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EFFECT OF REPETITIVE ACTIVITY UPON INTRACELLULAR pH, SODIUM AND CONTRACTION IN SHEEP CARDIAC PURKINJE FIBRES

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SUMMARY

1. The influence of repetitive activity upon intracellular pH (pH_i), intracellular Na^+ activity (a^i_{Na}) and contraction was examined in isolated sheep cardiac Purkinje fibres. Ion-selective microelectrodes were used to measure intracellular Na^+ and H^+ ion activity. Twitch tension was elicited by field stimulation or by depolarizing pulses applied using a two-microelectrode voltage clamp. Experiments were performed in HEPES-buffered solution equilibrated either with air or 100% O_2.

2. An increase in action potential frequency from a basal rate of 0·1 to 1–4 Hz induced a reversible fall in pH_i and a reversible rise in a^i_{Na}. These effects reached a steady state 3–10 min following an increase in stimulation frequency, and showed a linear dependence on frequency with a mean slope of 0·023 pH units Hz^{-1} and 0·57 mmol l^{-1} Hz^{-1}, respectively. The rise in total intracellular acid and a^i_{Na} associated with a single action potential was estimated as 5·3 μequiv l^{-1} of acid and 3·5 μequiv l^{-1} of Na^+.

3. At action potential frequencies greater than 1 Hz, the rate-dependent rise in a^i_{Na} was usually accompanied by a positive force staircase.

4. The fall in pH_i following a rate increase also occurred when fibres were bathed in Tyrode solution equilibrated with 23 mM-HCO^-_3 plus nominally 5% CO_2/95% O_2. In these cases, however, the fall in pH_i was halved in magnitude.

5. In fibres exposed to strophanthidin (0·5 μM), the rate-dependent fall in pH_i was doubled in magnitude and its time course was more variable than under drug-free conditions. The rate-dependent rise in a^i_{Na} was also usually larger in strophanthidin.

6. In order to examine the influence of the rate-dependent acidosis on developed tension, the acidosis was reversed experimentally by adding 2 mmol l^{-1} NH_4Cl to the bathing solution. This produced a rise in pH_i accompanied by a large increase in twitch tension. Such an effect of pH_i upon tension was quantitatively similar to that observed in previous work on Purkinje fibres (Vaughan-Jones, Eisner & Lederer, 1987).

7. It is concluded that the rate dependence of pH_i will influence both the magnitude and the time course of an inotropic response to a change in heart rate.

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INTRODUCTION

The force of contraction of cardiac muscle is influenced by the frequency and pattern of stimulation (Koch-Weser & Blinks, 1963; Fozzard, 1977). The influence of rate upon developed tension (the force–frequency relationship) is usually attributed to rate-dependent changes in the systolic and diastolic levels of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (e.g. Allen & Blinks, 1978; Lado, Sheu & Fozzard, 1982). Many factors have been suggested to play a role including changes in the inward Ca^{2+} current (Noble & Shimoni, 1981, but cf. Boyett & Fedida, 1984), changes in the time available between beats for Ca^{2+} reuptake by the sarcoplasmic reticulum (Allen, Jewell & Wood, 1976) and rate-dependent changes in the intracellular activity of Na^+ (a^i_{Na}) (Cohen, Fozzard & Sheu, 1982; Boyett, Hart, Levi & Roberts, 1987*b*). This last factor is of particular interest since a^i_{Na} is a major controller, via sarcolemmal Na^+–Ca^{2+} exchange, of diastolic $[Ca^{2+}]_i$ which, in turn, determines Ca^{2+} loading of the sarcoplasmic reticulum (Fabiato & Fabiato, 1978) and thus contractility (e.g. Eisner, Lederer & Vaughan-Jones, 1984).

Alteration of $[Ca^{2+}]_i$ is not, however, the only way in which contraction can be modified in cardiac muscle. Alteration of the Ca^{2+} *sensitivity* of the contractile proteins can also, at constant $[Ca^{2+}]_i$, produce a change in force development. One important modulator of Ca^{2+} sensitivity is intracellular pH. In both skinned (Fabiato & Fabiato, 1978) and intact fibres (Allen & Orchard, 1983; Vaughan-Jones *et al.* 1987) a fall in pH_i can lead to a decrease in contraction and much of this influence is attributable to a reduction in the Ca^{2+} sensitivity of the myofibrils.

In the present work, we have investigated if changes in pH_i occur following changes in stimulation rate. We have measured pH_i, a^i_{Na} and contraction simultaneously in isolated sheep Purkinje fibres while varying their frequency of stimulation. The measurements of pH_i and a^i_{Na} were made with liquid-sensor, ion-selective microelectrodes while the fibres were paced either with field stimulation to elicit action potentials or with depolarizing pulses under voltage-clamp control. We find that pH_i falls reversibly following an increase in rate (cf. Vanheel & de Hemptinne, 1985) and that this fall in pH_i has a depressant effect on contraction. We conclude that the force–frequency relationship of this tissue is influenced significantly by variations in pH_i. Preliminary reports of this work have appeared (Kaila & Vaughan-Jones, 1985; Kaila, Vaughan-Jones & Bountra, 1987).

METHODS

The basic methods have been described previously (Eisner, Lederer & Vaughan-Jones, 1981; Kaila & Vaughan-Jones, 1987). Briefly, experiments were carried out on Purkinje fibres dissected from sheep hearts, obtained from a local slaughter house. The preparations were attached to a tension transducer in the experimental bath. They ranged from 1·5–2 mm in length and 100–300 μm in diameter (this includes the sheath of connective tissue surrounding a fibre).

Conventional and ion-selective microelectrodes were constructed as described previously (Vaughan-Jones & Kaila, 1986; Kaila & Vaughan-Jones, 1987). All microelectrodes were dry-bevelled before filling (Kaila & Voipio, 1985). The microelectrodes used for measuring membrane potential or for current injection were filled with 3 M-KCl (resistance 3–4·5 MΩ). The current-passing microelectrode was wrapped up to its shank with aluminium foil which was earthed. Such earthing reduced the appearance of capacitative artifacts on the ion-electrode signal during current

injection. The ion-selective microelectrodes contained a short column (50–100 μm) of either liquid sodium sensor (ligand 227; Fluka) or liquid proton sensor (Fluka). They were calibrated as described by Kaila & Vaughan-Jones (1987).

Twitches were elicited by evoking action potentials with extracellular stimuli from platinum electrodes (pulse duration 0·1 ms, amplitude twice threshold) or by depolarizing voltage-clamp pulses applied using a conventional two-microelectrode voltage clamp.

