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Actions of the digitalis analogue strophanthidin on action potentials and L-type calcium current in single cells isolated from the rabbit atrioventricular node

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1 The atrioventricular node (AVN) of the heart is vital to normal cardiac function and is a major site of antiarrhythmic drug action. This study describes the effects of the cardiac glycoside analogue strophanthidin on spontaneous action potentials and L-type calcium current recorded from single AVN cells isolated from the rabbit heart.

2 With a standard KCl-based internal dialysis solution, exposure to 50 μM strophanthidin produced a progressive depolarization of the maximum diastolic potential and a reduction in action potential amplitude and upstroke velocity. Sustained application resulted in the loss of action potentials and occurrence of spontaneous 'bell-shaped' depolarizations.

3 Cells were whole-cell voltage clamped at -40 mV and depolarizing voltage clamps applied. With a standard KCl-based internal dialysis solution, exposure to 50 μM strophanthidin caused a large reduction of $I_{Ca,L}$ at all potentials between -30 and $+40$ mV ($n=4$). At $+10$ mV, the mean $I_{Ca,L}$ amplitude was reduced from -232 ± 65 pA to -48 ± 26 pA ($P<0.05$; t test; $n=5$ cells).

4 To record $I_{Ca,L}$ more selectively, cells were dialysed with a Cs-based pipette solution. A short strophanthidin exposure reduced $I_{Ca,L}$ amplitude from -250 ± 31 pA to -88 ± 19 pA ($P<0.001$; $n=8$ cells). For both KCl and CsCl-based solutions it was observed that sustained exposure to strophanthidin for several minutes caused spontaneous inward fluctuations in the membrane current record similar to the 'I_{TI}' (arrhythmogenic oscillatory transient inward) current shown for other cardiac cells.

5 When the calcium chelator BAPTA was added to the pipette solution (10 mM), the reduction in $I_{Ca,L}$ by strophanthidin was largely eliminated ($P>0.1$), and no spontaneous inward current fluctuations were observed after sustained exposure to strophanthidin ($n=8$ cells).

6 When external Ca in the perfusate was replaced with Ba, strophanthidin did not significantly reduce the Ba current through L-type calcium channels ($n=5$ cells).

7 We conclude that strophanthidin reduces $I_{Ca,L}$ by an indirect action, mediated by the rise in intracellular calcium (Ca_i) which follows inhibition of the Na/K pump caused by cardiac glycosides. The appearance of spontaneous I_{TI} with strophanthidin would also seem to be mediated by a rise in Ca_i, and may contribute to the spontaneous oscillations in membrane potential observed after prolonged strophanthidin exposure.

Keywords: Atrioventricular node; single cell; L-type calcium current; strophanthidin; cardiac glycoside

Introduction

Cardiac glycosides (cardiotonic steroids) have been used to treat congestive heart failure and abnormal heart rhythms for over two centuries (e.g. McMichael, 1982; Wray *et al.*, 1985; Levi *et al.*, 1994). Despite this, very little is known about the mechanism of antiarrhythmic action of these compounds on the atrioventricular node. The atrioventricular node (AVN) is normally the only route for conduction of the cardiac impulse from atria to ventricles (Tawara, 1906). Electrical conduction is slowed through this region (De Carvalho & De Almeida, 1960) allowing adequate ventricular filling prior to contraction and cells within the AVN can take over pacemaking of the ventricles should the primary pacemaker, the sinoatrial node (SAN), fail (Watanabe & Dreifus, 1968). The conduction properties of the AVN make it a vital region of the heart: abnormal AVN conduction can result in arrhythmogenesis (Goy & Fromer, 1991), whilst the AVN can provide a protective function during atrial fibrillation and flutter, since the slow conduction properties of the AVN means that only some impulses are transmitted, thereby protecting the ventricles from too fast a rate (see Levi *et al.*, 1994).

It has been known for many years that digitalis glycosides depress conduction through the AVN, without significantly altering conduction in atrial muscle or in the His-Purkinje system (e.g. Castellanos & Lemberg, 1963; Watanabe & Dreifus, 1966; 1970) and because of this these drugs are used to prolong AVN refractoriness in the treatment of atrial fibrillation and flutter. However, the cellular mechanism which underlies this antiarrhythmic action of digitalis glycosides on the AVN is almost completely unknown at present. There has only been one study of the cellular mechanisms of digitalis glycosides on the AVN thus far, utilising a small multicellular AVN preparation (Watanabe *et al.*, 1988). While this study provided useful qualitative information regarding effects of glycosides on the AVN, the widely accepted difficulties in using multicellular cardiac muscle preparations for quantitative voltage clamp studies (see Noble, 1984) indicate that a single-cell preparation is more suitable for electrophysiological investigations of the underlying mechanism.

In this study we have investigated the actions of the digitalis analogue strophanthidin on single cells isolated from the AVN of the rabbit heart (Hancox *et al.*, 1993). This preparation is ideal for electrophysiological studies since it has none of the disadvantages of the multicellular preparation for voltage clamp experiments. Whilst there is some heterogeneity in the action potential throughout the AVN (Akiyama and Fozzard, 1979; Kokubun *et al.*, 1980), evidence from single AVN cell

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experiments, as well as from multicellular preparations and the intact perfused heart, suggests that in a proportion of AVN cells the action potential upstroke is largely carried by L-type calcium current $I_{Ca,L}$ (Zipes & Fischer, 1974; Kokubun *et al.*, 1982; Hancox & Levi, 1994). The primary objective of the present study, therefore, was to investigate the action of strophanthidin on $I_{Ca,L}$ in the single AVN myocyte preparation.

