Br. J. Pharmac (1983), **78**, 151–158

Seasonal variations in drug response and staircase phenomena in atrial muscle from a hibernating rodent (*Spermophilus richardsonii*)

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1 Electrically driven atrial muscle from a hibernating rodent (*Spermophilus richardsonii*) showed marked seasonal variations in inotropic responsiveness to both the cardiac glycoside actodigin and to raised extracellular calcium concentration. In contrast, glycoside toxicity was apparent and comparable in all tissues.

2 These variations were accompanied by changes in force-frequency ('staircase') characteristics of the tissues.

3 In contrast, no seasonal variation was observed in either positive inotropic responses to rubidium (a non-glycoside inhibitor of $Na^+ + K^+$ ATP-ase) or negative inotropic responses to methacholine.

4 In summer atria the calcium antagonist, verapamil (6×10^{-7} M) abolished the positive staircase phenomenon but did not modify the positive inotropic actions of actodigin.

5 These data do not support the hypothesis that 'staircase' and glycoside-induced inotropism are mediated through a common mechanism.

Introduction

Previous studies from this laboratory (Charnock, Dryden, Skoog & Lauzon, 1980a; Charnock, Dryden, Marshall & Lauzon, 1980b) have demonstrated that during hibernation, atrial and ventricular muscle from a normally glycoside-sensitive species, the Richardson ground-squirrel (*Spermophilus richardsonii*) respond weakly to ouabain and actodigin. Parallel biochemical studies (Charnock, Simonson & Dryden, 1980c) have shown that this loss in glycoside-sensitivity during hibernation is accompanied by a 40% reduction in myocardial $(Na^+ + K^+)$-ATP-ase activity and by a corresponding decrease in the amount of labelled ouabain binding. However, since the β-adrenoceptor-mediated positive inotropic responses to noradrenaline are also depressed during the hibernation phase (Charnock *et al.*, 1980a), the possibility remains that hibernation induces some non-specific change in membrane structure or the availability of Ca^{2+} ions which affects the fundamental excitation-contraction coupling properties of ground-squirrel myocardium.

The aim of the present study was to re-examine the physiological and pharmacological properties of ground-squirrel cardiac muscle throughout both the hibernation and active phases with a view to throwing some light on the mechanisms underlying the seasonal variation in positive inotropic responses to cardiac glycosides in this species. Atrial preparations were used since this tissue exhibits the more marked variations in glycoside responses between hibernation and activity (Charnock *et al.*, 1980a, b).

It was also of interest to know whether other drug-receptor systems (e.g. muscarinic cholinoceptors) in the heart were depressed during hibernation and whether responses to non-glycoside inhibitors of $(Na^+ + K^+)$-ATP-ase (e.g. rubidium ions) showed seasonal variation in this species.

Methods

Tissue preparation

The details concerning the trapping of the Richardson ground squirrels, their subsequent maintenance in the laboratory and the monitoring of the hibernation state by the 'sawdust' method of Lyman (1948)

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0007-1188/83/010151-08-$01.00

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have all been described in full in previous publications from this laboratory (Charnock & Simonson, 1978; Charnock *et al.*, 1980a, c).

Animals of either sex trapped during the previous May and June were introduced to a darkened cold (4°C) room once preparations for hibernation (e.g. nesting behaviour) were observed in the animals' behaviour (usually September/October). Animals were assigned to one of 5 groups and a sixth group comprising freshly trapped animals was also included in the experimental design. Animals in Groups I, II, III and V were kept in the dark at 4°C until they were killed.

Group I – (Hibernation, $n = 5$): These animals (250–445 g) were killed in February during a cyclical period of hibernation. The inclusion criterion was that any animal was at least 5 days into a hibernation/arousal cycle. Under light ether anaesthesia, these animals had a heart rate of < 1 beat/min and a mean rectal temperature of 6 ± 1°C.

Group II – (Immediate post-hibernation, $n = 5$): These animals (270–390 g) were killed (March) 6–12 days after final arousal from hibernation. Under anaesthesia these animals exhibited heart rates between 200–300 beats/min and rectal temperature of 35 ± 1°C.