Measurement of intracellular ion activity in electrically active fibres

The high resistance of an ion-selective microelectrode (about 10^{11} Ω) coupled with an appreciable stray capacitance results in an electrical time constant much longer than that of a conventional microelectrode. We find that the electrical time constant of Na^+ and pH-selective microelectrodes can be minimized to 150–200 ms providing (i) the electrodes are bevelled (see above), (ii) the column of liquid ion sensor at the tip is short (< 100 μm; see Vaughan-Jones & Kaila, 1986), (iii) leads connecting the ion-selective electrodes to the input stage of the recording amplifier are not screened and are as short as possible. Nevertheless, the ion-selective microelectrodes are too slow to follow faithfully the changes in membrane potential during action potentials or voltage-clamp pulses. Valid measurements, however, of intracellular ion activity require that changes in the membrane potential be subtracted from the signal of the ion-selective microelectrode with a high common-mode rejection ratio (CMMR). During a train of action potentials or voltage-clamp pulses, the mismatch in the frequency response of the conventional and ion electrode therefore produces artifactual voltage transients on the derived recording of ion-activity. These voltage spikes can be minimized by low-pass filtering (passive RC filter; time constant, about 200 ms) the signal from the conventional microelectrode before subtracting it from the ion-selective electrode signal. Thus the frequency response of the conventional microelectrode is reduced to match that of the ion electrode, thereby improving the apparent CMMR. This procedure rarely removes all of the artifactual voltage transients since the time constants of the two electrodes can rarely be matched exactly (the distributed capacitance of an ion-selective microelectrode means that it does not conform electrically to a simple RC network). Further suppression of artifacts can be achieved by passing the derived signal of ion activity through a second low-pass passive filter (time constant 1–2 s). The resulting trace is displayed in the Figures that follow (see also Vaughan-Jones & Aickin, 1987, for further details). The above filtering procedure was checked by comparing the time course of the filtered signal with that obtained with no filtering. In addition, when stimulation was terminated and all filtering then removed, there was no immediate change in the recording of ion activity. This latter observation confirms that the mean level of a^i_{Na} or pH_i is not distorted by the filtering procedure. Clearly, however, such filtering does not permit resolution of ion activity changes during a *single* action potential (see Discussion).

Solutions

In most experiments, the Tyrode solution was gassed with 100% O_2 or air and its composition was (mmol l^{-1}): NaCl, 140; KCl, 4·5; $CaCl_2$, 2·5; $MgCl_2$, 1·0; glucose, 11; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 10 (adjusted to pH 7·4 with 4 M-NaOH at 37 °C). In some experiments, a physiological solution equilibrated with 95% O_2 + nominally 5% CO_2 was used. It contained (mmol l^{-1}): NaCl, 140; KCl, 4·5; $MgCl_2$, 1·0; glucose, 11; $NaHCO_3$, 23 (pH 7·4 at 37 °C). NH_4Cl (2–5 mmol l^{-1}), applied to produce changes in pH_i (cf. Roos & Boron, 1981), was added to the Tyrode solution with no osmotic compensation. Strophanthidin (Sigma chemicals) was diluted from a stock stolution of 10^{-2} mol l^{-1} made in ethanol.

RESULTS

The rate dependence of pH_i, a^i_{Na} and twitch tension

Figure 1 shows the effect on pH_i, a^i_{Na}, contraction and membrane potential of increasing the frequency of stimulation from 0·1 to 2·0 Hz. Twitch tension displays a biphasic change with time which is typical of many cardiac tissues (Koch-Weser & Blinks, 1963). Following an increase in frequency there is, initially, a fall in twitch tension followed by a slower, secondary recovery (the positive staircase). Figure 1*B*

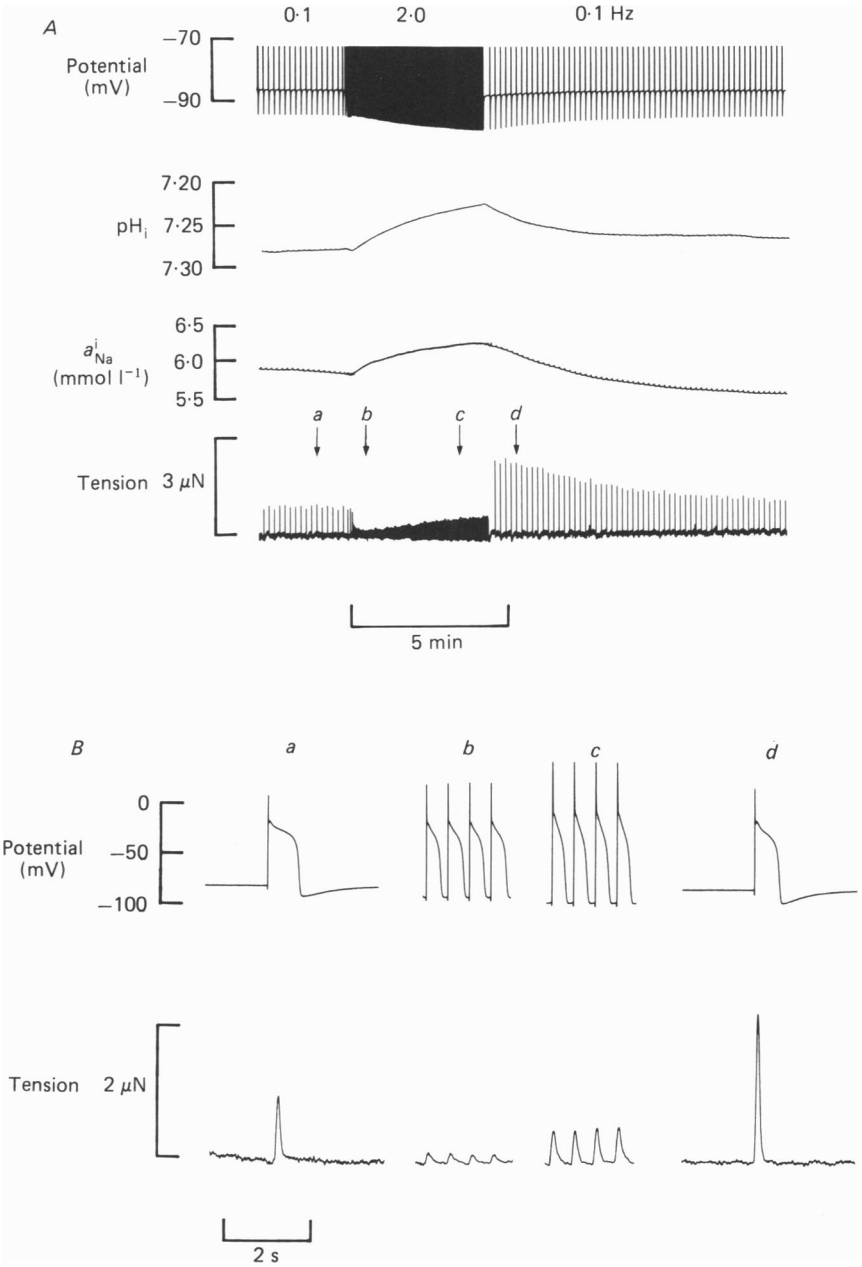

Fig. 1. A rate increase affects pH_i, a^i_{Na} and twitch tension. *A*, traces show from top to bottom, membrane potential, pH_i, a^i_{Na} and twitch tension in a Purkinje fibre stimulated extracellularly at a basal frequency of 0·1 Hz. The fibre was then stimulated at 2·0 Hz for a period of 4·3 min. The full height of the action potentials is off scale on the potential trace, which therefore shows only the changes in diastolic membrane potential (also in Figs 2, 5 and 6). The tension trace has been bandpass filtered (0·1–10 Hz) in order to facilitate comparison of twitches (also Figs 2, 5, 6 and 9). Records of action potentials and associated twitch tension are shown in *B*, on a faster time scale. Tension records here are not bandpass filtered. Letters *a–d* correspond to the times indicated in *A*.

shows sample records of tension and membrane potential obtained at the times indicated in Fig. 1*A* and displayed on a faster time scale. It is evident that, at 2·0 Hz, the action potential shortens considerably in parallel with a progressive diastolic hyperpolarization. Upon return to 0·1 Hz stimulation rate, there is an immediate, initial increase in twitch amplitude to a level which is higher than the

