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Digoxin activates sarcoplasmic reticulum Ca^{2+}-release channels: a possible role in cardiac inotropy

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1 The effect of digoxin on rapid $^{45}Ca^{2+}$ efflux from cardiac and skeletal sarcoplasmic reticulum (SR) vesicles was investigated. Additionally the interaction of digoxin with single cardiac and skeletal muscle SR Ca^{2+}-release channels incorporated into planar phospholipid bilayers and held under voltage clamp was determined.

2 Digoxin (1 nM) increased the initial rate and amount of Ca^{2+}-induced release of $^{45}Ca^{2+}$ from cardiac SR vesicles, passively loaded with $^{45}CaCl_2$, at an extravesicular [Ca^{2+}] of 0.1 μM. The efflux in the presence and absence of digoxin was inhibited at pM extravesicular Ca^{2+} and blocked by 5 mM Mg^{2+}.

3 To elucidate the mechanism of action of digoxin, single-channel recording was used. Digoxin (1–20 nM) increased single-channel open probability (P_o) when added to the cytosolic but not the luminal face of the cardiac channel in the presence of sub-maximally activating Ca^{2+} (0.1 μM–10 μM) with an EC_{50} of 0.91 nM at 10 μM Ca^{2+}. The mechanisms underlying the action of digoxin appear to be concentration-dependent. The activation observed at 1 nM digoxin appears to be consistent with the sensitization of the channel to the effects of Ca^{2+}. At higher concentrations the drug appears to interact synergistically with Ca^{2+} to produce values of P_o considerably greater than those seen with Ca^{2+} as the sole activating ligand.

4 Digoxin had no effect on single-channel conductance or the Ca^{2+}/Tris permeability ratio. In channels activated by digoxin the P_o was decreased by Mg^{2+}. Single-channels were characteristically modified to a long lasting open, but reduced, conductance state when 100 nM ryanodine was added to the cytosolic side of the channel.

5 Activation of the cardiac SR Ca^{2+}-release channel was observed with similar concentrations of digitoxin, however, higher concentrations of ouabain were required to increase P_o. In contrast, a steroid which is not positively inotropic, chlormadinone acetate, had no effect on either cardiac or skeletal SR Ca^{2+}-release channel activity.

6 At concentrations up to 1 μM, digoxin had no effect on Ca^{2+}-induced $^{45}Ca^{2+}$ efflux from skeletal muscle SR vesicles nor did it affect skeletal SR Ca^{2+}-release channel P_o, reflecting a difference between the cardiac and skeletal isoforms of the Ca^{2+}-release channel.

7 Since activation of the cardiac SR Ca^{2+}-release channel occurs within the range of concentrations of digoxin encountered therapeutically, it is possible that activation of this channel contributes to the positive inotropic effect observed with this drug. Further, activation of the channel by higher concentrations of digoxin may contribute to the toxic effects seen clinically.

Keywords: Sarcoplasmic reticulum; cardiac Ca^{2+}-release channel; digoxin; cardiac glycosides

Introduction

Cardiac glycosides increase the force of contraction of cardiac muscle and are used clinically in the treatment of heart failure, yet how these drugs achieve their positive inotropic effect remains controversial. Digoxin and other cardiotonic steroids cause an increase in intracellular free calcium when applied to cardiac muscle (Wier & Hess, 1984; Allen *et al.*, 1984; 1985). The mechanism of this increase is thought to be due primarily to Na^+/K^+ ATPase inhibition by the glycoside, which raises intracellular sodium thereby modulating Na^+/Ca^{2+} exchange and increasing the uptake and subsequent release of calcium by the sarcoplasmic reticulum (SR) (Eisner & Lederer, 1979; Wier & Hess, 1984; Morgan, 1985; Bers & Bridge, 1988; Eisner, 1990). An enhancement of the transient inward current by glycosides has also been reported and may contribute to the increase in intracellular calcium (Weingart *et al.*, 1978; Marban & Tsien, 1982). However, in some experiments glycosides stimulate, rather than inhibit, the Na^+/K^+ ATPase at positively inotropic concentrations (Cohen *et al.*, 1976; Godfraind & Ghysel-Burton, 1977; Ghysel-Burton & Godfraind, 1979); in others, compounds which inhibit the Na^+/K^+ ATPase do not cause an increased contraction even though they displace [^{3}H]-ouabain from binding sites (LaBella *et al.*, 1979). Further, it has been reported that strophanthidin may cause an increase in developed tension prior to an increase in intracellular sodium (Boyett *et al.*, 1986), suggesting that inhibition of the Na^+/K^+ ATPase is not the initial mechanism of inducing positive inotropy. Given these, at times, contradictory reports of the mechanisms of action of cardiotonic steroids, there may be an additional action of these drugs to raise intracellular calcium and increase tension in cardiac muscle (Hart *et al.*, 1983). Since glycosides are able to cross the sarcolemma (Dutta *et al.*, 1968; Fricke & Klaus, 1978), and it has been shown that a range of positively inotropic agents directly activate the cardiac SR Ca^{2+}-release channel (Williams, 1992), the possibility exists that glycosides induce positive inotropy by acting at this intracellular site. In view of this possibility we have investigated the effects of therapeutic and toxic concentrations of cardiac glycosides on the release of calcium from SR vesicles and on the open probability of the SR Ca^{2+}-release channel.