Group III – (Post hibernators, $n = 6$): This group comprised animals (310–529 g) killed (April) 73–107 days after arousal from the final hibernation phase. Under anaesthesia, these animals had heart rates of 250–300 beats/min and mean rectal temperature of 37 ± 2°C.

Group IV – (Active, $n = 8$): This group comprised animals (495–615 g) removed from cold room in April and kept at room temperature. They were killed during June when activity is at a maximum. Under anaesthesia these animals had heart rates between 250–350 beats/min and mean rectal temperature of 37 ± 1°C. This group had therefore been kept in captivity for 10 months.

Group V – (Cold adapted active, $n = 5$): This group was kept in the dark at 4°C throughout the study (Feb–June) and was killed at the same time as Group IV. This group had heart rates of between 200–300 beats/min and a mean rectal temperature of 35 ± 2°C. Daily inspection showed that physical activity and food consumption were not obviously different from animals in Group IV.

Group VI – (Fresh active, $n = 6$): These animals were trapped in June and killed after a stabilization period of 3–4 weeks. They weighed between 230–360 g. This group exhibited heart rates of between 230–290 beats/min and a mean rectal temperature of 36 ± 2°C.

All animals were killed by decapitation after light ether anaesthesia, the hearts were rapidly excised and placed in cold Krebs-Henseleit solution equilibrated with 95% O_2 and 5% CO_2. The composition of the medium was (mmol/litre): NaCl 143, KCl 3.7, $NaHCO_3$ 24.9, KH_2PO_4 2.2, $MgSO_4$ 1.2, $CaCl_2$ 3.6 and glucose 10.0. Left atria were dissected free of other tissue, were mounted on punctate platinum electrodes and suspended in 4 ml tissue baths containing Krebs-Henseleit solution maintained at 34.0 ± 0.5°C by means of circulating warm water. Mounted tissues were allowed to equilibrate for at least 30 min before the threshold voltage was determined using square-wave pulses of 5 ms duration at 1.0 Hz, delivered from a Grass SDA stimulator. A stimulation voltage of twice threshold (0.4–2.0 V) was used and the isometric tension developed was measured with Grass force-displacement transducers (FTO3B) coupled to a multichannel Grass polygraph (model 5 DWCB). The atrial preparations were set up under an initial resting tension of 500–600 mg which resulted in 80–90% maximal contractions at that frequency. Twitch tension was monitored continuously. The recorder was run at a high paper speed (100 mm/s) before and during drug responses to enable the accurate determination of time to peak tension.

Force-frequency curves

After further stabilization for 30 min at a frequency of 1.0 Hz the frequency of stimulation was changed to 0.017 Hz (1 pulse per min) and subsequent twitches monitored. When twitch heights were again stable, the frequency of stimulation was increased stepwise (0.034, 0.1, 0.2, 0.5, 1.0, 2.0) to 4.0 Hz. Care was taken to ensure that twitch heights had stabilized at any given frequency. The tension developed at any frequency was expressed as a percentage of that developed at 0.017 Hz (the rested state contraction). In some animals in Group VI the effects of verapamil were studied on the contractile response produced by changing the frequency of stimulation from 0.5 to 2.0 Hz for 5 min.

Drug administration

Cumulative dose-response curves were obtained to acetyl-β-methyl choline (Sigma), rubidium hydrochloride (Sigma) and calcium chloride (B.D.H.). Dose-response curves to actodigin (Ayerst) were constructed from single administrations of this drug to the bath for 30 min, using a 45 min washout period between doses. Verapamil (Knoll A.G.) was administered cumulatively at intervals of 20–30 min depending on the time taken for developed tension to attain a new steady state. All drugs were dissolved in distilled water and were administered to the bath in volumes < 0.3 ml. Twice this volume of distilled water (0.6 ml) alone exerted no effects on the atrial