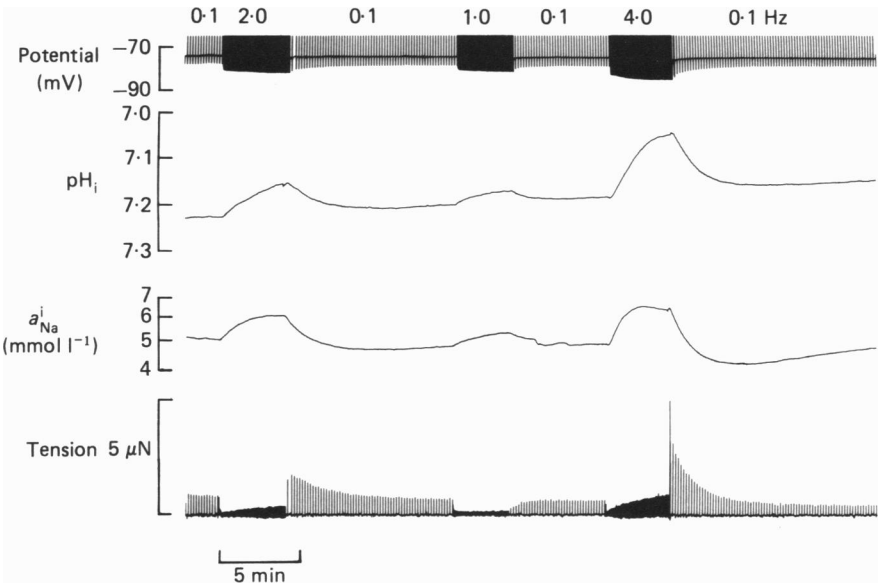

Fig. 2. The fall in pH_i and the rise in a^i_{Na} following a rate increase are frequency dependent. The fibre was stimulated extracellularly at a basal frequency of 0·1 Hz and then for short periods at 2·0, 1·0 and 4·0 Hz, as indicated above the membrane potential trace. A rate increase elevates a^i_{Na} and reduces pH_i and these effects are larger at higher frequencies. The tension record has been bandpass filtered (0·1–10 Hz).

steady-state value at 0·1 Hz. This is then followed by a slow secondary decline in force back to the steady-state level (the negative staircase), accompanied by a slow diastolic depolarization (Fig. 1*A*) and an increase in action potential duration Fig. 1*Bd*).

Figure 1*A* also shows that, at 2·0 Hz, the slow positive force staircase is accompanied by a small rise in a^i_{Na} of about 0·5 mmol l^{-1}. Similarly the slow negative force staircase observed upon return to 0·1 Hz is accompanied by a fall in a^i_{Na}. These changes in tension and a^i_{Na} are similar to those observed previously in sheep Purkinje fibres (Lederer & Sheu, 1983; Boyett *et al.* 1987*b*). Figure 1 also shows that an increase in frequency leads to a progressive fall in pH_i (*ca.* 0·05 units). This acidosis is reversed upon return to 0·1 Hz. A similar, reversible acidosis is also seen when a fibre is stimulated with trains of depolarizing voltage-clamp pulses (see Fig. 9).

Figure 2 shows that the magnitude of the changes in pH_i and a^i_{Na} is frequency dependent. In this experiment the tissue was paced (field stimulation) at frequencies varying from 0·1–4·0 Hz. It is apparent that an increase in frequency from 0·1 to 4·0 Hz produced the largest fall in pH_i and rise in a^i_{Na}. The changes in pH_i and a^i_{Na} observed in Fig. 2 are plotted in Fig. 3 as a function of frequency. The changes

increase roughly linearly with frequency in the range 0·1–4·0 Hz. Similar results were observed in a total of eight fibres from which we calculate that a mean acidification of 0·023 pH_i units and a rise in a^i_{Na} of 0·57 mmol l^{-1} occurs for each 1·0 Hz increase in rate (see Table1).

In most fibres tested, a^i_{Na} approached a steady state within 5 min following a rate change, see e.g. Figs 1, 2, 5 and 6*A*. Occasionally, as reported by Boyett *et al.* (1987*b*), biphasic changes in a^i_{Na} were evident as can be seen during the 4 Hz stimulation period in Fig. 2. In some cases, the changes in pH_i also approached a steady state within the 5 min period (see e.g. Bountra, Kaila & Vaughan-Jones, 1986) but, in other cases, pH_i took up to 10 min to settle.

TABLE 1. The intracellular acidosis and rise of a^i_{Na} associated with an increase in action potential frequency

A	ΔpH_i (Hz^{-1})	Initial rate of acid loading (mequiv l^{-1} min^{-1} Hz^{-1})	Acid load per action potential (mequiv l^{-1})	ΔpH_i per action potential
	0·023 ± 0·004 (n = 8)	0·32 ± 0·1 (n = 8)	0·0053	0·0002
B	Δa^i_{Na} (mmol l^{-1} Hz^{-1}	Initial rate of Na^+ loading (mmol l^{-1} min^{-1} Hz^{-1})	Na^+ load per action potential (mmol l^{-1})	Na^+ influx per action potential (pmol cm^{-2})
	0·57 ± 0·12 (n = 5)	0·21 ± 0·04 (n = 5)	0·0035	1·2

A, following a 1·0 Hz rate increase is shown the fall in steady-state pH_i (ΔpH_i), the initial rate of net acid loading (calculated as $-\beta\, dpH_i/dt$ see text and legend to Fig. 4), the net acid load per stimulus (calculated by $-\beta(dpH_i/dt)/60$) and the initial fall in pH_i per stimulus (calculated as $(dpH_i/dt)/60$). *B*, following a 1·0 Hz rate increase is shown the rise in steady-state $a^i_{Na}(\Delta a^i_{Na})$, the initial rate of rise in $a^i_{Na}(da^i_{Na}/dt)$, the rise in $[Na^+]_i$ (i.e. $da^i_{Na}/dt\ \gamma^{-1}_{Na}$ where γ_{Na} is the intracellular activity coefficient for Na^+) per stimulus and the net Na^+ influx per stimulus, estimated using a volume : surface area ratio for a Purkinje fibre of $2{\cdot}56 \times 10^{-4}$ cm (Mobley & Page, 1972). n = number of fibres tested ± S.E.M.

Following a rate increase, the *speed* at which pH_i falls and a^i_{Na} rises is also frequency dependent. Both the initial rate of acidification and the initial rate of rise in a^i_{Na} increase roughly linearly with frequency in the range 0·1–4 Hz. This is illustrated in Fig. 4 which plots data obtained from an experiment similar to that of Fig. 2. Note that the initial rate of acidification shown in Fig. 4*B* has been expressed as the initial rate of production of intracellular acid equivalents (mequiv l^{-1} min^{-1}) calculated as $-\beta\, dpH_i/dt$ where β is the intracellular, 'non-CO_2' buffering power assumed to be 35 mequiv l^{-1} (Ellis & Thomas, 1976) and $-dpH_i/dt$ is the initial rate of acidification. Similar data from a total of eight fibres have been pooled in Table 1. Here the initial rates of rise of a^i_{Na} and acid equivalents have been used to estimate the mean increment in intracellular acid and $[Na^+]_i$ for a single stimulus, i.e. for one action potential plus associated contraction. Both $[Na^+]_i$ and intracellular acid appear to increase by roughly similar amounts, i.e. 3–5 μequiv l^{-1} per stimulus. Expressed another way, a 1·0 Hz rate increase leads initially to intracellular acid accumulation which reduces pH_i by 0·0002 per stimulus while the initial rise in a^i_{Na} is equivalent to a net Na^+ influx of 1·2 pmol cm^{-2} per action potential (see legend to