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Methods

Isolation of SR membrane vesicles

The methods for the isolation of SR membrane vesicles were as previously described (Sitsapesan & Williams, 1990). Briefly, sheep hearts or skeletal muscles were obtained from a local abattoir and transported to the laboratory in cold modified cardioplegic solution (Tomlins *et al.*, 1986), which contained (mM): NaCl 102, KCl 21.4, $MgCl_2$ 16, sodium lactate 28.7, $CaCl_2$ 2.4 and procaine 1.0. Tissue was stripped of fat and minced prior to homogenization in a solution containing phenylmethyl-sulphonide chloride (PMSF) 1 mM, sucrose 300 mM and piperazine-N'N'-*bis*-2-ethanesulphonic acid (PIPES) 20 mM, pH 7.4. The homogenate was centrifuged at 8500 g_{av} in a Sorvall GSA rotor for 20 min and the pellet discarded. The supernatant was then centrifuged at 100000 g_{av} for 40 min in a Sorvall A641 or T647.5 rotor. The mixed membrane population sedimented by this step was resuspended in a solution containing mM: KCl 400, $MgCl_2$ 0.5, $CaCl_2$ 0.5, 1,2 di(2-aminoethyl)ethane-N,N,N',N'-tetra-acetic acid (EGTA) 0.5 and PIPES 25, pH 7.0 plus 10% sucrose w/v and fractionated on discontinuous sucrose-density gradients. The membrane suspension was layered over identical salt solutions containing 20, 30 and 40% sucrose w/v and sedimented at 100000 g_{av} for 120 min in a Sorvall AH629 rotor. Heavy sarcoplasmic reticulum (HSR) membrane vesicles collecting at the 30–40% interface were diluted in KCl 400 mM and pelleted by centrifugation at 100000 g_{av} for 40 min in a Sorvall T647.5 rotor before resuspension in a solution containing sucrose 400 mM and N'-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 5 mM titrated to pH 7.4 with tris(hydroxymethyl)-methylamine (Tris). Membrane vesicles were snap frozen in liquid nitrogen and stored at − 80°C.

Rapid $^{45}Ca^{2+}$ efflux from SR vesicles

The kinetics of $^{45}Ca^{2+}$ efflux from SR vesicles passively loaded with $^{45}Ca^{2+}$ were investigated by use of the Bio-Logic rapid filtration apparatus (Intracel, Cambridge, U.K.), which allows resolution of ionic fluxes in the millisecond time range, utilizing a modification of the methods of DuPont (DuPont, 1984; Moutin & DuPont, 1988). HSR vesicles were thawed, resuspended in KCl 100 mM and HEPES 20 mM, pH 7.4 and pelleted by centrifugation in a Sorvall T647.5 rotor at 100000 g_{av} to remove residual sucrose prior to resuspension and incubation at a protein concentration of 4 mg ml^{-1} in an uptake solution containing KCl 100 mM, HEPES 20 mM, $CaCl_2$ 4.95 mM and $^{45}CaCl_2$ 50 μM for 120 min at 22°C. $^{45}Ca^{2+}$-loaded vesicles were used within 1 h following loading, during which time uptake remained constant. Under the conditions described, uptake of 25–35 nmol of $^{45}Ca^{2+}$ into the vesicles mg^{-1} protein for cardiac and 45–55 nmol of $^{45}Ca^{2+}$ mg^{-1} protein for skeletal vesicles was consistently achieved, with 50% of maximal loading occurring after approximately 1 min. Maximum uptake occurred after 1 h and remained constant thereafter. This loading is higher than has been achieved by passive loading of canine cardiac SR vesicles under similar conditions; however, the loading for skeletal vesicles is similar to that obtained by others (Meissner & Henderson, 1987). Aliquots of vesicles containing 200 μg protein were placed on 0.45 μm pore diameter Millipore filters, pre-soaked in non-release buffer, which contained KCl 100 mM, HEPES 20 mM, EGTA 1 mM, $MgCl_2$ 5 mM and $CaCl_2$ 5 mM. Vesicles and filter were washed with 5 ml of non-release buffer under vacuum to remove extravesicular $^{45}Ca^{2+}$. The filter was then placed in the efflux apparatus and washed for controlled times (10–1000 ms) with a solution consisting of KCl 100 mM, HEPES 20 mM, EGTA 1 mM and $CaCl_2$ 695 μM (free $[Ca^{2+}] = 0.1$ μM). Digoxin was added to the injection syringe from a stock solution of 100 nM. After the efflux was completed, filters were removed and dissolved in 10 ml Packard scintillant and the remaining radioactivity on the filters counted in a liquid scintillation counter. Initial efflux rates were calculated from the derivative of the single exponential decay curve fitted to each set of data.

Planar lipid bilayer methods

Experiments were performed essentially as previously described by Sitsapesan & Williams (1990). Bilayers, composed of phosphatidylethanolamine dispersed in decane (30 mg ml^{-1}) were painted across a 200 μm diameter hole in the partition between two fluid-filled styrene co-polymer chambers designated *cis* and *trans*. The *trans* chamber was held at ground and the *cis* chamber clamped at holding potentials relative to this. Current fluctuations were monitored with an operational amplifier as a current-voltage converter (Miller, 1982). Initially, both chambers contained choline chloride 50 mM, HEPES 10 mM and $CaCl_2$ 5 mM, with the pH adjusted to 7.4 with Tris. HSR vesicles were added to the *cis* chamber and the choline chloride concentration increased to give a 7:1 gradient *cis-trans* to promote vesicle fusion with the bilayer. Fusion was marked by the appearance of Cl^--selective channels (Smith *et al.*, 1985). SR vesicles incorporated into the planar bilayer in a fixed orientation such that the cytosolic face of the Ca^{2+}-release channel was directed towards the *cis* chamber and the luminal face to the *trans* chamber. Following vesicle fusion, the *cis* and *trans* chambers were perfused with solutions allowing resolution of Ca^{2+}-release channel currents. The *cis* chamber was perfused with HEPES 250 mM, Tris 125 mM, pH 7.4; and the *trans* with glutamic acid 250 mM, HEPES 10 mM with the pH adjusted to 7.4 with $Ca(OH)_2$, giving a $[Ca^{2+}]$ on the *trans* side of 67 mM. The concentration of Ca^{2+} in the *cis* chamber was buffered to the desired level by the addition of $CaCl_2$ and EGTA and calculated using EQCAL (BioSoft, Cambridge). Experiments were performed at room temperature (22 ± 1°C).

Data acquisition and analysis

Channel data were displayed on an oscilloscope and recorded on video tape. Current recordings were replayed, low pass filtered at 1 kHz using an eight pole Bessel filter and digitized at 2 kHz with either an AT-based system (Intracel, Cambridge) or a PDP 11/73 based system (Indec, Sunnyvale, U.S.A.). Channel open probability (P_o) and the lifetimes of open and closed events were monitored by 50% threshold analysis at a holding potential of 0 mV from 3 min of continuous recordings. P_o was determined by the equation $P_o = T_{open}/(T_{open} + T_{closed})$, where T_{open} and T_{closed} are the mean open and closed lifetime durations. Lifetimes, accumulated from 3 min steady-state recordings were stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to a probability density function by the method of maximum likelihood (Colquhoun & Sigworth, 1983). Lifetimes lasting less than 1 ms were not fully resolved under these conditions of data acquisition and were therefore excluded from the fitting procedure. A missed events correction was applied (Colquhoun & Sigworth, 1983) and a likelihood ratio test was used to compare fits to double and triple exponentials (Blatz & Magleby, 1986).