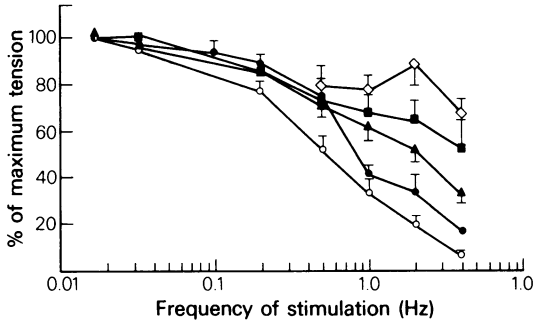

Figure 1 Force-frequency relationship in ground squirrel electrically driven atria. (○)–Group I, (●)–Group II, (▲)–Group III, (■)–Group IV and (◇)–Group V. Each point is mean of at least 5 values and the standard errors are shown by the vertical bars.

preparations. EC_{50} values (concentrations producing 50% maximal responses) were calculated from individual concentration-response curves using computerised regression analysis.

Results

Force-frequency curves

There were significant differences between the magnitudes of the 'rested state contraction' in the hibernation and immediately post-hibernation groups compared wih the summer animals. Tissues in groups I–III developed significantly greater tension (Table 1). In addition, marked differences in the force-frequency relationship became apparent as the animals recovered from hibernation (Figure 1). During and shortly following hibernation, ground-squirrel atria showed a clear-cut negative staircase (i.e. decreased tension with increasing frequency) which changed progressively throughout the year so that tissues taken from fully active animals in the summer exhibited the classical biphasic force-frequency relationship seen in most other mammals (Koch-Weser & Binks, 1963). In these latter tissues, increasing the frequency of stimulation up to 0.5 Hz caused a decrease in developed tension but at higher frequencies, steady state developed tension was maintained and even increased. This is well illustrated in Figure 2 which shows typical traces of a force-frequency experiment in a hibernator (Group I) and in a fully active summer animal (Group IV). Tissues taken from cold-adapted animals (Group V) and from freshly-caught animals (Group VI) exhibited identical force-frequency response curves to those obtained with Group IV in that increasing frequency first caused a decrease in steady state twitch height which was followed (at frequencies > 0.5 Hz) by an increase.

Table 1 A comparison of the rested state contraction size and times to peak tension in ground squirrel atria

Group	*Rested state contraction* (mg tension/mg wet weight)	*Time to peak tension* (ms)
I	19.3±2.1	56±2
II	18.0±2.1	63±6
III	19.7±4.3	51±1
IV	9.2±3.0	49±1
V	8.5±0.8*	Not tested
VI	10.8±2.0*	Not tested

*$P < 0.05$ compared to Group I values.

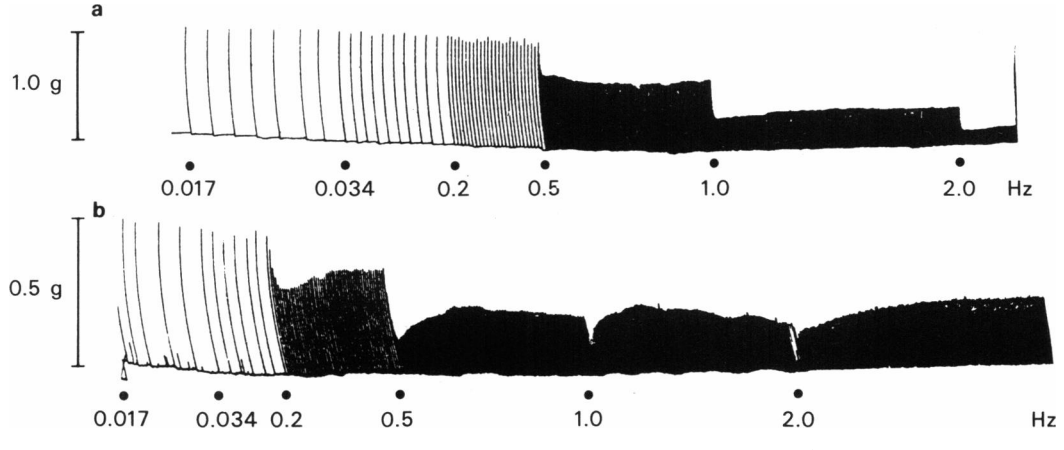

Figure 2 Typical force-frequency traces obtained from atria taken from a hibernator (Group I) (a) and an active summer ground squirrel (Group IV) (b).