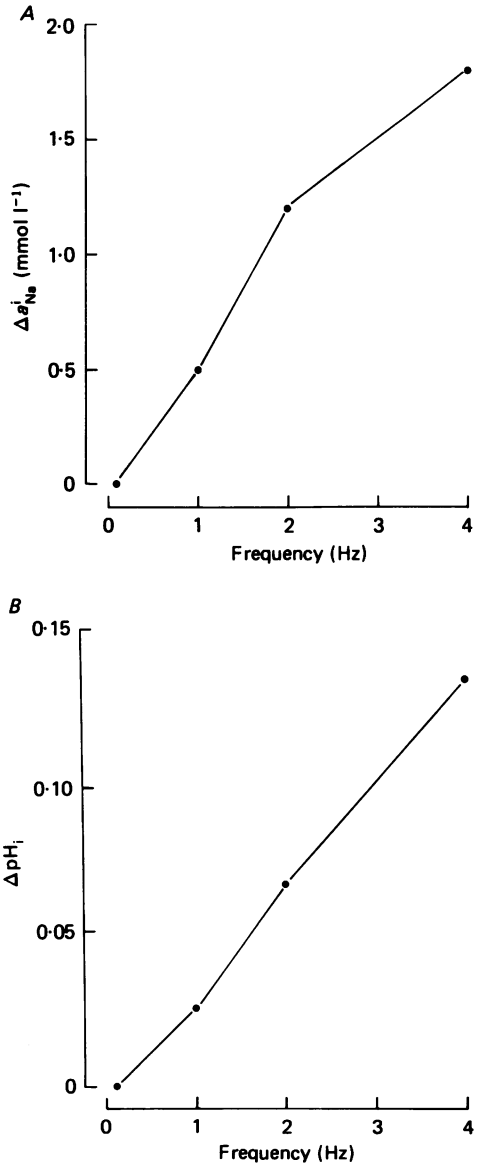

Fig. 3. Changes in a^i_{Na} (Δa^i_{Na}) and pH_i (ΔpH_i) plotted as a function of stimulation frequency. The rise in a^i_{Na} (*A*) and the simultaneous fall in pH_i (*B*) observed as a consequence of stimulating a Purkinje fibre at three different test frequencies (1·0, 2·0 and 4·0 Hz) from a basal frequency of 0·1 Hz. Measurements of Δa^i_{Na} and ΔpH_i were made 4 min after commencing the test frequency. Data obtained from experiment shown in Fig. 2.

Table 1 for further details). This value for Na^+ influx per action potential is similar to the value of 1·7 pmol cm^{-2} calculated recently by Ellis (1985).

The rate-dependent changes in a^i_{Na} and tension observed in the present work are therefore consistent with previous reports. However, we find that there are also significant, simultaneous rate-dependent changes in pH_i.

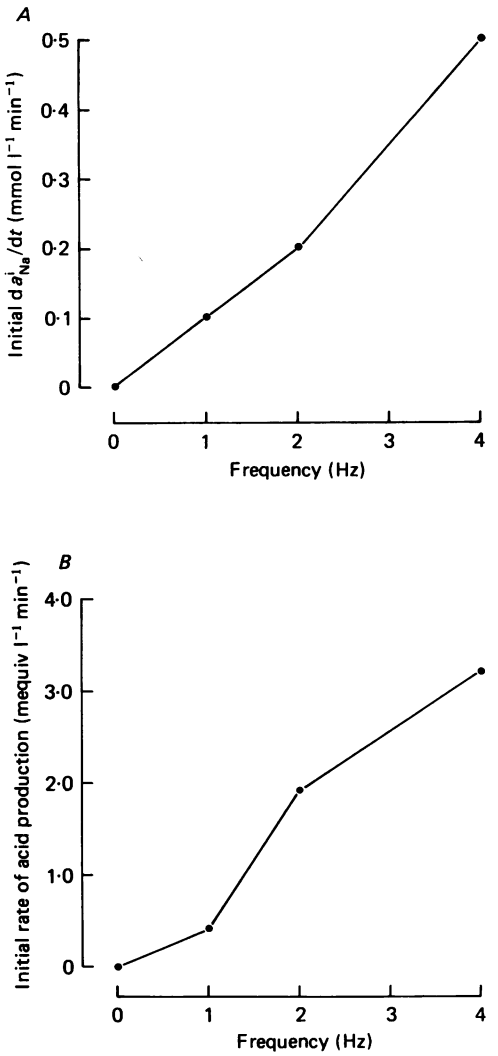

Fig. 4. Following an increase in stimulation frequency, the initial rate of rise of both a^i_{Na} (da^i_{Na}/dt) and intracellular acid equivalents is frequency dependent. Initial da^i_{Na}/dt and initial rate of acid production are plotted in *A* and *B* respectively as a function of stimulation frequency. The initial rate of acid production was calculated as $-\beta\, dpH_i/dt$ where β is the intracellular, 'non CO_2' buffering power, assumed to be 35 mequiv l^{-1} and $-dpH_i/dt$ is the initial rate of acidification. In this experiment the fibre was stimulated at 0, 1·0, 2·0 and 4·0 Hz. Data taken from an experiment similar to that shown in Fig. 2, i.e. a fibre driven by extracellular stimulation.

Effect of using CO_2–HCO_3^--buffered Tyrode solution

In the present work, Purkinje fibres were perfused with HEPES-buffered Tyrode solution equilibrated with oxygen (see Methods). We have also investigated if a similar frequency-induced fall in pH_i occurs in fibres equilibrated in Tyrode solution buffered with CO_2–HCO_3^-. Figure 5 compares the intracellular acidosis induced at 2 Hz in a fibre bathed first in HEPES and then in CO_2–HCO_3^--buffered Tyrode

solution. It is apparent that, in both cases, there is a frequency-induced fall in pH_i. However, the acidosis is reduced by about 50% in the CO_2–HCO_3^--buffered Tyrode solution. A similar result was observed in two other fibres.

The reduction in the frequency-induced acidosis with CO_2–HCO_3^--Tyrode solution is consistent with the increase in intracellular buffering power. In Fig. 5, we estimate that intracellular buffering power is doubled from 35 to 62 mequiv l^{-1} in the presence

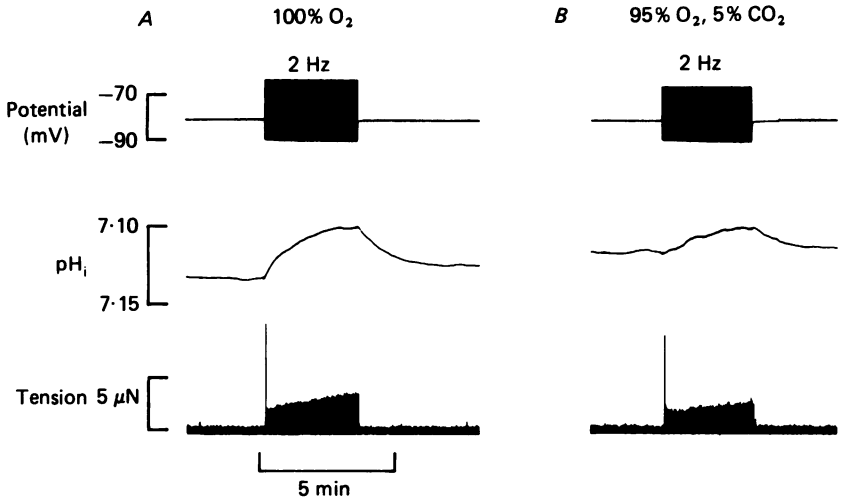

Fig. 5. The intracellular acidosis following a rate increase is smaller in CO_2–HCO_3^--buffered solution when compared with O_2–HEPES-buffered solution. A quiescent fibre was equilibrated first in Tyrode solution buffered with 10 mM-HEPES saturated with 100% O_2 (*A*) and then in Tyrode buffered with 23 mM [HCO_3^-] saturated with nominally 5% CO_2+95% O_2 (*B*) (pH of both solutions was 7·4). In each solution, the fibre was subjected to a 2·0 Hz test train (extracellular stimulation) for 3–4 min. Records in *A* and *B* were obtained from a continuous recording in the same fibre; traces in *B* begin after 30 min equilibration in CO_2–HCO_3^- Tyrode solution. Note that resting pH_i is more acidic than in O_2–HEPES Tyrode solution (cf. Ellis & Thomas, 1976). Tension trace has been bandpass filtered (0·1–10 Hz).