Methods

Preparation of cells for recording

Rod and spindle shaped AVN myocytes were isolated from the hearts of male New Zealand White rabbits (1.5–2.5 kg) by the method described by Hancox *et al.*, (1993). (Note that [EGTA] added to the perfusate during the isolation was 100 μM and not 100 mM as published). AVN cells were placed in a chamber mounted on an inverted microscope (Nikon Diaphot) and superfused with Tyrode solution containing (in mmol l^{-1}): NaCl 130, KCl 2.7, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES 5, titrated to a pH of 7.4 with 4 mmol of NaOH. The experimental chamber had a volume of 75 μl and a flow rate of 2–4 ml min^{-1} (Levi, 1991). AVN cells were placed in the chamber as a cell suspension in 'KB' medium (Isenberg & Klockner, 1982), and allowed to settle on the glass bottom for 20 s. Perfusion was then started at a slow rate and gradually increased. In this way, flow rate could be increased without cells becoming dislodged and as the Ca-containing bath solution replaced KB, the cells were slowly re-exposed to Ca.

Electrophysiological recording

For electrophysiological recording, the whole cell patch clamp technique was used (Hamill *et al.*, 1981). Patch-pipettes (Corning 7052 glass, AM Systems Inc.) were pulled to resistances of 2.5–3 MΩ (Narashige PP83) and fire-polished to 3–6 MΩ (Narishige, MF83). The K-based internal dialysis solution contained (in mmol l^{-1}): KCl 110, NaCl 10, K_2ATP 5, $MgCl_2$ 0.4, HEPES 10, glucose 5, titrated to pH of 7.1 by adding 10 mmol KOH. The 'pipette-to-bath' liquid junction potential was measured for this filling solution and was found to be −2.7 mV. Since this value was small, no corrections of membrane potential were made. It has been shown previously that a caesium (Cs^+)-based internal dialysis solution blocks effectively outward potassium currents in single AVN myocytes, and this was used for selective recording of L-type calcium current ($I_{Ca,L}$; Hancox *et al.*, 1993). The Cs-based internal dialysis solution contained (in mmol l^{-1}): CsCl 113, NaCl 10, K_2ATP 5, $MgCl_2$ 0.4, HEPES 10, glucose 5, titrated to pH 7.1 by adding 10 mmol CsOH. The calcium chelator BAPTA (1,2,bis(2 aminophenoxy) ethane N,N,N′,N′-tetraacetic acid (10 μM Sigma) was included in the Cs-based internal dialysis solution for some experiments since this eliminates the intracellular calcium (Ca_i) transient and resulting sarcolemmal Na-Ca exchange in cardiac myocytes (e.g. Terrar & White, 1989; Hancox & Levi, 1995). Thus, inclusion of BAPTA blocked contamination of $I_{Ca,L}$ with Na-Ca exchange and other calcium-activated membrane currents (e.g. Giles & Imaizumi, 1989; Zygmunt & Gibbons, 1991). For all experiments a holding potential of −40 mV was used, since this lies near the 'zero-current' potential for AVN cells (Hancox *et al.*, 1993) and inactivates fast sodium current and transient outward potassium current. All recordings of membrane currents and action potentials were made using an Axopatch 200 amplifier (Axon Instruments) and a CV-201 headstage. Normally 80 to 90% of the electrode series resistance could be compensated. All experiments were performed at temperatures between 35–37°C.

Experimental solutions

Strophanthidin (Sigma) was dissolved in ethanol to give a 10 mM stock solution, which was kept in a light-tight container at 4°C. Aliquots of this were added to normal Tyrode solution to produce a solution with a final concentration of 50 μM. For barium-containing Tyrode solution 2 mM $CaCl_2$ was replaced with 2 mM $BaCl_2$. All test solutions were applied to cells using a multi-barelled 'in-house' built solution application device which could change bulk solution around a single myocyte within 2 s, whilst maintaining temperature at 35°C (Hancox *et al.*, 1995).

Generation of protocols, data acquisition and analysis

For voltage-clamp experiments, 500 ms duration voltage steps were applied at a frequency of 0.33 Hz. Specific protocols are detailed in the relevant 'results' sections. The voltage clamp command signal was generated by a Cambridge Electronic Design (CED) 1401 interface and 'WCP', a program written and supplied free of charge by John Dempster of Strathclyde University. Data were recorded onto the 'hard-disk' of a Viglen EX personal computer for off-line analysis using 'WCP'. Membrane currents were sampled at 2 kHz. Calcium current amplitudes were measured as the difference between the peak inward current at the start of the test pulse and the steady-state value at the end of the pulse. Graphs were drawn by use of 'FigP for Windows' software (Biosoft) and statistical analysis was performed with 'Systat for Windows'. Data are presented as means ± s.e.mean and, unless stated otherwise in the text, statistical comparisons were made by Student's *t*-test.

Results

Effects of strophanthidin on spontaneous action potentials

Figure 1 shows the effects of strophanthidin on spontaneous action potentials recorded from an AVN myocyte. Recordings were made from spontaneously beating AVN cells with the KCl-based internal dialysis solution. In normal Tyrode solution the maximum diastolic potential was near −65 mV and the action potential overshoot reached +15 mV in the cell shown in Figure 1. (These values are typical for AVN cells – see Hancox *et al.*, 1993.) After 30 seconds exposure to 50 μM strophanthidin, the maximum diastolic potential depolarized to −55 mV and the action potential amplitude decreased, such that the action potentials did not overshoot 0 mV significantly. There was also a small increase in spontaneous action potential rate, and a reduced action potential upstroke rate. With fur-