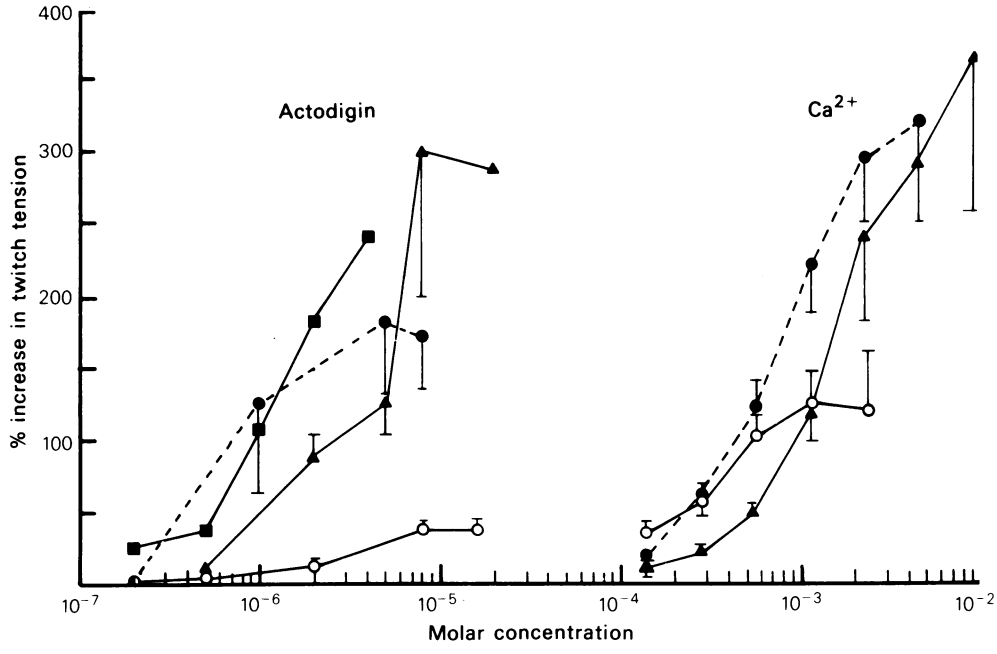

Figure 3 Concentration-response curves for the positive inotropic effects of actodigin and Ca^{2+} in atria taken from ground squirrels throughout the year. (○)–Group I, (●)–Group II, (▲)–Group III and (■)–Group IV. Each point is the mean of 5–9 determinations and the standard errors are shown by the vertical bars.

Responses to actodigin and to increasing Ca^{2+} concentration

As previously reported (Charnock *et al.*, 1980b), atrial tissue from summer ground squirrels (Group IV) was extremely sensitive to the positive inotropic actions of 10^{-7}–10^{-6} M actodigin (Figure 3). In contrast, even high concentrations of actodigin (1.6×10^{-5} M) caused only a slight increase in developed tension of tissue from hibernators. There was evidence that the positive inotropic effects of increasing Ca^{2+} concentration were also significantly reduced in hibernating animals although this reduction was not so marked as that seen with the glycoside (Figure 3). A clearer picture of the gradual changes in the magnitude of the responses to actodigin can be obtained in Figure 4 where the responses to actodigin are plotted as a percentage of the maximal response to Ca^{2+}. Tissues taken from animals in Groups V and VI were also sensitive (100% max. response) to the effects of actodigin and the calculated mean EC_{50}s (4×10^{-7} M and 6×10^{-7} M respectively) were not significantly different from that calculated for Group IV (6×10^{-7} M). In spite of the marked differences in the positive inotropic activity of actodigin in some of the groups, toxic symptoms (manifested as a steady increase in baseline tension) were observed in tissues of all groups at the same range of concentration (Table 2).