of CO_2–HCO_3^--buffered Tyrode solution (buffering power in CO_2–HCO_3^--Tyrode solution is calculated as $\beta + 2{\cdot}3\,[HCO_3^-]_i$, where intracellular HCO_3^- concentration is estimated from $[HCO_3^-]_i = 10^{(pH_i - pH_o)}\,[HCO_3^-]_o$; see Vaughan-Jones, 1986). For a review of buffering power see Roos & Boron, 1981; Vaughan-Jones & Aickin, 1987. The results therefore suggest that the rate-dependent production of acid within the Purkinje fibre has not been significantly affected by the CO_2–HCO_3^--buffered Tyrode solution.

Effect of strophanthidin

Figure 6 shows the effect of the cardioactive steroid, strophanthidin, on the frequency-dependent changes in pH_i, a^i_{Na} and contraction. In the presence of a suitable dose of strophanthidin, the Na^+–K^+ pump is inhibited so that a^i_{Na} and hence $[Ca^{2+}]_i$ are elevated and there is a large increase in twitch tension (Eisner *et al.* 1984). In the experiment shown, a relatively low dose of strophanthidin was applied

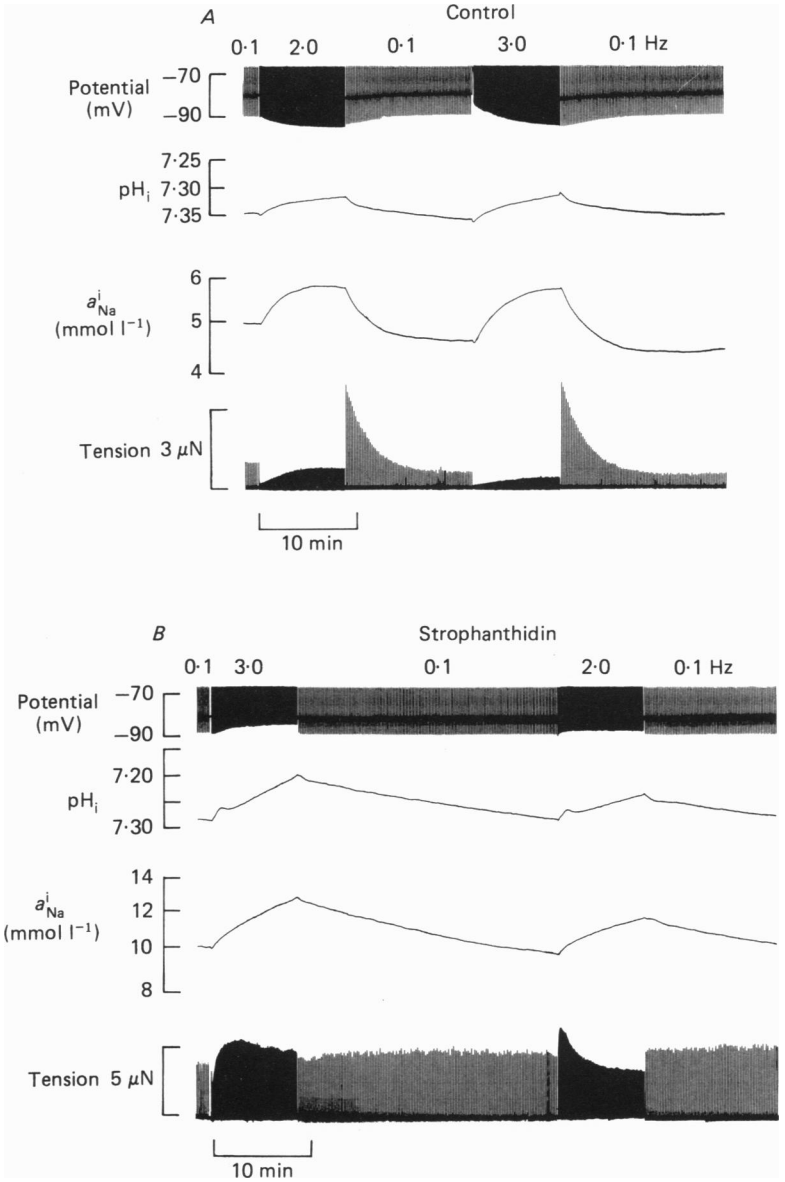

Fig. 6. The rate-dependent changes in pH_i and a^i_{Na} are larger following the application of strophanthidin. A fibre was stimulated extracellularly at two different test frequencies (2·0 and 3·0 Hz) in the absence (*A*, control) and in the presence (*B*) of 0·5 μmol strophanthidin l^{-1}. Throughout the rest of the experiment the fibre was stimulated at 0·1 Hz. Simultaneous recordings of pH_i, a^i_{Na} and tension are shown. Both *A* and *B* are from a continuous recording in the same fibre. The record in *B* begins 30 min after perfusion with strophanthidin. The tension trace has been bandpass filtered (0·1–10 Hz).

($0{\cdot}5 \times 10^{-6}$ mol l^{-1}) which does not maximally inhibit the Na^+–K^+ pump (Deitmer & Ellis, 1978). Intracellular Na^+ activity rose from 5 mmol l^{-1} in Fig. 6*A* to 10 mmol l^{-1} in 6*B* and this was associated with an increase in the level of twitch tension. Inhibition of the Na^+–K^+ pump in the Purkinje fibre can also reduce pH_i (Deitmer & Ellis, 1980; Vaughan-Jones, Lederer & Eisner, 1983; Kaila & Vaughan-Jones, 1987) which explains why pH_i at the start of the record shown in Fig. 6*B* was 0·06 pH units more acid than at the end of the record in Fig. 6*A*. Figure 6*B* shows that rate-dependent changes in a^i_{Na} and pH_i still occurred in the presence of strophanthidin. However, for a given period of stimulation, the changes in pH_i were nearly twice as large as those seen in control conditions. This is illustrated more clearly in Fig. 7*B* which plots as a function of frequency, the changes of pH_i measured in both the presence and absence of 0·5 μmol l^{-1} strophanthidin. The fall in pH_i was linearly related to frequency but the slope of the relationship was steeper in strophanthidin (*ca.* 0·025 pH_i units Hz^{-1} in strophanthidin and 0·01 Hz^{-1} in control conditions). In six fibres, the mean fall in pH_i per 1·0 Hz increase in frequency was 2·2-fold $\pm$ 0·46 (S.E.M.) larger in the presence of 0·5 μM-strophanthidin (drug applied for at least 20 min before applying test frequency). In four out of five experiments, the rate-induced changes in a^i_{Na} were also larger in the presence of 0·5 μmol l^{-1} strophanthidin (see e.g. Fig. 6).