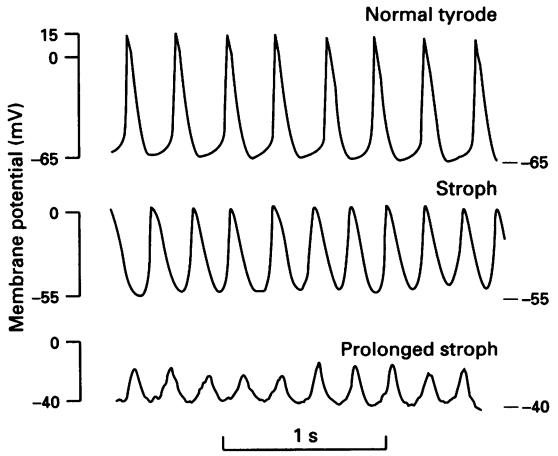

Figure 1 Spontaneous action potentials recorded from an AVN cell: in normal Tyrode solution (upper panel); 30 s after exposure to 50 μM strophanthidin (Stroph; middle panel) and after 4 min exposure to strophanthidin. The horizontal bar to the right of each trace represents a reference potential level (given numerically in mV).

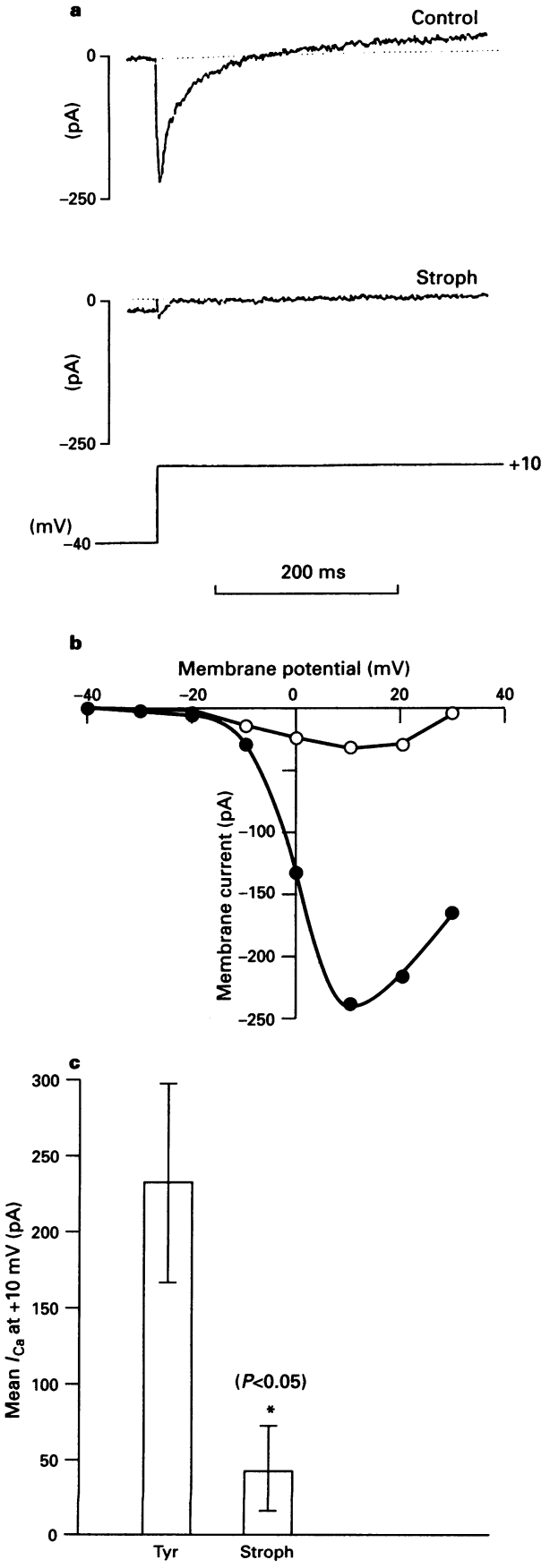

Figure 2 Effects of 50 μM strophanthidin (Stroph) on $I_{Ca,L}$ recorded with KCl-based pipette dialysis solution. (a) $I_{Ca,L}$ elicited by a pulse from −40 mV to +10 mV in normal Tyrode solution (Control; upper record) and after 2 min of Stroph (middle record). Voltage clamp test pulse shown in lower record. (b) Current-voltage relation for $I_{Ca,L}$ for the same cell in normal Tyrode solution (●) and after 2 min of Stroph (50 μM, ○). (c) Histogram showing mean amplitude of $I_{Ca,L}$ activated by pulses from −40 mV to +10 mV in normal Tyrode solution (Tyr) and after 2 min of Stroph. *P* value marks level of significance for *t* test comparison of data ($n=5$ cells).

ther exposure to strophanthidin, these effects became more marked. After four minutes exposure to strophanthidin, the membrane potential was further depolarized and the cell no longer exhibited clear diastolic pacemaker depolarizations, or clear action potentials – there were 'bell-shaped' depolarizations from the maximum diastolic potential of −40 mV. These effects are similar to those obtained by Watanabe *et al.*, (1988) for a multicellular AVN preparation. We observed similar results in each of three cells. After a prolonged exposure, the effects of strophanthidin were irreversible and cells eventually went into contracture.