Positive inotropic effects of rubidium

In contrast to the effects of actodigin, administration of rubidium chloride (1–20 mM) resulted in immediate increases in developed tension in atria taken from both hibernating as well as non-hibernating animals (Figure 5). The maximal positive inotropic

Table 2 Actodigin concentrations (M) causing toxicity in ground squirrel atria

Group I	*Group II*	*Group III*	*Group IV*
2×10^{-6}	8×10^{-6}	2×10^{-5}	8×10^{-6}
5×10^{-7}	5×10^{-6}	1.4×10^{-5}	4×10^{-6}
8×10^{-6}	5×10^{-6}	1.4×10^{-5}	4×10^{-6}
1.6×10^{-5}	5×10^{-6}	2×10^{-5}	4×10^{-6}
1.6×10^{-5}	5×10^{-6}		2×10^{-6}
1.6×10^{-5}			2×10^{-6}

†Toxicity to actodigin was manifest in 20 out of 21 atria tested as a steady increase in baseline tension (i.e. partial contracture). Occasionally this was preceded by bursts of irregular firing. Development of contracture always occurred after maximum increase in developed tension.

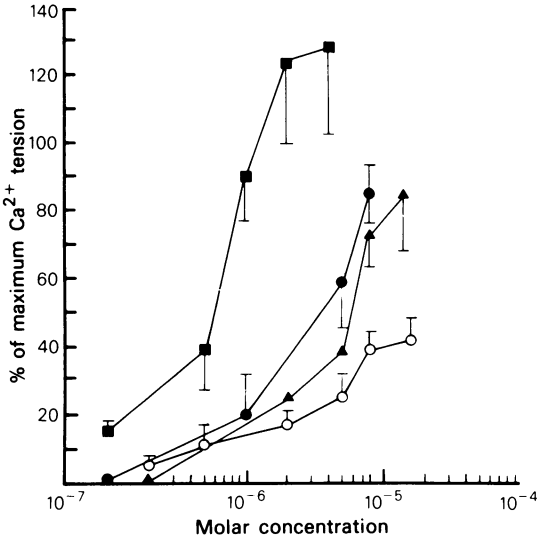

Figure 4 Concentration-response curves for the positive inotropic effects of actodigin, expressed as % of maximum response produced by increasing the Ca^{2+} concentrations. (○)–Group I, (●)–Group II, (▲)–Group III and (■)–Group IV.

effect produced by rubidium was only about 40–60% of that produced by increasing Ca^{2+} concentration and higher concentrations of rubidium rendered the tissues temporarily inexcitable to electrical stimulation.

The effects of acetyl β-methyl choline

Cumulative administration of acetyl β-methyl choline (0.01–3.0 μg/ml) caused dose-dependent decreases in developed tension which could be antagonized by the prior administration of atropine (2 μM). Tissues from hibernators (Group I) from immediate post-hibernators (Group II) and from non-hibernating summer animals (Group IV) were equally sensitive to the negative inotropic actions of this muscarinic cholinoceptor agonist (Figure 6).

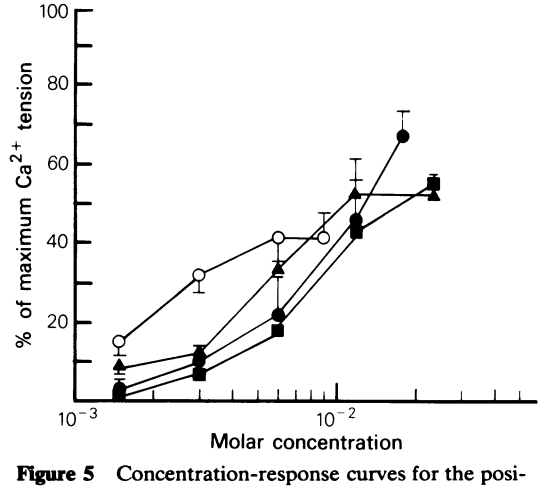

Figure 5 Concentration-response curves for the positive inotropic effects of rubidium ions in ground squirrel electrically driven atria. Symbols as for Figure 3.