Inspection of Fig. 6*A* and *B* reveals that the *time course* of the frequency-induced changes in pH_i and a^i_{Na} was also altered in strophanthidin. A consistent observation was that, unlike under control conditions, pH_i during a frequency train did not settle completely within 10 min of a rate increase; there was a slow fall in pH_i that continued for 15 min or more (longer periods were not tested routinely). In strophanthidin, the profile of the initial frequency-induced acidosis was also somewhat variable. In three experiments there was an initial rapid acidification of about 0·025 pH_i units followed by a slower component of acidosis. In two experiments (e.g. Fig. 6*B*) the initial rapid acidosis was transient and was followed later by a slower component of acidosis, whereas in one other experiment only the slow component of acidosis was evident. The reason for this variability is not known. The main point to note, however, is that the rate-induced acidosis was larger in strophanthidin when examined over a 10 min period.

One consequence of adding 0·5 μmol strophanthidin l^{-1} is that, at a constant frequency, it produces a fall in pH_i (cf. Vaughan-Jones *et al.* 1983, 1987, using higher doses of strophanthidin). This is evident from Table 2 which shows data pooled from six fibres. In each fibre, pH_i was recorded first at a low frequency (0 or 0·1 Hz) and then at 2 Hz (pH_i was recorded after 6–10 min of stimulation, a time sufficient to allow it to stabilize). Strophanthidin was then added for 20–30 min and recordings of pH_i were again taken at low frequency (0 or 0·1 Hz) and at 2 Hz (measurements at 2 Hz again taken after a 10 min stimulation period, see legend to Table 2). Table 2 shows that pH_i fell following the addition of strophanthidin (final column). Furthermore the fall in pH_i (ΔpH_i) was about 50% larger when recorded at the higher frequency of 2 Hz.

In summary, the application of a moderate dose of strophanthidin (0·5 μmol l^{-1}) has two effects (i) it decreases pH_i, this decrease being larger in fibres driven at 2 Hz

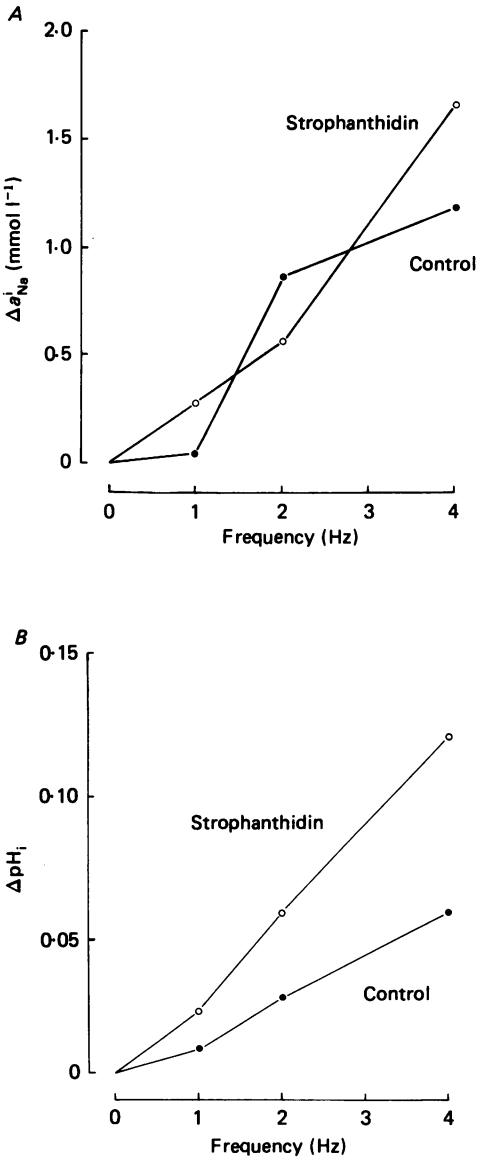

Fig. 7. Comparison of the frequency-dependent changes in a^i_{Na} (Δa^i_{Na}) and pH_i (ΔpH_i) in the presence and absence of strophanthidin (0·5 μmol l^{-1}). Plots of Δa^i_{Na} (*A*) and ΔpH_i (*B*) at three different stimulation frequencies in the absence and presence of strophanthidin. Data taken from a continuous recording of a^i_{Na} and pH_i in an experiment similar to that shown in Fig. 6. Measurements were made 6 min after commencement of test frequency. Test frequencies in the presence of strophanthidin were applied 20 min after addition of the drug.

rather than 0·1 or 0 Hz; (ii) it increases the magnitude of the intracellular acidosis induced by a rate increase.

Effect of changes in pH_i on developed tension

Tension in the Purkinje fibre is sensitive to changes in pH_i (Vaughan-Jones *et al.* 1987). It is therefore important to determine if the rate-dependent changes in pH_i affect twitch tension. In previous work, a decrease in pH_i, induced by the addition and subsequent removal of extracellular NH_4Cl, was found to decrease twitch tension roughly in accordance with the equation: log tension $\propto$ 2·0 pH_i (see Fig. 3 of Vaughan-Jones *et al.* 1987). We have therefore used this relationship to estimate the influence of the rate-dependent acidosis upon twitch tension. Figure 8 shows (filled circles) the relationship between steady-state twitch tension and frequency,

TABLE 2. Intracellular pH decreases in the presence of 0·5 μmol l^{-1} strophanthidin and the decrease is larger in fibres stimulated at 2 Hz than at 0–0·1 Hz

Frequency (Hz)	pH_i (control)	pH_i (strophanthidin)	ΔpH_i
0–0·1†	7·27 ± 0·03	7·20 ± 0·03	0·08 ± 0·01*
2	7·22 ± 0·04	7·11 ± 0·03	0·12 ± 0·01*

Data pooled from six fibres ± S.E.M. The pH_i was measured at 0 or 0·1 Hz and then at 2 Hz (after a 10 min stimulation period) in the absence and presence of 0·5 μmol strophanthidin l^{-1} (drug applied for 20–30 min). The resulting fall in pH_i(ΔpH_i) produced by the drug and measured at a given frequency is shown in the final column. In the presence of strophanthidin, pH_i recorded at 2 Hz had not always stabilized completely after a 10 min train of stimuli. The mean acidosis (0·12 units) shown in the last column may therefore be somewhat underestimated.

* Significant difference, $P < 0·05$. † pH_i does not change in the range 0–0·1 Hz.

determined for the experiment shown in Fig. 2. In the steady state, there is a biphasic dependence of tension on frequency, displaying a minimum at 1 Hz. The relationship described by the open circles is that predicted if no rate-dependent change in pH_i had occurred (from 0·1–4 Hz). The upper curve is thus the predicted force–frequency relationship when corrected for the effects of the observed changes in pH_i. Corrections were applied using eqn (2). Assuming that log tension $\propto$ 2·0 pH_i, it can be shown that

$$f_{corr} = f_{obs}\, 10^{2·0(pH_1 - pH_2)}, \quad (1)$$

where f_{obs} is 'uncorrected force' observed at a given test frequency; f_{corr} is the force when corrected for the effect of the pH_i change; pH_1 is the control value of pH_i and pH_2 is intracellular pH observed at the test frequency. Although the corrected force–frequency relationship is still biphasic, tension predicted at 4 Hz is now nearly 100% larger than observed force whereas force predicted at 1 Hz is 20% larger. This example therefore emphasizes that the rate-dependent decreases in pH_i are likely to exercise a considerable inhibitory influence upon the force–frequency relationship, this influence becoming more pronounced at the higher frequencies.

