Positive inotropic effect of porcine left ventricular extract on canine ventricular muscle

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1 We previously isolated an extract from porcine left ventricle that possessed digitalis-like properties such as inhibition of cardiac and kidney Na⁺, K⁺-ATPase, displacement of [³H]-ouabain from its binding sites and cross reactivity with digoxin antibodies. The extract also had a positive inotropic effect on the guinea-pig heart.

2 In the present study the positive inotropic response of the extract was characterized in canine right ventricular trabeculae. Maximum inotropic response (501 ± 20%) was produced by 300 µl and the half maximal increase occurred with 125 µl of the extract.

3 Ouabagenin produced aftercontractions in rapidly paced trabeculae. Equipotent and even greater amounts of the extract did not produce aftercontractions.

4 The extract increased the amplitude of the delayed component (P2) of biphasic contractions produced by replacing about 92–96% of the external Ca with Sr. A smaller increase in the size of the early component (P1) was also seen.

5 The extract decreased post-rest potentiation after rest for 30 s and 2 min. After 8 min of rest, post-rest potentiation was converted to post-rest depression.

6 The extract (20 µl) produced a decrease in the amplitude of the post-rest rapid cooling contracture (RCC) at all rest intervals. The steady state RCC, although greater than that in the control muscle, was increased to a lesser extent than the size of the steady state electrically driven contractions.

7 It is suggested that the extract from porcine left ventricle produces a positive inotropic response by increasing the trans-sarcolemmal influx of Ca. It also has additional effect(s) on the sarcoplasmic reticulum in that it may facilitate the loss of Ca from the sarcoplasmic reticulum and/or inhibit the uptake of Ca by the organelle.

Introduction

Isolation of a digitalis-like substance (DLS) from biological materials such as plasma, cardiac tissue, placenta etc. has been attempted by many laboratories (Gruber *et al.*, 1980; Kelly *et al.*, 1985; Balzan *et al.*, 1986; Ebara *et al.*, 1986). In our laboratory, we isolated a crude fraction from porcine left ventricle, after extraction of lipids, chromatographic separation and desalting (Khatter *et al.*, 1986). The extract had digitalis-like properties such as inhibition of cardiac and kidney Na⁺, K⁺-ATPase dose-dependently, displacement of [³H]-ouabain from its binding sites and cross reactivity with antibodies to digoxin (Khatter *et al.*, 1986; Agbanyo & Khatter 1990; Khatter *et al.*, 1990). We also investigated the effect of this extract on ⁸⁶Rb uptake, which is a representation of functional Na⁺ pump activity. The extract inhibited the uptake of ⁸⁶Rb in a dose-dependent fashion, indicating that it inhibits the Na pump.

Even though many laboratories have been able to demonstrate the presence of digitalis-like substance(s) either immunologically or biochemically, the functional characterization of positive inotropism was not done in these studies (Gruber *et al.*, 1980; Kelly *et al.*, 1985; Balzan *et al.*, 1986; Ebara *et al.*, 1986). The crude extract obtained in our laboratory from porcine ventricle was consistently inotropic in guinea-pig isolated hearts (Khatter *et al.*, 1986). Furthermore, in our previous studies we also excluded the possibility of catecholamines being responsible for the inotropic effect because: (a) it was not blocked by a β -adrenoceptor antagonist, (b) the extract did not compete for [³H]-(-)-dihydroalprenolol binding (Khatter *et al.*, 1986) and (c) in high performance liquid chromatography (h.p.l.c.) analysis it did not

have similar peaks as those of adrenaline, noradrenaline or dopamine (Agbanyo & Khatter, 1990). Essentially the extract obtained from porcine left ventricle had contractile, immunological and biochemical properties like those of digitalis. The present study was undertaken to characterize further the positive inotropic response of the extract on canine ventricular trabeculae, in order to investigate its possible mechanism of action.

Methods

Isolation of digitalis-like substance (DLS)-containing extract from porcine left ventricle

The detailed methodology for the isolation of the crude porcine ventricular extract has been described previously (Khatter *et al.*, 1986). A homogenate of the porcine left ventricle was obtained, lipids were extracted with methanol and diethyl ether and the fraction subjected to column chromatography (Sephadex G-25-80 fine bead) and desalting. The final extract from 150 g porcine left ventricle was neutralized to pH 7.4 with 1 M Tris and adjusted to a total volume of 5 ml.