Single-channel conductance was obtained from the slope of linear regression lines drawn through single-channel current amplitude data monitored at holding potentials within the range (+40 to −40 mV). Reversal potentials (E_{rev}) were obtained from extrapolations of these lines. The $Ca^{2+}/Tris^+$ permeability ratio was calculated from the equation given by Fatt & Ginsborg (1958):

$$pX^{2+}/pY^+ = [Y^+]/4[X^{2+}].\exp(E_{rev}F/RT).[\exp(E_{rev}F/RT) + 1]$$

Drugs used

Drugs used in the study were: digoxin, digitoxin, ouabain octahydrate, chlormadinone acetate and spironolactone (Sigma). Stock solutions of digoxin, digitoxin, chlormadinone and spironolactone were prepared in ethanol. The final concentration of ethanol did not exceed 0.03% in either the rapid efflux experiments or the single-channel experiments. In both cases, this concentration of ethanol was without effect. Ouabain was prepared in distilled water. Phosphatidylethanolamine was from Avanti Polar Lipids, Birmingham, U.S.A.

Results

Enhancement of Ca^{2+}-induced $^{45}Ca^{2+}$ efflux from cardiac SR vesicles by digoxin

Isolated SR vesicles are of a fixed orientation with the outside of the vesicle equivalent to the cytosolic face of the membrane. Efflux from cardiac (and skeletal) SR vesicles was dependent upon the extravesicular Ca^{2+} concentration. There was no measurable efflux of $^{45}Ca^{2+}$ at pM Ca^{2+} in the presence or absence of digoxin. However, the initial rate and total amount of $^{45}Ca^{2+}$ release increased as extravesicular [Ca^{2+}] was raised. In all cases efflux could be described by a single exponential. Release was inhibited by 5 mM Mg^{2+} (data not shown). In the presence of digoxin, the Ca^{2+}-induced release of $^{45}Ca^{2+}$ was enhanced (Figure 1). At 0.1 μM Ca^{2+} the initial rate of efflux increased from 144 ± 20 to 258 ± 24 nmol $^{45}Ca^{2+}$ mg^{-1} protein s^{-1} (± s.e.mean, $n = 5$) when 1 nM digoxin was present. The digoxin-enhanced efflux was also blocked by 5 mM Mg^{2+} (not shown).

Lack of effect of digoxin on Ca^{2+}-induced $^{45}Ca^{2+}$ efflux from skeletal SR vesicles

Digoxin failed to increase the initial rate or total amount of $^{45}Ca^{2+}$ released from skeletal SR vesicles in the same conditions. Initial efflux rates at an extravesicular [Ca^{2+}] of 0.1 μM were 502 ± 32 nmol $^{45}Ca^{2+}$ mg^{-1} protein s^{-1} (control) and 453 ± 59 nmol $^{45}Ca^{2+}$ mg^{-1} protein s^{-1} in the presence of 1 nM digoxin (± s.e.mean, $n = 4$).

Whilst these experiments establish that therapeutic concentrations of digoxin increase the rapid efflux of $^{45}Ca^{2+}$ from cardiac, but not skeletal SR vesicles this method does not elucidate the exact route and mechanism of this enhanced $^{45}Ca^{2+}$ efflux in the presence of digoxin.

Single-channel recording

To study the mechanism of action of digoxin, HSR vesicles from at least six different preparations were incorporated into planar phospholipid bilayers and Ca^{2+}-release channel conduction and gating properties studied under voltage clamp conditions.

Cardiac SR Ca^{2+}-release channels

Single Ca^{2+}-release channels were activated by adding Ca^{2+} 0.1 μM–10 μM to the cytosolic side of the channel (Figure 2). Digoxin (1 nM) further increased single-channel P_o when added to the cytosolic, but not the luminal face of the channel. At an activating [Ca^{2+}] of 0.1 μM, 1 nM digoxin increased P_o from 0.012 ± 0.003 to 0.110 ± 0.029 (± s.e.mean, $n = 8$). At 10 μM Ca^{2+}, 1 nM digoxin increased P_o from 0.048 ± 0.012 to 0.281 ± 0.065 ($n = 4$). As digoxin caused an increase in P_o when added from only one side of the bilayer, it is likely that it is acting at a site on the cytosolic face of the Ca^{2+}-release channel rather than indirectly affecting the channel by partitioning into the lipid bilayer.

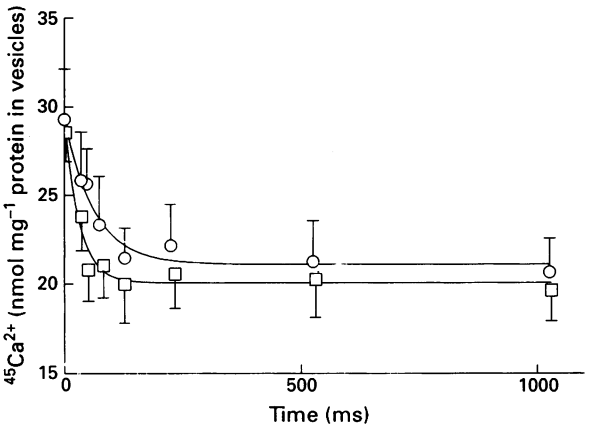

Figure 1 Rapid Ca^{2+}-induced $^{45}Ca^{2+}$ efflux at 0.1 μM Ca^{2+} (○) and at 0.1 μM Ca^{2+} + 1 nM digoxin (□). Digoxin increased both the initial rate of efflux and total amount of $^{45}Ca^{2+}$ released at an extravesicular [Ca^{2+}] of 0.1 μM. Each set of efflux data was best fitted by a single exponential curve, using non-linear regression and initial efflux rates were calculated from the differential of the single exponential decay equation. Fits to single and double exponentials were compared by an F test. Each point is the mean of five experiments conducted in duplicate and results are displayed as means ± s.e.mean.