In the experiment shown in Fig. 9, we examined more directly the inhibitory influence on force of the rate-dependent acidosis. In this experiment, depolarizing voltage-clamp pulses were applied first at 0·2 or 0·1 Hz and then at 3 Hz. This

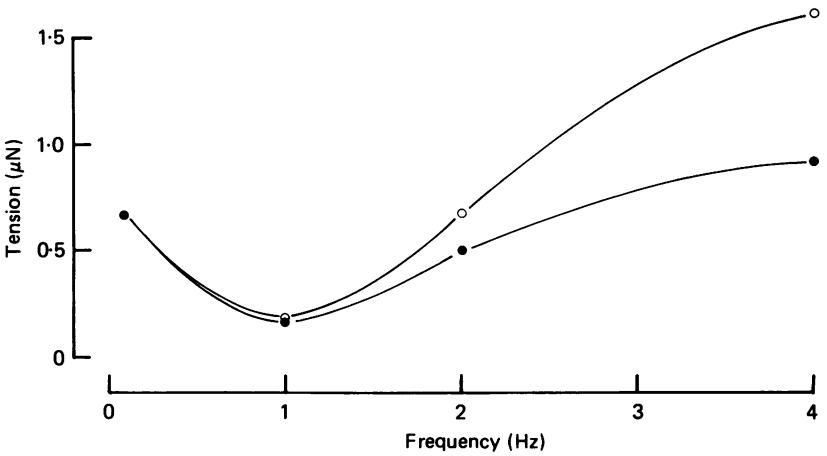

Fig. 8. Influence of rate-dependent acidosis upon the steady-state force–frequency relationship of a Purkinje fibre. Closed circles show steady-state twitch tension plotted as a function of frequency for data obtained from the experiment shown in Fig. 2. Open circles show the predicted relationship if the rate-dependent acidosis observed in Fig. 2 had not occurred. Predicted relationship calculated using eqn (1) in text.

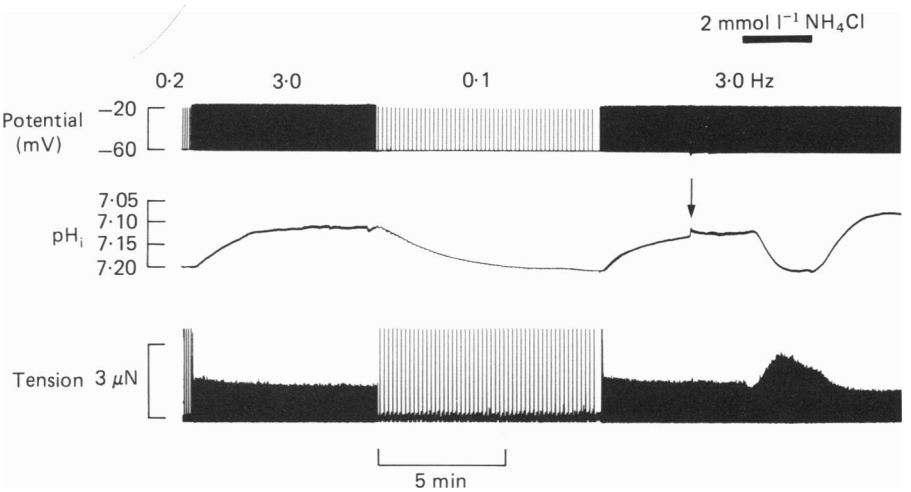

Fig. 9. The frequency-induced acidosis causes a marked reduction in developed tension. Depolarizing voltage-clamp pulses (40 mV, 120 ms) were applied at the frequencies indicated above the potential trace. An increase in stimulation frequency from 0·2 or 0·1 to 3·0 Hz caused an acidosis. During the second of the 3·0 Hz trains, superfusion of NH_4Cl (2 mmol l^{-1}), for the period denoted by the bar, caused an alkalosis which was sufficient to remove entirely the rate-dependent acidosis. This alkalosis was associated with an increase in twitch tension. The twitches at 0·1 Hz are off scale. The tension trace has been bandpass filtered (0·1–10 Hz). The arrow above the pH_i trace indicates a momentary disturbance of the intracellular pH-selective microelectrode.

increase in rate was accompanied by a fall in pH_i of 0·09 units. External NH_4Cl (2 mmol l^{-1}) was then superfused for 2 min. This produced a small alkalosis thus returning pH_i to its original level. This removal of the rate-induced acidosis produced an increase in twitch tension from 1·6 to 2·7 μN. The experiment thus provides direct evidence that the frequency-induced acidosis significantly attenuates developed tension.

DISCUSSION

Effect of frequency on pH_i

The present work shows that intracellular pH in contracting sheep cardiac Purkinje fibres is not constant, but varies with stimulation frequency. When a fibre is activated by trains of action potentials, the dependence of pH_i upon frequency is roughly linear over the physiological range (about 1–4 Hz) and, in HEPES-buffered media, amounts to an average decrease in pH_i of 0·023 units for each 1·0 Hz increase in rate. The fall in pH_i is reversible and is usually expressed fully within 10 min of a rate change. A qualitatively similar frequency dependence of pH_i can be seen when the fibre is stimulated by trains of depolarizing voltage-clamp pulses (see e.g. Fig. 9 of present work). These changes in pH_i are in reasonable agreement with those reported by Vanheel & de Hemptinne (1985) who detected a post-stimulus acidification in sheep Purkinje fibres using double-barrelled, recessed-tip pH microelectrodes (they observed a fall in pH_i of 0·03 after a 2 min train of action potentials elicited at 2·0 Hz). Allen, Elliott & Smith (1986*b*) have also measured, using the technique of ^{31}P-NMR, a 0·02 decrease in pH_i in perfused ferret hearts following a rate increase of 0·1–2·0 Hz. This latter observation is of particular interest since the intrinsic buffering power of ferret myocardial tissue is reported to be more than double that of the Purkinje fibre (Ellis & Thomas, 1976). In addition, the whole-heart experiments were carried out using CO_2–HCO_3^--buffered media and, under these conditions, the intracellular buffering power is increased further (see Roos & Boron, 1981; Vaughan-Jones & Aickin, 1987). A frequency-dependent decrease in pH_i of a similar amplitude in both Purkinje fibres and whole ferret hearts may therefore imply that twice or three times as much acid (or its ionic equivalent) is produced per unit volume in myocardial tissue at a given test frequency. More importantly, frequency-dependent changes in pH_i have now been shown to occur in intact perfused hearts as well as in isolated Purkinje fibres bathed in HEPES-buffered or CO_2–HCO_3^--buffered media although, in this latter case, the pH_i changes are reduced by roughly 50% (Fig. 5). It is possible, therefore, that rate-dependent changes in pH_i occur in all types of cardiac tissue.

The initial rate of intracellular acidification following an increase in frequency suggests that a net intracellular acid load of 0·0002 units is imposed by a single twitch (Table 1). The fact that pH_i eventually stabilizes at higher frequencies may therefore indicate that the efflux of acid equivalents from the cell (or into intracellular organelles) increases to match the increased delivery of acid to the cytoplasm.

Effect of frequency-dependent pH_i changes on developed tension

Although relatively small, the frequency-dependent decrease in pH_i has a significant inhibitory effect on developed tension. For example, the fall in pH_i

observed in Fig. 9 (0·09 units at 3 Hz) appears to have attenuated force production by 41 %. Previous work has shown that twitch tension in the Purkinje fibre decreases roughly exponentially with pH_i (see Fig. 3 of Vaughan-Jones *et al.* 1987) and a similar influence of pH_i on force is also evident in skinned ventricular fibres providing the myofilaments are less than 50 % saturated by Ca_i^{2+} (Fabiato & Fabiato, 1978). In both cases, the influence of pH_i on tension can be described empirically as:

$$\text{tension} = k a_H^{i\,-2}, \tag{2}$$

where a_H^i is the intracellular activity of H^+ ions and where k is independent of a_H^i. This sensitivity of force to pH_i predicts a 34 % attenuation of twitch tension in Fig. 9 which is very similar to the 41 % attenuation that we measured experimentally. Such agreement would then suggest that the predicted influence of pH_i on contraction shown at different stimulation rates in Fig. 8 (the steady-state force–frequency curve) is reasonably accurate.

