The effects of calcium antagonists on calcium overload contractures in embryonic chick myocytes induced by ouabain and veratrine

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1 The protective effects of some calcium antagonists against different forms of calcium overload contracture were investigated in embryonic chick cardiac myocytes.

2 Tetrodotoxin-sensitive sodium currents were recorded from the myocytes by the whole-cell voltage-clamp technique. Although the peak current was attenuated by veratrine, the inactivation process was markedly inhibited, resulting in a large increase in the total inward current. Action potentials were prolonged by veratrine, automaticity was inhibited and the membrane potential depolarized from -79 to around -45 mV.

3 Measurements of contraction were made from aggregates of myocytes using a video edge detection technique which quantified edge movement. Veratrine caused an initial positive inotropism then inhibited automaticity of aggregates with subsequent development of a tonic contracture to around 300% of the twitch contraction.

4 Veratrine-induced contractures were not significantly affected by $10\text{ }\mu\text{M}$ diltiazem or verapamil. Nifedipine ($5\text{ }\mu\text{M}$), nimodipine ($5\text{ }\mu\text{M}$) and ryanodine ($5\text{ }\mu\text{M}$) also had little effect whilst nicardipine and flunarizine caused a concentration-dependent inhibition of veratrine-induced contractures with IC_{50} s of $3\text{ }\mu\text{M}$ and $2\text{ }\mu\text{M}$ respectively.

5 Veratrine-induced contractures were found to be very sensitive to extracellular calcium concentration with an EC_{50} of $32\text{ }\mu\text{M}$. Edge movement associated with beating of the myocytes was much less sensitive to calcium ($\text{EC}_{50} = 1\text{ mM}$). Submaximal veratrine contractures in $20\text{--}50\text{ }\mu\text{M}$ extracellular calcium were not potentiated by $1\text{ }\mu\text{M}$ Bay K 8644.

6 Tetrodotoxin also inhibited veratrine-induced contractures but did not affect contractions induced by ouabain in the presence of $10\text{ }\mu\text{M}$ diltiazem.

7 Ouabain-induced contractures were also inhibited by nicardipine and flunarizine indicating that these drugs can protect against calcium overload in embryonic chick heart by a mechanism independent of the normal form of voltage-sensitive sodium or calcium channels.

Introduction

The level of intracellular calcium in quiescent 13 day-old embryonic chick myocytes has been shown to be around 105 nM (Kim & Smith, 1988). These low levels are maintained by sequestration into intracellular stores such as mitochondria and the sarcoplasmic reticulum and extrusion from the cell, predominantly by the sodium-calcium exchanger (Barry *et al.*, 1985). Since the activity of this exchange mechanism is dependent on the gradient of sodium across the plasma membrane, its ability to extrude calcium is dependent on maintaining a low level of intracellular sodium. Intracellular sodium in myocytes is normally 10 mM at rest (Chapman &

Rodrigo, 1987) and this can increase with contractile activity to 20 mM (Mullins, 1981). Under conditions of ischaemia where the ATP supply is compromised and the activity of the Na-K-ATPase reduced it has been shown that intracellular sodium levels rise (Turnstall *et al.*, 1986). This will result in a reduction of the sodium gradient across the cell membrane and thus a reduction of extrusion of calcium by the calcium-sodium exchanger. Such a process may lead to calcium overload which is known to result in cell death (Allen *et al.*, 1985). Thus the basic process preceding calcium overload may be sodium overload of the cell. We were interested in the effect of calcium antagonists on calcium overload caused by sodium overload and have followed up the work of Ver

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Donk *et al.* (1986) who caused rounding up of rat myocytes with veratrine, a mixture of the ceveratrum alkaloids which are known to inhibit sodium channel inactivation and thus increase membrane sodium permeability in nerve (Ulbricht, 1969) and heart muscle (Horakova & Vassort, 1973; see Honerjäger, 1982 for a review). Sperelakis & Pappano (1969) and later Romey *et al.* (1980) showed that veratrine and veratridine (the active component of veratrine) caused a depolarization and cessation of beating in chick myocytes. Ver Donk *et al.* (1986) demonstrated selective protection against veratrine-induced rounding-up of myocytes by calcium antagonists. Embryonic chick myocytes have been used in the study of calcium transport and homeostatic mechanisms. In common with other heart muscle these cells have two types of calcium current (Anderson *et al.*, 1988; Anderson & Patmore, 1988) and have been extensively used in the study of cardiac ionic transport processes. Aggregates of embryonic chick myocytes display automaticity and develop a 'tone' which increases when the rate of beating is increased or in the presence of positive inotropic agents and is decreased if automaticity is abolished, if extracellular calcium is reduced or in the presence of calcium antagonists (Patmore & Whiting, 1985; Patmore, 1987). The diastolic tone of these cells thus appears to reflect the level of intracellular calcium which cannot be extruded from the cell or sequestered before the next beat. This preparation is thus a useful model for investigating calcium homeostasis in cardiac cells and we have assumed that the contractile state is a reflection of free intracellular calcium.