Effects of strophanthidin on $I_{Ca,L}$ recorded with KCl-based dialysis solution

To investigate the actions of strophanthidin in more detail, we performed voltage-clamp experiments with the KCl-based internal dialysis solution. The action potential upstroke in at least a proportion of AVN cells and multi-cellular tissue is carried predominantly by L-type calcium current, $I_{Ca,L}$ (Zipes & Fischer, 1974; Kokubun *et al.*, 1982; Hancox & Levi, 1994). Figure 2 illustrates the effects of strophanthidin application on this current. Figure 2a shows the current elicited by a depolarizing pulse from −40 mV to +10 mV in normal Tyrode solution (upper panel) and after 2 min application of strophanthidin (lower panel). In normal Tyrode solution a rapidly activating inward $I_{Ca,L}$ was observed, and after 2 min exposure to strophanthidin the amplitude of this current was decreased greatly. There was also an inward shift in the current with a holding potential of −40 mV, following strophanthidin exposure. Figure 2b shows the effects of strophanthidin on $I_{Ca,L}$ in the same cell over a range of potentials between −40 mV and +30 mV. $I_{Ca,L}$ is known to exhibit a bell-shaped voltage dependence in AVN and other cardiac cells (Pelzer *et al.*, 1992; Hancox & Levi, 1994), attaining a maximum amplitude in AVN cells with pulses to +10 mV. After 2 min exposure to strophanthidin the inward current elicited at all potentials was much smaller than in normal Tyrode solution. Figure 2c shows the mean effect of strophanthidin application on peak $I_{Ca,L}$ (elicited by pulses from −40 mV to +10 mV) in five cells in normal Tyrode solution and after 2 min of strophanthidin application. Strophanthidin inhibited $I_{Ca,L}$ at +10 mV by over 80%, under these recording conditions.

Effects of strophanthidin on $I_{Ca,L}$ with a Cs-based dialysis solution

The results in Figure 2, indicate strongly that strophanthidin reduces $I_{Ca,L}$ amplitude in single AVN cells, with a pipette dialysis solution identical to that used to record spontaneous action potentials. However, with a KCl-based dialysis solution it is possible that recordings of $I_{Ca,L}$ might be contaminated with outward potassium currents, therefore further experiments were performed with a Cs-based pipette solution to make selective recordings of $I_{Ca,L}$ in AVN cells (Hancox & Levi, 1994).

Figure 3a shows the effects of strophanthidin application on $I_{Ca,L}$ elicited by a depolarizing pulse from −40 mV to +10 mV, with the Cs-based dialysis solution. The upper panel illustrates the current activated by the test pulse in normal Tyrode solution, and the lower panel shows the current one minute after the perfusate had been rapidly switched to one containing 50 μM strophanthidin. Clearly, the amplitude of $I_{Ca,L}$ recorded with the Cs-based solution was markedly re-

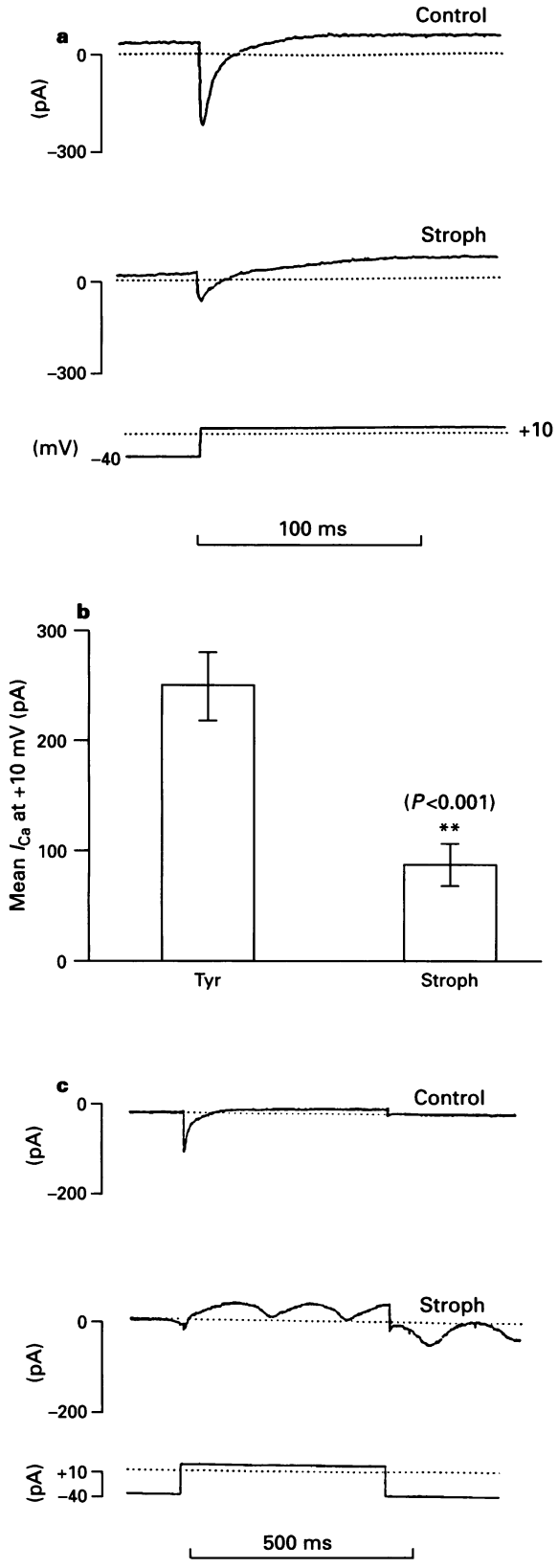

Figure 3 Effects of 50 μM strophanthidin (Stroph) on $I_{Ca,L}$ recorded in CsCl-based pipette dialysis solution. (a) $I_{Ca,L}$ elicited by a pulse from −40 mV to +10 mV in normal Tyrode solution (Control; upper record) and after 1 min of Stroph (middle record). Voltage clamp test pulse shown in lower record. (b) Histogram showing mean amplitude of $I_{Ca,L}$ activated by pulses from −40 mV to +10 mV in normal Tyrode solution (Tyr) and after 1 min of Stroph. P value marks level of significance for t test comparison of data ($n=8$ cells). (c) Effect of sustained application of strophanthidin on membrane current. Upper record: control; middle record: 3 min of Stroph; lower record: voltage clamp test pulse.