Lifetime analysis of the channel open and closed states in the presence and absence of digoxin (Figure 3) indicated that the increase in P_o caused by 1 nM digoxin at 0.1 μM and 10 μM activating Ca^{2+} appeared to be similar to that observed as the concentration of activating cytosolic Ca^{2+} is increased. That is, P_o was increased as a result of increased frequency of channel opening with no significant increase in channel open lifetimes.

The interpretation that 1 nM digoxin increased P_o by increasing the frequency of channel opening and hence sensitized the channel protein to Ca^{2+}, is supported by the observation that digoxin failed to activate the channel at sub-activating (pM) Ca^{2+}. Similarly at a [Ca^{2+}] of 100 μM, digoxin induced no further increase in P_o. At 100 μM Ca^{2+}, P_o was 0.160 ± 0.043 in the absence and 0.143 ± 0.038 (± s.e.mean, $n = 3$) in the presence of 1 nM digoxin. In addition Mg^{2+}, which is thought to compete with Ca^{2+} for the activation site on the Ca^{2+}-release channel (Ashley & Williams, 1990), reduced P_o following activation by digoxin (not shown).

Digoxin had no effect on single-channel conductance (86 ± 3 pS, control vs. 88 ± 2 pS + 1 nM digoxin, ± s.e.mean, $n = 5$) or the $Ca^{2+}/Tris^{+}$ permeability ratio (12.7 ± 0.3, control vs. 13.0 ± 0.3 + digoxin, $n = 5$). Channels activated by Ca^{2+} alone or by Ca^{2+} and digoxin together were characteristically modified to a long lasting open, but reduced conductance state when 100 nM ryanodine was added to the cytosolic side of the channel (Figure 4).

Table 1 summarizes the effects of increasing cytosolic Ca^{2+} and digoxin on single SR Ca^{2+}-release channel P_o and mean open and closed lifetimes.

Higher concentrations of digoxin (10 nM and 20 nM) increased P_o at 10 μM Ca^{2+} to 0.480 ± 0.049 (± s.e.mean, $n = 5$) and 0.531 ± 0.032 ($n = 4$) from control values. Given these results, an approximate apparent EC_{50} of 0.91 nM is obtained for digoxin from this data. At these higher concentrations of digoxin, there was an increase in channel open lifetime duration as well as an increase in the frequency of opening (not shown). Single-channel open and closed lifetimes could be best fitted by triple exponential curves, indicating that digoxin interacts with at least one of the open states as well as at least one of the closed states of the Ca^{2+}-release channel. However, the interaction with the open state, resulting in an increase in the channel open lifetime

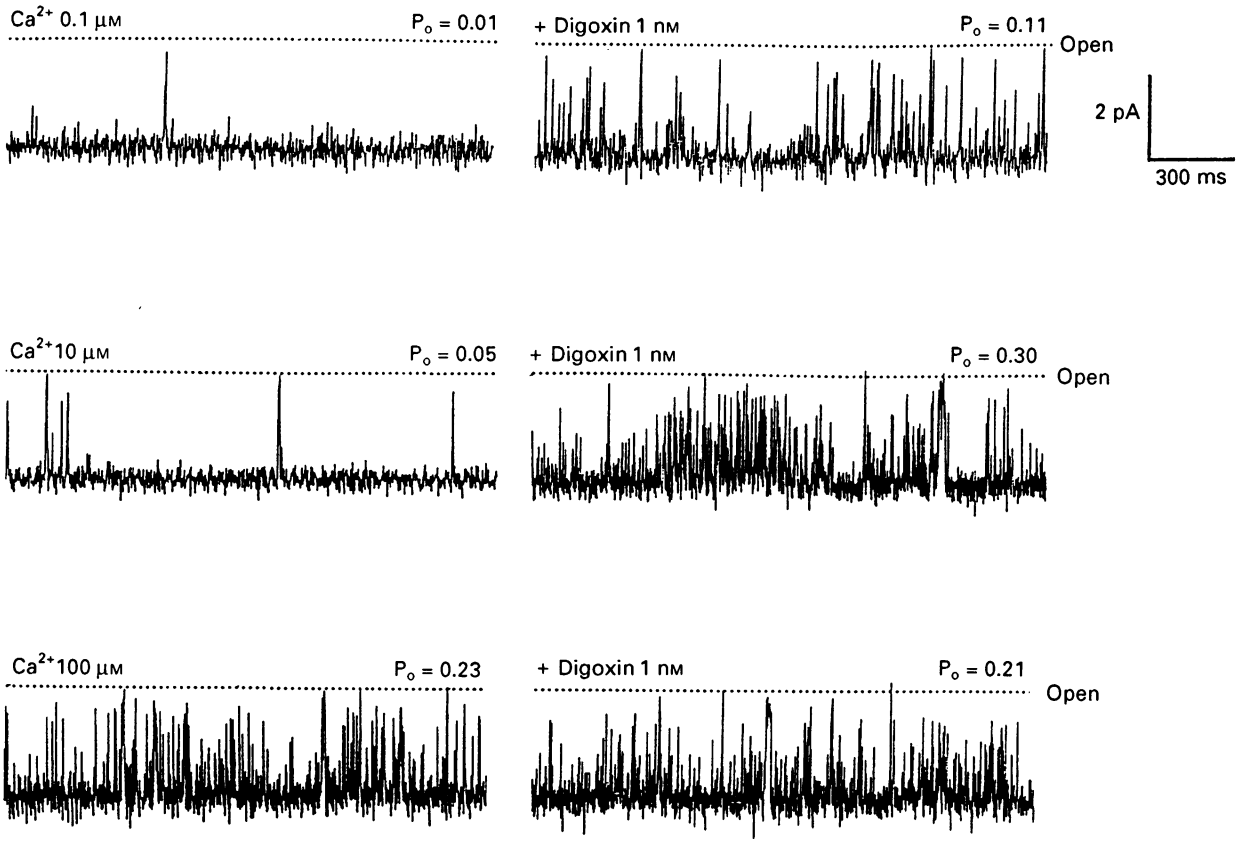

Figure 2 Representative portions of data from single SR Ca^{2+}-release channels held at 0 mV. Channels were first activated by Ca^{2+} and then 1 nM digoxin was added to the *cis* chamber. Single-channel P_o, determined from 3 min of continuous recordings, increased as cytosolic Ca^{2+} was raised. In the presence of 1 nM digoxin, P_o increased further at 0.1 μM and 10 μM Ca^{2+}, but not at 100 μM Ca^{2+}. Digoxin had no effect on single-channel conductance. Single-channel P_o values were calculated as described in the Methods section and are indicated above each respective portion of recording. Dotted lines indicate channel open level.

duration, only occurs at these higher concentrations of digoxin and therefore may be with a lower affinity site.