In the present work we have looked at the effect of veratrine on contractions of embryonic chick myocytes and give an account of the underlying electrophysiological changes which occur. Sodium overload and subsequent calcium overload of embryonic chick myocytes has also been induced by the cardiac glycoside, ouabain. The effects of several different calcium antagonists on veratrine- and ouabain-induced contractures are described.

Methods

Cell cultures

Hearts were obtained from 11 day old Leghorn K chicken embryos by sterile dissection. Cell dispersion and culture techniques were as described by Clusin (1981). Briefly, ventricular fragments were washed in Hanks calcium- and magnesium-free balanced salt solution (GIBCO) and thereafter in 0.05% trypsin solution. This digestion process was repeated 3–4 times and trypsinisation was terminated after 5 min

by dilution of the cells in an equivalent volume of Hanks balanced salt solution containing 20% foetal calf serum. The resulting cell suspension was pooled, centrifuged and the pellet resuspended in culture medium to give a final cell density of $3\text{--}4 \times 10^5$ cells ml^{-1} . This cell suspension was then dispensed into 96 well trays (Costar), the bottom of each well having been previously coated with a layer of Sylgard 184 (Dow Corning) silicon elastomer resin. This treatment prevents the formation of monolayers and promotes the aggregation of cells into spherical clusters (80–160 μm diameter) over 3 days in culture. The culture medium contained 20% M-199 (GIBCO), 6% heat-inactivated foetal calf serum (Northumbria Biologicals), 1% penicillin-streptomycin solution and 73% potassium-free salt solution which was composed of (mM): NaCl 116, NaHCO_3 26.2, MgSO_4 0.8, NaH_2PO_4 0.9, CaCl_2 1.8 and dextrose 5.5, adjusted to pH 7.3.

Solutions

The perfusion solution was designed to resemble the ionic composition of the culture media and contained (mM): NaCl 110, KCl 1, MgSO_4 0.8, HEPES 19.3, NaHCO_3 0.83 and dextrose 5.5, pH was adjusted to 7.4. The calcium concentration was routinely 2 mM CaCl_2 . In some experiments the calcium concentrations were varied by either adding more CaCl_2 or buffering the calcium with EGTA. Free calcium concentrations were calculated taking into account the ionic effects of the salt solution, temperature and pH on the dissociation constant for EGTA according to Miller & Smith, 1984 and Miller, D.J. (personal communication) ($-\log K_{\text{app}} = 6.44$). In some experiments the superfusate Na level was also raised to 150 mM by adding more NaCl to increase the extra/intracellular gradient for sodium ions. When 40 mM TMA was added in place of sodium no osmotic contracture was observed.

Electrical measurements

Whole-cell voltage-clamp recordings were made on single myocytes which were typically around 5 μm wide and 20 μm in length. Pipettes were made from Garner glass (Corning No. 7052) and were fire polished and filled with a solution containing (mM): KCl 140, NaCl 10, MgCl_2 1, EGTA 11, CaCl_2 2.43, HEPES 10, glucose 5 at pH 7.2. Experiments were carried out at room temperature and under these conditions the free calcium ion concentration was estimated to be 10^{-7} M. Electrodes had resistances of 2–3 M Ω . Recordings were made according to the methods of Hamill *et al.* (1981) using an EPC-7 amplifier (List Electronics, FRG). Both current clamp and voltage clamp recordings were made.

Intracellular recordings were made from aggregates of embryonic chick myocytes which were prepared as described above. Micropipettes were pulled from 1.5 mm o.d. borosilicate glass and filled with 3 M KCl. Differential recordings were made with reference to a bath micropipette using a high impedance differential amplifier (Firbank Electronics, Norwich). Electrical signals were recorded on a galvanometric chart recorder.