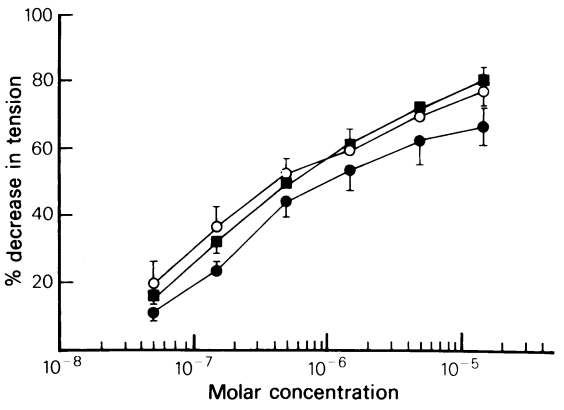

Figure 6 Concentration-response curves for the negative inotropic effects of acetyl-β-methyl choline in ground squirrel electrically-driven atria. (○)–Group I, (●)–Group II and (■)–Group IV.

The effects of verapamil

Verapamil (10^{-7}–10^{-4} M) caused sustained negative inotropic effects in every tissue studied. The calculated EC_{50} values are shown in Table 3 and demonstrate that there were no significant differences between the groups as regards their sensitivity to this calcium antagonist drug. However in atria taken from hibernating animals, the negative inotropic effects of low concentrations of verapamil (<2 μM) were always preceded by a transient positive inotropic response (4–18%) which was also seen in 66% of tissues in Group II (immediately post-hibernation) but never seen in tissues in other Groups (Figure 7).

In tissues of Groups IV and VI which showed a secondary positive staircase at frequencies greater

Table 3 A comparison of the sensitivity (EC_{50}) of ground squirrel atrial muscle to the negative inotropic effects of verapamil when tested during, immediately after and 6 months after hibernation

Group	n	*EC_{50} value* (μM)
Group I	5	6±3
Group II	5	2±3
Group III	6	2±2
Group IV	8	1±2
Group V	3	5±2
Group VI	6	7±3

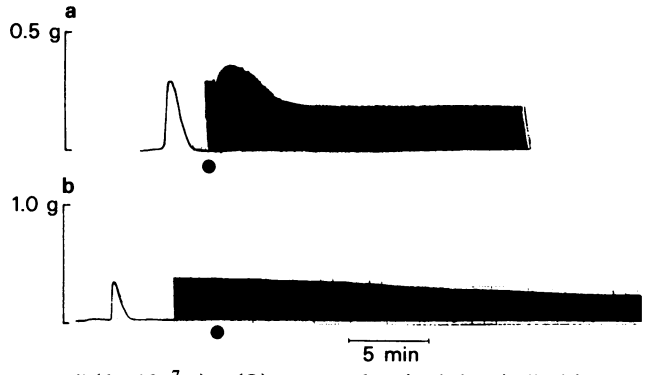

Figure 7 Effects of verapamil (6×10^{-7} M) at (●) on ground squirrel electrically driven atrial preparations from a hibernator (a) and from a fully active summer animal (b). The initial positive inotropic action of verapamil was seen in all tissues from hibernators (Group I), in 4/6 tissues immediately post-hibernation (Group II) but was never seen in tissues taken from any other groups.

than 0.5 Hz (Figure 1), a low concentration of verapamil (6×10^{-7} M) reversed this portion of the force-frequency curve to a negative staircase (Table 4) so that the force-frequency curve now resembled that obtained in hibernating animals. However, this concentration of verapamil did not reduce the positive inotropic effects of a submaximal concentration (1 μM) of actodigin (Table 4).