duced by exposure to strophanthidin. Figure 3b shows mean data from eight cells. The histogram shows the mean amplitude of $I_{Ca,L}$ elicited by test pulses from −40 mV to +10 mV in normal Tyrode solution and after a one minute exposure to strophanthidin. In eight cells the amplitude of $I_{Ca,L}$ was reduced by nearly 60% and the difference between the control amplitude of $I_{Ca,L}$ and that in strophanthidin was highly significant. When the mean amplitude of $I_{Ca,L}$ after exposure to strophanthidin was compared in cells dialysed with the KCl-based solution and cells dialysed with the CsCl-based solution, the difference in amplitude was not found to be statistically significant ($P>0.2$), indicating that $I_{Ca,L}$ was reduced to a similar level with K- and Cs-based internal dialysis solutions.

Figure 3c shows an additional effect of strophanthidin application. We observed (for both KCl and Cs-based dialysis solutions) that sustained application of strophanthidin resulted in the development of oscillations in the membrane current record. The time taken for such oscillations to develop varied from cell-to-cell, but in general these appeared after 3 to 5 min exposure to strophanthidin. The cell illustrated in Figure 3c exhibited a clear $I_{Ca,L}$ in normal Tyrode solution. However, after 3 min of exposure to 50 μM strophanthidin, not only was the inward $I_{Ca,L}$ greatly reduced in amplitude, but clear oscillations of the current record were apparent during the current elicited by the depolarizing test pulse, and in the current record after the pulse. Similar observations were made from each of five cells.

Effects of strophanthidin on cells dialysed with 10 mM BAPTA- containing pipette solution

The experiments with K- and Cs-based dialysis solutions indicate that $I_{Ca,L}$ in AVN cells becomes markedly reduced by exposure to strophanthidin, under these conditions. This could result from a *direct* effect of strophanthidin on the calcium channel, or an *indirect* effect of raised intracellular sodium (Na) produced by Na-K pump inhibition with strophanthidin. A rise in intracellular Na will result in an increased intracellular calcium (Ca_i) via Na-Ca exchange, and the rise of Ca_i could then produce Ca-induced inactivation of the Ca channel (e.g. Boyett *et al.*, 1988; Levi, 1991; Levi *et al.*, 1994).

In order to distinguish between these two possibilities we added 10 mM BAPTA to the Cs-based dialysis solution, to attenuate the rise in Ca_i which might occur following exposure to strophanthidin. In cells dialysed with a Cs-based solution alone, it was possible to observe phasic contractions with each test pulse. In cells dialysed with a Cs-based solution containing 10 mM BAPTA for several minutes no contractions were evident when test pulses were applied, indicating that BAPTA dialysis was effective in attenuating rises in Ca_i under our recording conditions. Figure 4 demonstrates the effects of adding BAPTA to the intracellular dialysis solution on the strophanthidin response. Figure 4a shows $I_{Ca,L}$ elicited by a pulse from −40 mV to +10 mV, with the Cs/BAPTA based pipette solution – in normal Tyrode solution (upper panel) and after a 1 min exposure to strophanthidin (lower panel). It is clear that $I_{Ca,L}$ was only slightly smaller in strophanthidin-containing solution compared to normal Tyrode solution. Figure 4b shows the peak $I_{Ca,L}$ amplitude measured at +10 mV in eight cells dialysed with Cs/BAPTA solution in normal Tyrode solution, and after one minute of strophanthidin exposure. Although there was a slight reduction in $I_{Ca,L}$ amplitude over this period, this was not statistically significant, suggesting that inclusion of BAPTA in the dialysis solution largely eliminated the $I_{Ca,L}$ blocking action of strophanthidin.

Figure 4c shows the effects of sustained application of strophanthidin application on the membrane current record, with BAPTA included in the pipette solution. In the absence of BAPTA, oscillations in the current record became apparent after 3–5 min exposure (Figure 3c). However, it is clear from Figure 4c, which shows records in normal Tyrode solution and after 3 min exposure to strophanthidin, that not only was $I_{Ca,L}$