Assessment of contractility

Contractility was estimated by measuring edge movement of aggregates of embryonic chick heart cells. Aggregates were initially grown on silicon elastomer and then transferred to normal tissue culture treated Lux select-a-well plates. After a period of 10–15 min the aggregates developed contacts with the dish such that they could be superfused quite rapidly. The wells contained approximately 200 μ l of fluid and were superfused at a rate of 2.5 ml min⁻¹ with salt solution prewarmed to 37°C. The well was mounted on the stage of an inverted microscope with a video camera (National Panasonic WV1500) attached to the camera port. A video image of an aggregate of cells magnified to 200 $\times$ was displayed on a monitor screen. An area of the aggregate which contained an edge which displayed significant movement was selected for analysis using a video motion detection system (Tephconics Ltd, Edinburgh). A window was superimposed on the image (see Patmore & Whiting, 1985), this was typically one-third the width of the screen and 20–30 video lines high. Each of these lines was scanned to determine the point where the image changed from light (background) to dark (image) and the area of the window occupied by the aggregate calculated by integration and updated every 20 ms. On contraction the area of window occupied by the aggregate changes and an analogue output of this area describes the waveform of edge movement of the aggregate which was typically 2–5 μ m. Responses were recorded on a potentiometric chart recorder and in some cases recorded on magnetic tape for reproduction. Contractures or relaxations of aggregates are expressed as % of the twitch edge movement of an individual aggregate.

Drugs

Tetrodotoxin, veratrine, veratridine, ouabain (strophanthin G), diltiazem, verapamil and flunarizine were all obtained from Sigma and dissolved in distilled water. Nicardipine (Syntex), nifedipine and nimodipine (Bayer) were dissolved in ethanol. Concentrated stock solutions of all drugs were prepared and aliquots were added to the salt solution to

achieve the concentrations required. Final ethanol levels were always less than 0.1%.

Statistical analyses

The effects of drugs on veratrine- or ouabain-induced contractures were analysed for significance by an unpaired Student's *t* test. The control group were responses obtained in the absence of drug on the same day. Data shown are mean values $\pm$ s.e. of mean for *n* experiments as shown.

Results

Effects of veratrine on sodium currents and action potentials

When single myocytes were held under voltage clamp at potentials more negative than -60 mV, applying step depolarizations evoked inward currents. Peak inward currents were observed with steps to around -35 mV. The action of veratrine on the inward current is shown in Figure 1A. In the presence of 100 μ g ml⁻¹ veratrine the peak inward current is attenuated and the inactivation process is inhibited. Under control conditions the sodium currents inactivated rapidly with a $t_{1/2} = 1.0 \pm 0.1$ ms (*n* = 3) and this process could be fitted by two exponentials with time constants of 0.85 ms and 5 ms. In the presence of veratrine there was an initial rapid inactivation but the second slower (5 ms) inactivation process was inhibited and this resulted in a very slow decay of the current which was not complete after 300 ms and produced a large increase in the total inward current. This effect is shown quite clearly on a longer time-scale in Figure 1B. Subsequent application of tetrodotoxin abolished the inward currents indicating that they were carried by sodium ions (Figure 1A). The effects of veratrine on the action potential are shown in Figure 1C. This cell was held under both voltage-clamp and current-clamp. In the current clamp mode the membrane potential, initially -64 mV, was held at -85 mV and small depolarizing current pulses of 1 ms duration were applied which evoked action potentials. Under control conditions the action potentials were 100 mV magnitude and around 70 ms long (duration to 90% repolarization). In the presence of veratrine the peak potential was reduced and the duration greatly increased such that complete repolarization took almost 1000 ms.

Effects of veratrine on aggregates of myocytes

Aggregates of embryonic chick myocytes display automaticity with a beating rate of 60–115

Figure 1 The effects of veratrine on sodium currents and action potentials in embryonic chick heart myocytes. (A) Averaged records of 4 current responses evoked from depolarizations from -85 mV to -35 mV, (a) control, (b) in the presence of $100 \mu\text{g ml}^{-1}$ veratrine, (c) with subsequent application of $1 \mu\text{M}$ tetrodotoxin (TTX). (B) Inward sodium currents on a slower timebase showing the very slow inactivation of the sodium current during the clamp pulse and then on repolarization a slowly decaying tail current. (C) In the same cell switched into current clamp with the membrane potential held at -85 mV, small depolarizing current pulses of 1 ms duration evoked action potentials. Action potential duration is prolonged in the presence of veratrine.