Discussion

Previous studies in this laboratory have demonstrated large seasonal variations in the positive inotropic response to the cardiac glycosides, ouabain and actodigin in isolated atrial and papillary muscle from hibernating and non-hibernating ground squirrels (Charnock *et al.*, 1980a; 1980b). Associated biochemical evidence suggested that there were accompanying changes in both ($Na^+ + K^+$)-ATP-ase activity and in ouabain binding (Charnock *et al.*, 1980c), although this was insufficient to account wholly for the loss in sensitivity to the inotropic actions of glycosides noted in tissues from hibernating animals. Thus an additional explanation is required. The present studies have confirmed that between the summer active state and hibernation of Richardon's ground squirrel, there is a marked difference in the positive inotropic response to the semi-synthetic fast-acting glycoside, actodigin, which is independent of the ambient temperature of the animals. The constancy of the atrial response to methacholine at all seasons, however, would suggest that fluctuation in the response to drugs is not a general phenomenon in these animals, and that only positive inotropic responses are affected.

Table 4 The influence of verapamil (0.6 μM) on positive inotropic responses induced by actodigin (1 μM) and by increasing the frequency of stimulation (0.5–2.0 Hz) in electrically-driven atria from summer ground squirrels ($n = 8$)

Pre-Verapamil		*Post-Verapamil*	
0.5–2.0 Hz	*Actodigin*	*0.5–2.0 Hz*	*Actodigin*
+16 ± 7	+77 ± 22	−34 ± 5*	106 ± 25 NS

Responses are expressed as % increase over control twitch height. This concentration of verapamil decreased twitches by 39 ± 3%.
*Significantly different from pre-verapamil response $P < 0.01$. NS Not significant.

Central to the consideration of inotropy is the role of calcium, in particular the calcium fluxes necessary to raise the level of contractile response during muscle activation. Current evidence suggests the existence of two important mechanisms: (1) an electrically neutral coupled exchange of calcium for sodium (Langer, 1980) and (2) a calcium influx occurring during and contributing to membrane depolarization (Reuter, 1973). The first of these mechanisms has been postulated to explain inotropy both of cardiac glycosides (Blesa, Langer, Brady & Serena, 1970) and of increasing frequency of stimulation (Langer, 1971) as a consequence of raised intracellular sodium levels. An alternative explanation for the positive staircase response is that it is directly related to depolarization-dependent calcium influx (Braveny & Subera, 1970; Kaufmann, Antoni, Hennekes, Jacob, Kohlhardt & Lab, 1971) during the slow inward current phase of the cardiac action potential. In either case, the ultimate source of calcium used in the inotropic response is the extracellular pool.

Our experiments conducted to determine the sensitivity of tissues from the various groups to extracellular calcium concentration revealed that here too

marked seasonal variation occurred with little change in contractile response in hibernator atria between 6×10^{-4} M and 3×10^{-3} M, whereas tissues from other groups showed marked sensitivity to external calcium concentration over this range. When actodigin concentration-response curves are corrected for the 'calcium-sensitivity' i.e. normalised in relation to the ability of the tissue to respond to external calcium (Figure 4) the reduction in the maximal positive inotropic effect ($\sim 50\%$) seen in hibernator atria corresponds well with the reported 40% decrease in ouabain binding capacity (Charnock *et al.*, 1980c). In contrast, concentration-effect curves for rubidium, when similarly assessed, were not significantly different between the various groups. Although rubidium inhibits cardiac $(Na^+ + K^+ +)$-ATP-ase by promoting premature dephosphorylation of the enzyme (Tobin, Akera, Han & Brady, 1974) and there is a good correlation between such ATP-ase inhibition and positive inotropic action in both guinea-pig and rat myocardium, the latter of which is insensitive to glycosides (Ku, Akera, Tobin & Brady, 1976), the mechanism of intropy seems to be different from that of glycosides although inhibition of $(Na^+ + K^+)$-ATP-ase seems to be common to both agents.