Effect of DLS containing extract on canine right ventricular trabeculae

Experimental preparation Tissue preparation and tension measurements were carried out as described previously (Hryshko *et al.*, 1989a). Briefly, mongrel dogs of either sex (8–12 kg) were anaesthetized with sodium pentobarbitone (35 mg kg⁻¹, i.v.), hearts were rapidly excised and thin, free, running right ventricular trabeculae were dissected from the wall of the heart. Isolated muscle preparations were transferred to a 5 ml vertical bath containing oxygenated (95% O₂, 5% CO₂) Krebs Henseleit (KH) solution (in mM: NaCl 118,

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KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, KH_2PO_4 1.4, NaHCO_3 25 and glucose 11) maintained at a pH of 7.4 and at a temperature of 37°C . Muscles were field stimulated by pulses delivered from a computer-controlled stimulus sequencer (Boyeckko & Bose, 1982) with a frequency of 0.5 Hz and amplitude of 75–100% above threshold. Muscles were stretched to an optimal length for maximal isometric force production and isometric contractions were recorded with a force transducer (Grass FT-03C). Periods of rest ranging from 30–480 s were imposed on an otherwise constant stimulus pattern. Dose-response of the porcine extract was tested by addition of increasing amounts of the extract to the tissue bath in a cumulative manner.

Rapid cooling contracture (RCC) The apparatus employed to elicit rapid cooling contracture (RCC) has been described in detail elsewhere (Hryshko *et al.*, 1989b). Briefly, the muscle chamber consists of single bath and two reservoirs containing bathing medium at 37°C and -4°C connected in parallel to the bath. This allowed instantaneous (<1 s) changing of bath solutions. RCCs were elicited by cooling the preparation from 37° to 0 – 2°C by changing the solution. RCCs were obtained in place of a normal electrical twitch (steady state RCC) or after variable periods of rest (post-rest RCC).

Biphasic contraction Biphasic contraction in canine trabeculae was produced as described by King & Bose (1983). Trabeculae were equilibrated in KH solution by stimulating at 0.5 Hz for 1 hour. This was followed by replacement of solution with SrKH (identical composition to KH except that it contained 2.5 mM Sr^{2+} instead of CaCl_2), which produced a series of changes in the shape of contractions. After 10–20 min in SrKH, the two components designated as phase 1 (P1) and phase 2 (P2) had approximately equal tensions. The biphasic contraction was maintained by the addition of 0.1–0.2 mM Ca^{2+} to the external medium containing 2.5 mM Sr^{2+} .

Drugs

Ouabagenin and sotalol were obtained from Sigma Chemical Co. and solutions were made in distilled deionized water. All chemicals for physiological solutions were reagent grade.

Statistics

Statistical significance was determined with Student's *t* test. The level of significance chosen for all experiments was $P < 0.05$. Data have been expressed as mean $\pm$ s.e.mean.

Results

Positive inotropic effect of extract

The dose-response relationship of the positive inotropic effect of the extract was studied by cumulative addition. Figure 1 shows the typical fast (1a and b) and slow (1c and d) recording of tension development in canine trabeculae. Addition of $50\text{ }\mu\text{l}$ of the extract produced a small initial negative inotropic effect followed by a marked positive inotropic response within a few seconds (Figure 1c). The maximum positive inotropic response was seen 2–3 min after addition of the extract. The log dose-response plot is shown in Figure 2 which clearly illustrates a sigmoid dose-response relationship with the maximum increase in developed tension of $501 \pm 20\%$. The half maximal increase in developed tension (ED_{50}) was produced by $125\text{ }\mu\text{l}$ of the extract. Under similar experimental conditions, the dose-response relationship of ouabagenin was also studied. Maximum inotropic effect was produced by 10^{-6} M ouabagenin. Higher concentrations of ouabagenin caused aftercontractions, increase in diastolic tension and decrease in active tension. The positive inotropic effect of the extract is expressed as a percentage of the maximum response to ouabagenin in Figure 2b. A volume of $100\text{ }\mu\text{l}$ of the extract was approximately equipotent to 10^{-6} M ouabagenin and 300 – $350\text{ }\mu\text{l}$ extract produced 300% greater inotropy compared to that produced by a maximum concentration of ouabagenin. In addition to the increase in developed tension, the duration of contraction was also prolonged in the presence of the extract (Figure 1a). The prolongation of the total contraction duration was caused by the prolonged time to peak tension as well as by a slowing of relaxation. The prolongation of contraction duration was dose-dependent and the maximum prolongation of contraction occurred with a similar dose (200 – $350\text{ }\mu\text{l}$) to that producing near maximum increase in developed tension. The duration of contraction was unchanged in the presence of ouabagenin (Figure 1b).

Figure 1 Effect of extract from porcine left ventricle on tension of electrically driven trabeculae from canine right ventricle. (a) Tension development in (i) control and (ii) with $50\text{ }\mu\text{l}$ extract and (iii) $350\text{ }\mu\text{l}$ extract. (b) Tension development in (i) control and (ii) ouabagenin 10^{-6} M ; (c) and (d) show the time course of positive inotropy in response to the extract ($5\text{ }\mu\text{l}$) and ouabagenin (10^{-6} M) respectively at slow recording speed.