(mean = 107 ± 4 , $n = 20$) beats min^{-1} . Intracellular recordings from these aggregates showed maximum diastolic potentials of -79 ± 4 mV and peak potentials of around 20 mV. Veratrine caused a progressive prolongation of the action potential and a concomitant depolarization until automaticity was abolished and the membrane potential stabilised at -45 ± 10 mV ($n = 3$). Simultaneous recordings of edge movement were made from a few cells and these changes in electrical behaviour were paralleled by an initial positive inotropism, a transient relaxation as beating ceased and then a slowly developing tonic contracture as shown in Figure 2(a). The magnitude of these contractures was $293 \pm 11\%$ ($n = 118$) of the initial twitch edge movement.

Effects of calcium antagonists on veratrine-induced contractures

The pharmacological sensitivity of these tonic contractures is of interest. Ver Donk *et al.* (1986) have shown that only certain calcium antagonists protected against veratrine-induced rounding-up of rat myocytes. We found similar differences between calcium antagonists. The effects of the dihydropyridine calcium antagonists nifedipine and nicardipine are shown in Figure 2. Pretreatment with nicardipine, but not nifedipine prevented veratrine-induced contraction. The effects of a range of calcium antagonists from the different subgroups (diltiazem, verapamil, nifedipine, nimodipine, nicardipine and

Figure 2 Effects of calcium antagonists on veratrine-induced contractions of embryonic chick myocytes. (a) Effect of veratrine ($100 \mu\text{g ml}^{-1}$) on edge movement recorded from an aggregate of embryonic chick myocytes. Effect of pretreatment for 10 min with (b) $5 \mu\text{M}$ nifedipine and (c) $5 \mu\text{M}$ nicardipine on veratrine-induced tonic contractions. Expanded sections are all calibrated by the 1s bar.

Figure 3 Effects of calcium antagonists on veratrine-induced contractions of embryonic chick myocytes. Responses to veratrine in the presence of calcium antagonists have been expressed as a percentage of the control response to veratrine obtained in parallel experiments on the same day. Aggregates were exposed to drugs for 10 min before application of veratrine. Data shown are mean values, with vertical lines indicating s.e.mean, for n experiments. Diltiazem (Dil) ($n = 6$), verapamil (Ver) ($n = 6$), nifedipine (Nif) ($n = 11$), nimodipine (Nim) ($n = 4$), nicardipine (Nic) ($n = 5$), ryanodine (Rya) ($n = 5$). Statistical significance is denoted as *** for $P \leq 0.001$ where drug-treated and control groups were compared using an unpaired Student's t test.

Figure 4 Concentration-response relationships for the effects of (a) nicardipine and (b) flunarizine on veratrine-induced contractions of embryonic chick myocytes. Responses are calculated as a % of the twitch response and values are expressed as mean, with vertical lines indicating s.e.mean; (a) $n = 4-5$, (b) $n = 4$. Contractions in the presence of the drugs were compared with control groups by use of an unpaired Student's t test. Significant differences from control groups are indicated by *** where $P \leq 0.001$.

flunarizine) are shown in Figure 3. Relatively high concentrations of these drugs in relation to their calcium channel blocking activity were chosen. Diltiazem ($10 \mu\text{M}$), verapamil ($10 \mu\text{M}$), nifedipine ($5 \mu\text{M}$) and nimodipine ($5 \mu\text{M}$) had no significant effect on veratrine-induced contractions. The inhibitory effects of nicardipine were concentration-dependent (Figure 4a) and were statistically significant at 5 and $10 \mu\text{M}$ with an IC_{50} of $3 \mu\text{M}$. Similar protection against veratrine-induced contracture was observed with the diphenylalkylamine calcium antagonist flunarizine. This compound significantly reduced contractures at concentrations between 2.5 and $10 \mu\text{M}$ and had an IC_{50} of $2 \mu\text{M}$ (Figure 4b). Calcium antagonists of different subgroups (Spedding, 1985) have different interactions with the calcium channel facilitator Bay K 8644 in smooth muscle (Spedding & Berg, 1984),

in embryonic chick heart (Patmore & Duncan, 1988) and rabbit heart (Bodekke *et al.*, 1987). These authors have found that the inhibitory effects of the class I (dihydropyridines) and class II (verapamil and diltiazem) calcium antagonists can be reversed by Bay K 8644. Bay K 8644 did not reverse the effects of the class III diphenylalkylamine calcium antagonists. This site of action of the class III drugs may be of importance in the protection against veratrine-induced contractions.