Activation of the cardiac SR Ca^{2+}-release channel was observed with similar concentrations of digitoxin (data not shown). However, ouabain was without effect at 1 nM. When the concentration of ouabain at the cytosolic side of the channel was increased to 10 nM, P_o increased from 0.040 ± 0.011 to 0.232 ± 0.061 at 10 μM Ca^{2+} ($\pm$ s.e.mean, $n = 3$) (Figure 5). Lifetime analysis of the channel open and closed states indicate that 10 nM ouabain activates the channel by the same mechanism as 1 nM digoxin (Figure 6) that is, it increases P_o mainly by a reduction in channel closed lifetimes. Activation by digoxin, digitoxin and ouabain was readily reversible on washout of the drug from the *cis* chamber.

Chlormadinone acetate, a progestin without inotropic activity, but which displaces [^{3}H]-ouabain from binding sites (LaBella *et al.*, 1979), had no effect on single channel P_o or conductance at up to 100 μM (not shown). It also did not affect the digoxin activation of the channel. Similarly spironolactone, which inhibits the Na^+/K^+ ATPase and also reduces digoxin binding to the ATPase (Musgrave *et al.*, 1977), had no effect on P_o or single-channel conductance. Like chlormadinone, it did not affect digoxin activation of the cardiac SR Ca^{2+}-release channel at up to 100 μM. These observations suggest that neither chlormadinone nor spironolactone bind to the cardiac SR Ca^{2+}-release channel.

Skeletal SR Ca^{2+}-release channels

Consistent with our findings that digoxin failed to enhance the rate or magnitude of Ca^{2+}-induced $^{45}Ca^{2+}$-release from sheep skeletal muscle SR vesicles, we observed no equivalent effect of digoxin on skeletal muscle SR Ca^{2+}-release channels: P_o at 0.1 μM $Ca^{2+} = 0.009 \pm 0.002$; P_o at 0.1 μM Ca^{2+} + 1 nM digoxin $= 0.011 \pm 0.004$ ($\pm$ s.e.mean, $n = 8$). There was no increase in P_o at concentrations up to 1 μM digoxin. Chlormadinone and spironolactone were also without effect on skeletal channels.

Discussion

Our data demonstrate a correlation between the effect of digoxin on rapid Ca^{2+}-induced $^{45}Ca^{2+}$ efflux from cardiac SR vesicles and its effect on single SR Ca^{2+}-release channel activity. Since other cardiac glycosides also activate the cardiac SR Ca^{2+}-release channel in the nM range, we propose that there is a high affinity binding site on this channel protein for cardiotonic steroids.

The mechanisms involved in the activation of the cardiac SR Ca^{2+}-release channel by digoxin are qualitatively similar to those seen with the positive inotropic phosphodiesterase inhibitors, caffeine (Sitsapesan & Williams, 1990) and sulmazole (Williams & Holmberg, 1990). In all cases low concentrations of the compounds (i.e. 1 nM digoxin) appear to increase channel P_o by sensitizing the channel to Ca^{2+} and hence increase the frequency of channel opening. This proposal is supported by the finding that 1 nM digoxin is without effect in the absence of activating Ca^{2+} and that following a digoxin-induced increase, P_o is reduced by the addition of Mg^{2+}. The observation that 1 nM digoxin, when added to channels activated by 10 μM Ca^{2+}, is capable of producing values of P_o in excess of those seen with 100 μM Ca^{2+} or 100 μM Ca^{2+} plus 1 nM digoxin may appear somewhat paradoxical. However, it is consistent with the relation-