Figure 2 (a) Cumulative log dose-effect of the extract on the contractile force of canine trabeculae. (b) Cumulative log-dose response to the extract as a percentage of the maximum positive inotropic response to ouabagenin (10^{-6} M). Results are shown as mean of 5 experiments; s.e.mean indicated by vertical bars in (a).

Absence of digitalis-like toxicity

As demonstrated in Figure 2, the maximum inotropic response was produced by 300–350 μ l of the extract. Further increase in the amount of extract did not produce any significant change in either developed tension or resting tension. More importantly, there were no signs of toxicity e.g. oscillatory aftercontractions, particularly at faster rates of stimulation. Isolated trabeculae were treated with 350 μ l of extract (Figure 3a) or 5×10^{-6} M ouabagenin (Figure 3b) and driven at a faster rate (basic cycle length of 300 ms). The last driven contraction was followed by after-contraction of the muscles that were treated with ouabagenin, which is characteristic of

Figure 3 Canine right ventricular trabeculae were driven rapidly (basic cycle length 300 ms). Cessation of drive in the presence of (a) 350 μ l of extract and (b) 5×10^{-6} M ouabagenin. Arrow with dashed line depicts active tension developed prior to addition of the agent. Solid arrow shows aftercontraction in the presence of ouabagenin.

digitalis toxicity. However, the muscles that were treated with the extract did not manifest these aftercontractions.

Biphasic response

The biphasic contractions were produced by replacing 92–96% of calcium in the bathing solution with strontium. These conditions produced a prolonged contraction with two distinct phasic components, the early component P1 and the late component P2. Addition of 20–40 μ l of extract in the presence of 10^{-5} M sotalol (a β -adrenoceptor antagonist) produced a substantial increase in the amplitude of the latter component P2 and a smaller increase in P1 (Figure 4b). The pooled data from 5–6 experiments are illustrated in Figure 4a. It demonstrates that the extract produces significant increase in the amplitude of P1 and P2 but the effect on P2 is greater than that on P1.

Rest potentiation

Figure 5 shows the contractile response of canine isolated right ventricular trabeculae to 30 s, 2 and 8 min rest periods. The control muscle (Figure 5a) shows post-rest potentiation after all three rest intervals. Addition of DLS containing extract produced rest depression after 8 min rest and the amplitudes of post-rest contraction after 30 s and 2 min were smaller than that of control muscle.

Rapid cooling contracture (RCC)

The typical response of the isolated trabeculae to rapid cooling is illustrated in Figure 6. The control muscle shows a progressive decrease in the amplitude of the cooling contracture with increasing duration of rest prior to cooling (Figure 6a). In the presence of extract (200 μ l) (Figure 6b), there was a decrease in the amplitude of the post-rest RCC at all rest durations. The steady state RCC after the addition of the extract was greater than that in the control muscle. However, its size when expressed as a percentage of the preceding steady state contraction, was decreased compared to control steady state RCC similarly expressed. The pooled data from 4–5 experiments are depicted in Figure 7. It can be clearly seen that the amplitudes of rapid cooling contracture at steady state and after periods of rest were significantly smaller in the presence of the extract as compared to control muscle.

Figure 4 Effect of extract on biphasic contraction produced by substituting 92–96% external Ca by Sr. (a) Pooled data from 5 experiments showing the effect of 20 μ l of extract on the two components (P1 and P2) of the biphasic contraction. (b) Effect of extract on biphasic contraction showing the predominant effect on P1.

Figure 5 Typical response of canine ventricular trabeculae to varying periods of rest in an untreated muscle (a) and after the treatment with extract (b).

Discussion

The present study was undertaken in order to characterize the positive inotropic effect of DLS-containing extract from porcine left ventricle. In agreement with our previous data (Khatter *et al.*, 1986), the present results confirmed that the extract is as potent a positive inotropic agent in the dog as it is on guinea-pig heart. As described earlier, the extract is an inhibitor of the Na pump and displaces [^3H]-ouabain from its high affinity binding sites (Khatter *et al.*, 1986; Agbanyo & Khatter, 1990). Hence it is reasonable to hypothesize that the inotropic response may also be produced by the inhibition of Na^+ , K^+ -ATPase and subsequent effect on Na^+ - Ca^{2+} exchange and elevation of intracellular Ca^{2+} . In support of our hypothesis, the studies on biphasic contraction have indicated that the extract primarily increases the delayed slow component (P2), which is caused by divalent cation entry across sarcolemma (King & Bose, 1983). The extract also increased the amplitude of the early component (although to a smaller extent than P2). This indicates an increase in the release of activator cation from the sarcoplasmic reticulum. The latter effect may in fact be a secondary response to increased sarcolemmal Ca^{2+} influx. Cardiac glycosides which cause inotropy via an effect on Na^+ - Ca^{2+} exchange also increase P2 (King, 1982). However, unlike cardiac glycosides which either decrease or do not change the duration of contraction, the extract significantly prolonged it. This was due to the prolonged time to peak tension and half maximal relaxation. The duration of contraction is determined by the duration of sarcolemmal Ca^{2+} influx, sarcoplasmic reticular Ca^{2+} release as well as Ca^{2+} re-uptake (Langer, 1973). Hence if the