Calcium sensitivity of veratrine-induced contractures

The twitch edge movement of embryonic chick myocyte aggregates is near maximal in 2 mM calcium and several authors have reduced extracellular calcium in order to observe positive inotropic responses to drugs (Laurent *et al.*, 1985; Duncan *et al.*, 1987). The relationship between extracellular calcium levels and twitch edge movement is shown in Figure 5. An EC_{50} value of 1 mM was calculated. The sensitivity of veratrine-induced contractures to external calcium is somewhat different. These contractures are very sensitive to extracellular calcium with an EC_{50} of $32 \mu\text{M}$. In the presence of submaximal concentrations of calcium ($20\text{--}50 \mu\text{M}$) the dihydropyridine calcium facilitator Bay K 8644 at

Figure 5 Calcium dependence of twitch and veratrine-induced contractions. The magnitude of the twitch response (■) has been expressed as a % of the control response in normal (2 mM) extracellular calcium. An EC_{50} of 1 mM was calculated from these data. Veratrine-induced contractures (●) are much more sensitive to extracellular calcium with an EC_{50} of $32 \mu\text{M}$. At submaximal concentrations of calcium ($20\text{--}50 \mu\text{M}$), application of $1 \mu\text{M}$ Bay K 8644 (△) did not significantly increase contractures. Data shown are means, with vertical lines indicating s.e.mean, for $n = 5\text{--}8$ determinations for the twitch response and $n = 3\text{--}7$ determinations for the veratrine (and Bay K 8644) responses.

10^{-6} M , a concentration which is positively inotropic in these cells (Patmore & Whiting, 1985), had no effect on the veratrine-induced contracture (Figure 5). The possibility that calcium from intracellular stores such as the sarcoplasmic reticulum (SR) is involved in veratrine-induced contractures was explored by treatment of aggregates with the intracellular calcium antagonist ryanodine which has been shown to block release of SR calcium (Sutko & Willerson, 1980; Mitchell *et al.*, 1984). Ryanodine (10^{-5} M) inhibited twitch edge movement by $65 \pm 3\%$ ($n = 4$). Similar effects were observed by Rasmussen *et al.* (1987). Subsequent application of veratrine caused contractures to 355% of the twitch magnitude which was not significantly different from those obtained under control conditions (Figure 3). A direct interaction at the sodium channel could explain the effects of these drugs. We therefore investigated the actions of the sodium channel blocker tetrodotoxin (TTX) on veratrine-induced contractures. The effects of adding $1 \mu\text{M}$ TTX to the superfusate 2 min before exposure to veratrine and up to 90 s after are shown in Figure 6. TTX applied before veratrine inhibited beating in these aggregates and blocked the contracture. Interestingly, even when applied up to 90 s after veratrine, TTX relaxed the veratrine-induced contracture.

Application of $5 \mu\text{M}$ nicardipine 60 s after exposure to veratrine failed to relax the already well established contracture (not shown). However, these data do not unequivocally show that the action of nicardipine was not at the sodium channel since there may be differences in the access time of nicardipine and TTX to the sodium channel. In order to differentiate between protection against veratrine-induced contraction and sodium channel blockade by these drugs we investigated the effects of nicardipine and flunarizine against ouabain-induced contractures.

Effects of calcium antagonists on ouabain-induced contractures

In embryonic chick myocytes ouabain causes a depolarization, arrest of automaticity and a small tonic contracture (Koidl & Tritthart, 1980; Hasin & Barry, 1984; Nathan & Bhattacharya, 1984). We found similar responses in our preparations. Inhibition of twitch contractions of aggregates of myocytes with diltiazem and subsequent exposure to ouabain caused a larger contracture of around 150% of the original twitch magnitude. These responses were not affected by $1 \mu\text{M}$ TTX and thus appear to be independent of TTX-sensitive, voltage-dependent sodium channels. The contracture evoked by ouabain was only about half of that induced by veratrine and somewhat variable. To enhance the rise of intracellular sodium caused by ouabain-induced inhibition of

Figure 6 Effects of tetrodotoxin (TTX) on veratrine-induced contractions of embryonic chick myocytes. TTX ($1 \mu\text{M}$) was applied to the superfusate at times shown by the closed circles before or after application of $100 \mu\text{g ml}^{-1}$ veratrine. Note the break in the top trace.