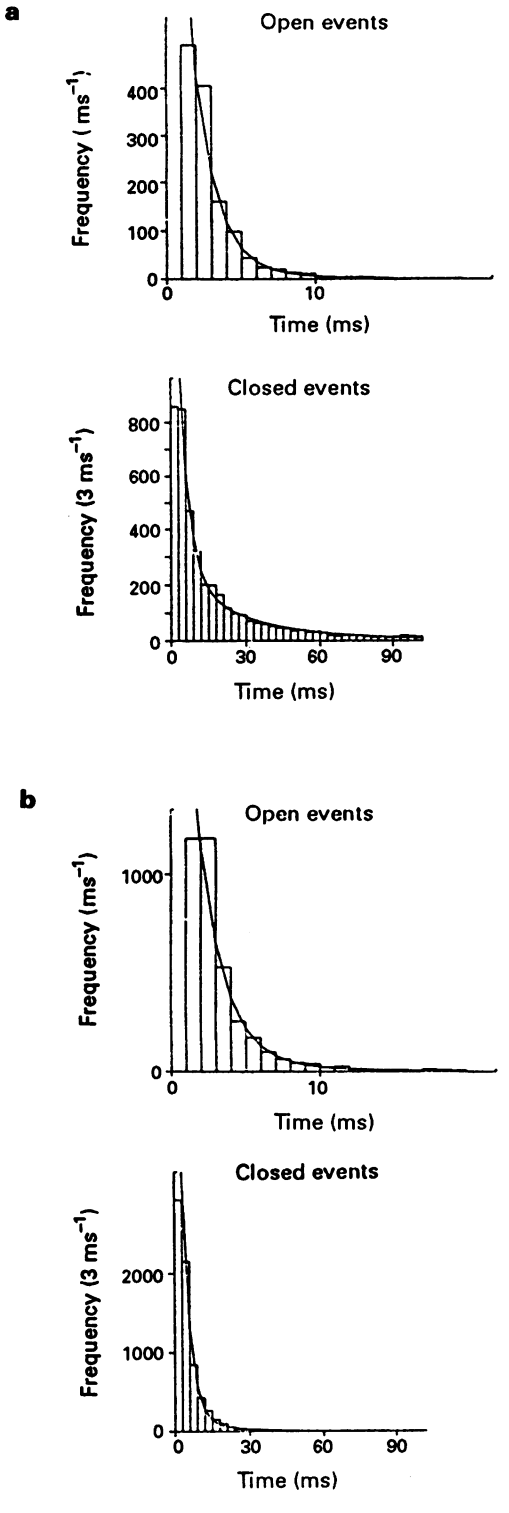

Figure 3 Open and closed lifetime histograms, with probability density functions obtained by maximum likelihood fitting to individual lifetimes for a single sheep cardiac SR Ca^{2+}-release channel activated by (a) Ca^{2+} 10 μM and (b) Ca^{2+} 10 μM + digoxin 1 nM on the cytosolic side of the channel. Lifetimes, obtained from continuous 3 min recordings, are displayed in non-cumulative histograms. Probability density functions, obtained by the method of maximum likelihood are drawn according to: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots\ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$. For open lifetimes most likely fits were obtained to double exponentials with (i) Ca^{2+} 10 μM: $a_1 = 93.37$, $a_2 = 6.63$, $\tau_1 = 1.32$, $\tau_2 = 12.51$ and (ii) Ca^{2+} 10 μM + digoxin 1 nM: $a_1 = 94.14$, $a_2 = 5.86$, $\tau_1 = 1.75$, $\tau_2 = 12.51$. For closed lifetimes, most likely fits were obtained to triple exponentials with (i) Ca^{2+} 10 μM: $a_1 = 21.91$, $a_2 = 56.67$, $a_3 = 21.42$, $\tau_1 = 4.67$, $\tau_2 = 28.09$, $\tau_3 = 162.21$ and (ii) Ca^{2+} 10 μM + digoxin 1 nM: $a_1 = 87.68$, $a_2 = 9.19$, $a_3 = 3.12$, $\tau_1 = 3.16$, $\tau_2 = 11.67$, $\tau_3 = 34.70$. Open and closed lifetime distributions are taken from the experiment shown in Figure 2.

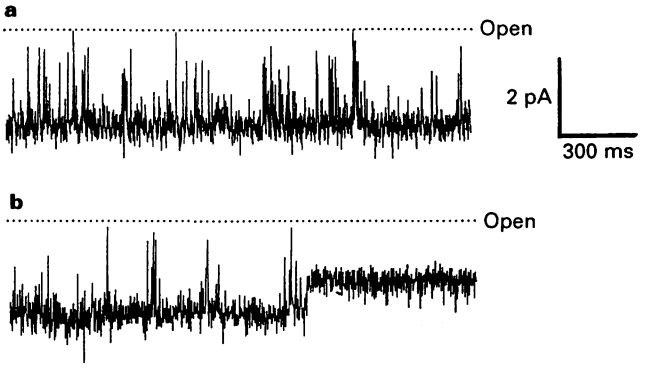

Figure 4 A single cardiac SR Ca^{2+}-release channel activated by digoxin (a) at Ca^{2+} 10 μM modified to a characteristic long-lasting open state of reduced conductance on addition of ryanodine 100 nM to the cytosolic side of the channel (b). The ryanodine-modified state has a conductance approximately 40% of the control value. Dotted lines indicate the fully open level of the channel.

Table 1 Summary of the effect of 1 nM digoxin on single-channel mean open and closed times and mean P_o at various concentrations of activating Ca^{2+}

	Mean open time (ms)	*Mean closed time* (ms)	*Mean* P_o
Ca^{2+} 0.1 μM	1.41 ± 0.45	138 ± 42	0.012 ± 0.003
+ digoxin 1 nM	1.36 ± 0.26	22 ± 3.87	0.110 ± 0.029
Ca^{2+} 10 μM	1.37 ± 0.20	34.80 ± 6.73	0.048 ± 0.012
+ digoxin 1 nM	1.73 ± 0.09	7.05 ± 1.54	0.281 ± 0.065
Ca^{2+} 100 μM	2.18 ± 0.38	14.35 ± 2.73	0.160 ± 0.043
+ digoxin 1 nM	2.05 ± 0.62	14.93 ± 3.14	0.143 ± 0.038

Values are mean ± s.e.mean.
For experiments at 0.1 μM Ca^{2+} the results are the mean of eight different single channels; at 10 μM Ca^{2+} the results are the mean of four channels and at 100 μM Ca^{2+}, the mean of three different channels. At 0.1 μM Ca^{2+} and 10 μM Ca^{2+} the increase in P_o caused by addition of 1 nM digoxin is mainly due to a reduction in the mean closed time of the channel, resulting in an increased frequency of channel opening. Mean open and closed lifetimes and mean P_o were calculated as described in the Methods section.

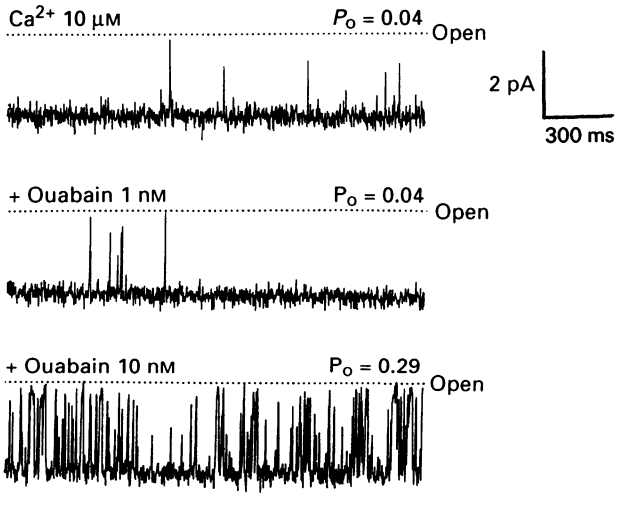

Figure 5 A single cardiac SR Ca^{2+}-release channel held at 0 mV, activated by Ca^{2+} and by Ca^{2+} and ouabain. The channel was activated by Ca^{2+} 10 μM added to the *cis* chamber, before addition of ouabain. Single-channel P_o was not affected by ouabain 1 nM, but was increased significantly on addition of ouabain 10 nM to the *cis* chamber. Ouabain, 1 nM and 10 nM, had no effect at Ca^{2+} 100 μM (not shown). Single-channel conductance was unaffected by ouabain. P_o values, indicated above each respective portion of data, were calculated as described in the Methods section. Dotted lines indicate channel open level.