extract increases the influx of Ca^{2+} via voltage-gated calcium channels, it may prolong the duration of the action potential in a similar manner to a calcium channel agonist (January *et al.*, 1988) and thus may also prolong the contraction duration. Alternatively, the extract may increase the influx of Ca^{2+} in a manner similar to digitalis but may also have an additional effect on the sarcoplasmic reticulum that may be responsible for the prolongation of contraction.

Aftercontractions after rapid pacing of the heart are characteristic of digitalis toxicity (Ferrier & Moe, 1973) and are caused by the oscillatory release of Ca^{2+} during diastole from the Ca^{2+} overloaded sarcoplasmic reticulum (Tsien *et al.*, 1978). The absence of digitalis-like toxicity even with the maximum dose of DLS-containing extract would indicate that either the increased trans-sarcolemmal influx of Ca^{2+} by a digitalis-like mechanism is matched by a concomitant increase in efflux, or the extract has an additional effect which protects the cardiac tissue from calcium overload. In any event, the absence of Ca^{2+} overload type toxicity would suggest that the extract may have better inotropic characteristics than digitalis.

In the present study we also investigated the effect of the extract on post-rest potentiation and rapid cooling contraction, which provide an estimate of the functional capacity of sarcoplasmic reticulum to store and release Ca^{2+} (Hryshko *et al.*, 1989a,b). In the presence of extract, there was a reduction in the extent of post-rest potentiation after 30 s and 2 min rest and a conversion of post-rest potentiation to post-rest depression after rest for 8 min. Much experimental evidence suggests that post-rest potentiation is due to the redistribution of calcium within the sarcoplasmic reticulum during diastole (Bers, 1985; Bridge, 1986; Bers *et al.*, 1987; Bose *et al.*, 1988)

Figure 6 Typical response of canine trabeculae to rapid cooling from 37°C to 0°C in less than a second. The rapid cooling contractions were obtained in place of a regularly driven beat or after 2 and 8 min of rest period. The arrows under the individual recordings represent the time of initiation of cooling. (a) Represents the control tracing and (b) tracing after administration of extract from porcine left ventricle.

Figure 7 Pooled data from 4 experiments demonstrating the effect of extract on rapid cooling contracture during steady state and after 2 and 8 min of rest. *Significantly different from appropriate control value ($P < 0.05$).

and the progressive reduction in the post-rest contraction amplitude with increasing duration of rest is due to the continuous but slow loss of calcium from the sarcoplasmic reticulum during diastole (Wohlfart & Noble 1982; Bose *et al.*, 1988). The present observation of enhancement of the latter

process by the extract, in spite of its potent positive inotropic response, indicates that it may also facilitate the loss of Ca^{2+} from sarcoplasmic reticulum. This was further supported by the observation with rapid cooling contracture. In the presence of the extract there was a decrease in rapid cooling contracture after all rest intervals, again indicating that the extract facilitated the loss of Ca^{2+} from sarcoplasmic reticulum. In addition, the amplitude of the steady state RCC immediately following a train of regular stimuli was a smaller percent of the preceding steady state contraction compared to control steady state RCC. This would mean that the extract may also impair the uptake of Ca^{2+} by the sarcoplasmic reticulum resulting in a relatively lower content of sarcoplasmic reticular Ca^{2+} (Bouchard *et al.*, 1989). This is in contrast to the effect of the Ca channel agonist, Bay K 8644 which increases the amplitude of steady state RCC even though it, like the extract, decreases the size of the RCC after various durations of rest.

In summary, the present study indicates that the DLS-containing extract from porcine left ventricle produces a positive inotropic response by increasing the influx of sarcolemmal Ca^{2+} . It also has additional effects on the sarcoplasmic reticulum which may facilitate the loss and/or inhibit the uptake of Ca^{2+} and which may also be responsible for the prolonged contraction duration. Electrophysiological studies to define the effect of the extract on transmembrane action potential and on whole cell ionic current(s), however, are necessary to elucidate further its mechanism of action.

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