and phloretin on the response of papillary muscles to calcium. Concentration-response curves are shown in the absence (●), and presence of cyto-B (▲); phloretin (○). $n = 9$.

papillary muscles in the absence of glucose. The results at the highest concentration were compared using the Mann-Whitney U-test and were found not to be significantly different. A full inotropic effect was also obtained in the presence of pyruvate (not shown).

The effects of Cyto-B (20 μM) and of phloretin (100 μM) on the inotropic responses to changes in the calcium concentration of the medium were tested in a separate series of experiments. Figure 5 shows that there was no difference between the responses of papillary muscles to calcium before and after 30 min treatment with Cyto-B. Since the effect of the highest concentration under control conditions was defined as 100%, we analyzed the results of phloretin only for the three lower concentrations by non-parametric analysis of variance, and found no significant difference. Entirely similar results were obtained in atria. The effect of Bay K 8644 was tested in these same preparations and the effect was the same as shown for the drug-treated groups in Figure 1.

We next tested the possibility that Cyto-B interfered with the binding of Bay K 8644 to the dihydropyridine binding site of the calcium channel. Only the highest concentration (20 μM) of the compound was tested in these experiments because that was the concentration at which blockade of the inotropic effect had been clearly determined in ventricular tissue and the broken cell preparation required for the binding studies was made from ventricular muscle. Paired data analysis indicates that there was

no significant change in the B_{max} (97 ± 12 control vs 110 ± 21 fmol mg^{-1} protein; $P > 0.05$) but that the change in the slope (i.e. the K_D) was statistically significant ($9.6 \pm 2 \times 10^{-11}$ M control vs 15.8 ± 10^{-11} M; $P < 0.05$).

Discussion

We have shown that Cyto-B and phloretin interfere with the positive inotropic effect of Bay K 8644 both in atria and in papillary muscles from rabbits. Further, we have demonstrated that the inotropic response to Bay K 8644 is greatly attenuated in atria, but not in papillary muscles, when glucose is omitted from the medium.

The immediate question arising from our results is whether they can be explained by a direct interaction of the drugs at the calcium channel. We have shown that Cyto-B causes an increase in the K_D for the binding of nitrendipine, a dihydropyridine which binds to the same site as Bay K 8644 (Sarmiento *et al.*, 1987). Were this causal to the blockade of the inotropic effect, one would expect a competitive inhibition of Bay K 8644 rather than the non-competitive inhibition observed. Further, were Cyto-B acting as a blocker of the calcium channel, one would expect a negative inotropic effect. This was not seen. Finally, we have shown here that neither phloretin nor Cyto-B significantly changed the concentration-response curve for calcium ion in the range of 0.45 to 3.6 mM.

This last observation is of special interest since Bay K 8644 acts specifically (Kokubun & Reuter, 1984; Hess *et al.*, 1984; 1986) to increase the 'open time' of the calcium channel. This action permits more calcium to enter the cell, and interference with channel function should be demonstrable by a decreased response to changes in calcium concentration. Intracellular modulation of the frequency of opening of calcium channels is by phosphorylation via a protein kinase known to require cyclic AMP for activity. We note that neither of these agents changes the positive inotropic effect of isoprenaline (Bailey & Dresel, 1971; Ogbaghebriel & Dresel, 1987), an observation which indicates that they do not interfere with this aspect of regulation of the calcium channel.

Cyto-B is known to have two major effects in intact cells. It combines with the sugar transporter protein and inhibits glucose transport at very low concentrations ($< 10^{-6}$ M) (Lin & Spudich, 1974). This appears to be a general action and certainly occurs at heart (Bihler *et al.*, 1985). At higher concentrations, it also changes a number of secretory processes by disorganizing the cytoarchitecture of many types of cells. This occurs because Cyto-B disrupts the structure and organization of microfilaments, especially those adjacent to the sarcolemma (Orci *et al.*, 1972; Howell & Tyhurst, 1982). The highest concentration used in the present experiments is in the higher of these ranges, but it should be emphasized that the effective concentration for blockade of Bay K 8644 in atria is well below that known to interfere with the cytoarchitecture. Further it is known that Cyto-B does not affect the actin filaments of the contractile apparatus (Croop & Holzer, 1975).

Phloretin and its glycoside phlorizin inhibit a large number of enzymes, including the sarcolemmal Na^+, K^+-ATPase (Robinson, 1969). However, phloretin in the concentration used in the present experiments has been shown to have considerable specificity for sugar transport in intact cardiac muscle (Bihler *et al.*, 1965).

The only known effects shared by these two compounds are thus their ability to inhibit the inotropic effects of Bay K 8644 described here and of digitalis (Ogbaghebriel & Dresel, 1987), and their action to block the transport of sugars. In view of the fact that the inotropic effects of digitalis and of Bay K 8644 are also decreased when glucose is omitted from the medium or when pyruvate is substituted for glucose (Figure 3 and Ogbaghebriel & Dresel, 1987), one must consider the possibility that glucose or one of the products of glycolysis are required for a full inotropic effect to develop.

Papillary muscles differed from atria in two respects: higher concentrations of Cyto-B were required to block the inotropic effect of Bay K 8644, and the absence of glucose from the medium did not significantly change the inotropic effect. These differences might be due to differences in the functions of the calcium channels, in the importance of the T-tubular system in the two kinds of tissues, or if one were to accept that glucose transport and subsequent glycolysis are directly connected to the inotropic effect of Bay K 8644, to differences in the levels of glycogen available for utilization. We have no measurements which bear on these possible explanations.

We suggest that the results can best be explained by a metabolic requirement for the inotropic action of Bay K 8644, perhaps for the primary effect of this drug to increase the open time of the calcium channel. We are testing this hypothesis at present by studying the effects of metabolic inhibitors.

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