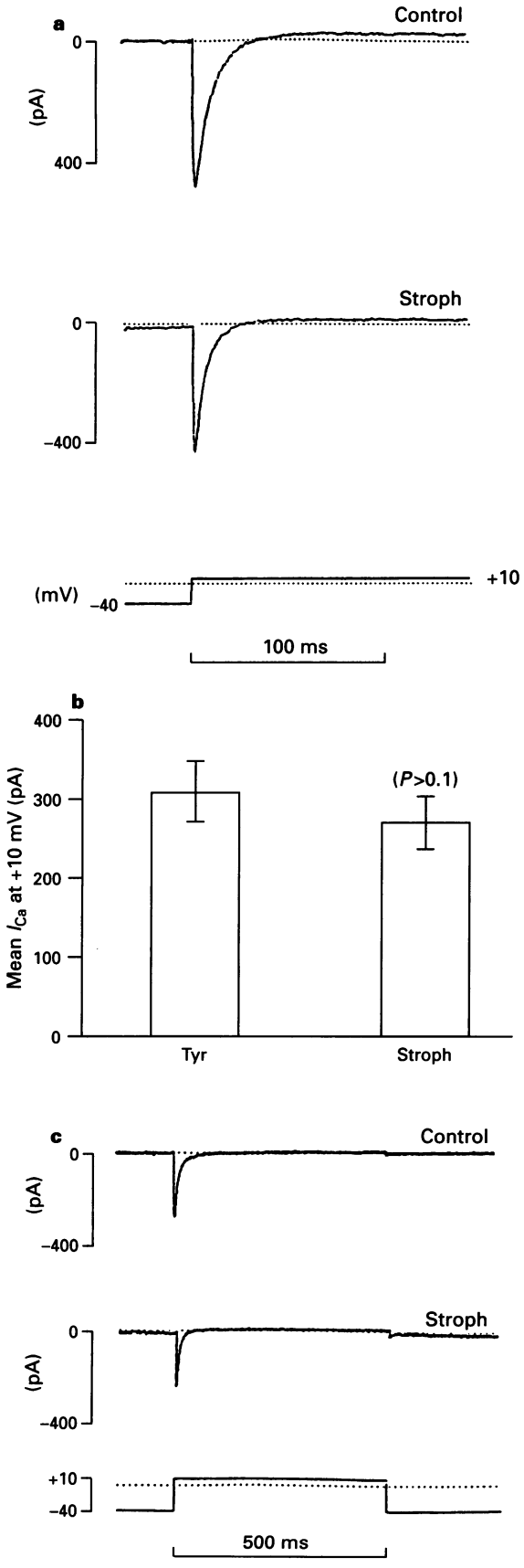

Figure 4 Effects of 50 μM strophanthidin (Stroph) on $I_{Ca,L}$ recorded with CsCl-based pipette dialysis solution containing 10 mM BAPTA. (a) $I_{Ca,L}$ elicited by a pulse from −40 mV to +10 mV in normal Tyrode solution (Control; upper record) and after 1 min of Stroph (middle record). Voltage clamp test pulse shown in lower record. (b) Histogram showing mean amplitude of $I_{Ca,L}$ activated by pulses from −40 mV to +10 mV in normal Tyrode solution (Tyr) and after 1 min of Stroph. P value marks level of significance for t test comparison of data ($n=8$ cells) (c) Effect of sustained application of strophanthidin on membrane current in a cell dialysed with BAPTA containing pipette solution. Upper record: control; middle record: 3 min of Stroph; lower record: voltage clamp test pulse.

well maintained but also that oscillations in membrane current were not observed. Similar results were observed in each of four cells.

Effect of replacing external Ca with Ba on the strophanthidin response

Adding BAPTA to the Cs-containing pipette solution appeared to eliminate largely the effects of strophanthidin application on $I_{Ca,L}$, suggesting that the effect of strophanthidin on this current might be an *indirect one*. To test this possibility further, we replaced calcium in the external solution by 2 mM barium (Ba) and used the Cs/BAPTA-based dialysis solution in order to eliminate entirely a rise in Ca_i. Figure 5 shows the effects of replacing external Ca with Ba on the strophanthidin response. Figure 5a shows barium current through the Ca channel ($I_{Ba,L}$) elicited by pulses from −40 mV to +10 mV in Tyrode solution (upper panel) and after 1 min of strophanthidin exposure (lower panel). Following strophanthidin exposure, the current amplitude became slightly reduced.

Figure 5b shows peak $I_{Ba,L}$ amplitude measured at +10 mV in Tyrode solution and following 3 min strophanthidin application ($n=5$ cells). Although $I_{Ba,L}$ became slightly reduced following strophanthidin exposure, this was not statistically significant. In fact, when Ba replaced Ca as the charge carrier through the channel, we observed that the amplitude of $I_{Ba,L}$ declined over time in normal Tyrode solution. This raised the possibility that the apparent reduction in $I_{Ba,L}$ following strophanthidin exposure might be simply a result of 'run down' of the current. Figure 5c shows the mean amplitude of $I_{Ba,L}$ in five cells after 3 min exposure to strophanthidin, compared with that for four cells at the same point in time after exposure to Ba-Tyrode alone. The mean $I_{Ba,L}$ amplitude in the two conditions was almost identical, and this suggests that strophanthidin did not have a significant blocking effect on $I_{Ba,L}$.

Discussion

Strophanthidin and spontaneous action potentials

In this study, application of strophanthidin produced a progressive depolarization of the maximum diastolic potential, a decrease in action potential amplitude and a decrease in action potential upstroke rate. With sustained application, spontaneous action potentials were abolished, giving way to spontaneous oscillations in membrane potential. These effects are similar to those obtained for the small multicellular AVN preparation used by Watanabe *et al.*, (1988). These workers observed also that 1 μM acetylstrophanthidin (AcS) reduced action potential amplitude and upstroke velocity. When they raised the concentration of AcS to 3 μM these effects were enhanced and spontaneous activity no longer exhibited a clear diastolic pacemaker depolarization, and changes in membrane potential became 'bell-shaped' oscillations. These dose-dependent effects (Watanabe *et al.*, 1988) are similar to the time-dependent effects we observed on single AVN cells with 50 μM strophanthidin. Toda and West (1969) observed complete block of AV nodal conduction with 10 $\mu g\,ml^{-1}$ (1.4 μM) ouabain, in an isolated preparation containing atrium, SAN, AVN and His-bundle. The same dose of ouabain was also observed to reduce the maximum diastolic potential, amplitude and upstroke velocity of action potentials in the SAN and AVN (Toda & West, 1969). A more recent study with a multicellular SAN preparation shows spontaneous activity giving way to membrane potential oscillations with a depolarized