The force-frequency studies provided further evidence of profound seasonal variation in the physiology of ground squirrel atria. The classical relationship seen in atria from species other than the rat (see Koch-Weser & Blinks, 1963) was noted only during the summer months. At other times the response was similar to that noted for rat atria (Henderson, Brutsaert, Parmley & Sonnenblock, 1969; Landmark, 1972), i.e. a negative staircase, with the steepest slope occurring during hibernation. The positive staircase seen in summer tissues was converted to a negative staircase by the calcium antagonist drug, verapamil (6×10^{-7} M). There is controversy over the detailed explanation for both the positive and negative staircase phenomena, although there is general agreement that they relate to the ability of the cardiac cell to mobilize and sequester calcium from and into various pools during the contraction cycle. The sensitivity of the positive staircase to verapamil and also to external calcium concentration (Teiger & Farah, 1968) suggests that it is dependent on a transmembranal calcium flux, at a time when the tissues are sensitive to external calcium concentrations. If, indeed, as is generally believed, this effect of verapamil is being exerted at the sarcolemma (Fleckenstein 1971), then this calcium permeation of the cell membrane could well be missing in tissues from the hibernating animals. However, it is unlikely that this verapamil-sensitive calcium current is directly associated with the inotropic effects of cardiac glycosides despite the temporal correlation. As has been shown previously in rabbit hearts (McCans, Lindenmayer, Munson, Evans & Schwartz, 1974) it was possible to dissociate the two phenomena and in summer tissues produce an unchanged response to actodigin afer verapamil conversion of the staircase phenomenon.

The 'negative staircase' characteristics of rat cardiac muscle have been proposed as a major explanation for the relative insensitivity of this species to cardiac glycosides (Blesa *et al.*, 1970). Langer (1971) took this idea further by proposing that the step in the excitation-contraction coupling mechanism responsible for the positive staircase phenomenon is also the site of action of cardiac glycosides. The present studies would negate this proposal in its direct form, and suggest any link between the two effects to be more tenuous. Verapamil, indeed, depressed contractions to the same extent in both hibernating and fully active animals suggesting that in hibernation there is no depletion of verapamil-sensitive superficial calcium binding sites (Nayler, 1973). Indeed the verapamil-induced initial transient positive inotropic effect may well be attributable to displacement of some membrane bound calcium by the drug.

Although the role of $(Na^+ + K^+)$-ATP-ase inhibition in the positive inotropic actions of cardiac glycosides is still controversial (see Akera & Brody, 1978; Noble, 1980) our studies have shown that glycoside binding, enzyme activity and positive inotropic effects are all depressed during hibernation. However, since the depression of the positive inotropic action of actodigin during hibernation is quantitatively greater than expected from the biochemical data, it is possible that more than one process is affected during the change from summer active to hibernating states. Our experiments point to a change in the regulation of calcium in addition to a frank loss of sodium pumping sites. There is a labile calcium flux, associated with a positive staircase, and an unchanging calcium flux, suppression of which leads to a slow loss in tissue contractility. Both calcium fluxes seem to be sensitive to verapamil, but the former is sensitive at much lower concentrations. The labile calcium flux may well be associated with a sodium-calcium exchange mechanism which too may disappear with the approach of hibernation or may simply become quiescent in the absence of a supply of calcium ions. Thus the inotropic response to cardiac glycosides varies with the seasons.

In contrast to the marked differences in the magnitude of positive inotropic responses to actodigin between summer and winter, toxicity (manifested as contracture) was apparent in all groups at glycoside concentrations of around 10 μM. A similar dissociation between inotropic response and toxicity has been reported for ouabain in perfused guinea-pig hearts incubated with neuraminidase (Bailey & Fawzi, 1980). These results, however, have not been

confirmed in guinea-pig isolated atria (Harding & Halliday, 1980). It would seem from our results that inotropy is dependent on secondary, and in our system, labile processes while toxicity is the result of the primary effect of cardiac glycosides, i.e. $(Na^+ + K^+)$-ATP-ase inhibition with a failure to maintain membrane potential and all that is consequent on this. Sodium pumping occurs year round, although the activity of the enzyme or relative amount of pumping may vary with the seasonal needs. Nonetheless, inhibition of pumping produces the seasonally invariant response of toxicity.

The authors would like to thank Mr Kevin Murphy for trapping the animals in the wild. The project was supported by a Grant-in-Aid from the Alberta Heart Foundation. The present studies were carried out during study leave (R.J.M.) from University of Strathclyde, Glasgow, partly supported by the Wellcome Travel Trust. Correspondence to R.J.M. please.

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(Received July 5, 1982.
Revised August 9, 1982.)