the Na-K-ATPase we increased the level of extracellular sodium. The contracture caused by ouabain can be explained by a reduction in the sodium gradient (due to an increased level of intracellular sodium) which drives the sodium-calcium exchange mechanism which normally extrudes calcium from the cell. Thus to maximize the contracture to ouabain we also increased extracellular calcium. In all experiments the twitch contractions were inhibited by $10 \mu\text{M}$ diltiazem which caused a relaxation of the tone of the aggregate. Raising calcium from 2 to 5 mM had little effect on tone. Interestingly when extracellular sodium was also increased from 110 to 150 mM the aggregate relaxed by $87 \pm 36\%$ ($n = 8$) of the twitch contracture (Figure 7a). Application of $10 \mu\text{M}$ ouabain in the presence of raised calcium and sodium caused a slowly developing tonic contracture of $266 \pm 38\%$ ($n = 37$) of the twitch magnitude (Figure 7b) and is comparable to that attained with veratrine. Diltiazem ($10 \mu\text{M}$) or verapamil ($10 \mu\text{M}$) had no effect on these contractions and responses to

Figure 7 Effects of calcium antagonists on ouabain-induced contractions of embryonic chick myocytes. In all the records the sodium and calcium concentrations of the superfusate were increased as shown in the top panel. The twitch contracture was inhibited by application of $10 \mu\text{M}$ diltiazem. In (a) increasing extracellular sodium and calcium resulted in a relaxation of the aggregate. (b) Simultaneous application of ouabain ($10 \mu\text{M}$ strophanthin G) caused a slowly developing tonic contracture. (c) Incubation with $5 \mu\text{M}$ flunarizine for 10 min before ouabain treatment inhibited the tonic contracture. Similar responses were obtained with $5 \mu\text{M}$ nicardipine. Note the break in the record in (c) for 8.5 min.

ouabain obtained in the presence of $1 \mu\text{M}$ TTX to inhibit the twitch were not significantly different from those obtained in the presence of diltiazem. Nicardipine and flunarizine both inhibited ouabain contractions (Figure 7c and Figure 8). Responses were not significantly different from those obtained to 150 mM Na and 5 mM Ca in the absence of ouabain, but were significantly different at the $P < 0.01$ level when compared with the control ouabain contracture.

Time-dependence of drug application

It is interesting to examine whether nicardipine and flunarizine can inhibit ouabain-induced contractions if applied after ouabain. Whilst established veratrine-induced contractions were relaxed by TTX, we found

Figure 8 Effects of nicardipine and flunarizine on ouabain-induced contractures of myocyte aggregates. Responses are expressed as a % of the twitch contraction and mean values are shown, with vertical lines indicating s.e.mean. Aggregates were treated with $10\ \mu\text{M}$ diltiazem to suppress beating. Increasing extracellular sodium from 110 to 150 mM and extracellular calcium from 2 to 5 mM ($150\ \text{Na}/5\ \text{Ca}$) caused a relaxation ($n = 8$). (a) Simultaneous application of ouabain ($10\ \mu\text{M}$ strophanthine G) caused a contraction to around $266 \pm 38\%$ of the twitch ($n = 37$). (b) Pretreatment for 10 min with $5\ \mu\text{M}$ nicardipine (Nic) ($n = 11$) (c) or $5\ \mu\text{M}$ flunarizine (Flu) ($n = 10$) (d) inhibited contraction to ouabain. Statistical significance is denoted by ** where $P \leq 0.01$ when compared with control (ouabain alone) responses.

that nicardipine did not relax established veratrine-induced contractures. To investigate the time-dependence of drug application, we applied ouabain to aggregates in low calcium solutions ($[\text{Ca}^{2+}]_o = 10^{-9}\ \text{M}$). Aggregates were pretreated with $10\ \mu\text{M}$ diltiazem to inhibit the twitch contracture. Under these circumstances, the aggregates remained relaxed. After 10 min nicardipine was added to the superfusate and a further 10 min later calcium was readmitted. Rapidly developing contractures to $293 \pm 41\%$ ($n = 6$) of the twitch magnitude were observed (Figure 9). These responses were not significantly different from those obtained after 20 min superfusion with ouabain alone ($248 \pm 26\%$) ($n = 6$).