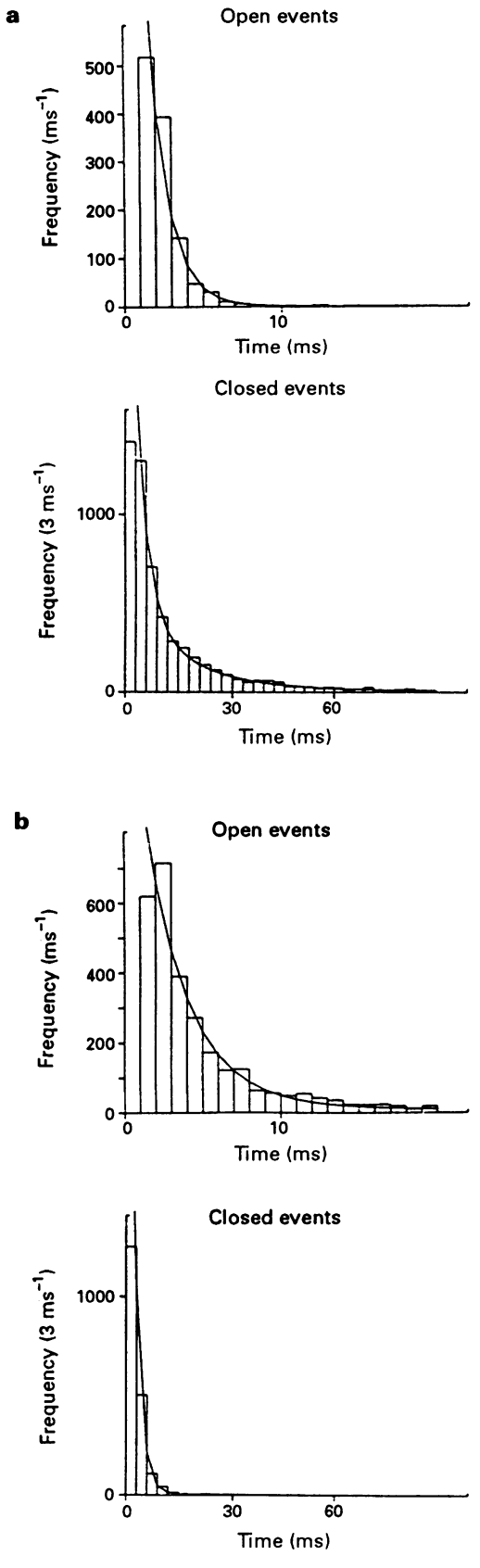

Figure 6 Single sheep cardiac SR Ca^{2+}-release channel open and closed lifetime histograms, with probability density functions obtained by maximum likelihood fitting to individual lifetimes. Open and closed lifetime distributions are taken from the experiment shown in Figure 5. Here, (a) corresponds to the channel activated by Ca^{2+} 10 μM and (b) to the channel activated by Ca^{2+} 10 μM + ouabain 10 nM. Lifetimes were obtained from 3 min of continuous recordings and are displayed in non-cumulative histograms. Probability density functions are drawn according to: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots\ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$. For open lifetimes most likely fits were obtained to double exponentials with (i) Ca^{2+} 10 μM: $a_1 = 98.09$, $a_2 = 1.91$, $\tau_1 = 1.24$, $\tau_2 = 14.40$ and (ii) Ca^{2+} 10 μM + ouabain 10 nM: $a_1 = 86.42$, $a_2 = 13.58$, $\tau_1 = 1.71$, $\tau_2 = 21.91$. For closed lifetimes, most likely fits were obtained to triple exponentials with (i) Ca^{2+} 10 μM: $a_1 = 51.67$, $a_2 = 38.27$, $a_3 = 10.06$, $\tau_1 = 3.28$, $\tau_2 = 14.83$, $\tau_3 = 96.89$ and (ii) Ca^{2+} 10 μM + ouabain 10 nM: $a_1 = 97.43$, $a_2 = 1.23$, $a_3 = 1.34$, $\tau_1 = 1.86$, $\tau_2 = 9.85$, $\tau_3 = 40.18$.

ship between SR Ca^{2+}-release channel P_o and activating Ca^{2+} concentration, which is complex and varies somewhat between individual channels. As the Ca^{2+} concentration at the cytosolic face of the channel is increased in the μM range P_o increases, reaches a maximum and then tends to decline as the concentration of Ca^{2+} at the cytosolic face of the channel is further increased (Sitsapesan *et al.*, 1991). Ca^{2+} does not fully activate the channel when it is present as the only activating ligand and the Ca^{2+} concentration producing maximal P_o will differ slightly from channel to channel. In the channels described here it appears probable that a Ca^{2+} concentration of 100 μM was greater than that required for maximal activation. The observation that P_o is unaffected or possibly slightly lowered by 1 nM digoxin at an activating Ca^{2+} concentration of 100 μM is, therefore, entirely consistent with Ca^{2+} sensitization.

The elevation of channel P_o by high concentrations of digoxin (10–20 nM) is associated with an increase in channel open lifetimes indicating a second mechanism of action of digoxin at these concentrations. A similar observation has been made with both caffeine (Sitsapesan & Williams, 1990) and sulmazole (Williams & Holmberg, 1990).

Since low concentrations of caffeine activate both the cardiac and skeletal SR Ca^{2+}-release channel by a similar mechanism to activation by digoxin of the cardiac channel, it may be suggested that these different compounds act at the same site on these channels. If this were so, digoxin would be expected to activate the skeletal, as well as the cardiac channel, however this does not occur. This presumably reflects the lack of a recognition site on the skeletal SR channel for digoxin, highlighting a functional difference in the two isoforms of this protein.