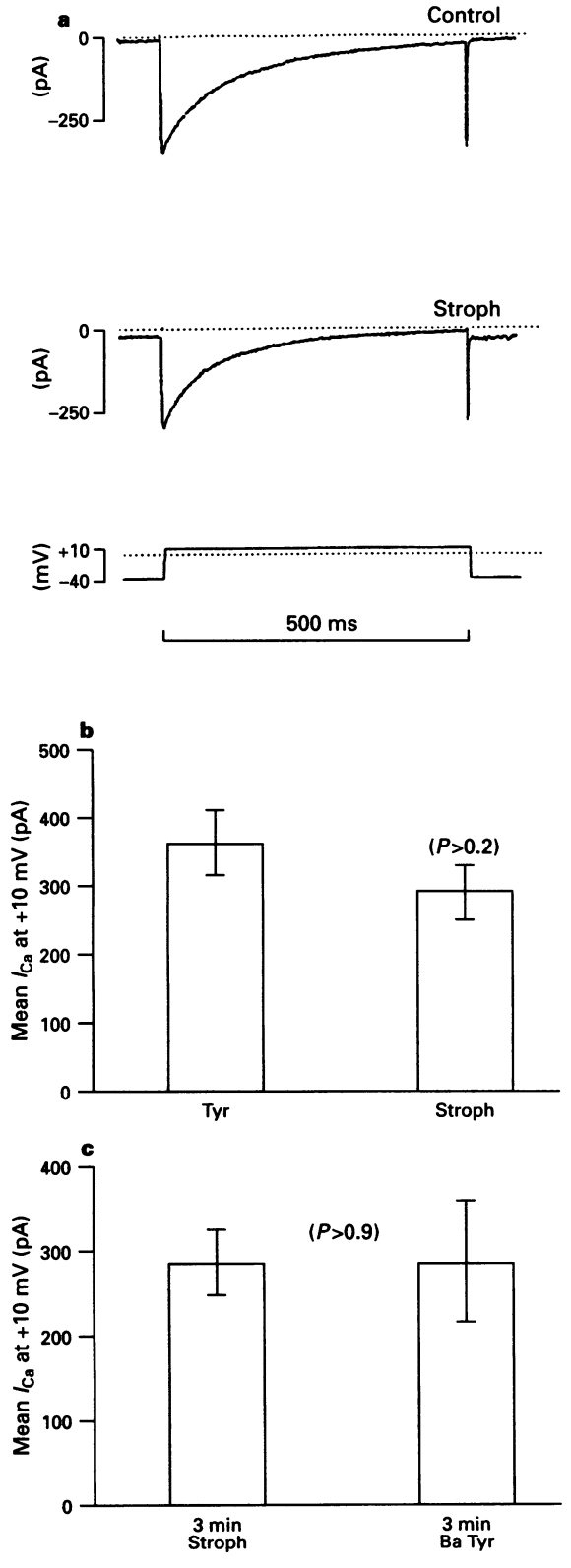

Figure 5 Effects of 50 μM strophanthidin (Stroph) on $I_{Ca,L}$ in cells exposed to a perfusate in which Ca is replaced by Ba as the charge carrier. Recordings made with CsCl-based pipette dialysis solution containing 10 mM BAPTA. (a) $I_{Ba,L}$ elicited by a pulse from −40 mV to +10 mV in normal Tyrode solution (Control; upper record) and after 1 min of Stroph (middle record). Voltage clamp test pulse shown in lower record. (b) Histogram showing mean amplitude of $I_{Ba,L}$ activated by pulses from −40 mV to +10 mV in normal Tyrode solution (Tyr) and after 3 min of Stroph. *P* value marks level of significance for *t* test comparison of data ($n=5$). (c) Histogram comparing mean amplitude of $I_{Ba,L}$ in cells exposed to Stroph for 3 min ($n=5$) with that from cells with no addition of Stroph to the superfusate ($n=4$). In both (b) and (c), the *P* values mark the level of significance for *t* test comparison of data.

maximum diastolic potential following sustained exposure to 1 μM ouabain (Satoh, 1994). Thus our results with strophanthidin are similar to those obtained with AcS and ouabain in previous studies in a variety of SAN and AVN preparations. Na-K pump inhibition by strophanthidin has been demonstrated previously to produce membrane potential depolarization in the AVN (Kurachi *et al.*, 1981), and this is consistent with the inward shift in holding current observed in our experiments (e.g. Figure 2a) following strophanthidin exposure. It is possible, therefore, that Na-K pump inhibition may account for the more depolarized maximum diastolic potential during spontaneous activity observed following glycoside exposure.

Mechanism of $I_{Ca,L}$ *blockade by strophanthidin*

The reduction in $I_{Ca,L}$ produced by strophanthidin in single AVN cells dialysed with K-based and Cs-based pipette solutions is consistent with previous studies showing that cardiac glycosides can reduce $I_{Ca,L}$ in ventricular myocytes (Fischmeister *et al.*, 1986; Le Grand *et al.*, 1990; Levi, 1991). Fischmeister *et al.* (1986) found that in some cells ouabain, dihydroouabain and strophanthidin produced an initial increase in $I_{Ca,L}$ followed by a decline in this current during sustained application. However, their results might have been complicated by progressive dephosphorylation of Ca channels (see Levi *et al.*, 1994). Subsequent studies (Le Grand *et al.*, 1990; Levi, 1991) have indicated that, under normal physiological conditions, application of glycoside results only in a reduction of $I_{Ca,L}$ in ventricular cells. In the multicellular AVN, 10^{-6} μM AcS blocked the early inward current by 70% (Watanabe *et al.*, 1988). This is similar to the degree of $I_{Ca,L}$ reduction we observed with a K-based pipette solution.