Discussion

The action of the veratrine on contractility and ionic currents of heart muscle has been well documented

and has been reported to inhibit the inactivation of the inward sodium current (see Honerjäger, 1982 for a review). In embryonic chick myocytes we have shown that veratrine also markedly slows the inactivation of the TTX-sensitive sodium current and inhibits one of the two processes which contribute to the bi-exponential decay of the sodium current. This results in an increase in the total inward sodium current with a concomitant increase in action potential duration and a depolarization of the membrane potential. Fosset *et al.* (1977) have shown that veratridine enhanced ^{22}Na uptake in embryonic chick heart cells, leading to a rise in intracellular sodium and enhanced calcium uptake, similarly Pang & Sperelakis (1982) demonstrated stimulation of calcium uptake by veratridine in chick myocytes. Calcium entry may occur through a number of different mechanisms.

The depolarization caused by sodium entry may increase the membrane permeability to calcium by activating voltage-sensitive calcium channels and these effects will result in a rise in the level of intracellular calcium and a tonic contracture. We find that the role of voltage-sensitive calcium channels in this process is questionable: firstly veratrine-induced contractures are insensitive to the calcium antagonists diltiazem, verapamil, nifedipine and nimodipine at concentrations which greatly exceed their calcium channel blocking activities in this and other tissues (Clarke *et al.*, 1984). Secondly, the sensitivity of veratrine-induced contractures to extracellular calcium and the sensitivity of beating is very different (see Figure 5) with EC_{50} values of $32\ \mu\text{M}$ and 1 mM respectively. Whilst twitch edge movement is dependent on voltage-sensitive calcium channels these differences suggest that veratrine-induced contractures are not dependent on calcium entry through these channels. This idea is further supported by the lack of potentiation of submaximal veratrine-induced contractures in $20\text{--}50\ \mu\text{M}$ calcium by the calcium channel facilitator Bay K 8644.

The possibility that the calcium which causes these contractures was released from intracellular stores such as the sarcoplasmic reticulum (SR) has been examined in experiments with ryanodine. Ryanodine has been shown by several authors to block the release of calcium from the SR (Sutko & Willerson, 1980; Clarke & Patmore, 1984). Marban & Wier (1985) have also used ryanodine to determine the contributions from calcium entry and calcium release to contractile calcium. In embryonic chick myocyte aggregates ryanodine ($10^{-5}\ \text{M}$) reduced contractions by 62% which suggests that almost two thirds of contractile calcium comes from the SR in this tissue. However, ryanodine had no significant effect on subsequent veratrine-induced contractures, indicating that calcium from the SR is not involved.

Figure 9 Time dependence of the protection against ouabain-induced contractures by nicardipine. Aggregates were treated with $10\text{ }\mu\text{M}$ diltiazem throughout to suppress twitch activity. Lowering extracellular calcium to approximately 10^{-9} M caused a relaxation. At the same time the aggregate was superfused with $10\text{ }\mu\text{M}$ ouabain and extracellular sodium was raised from 110 to 150 mM. After 10 min $5\text{ }\mu\text{M}$ nicardipine was added to the superfusate and after a further 10 min the calcium concentration was increased to 5 mM which evoked a rapidly developing tonic contracture.

Raised levels of intracellular sodium will cause a reduction in the extrusion of calcium via the calcium-sodium exchanger. The sensitivity of veratrine-induced contractures to extracellular calcium also suggests that the calcium-sodium exchanger may itself contribute to calcium entry by working in the reverse direction extruding sodium ions and allowing calcium to enter. Whilst this would be down the concentration gradient for calcium ions this direction of the exchanger would be against the concentration gradient for sodium and since the exchange process is electrogenic $-3\text{Na}^+ : 1\text{Ca}^{2+}$ (in the normal calcium extrusion mode positive charge enters the cell), this reverse process would result in the exit of positive charge from the cell which is against the electrochemical gradient. However, whilst the normally highly negative membrane potential of these cells favours the calcium extrusion mode of the exchanger, the depolarization caused by veratrine and the reduction of the sodium gradient may shift the equilibrium between calcium extrusion and calcium entry modes and significantly more time will be spent transporting calcium into the cell. The sodium calcium exchange mechanism has been extensively studied in mammalian ventricular muscle (Miller & Moiescu, 1976) and has been shown to exist in embryonic chick heart (Kim *et al.*, 1987). Of the two drugs which have been shown to inhibit veratrine-induced contractures nicardipine has been found to have no effect on low sodium contractures of embryonic chick myocyte aggregates (Patmore, 1987). It thus

seems that calcium must come from another source independent of the calcium sodium-exchanger, the SR or the normal form of voltage-sensitive calcium channels.