That chlormadinone does not affect the cardiac SR Ca^{2+}-release channel is an interesting finding. As it has no positive inotropic activity, it would not be expected to activate the channel. It acts as an antagonist of ouabain at the Na^+/K^+ ATPase yet it has no such effect on glycoside-induced effects on the cardiac SR Ca^{2+}-release channel, even at high concentrations. In view of the results obtained with digoxin and ouabain, these observations could be interpreted as confirming the findings of LaBella *et al.* (1979) that although chlormadinone antagonized ouabain binding to the ATPase, it did not affect the positive inotropy induced by the glycoside. The authors in that sutdy concluded that ouabain increased contraction of cardiac muscle by acting at a chlormadinone-insensitive site distinct from the ATPase. This chlormadinone-insensitive site may have been the SR Ca^{2+}-release channel. Similarly, the lack of effect of spironolactone on digoxin-induced inotropy, despite spironolactone inhibiting digoxin binding to the ATPase, was interpreted by Musgrave *et al.* (1977) as there being another site of action for digoxin in inducing inotropy, possibly the SR. Again, as with chlormadinone, although spironolactone antagonized glycoside binding to the Na^+/K^+ ATPase, it did not affect glycoside-induced inotropy. These observations again suggest that digoxin increases contraction of cardiac muscle by acting at a site distinct from the ATPase. This lends further credence to the theory, arising from the results presented here, that activation of the cardiac SR Ca^{2+}-release channel by glycosides, and therefore increased release of intracellular Ca^{2+}, contributes to their positive inotropic effect. Addi-

tionally, Musgrave *et al.* (1977) noted that spironolactone decreased digoxin toxicity by antagonizing digoxin binding to the ATPase. Since we contend that positive inotropy may, in part, occur as a result of digoxin binding to a site on the cardiac SR Ca^{2+}-release channel, this observation is even more interesting as it would seem to suggest that inhibition of the Na^+/K^+ ATPase by glycosides, possibly thereby inducing Ca^{2+} overload, contributes to glycoside toxicity, if not inotropy.

Structurally, chlormadinone and spironolactone are similar to digoxin and ouabain with respect to their steroid nucleus. However, neither possesses sugar residues attached to the hydroxyl at the C3 position of the molecule and chlormadinone does not contain a lactone ring. This may indicate that only aglycones or steroids with these sugar attachments may bind to the activation site on the cardiac SR Ca^{2+}-release channel and that the presence or composition of these sugar residues may be determinants of activity. The presence of the lactone ring on the steroid would appear not to be necessary for activity.

The concentration of digoxin achieved therapeutically in the blood is in the range of 0.75–2 nM, taking into account binding to plasma proteins (Noble, 1980). Given that digoxin and other glycosides are able to cross the sarcolemma (Dutta *et al.*, 1968; Fricke & Klaus, 1978) and that we calculate an EC_{50} of <1 nM for digoxin, we feel that the results presented here provide compelling evidence that activation of the SR Ca^{2+}-release channel by glycosides is a contributory factor to the positive inotropic effect seen with these drugs. This may not, however, be the case with all cardiotonic steroids, especially those that are more lipophobic than digoxin e.g. ouabain, or those which show a consistent correlation between Na^+/K^+ ATPase inhibition and positive inotropy. This raises the possibility of the existence of different mechanisms of action for therapeutic concentrations of different cardiac glycosides, with the lipid solubility of the drug (and therefore the ability to reach the SR Ca^{2+}-release channel) the determinant of which mechanism induces inotropy. The more lipophilic drugs such as digoxin and digitoxin may increase contraction of cardiac muscle by direct activation of SR Ca^{2+}-release, since they could easily gain access intracellularly, whereas relatively more lipophobic compounds like ouabain, although able to activate the SR Ca^{2+}-release channel, may indirectly increase contraction by inhibition of the Na^+/K^+ ATPase, as it is unlikely that ouabain could gain access to the cytosol unless it was actively transported. These observations support the suggestion that there may be several mechanisms by which cardiac glycosides induce positive inotropy. It is possible that these different mechanisms interact to increase intracellular Ca^{2+} and cardiac muscle contraction. However, as high concentrations of digoxin also activate the cardiac SR Ca^{2+}-release channel, it is also possible that toxic concentrations of digoxin in the blood i.e. >2 nM may contribute to Ca^{2+} overload and glycoside toxicity by acting at this site.

Glycoside-induced release of Ca^{2+} from the cardiac SR would result in only a transient positive inotropic effect if it occurred in the absence of processes which would produce an overall increase in cytosolic and hence SR Ca^{2+}. As glycosides induce a long-lasting increase in cardiac contraction, this may indicate the importance of other effects of these compounds on cellular Ca^{2+} entry and/or removal mechanisms. In support of this argument, Marban & Tsien (1982) concluded that glycosides increased the transient inward current. If this were to occur at the same time as the enhancement of Ca^{2+} release from the SR, this might account for an elevation of the steady state cytosolic Ca^{2+} concentration and a sustained increase in inotropy. Again it seems probable that there are several mechanisms by which the inotropic state of cardiac muscle may be increased by glycosides. Prolonged increases in cytosolic Ca^{2+} and hence contraction, may require that more than one of these mechanisms be activated by the drug.

As glycosides increase single Ca^{2+}-release channel open probability and also inhibit the Na^+/K^+ ATPase there appears to be a site on both proteins with a similar affinity for this class of drugs. However, there are differences in the glycoside binding sites between these proteins, as chlormadinone and spironolactone bind to the ATPase but not to the glycoside site on the channel. As we contend that activation of the SR Ca^{2+}-release channel by lipophilic glycosides contributes to, or is responsible for, the positive inotropy seen with these drugs, there exists a rationale for the development of drugs which are agonists at the glycoside site on the release channel but which have minimal or no ability to inhibit the ATPase. This would constitute a new class of positively inotropic drugs. Additionally, given the suggestion of Musgrave *et al.* (1977) that inhibition of the ATPase by glycosides contributes to toxicity but not to inotropy, then this new class of compound could be positively inotropic with less toxic side effects, due to lack of ATPase inhibition. These new drugs would be potentially more safe due to their decreased toxicity. However, as there may be more than one mechanism by which cardiac glycosides can increase contraction, it is equally feasible that these drugs may cause toxic side effects by more than one mechanism e.g. by excessive activation of the SR Ca^{2+}-release channel, as suggested above.

In conclusion, the present study has three main findings: First, nanomolar concentrations of cardiac glycosides activate the cardiac SR Ca^{2+}-release channel. The increase in P_o induced by therapeutic concentrations of the glycosides is Ca^{2+}-dependent and is achieved by increasing the frequency of channel opening. Secondly, there is no equivalent effect of glycosides on skeletal muscle SR Ca^{2+}-release channels, reflecting a difference in the cardiac and skeletal isoforms of the channel. Thirdly, as direct activation of the cardiac channel occurs within the range of concentrations of glycosides encountered therapeutically, we propose that activation of the cardiac SR Ca^{2+}-release channel by digoxin and related compounds contributes to the positive inotropy seen with these drugs.

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