There are two main possible mechanisms by which strophanthidin could have reduced $I_{Ca,L}$ in AVN cells. First, it could have a direct blocking effect on the L-type calcium channel. Second, it could exert its effect *indirectly*, as a result of Na-K pump blockade leading to a rise in Ca_i and to greater Ca-induced inactivation of $I_{Ca,L}$ (e.g. Chad *et al.*, 1984; Boyett *et al.*, 1988; Levi, 1991). Watanabe *et al.*, (1988) attempted to record $I_{Ca,L}$ selectively in their small multicellular AVN preparation by loading the preparation with Cs to block K currents and using caffeine to block the Ca_i transient. These workers found that $I_{Ca,L}$ was still reduced under these conditions – but caffeine would not have eliminated rises in Ca_i due to increased Ca-loading and so it is not possible to make mechanistic conclusions from their experiments. Our data, with a Cs-based dialysis solution to record $I_{Ca,L}$ selectively, appears to indicate that the action of strophanthidin on $I_{Ca,L}$ in AVN cells is an indirect one. When 10 mM BAPTA was added to the internal dialysis solution to minimize a rise in Ca_i, there was no significant blockade of $I_{Ca,L}$ by strophanthidin. This suggests that a rise in Ca_i is required for strophanthidin to exert an action on $I_{Ca,L}$. Consistent with the present data, in a microelectrode study of guinea-pig ventricular myocytes. Levi (1991) found that diffusing BAPTA from the microelectrode into the cell attenuated markedly the reduction of $I_{Ca,L}$ with glycoside. Moreover, when Ba was substituted for Ca in the extracellular perfusate no reduction of $I_{Ca,L}$ with glycoside could be observed (Levi, 1991). We observed only a small (statistically insignificant) effect of strophanthidin on $I_{Ba,L}$. When the current amplitude in the presence of strophanthidin was compared with that from cells exposed only to Ba-solution for the same length of time, there was no difference in current amplitudes. Thus, this study is consistent with an indirect mode of action for strophanthidin on $I_{Ca,L}$.

Strophanthidin has been shown clearly to block the Na-K pump in the small multicellular AVN preparation (Kurachi *et al.*, 1981) and we have shown directly that AVN cells have sarcolemmal Na-Ca exchange, since removing extracellular Na_i raises Ca_i (Hancox *et al.*, 1994). It is likely, therefore, that strophanthidin application increases Na_i since sodium extrusion is reduced and that this, in turn, raises Ca_i by increasing

calcium influx (or reducing Ca efflux) *via* the Na-Ca exchange. This mechanism is supported by the results of other studies where low external potassium (K_O) was used to inhibit the Na-K pump. Low K_O causes an increase in Na_i (Ellis, 1977) which raises Ca_i and increases contraction (Eisner *et al.*, 1981; Levi & Boyett, 1991). Interestingly, low K_O also leads to a reduction in $I_{Ca,L}$ (e.g. Fischmeister *et al.*, 1986; Boyett *et al.*, 1988) and the mechanism for this effect may be similar to that for strophanthidin – a rise in Ca_i and Ca-induced inactivation of $I_{Ca,L}$.

Oscillatory inward currents following sustained strophanthidin application

After several minutes exposure to strophanthidin, oscillations in current could be observed – both during a test pulse and after repolarization. These oscillatory inward currents were not present in cells dialysed with a BAPTA-containing pipette solution, suggesting that they were mediated in some way by a rise in Ca_i. This current, therefore, appears to be the arrhythmogenic oscillatory transient inward current (I_{TI}) which has been described in other cardiac cells when Ca_i is elevated. I_{TI} has been induced in Purkinje fibres by cardiac glycosides and reduced K_O (both of which inhibit Na-K pump activity; e.g. Lederer & Tsien, 1976; Eisner & Lederer, 1979; Eisner *et al.*, 1983) and glycoside application to the small multicellular SAN preparation also induced I_{TI} and arrhythmias in response to Ca_i overload (e.g. Satoh, 1994). Inward current fluctuations are also evident in some records following AcS application to a small multicellular AVN preparation (Watanabe *et al.*, 1988). Ca_i oscillations produced by strophanthidin are abolished by ryanodine (Eisner & Valdeolmillos, 1986), implicating spontaneous release of Ca from the sarcoplasmic reticulum (SR) in response to Ca_i overload as the mechanism. We have obtained evidence previously for SR Ca release in AVN cells (Hancox *et al.*, 1994). The most likely mechanism for the spontaneous current oscillations we observed thus involves progressive Ca_i-loading of the cell during exposure to strophanthidin, leading to oscillatory release of Ca from the SR and a resulting oscillatory current generated by the Na-Ca exchange.

Implications

The effect of strophanthidin to reduce $I_{Ca,L}$ described in this study is likely to be an important mechanism for the decrease in the AVN action potential amplitude and upstroke rate which occur with digitalis glycoside, since $I_{Ca,L}$ is an important current in generating the AVN action potential upstroke (Zipes & Fischer, 1974; Kokubun *et al.*, 1982; Hancox & Levi, 1994). Action potential upstroke velocity influences directly conduction velocity, and the depression of AVN conduction is likely to result in part from these changes to the action potential (Watanabe & Dreifus, 1966; 1970; Watanabe *et al.*, 1988). The rise in Ca_i following Na-K pump inhibition by glycoside might also be expected to decrease conduction through the AVN, since rises in Ca_i can lead to closing of gap junctions between cells, and thereby reduce cellular coupling (Ikeda *et al.*, 1980). The spontaneous 'bell shaped' oscillations in membrane potential following sustained exposure to strophanthidin are likely to result from oscillatory inward currents generated by the Na-Ca exchange in response to Ca_i overload. It is difficult to extrapolate from the present data, with a single large dose of an experimental glycoside, to the clinical situation in which actions result from the cumulative effects of repetitive low doses of clinically used compounds. Cardiac glycosides do have a low therapeutic index, however, and consequently their use is associated with a risk of cardiotoxicity and arrhythmogenesis. Oscillatory currents in the AVN following toxic levels of glycoside application might be expected to produce abnormal rhythms in this region, and it is possible that such a mechanism could contribute to the proarrhythmogenic effects of toxic digitalis glycoside levels.

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