The possibility that the protective action of nicardipine and flunarizine against veratrine-induced contractions is a direct action on sodium channels cannot be entirely discounted. We have shown that veratrine-induced contractures are blocked by TTX and can be relaxed after they have already developed by subsequent application of TTX, although nicardipine did not relax established contractures. The protection of these drugs against ouabain-induced contractures which are insensitive to TTX does, however, indicate that nicardipine and flunarizine can protect against calcium overload by a mechanism independent of TTX-sensitive, voltage-dependent sodium channels.

The dependence on time of application of these drugs throws an interesting light on their mechanism of action. If nicardipine was applied before ouabain in the presence of external calcium it prevented contracture. When applied after ouabain in the absence of external calcium nicardipine did not prevent the contracture following calcium re-admission.

This result has several implications: (i) nicardipine does not affect the calcium sensitivity of the contractile proteins or act on the contraction pathway at some point subsequent to a rise in calcium since contractures were of similar magnitude in the presence and absence of nicardipine. Thus it must act on processes which occur before calcium

entry. (ii) These data also provide further clear evidence against a direct effect on the calcium-sodium exchange process operating in the intrusion or calcium entry mode. We therefore suggest that nicardipine and flunarizine block sodium entry or sodium overload which precedes calcium overload.

In guinea-pig myocytes, Chapman & Rodrigo (1987) observed a rise in intracellular sodium from 10.2 to 49.5 mM when exposed to low calcium solutions and on reapplication of calcium the cells irreversibly rounded-up. This rise in intracellular sodium was associated with a prolongation of action potentials and depolarization to -20 mV indicative of increased sodium permeability (Chapman *et al.*, 1984; 1986). When these cells were superfused with low calcium solutions under voltage-clamp, Chapman & Rodrigo (1988) observed the development of a large sustained inward current and they suggested that sodium enters the cells in this process through the voltage-sensitive calcium channels. These phenomena may only occur in very low extracellular calcium and have not been observed in normal calcium solutions (Almers & McCleskey, 1984a, b). However, contractures of myocytes and ventricular fibres have been shown to occur following metabolic blockade (Lederer *et al.*, 1987; Allen *et al.*, 1987) and it is difficult to determine what conditions occur during ischaemia and, thus, whether these may modify the ionic selectivity of the calcium channel. In neuronal tissue modifications of calcium channels to conduct sodium ions have also been described (Carbone & Lux, 1987) in low calcium conditions and by Konnerth *et al.* (1987) on transient acidification of dorsal root ganglion cells. This effect is in response to a step change in pH which is unlikely to occur during ischaemia, although prolonged acidosis may play a role in modifying calcium channels during ischaemia in both heart and cerebral tissue. In both these tissues similar changes in the

ionic selectivity of the calcium channel may give rise to sodium entry which may be blocked by nicardipine and this could explain why nicardipine has been shown to be more effective in protecting ischaemic heart and cerebral tissue than other dihydropyridines (Alps *et al.*, 1983; 1988).

Sodium overload associated with ouabain-induced contractures of embryonic chick myocytes will occur due to inhibition of sodium efflux and sodium entry through a transport process independent of the TTX-sensitive sodium channel. One possibility may be sodium entry through voltage-sensitive calcium channels. These may be stimulated to translocate sodium by raised intracellular sodium directly or raised intracellular sodium may reduce the rate of the sodium-hydrogen ion exchanger in these myocytes (Kim & Smith, 1988) which would lead to intracellular acidosis and thus acidosis may modify the selectivity of the calcium channel. Such channels (or states of channels) would be resistant to diltiazem and nifedipine but sensitive to nicardipine and flunarizine. The same process may also contribute in the contractures induced by veratrine even though it is clear that the majority of sodium entry occurs via TTX-sensitive sodium channels. Another possible route for sodium entry may be through non-specific cation channels and it may be at these or at modified voltage-sensitive calcium channels that nicardipine and flunarizine act to prevent sodium overload and thus calcium overload in embryonic chick myocytes. Should non-specific cation channels be involved in calcium entry as well as sodium entry, then one would have to envisage some selectivity in the action of these drugs to block sodium but not calcium entry through these channels.

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