It should be noted that the pH_i sensitivity of tension will not necessarily be constant but may decrease when Ca_i^{2+} rises. The data of Fabiato & Fabiato (1978) indicate that the sensitivity of force to H^+ ions becomes less steep than predicted by eqn (2) once the myofilaments are > 50 % saturated by Ca_i^{2+} (see Discussion of Vaughan-Jones *et al.* 1987). In the present work maximal tension was not determined, but it is likely that, under most conditions, twitch tension was < 50 % of maximum. This is because the application of high concentrations of cardiotonic steroids to a Purkinje fibre usually increases twitch tension by tenfold or more (e.g. Eisner *et al.* 1984). Thus it is unlikely that the myofilaments are normally > 50 % saturated by Ca_i^{2+}. Consequently the rise in systolic Ca_i^{2+} that occurs following a rate increase is unlikely to influence greatly the pH_i sensitivity of force. In our experiments with strophanthidin (e.g. Fig. 6), however, systolic Ca_i^{2+} will have been elevated greatly so that force may have approached its maximum. In these cases, therefore, the pH_i sensitivity of force may have been reduced (see p. 357). Finally, we cannot exclude the possibility that, following a rate increase, intracellular factors other than Ca_i^{2+} may also affect the pH_i sensitivity of tension. Nevertheless the experiment shown in Fig. 9 indicates that, at 3 Hz stimulation rate, a change of pH_i exerts a clear effect on tension that is quantitatively similar to that observed previously in fibres stimulated at 0·1 Hz (Vaughan-Jones *et al.* 1987).

Because the magnitude of the rate-dependent fall in pH_i is linearly related to frequency, the steady-state force–frequency relationship of the Purkinje fibre will be increasingly influenced by an intracellular acidosis as one progresses from low to high frequencies (see e.g. Fig. 8). The effects of other inotropic factors will therefore become increasingly attenuated. Furthermore, the *time course* of fall in pH_i during a frequency train will affect the kinetics of a force staircase. For example, it is of interest to note in Fig. 2 that a slow positive force staircase is not visible at 1 Hz whereas at 2 and 4 Hz, a positive staircase is clearly evident. If, however, allowance is made for the inhibitory influence of pH_i, then one predicts a positive force staircase, even at 1 Hz. Moreover, the presence of a clear positive force staircase at 2 and 4 Hz indicates that a positive inotropic mechanism is more than compensating for the negative inotropic influence of the intracellular acidosis. Such a positive inotropic mechanism could be the rise in a_{Na}^i (Lederer & Sheu, 1983; Boyett *et al.* 1987*b*; see page 358 which, like the fall in pH_i, increases roughly linearly with frequency (Fig. 3). The rate dependence of pH_i will therefore be expected to influence not only the magnitude, but also the time course and possibly even the direction of an inotropic response.

Finally, it should be stressed that the present work does not define the mechanism

whereby pH_i affects tension. As discussed previously (Vaughan-Jones *et al.* 1987) it is likely that a fall in pH_i reduces force via more than one mechanism, e.g. via a reduction in myofibrillar Ca^{2+} sensitivity (Fabiato & Fabiato, 1978), a reduction in Ca^{2+} release from the sarcoplasmic reticulum (Fabiato & Fabiato, 1978; Orchard, 1987) and/or interference with Ca^{2+} movement across the sarcolemma (Fry & Poole-Wilson, 1981; Philipson, Bersohn & Nishimoto, 1982). Consequently, the quantitative influence of pH_i on force (eqn (2)) must be regarded as an empirical description which does not imply any particular mechanism. However, the fact that, in skinned fibres, pH_i can reduce tension at a constant $[Ca^{2+}]$ implies that a major route of action of pH_i upon force is via a reduction in myofibrillar Ca^{2+} sensitivity.

Effect of strophanthidin on rate dependence of pH_i and force

The inhibitory influence of pH_i upon the frequency dependence of twitch tension will become particularly important following the application of cardioactive steroids (e.g. strophanthidin) since rate-dependent changes in pH_i are more than doubled under these conditions. As noted already (p. 356) the fractional inhibition of force by a given intracellular acidosis may not be as large in the presence of 0·5 μmol strophanthidin l^{-1}, when systolic Ca_i^{2+} is greatly increased, as under control conditions. Nevertheless, previous work has shown that, even in the presence of high doses of strophanthidin (10 μmol l^{-1}), changes of pH_i in a Purkinje fibre can still influence contraction (Vaughan-Jones *et al.* 1987).

The present work also shows that pH_i, measured at a given frequency, falls in the presence of a moderate dose of strophanthidin (0·5 μmol l^{-1}) and that the fall appears to be larger in fibres stimulated at higher frequencies (Table 2). These observations conflict with a recent report of Boyett, Hart & Levi (1986) which states that the same drug concentration has no effect on pH_i. One explanation for this discrepancy may be that, in the earlier work, the drug was applied typically for 8–12 min whereas in the present work, it was applied for 20–30 min. The changes in pH_i may therefore have been smaller following the shorter exposure time. Nevertheless, the present data emphasize that possible changes in pH_i must be considered when assessing the inotropic effects of moderate doses of cardiotonic steroids.

Mechanism of the rate-dependent fall in pH_i

An investigation of the mechanism producing the rate dependence of pH_i will be presented in a separate paper (C. Bountra, K. Kaila & R. D. Vaughan-Jones, in preparation; a brief discussion of the possible mechanisms has also been presented by Vanheel & de Hemptinne, 1985). One possible explanation of the acidosis, however, should be considered here. It might be argued that, following a rate increase, the increased frequency of contraction places a metabolic demand upon the tissue that cannot be sustained by aerobic respiration alone and that this situation arises because the tissue is hypoxic. Hypoxia is known to reduce pH_i (Allen, Morris, Orchard & Pirolo, 1985; Ellis & Noireaud, 1987), largely because of a build-up of intracellular lactic acid generated anaerobically (Allen, Eisner, Morris, Pirolo & Smith, 1986*a*). Hypoxia is an unlikely cause, however, of the present acidoses since the fibres were small (100–300 μm core diameter; see Methods) and well perfused in the experimental chamber. Furthermore, we have found that the rate-dependent

changes in pH_i are identical in a fibre perfused with Tyrode solution equilibrated with 100% O_2, or Tyrode solution equilibrated with air (C. Bountra, K. Kaila & R. D. Vaughan-Jones, unpublished results). While these results do not rule out lactic acid production as a cause of the acidosis, they suggest that any such acid production is not primarily a result of hypoxic conditions in the experimental chamber.

Role of a^i_{Na} in determining rate-dependent changes in tension

The present work shows that twitch tension elicited at any frequency is attenuated by a simultaneous fall in pH_i. This means that the *positive* force staircase following a rate increase must be produced by factors other than pH_i. In agreement with previous work (Lederer & Sheu, 1983; Boyett *et al.* 1987*b*), we find that the positive staircase is usually accompanied by a rise in a^i_{Na}, particularly when the tissue is paced using field stimulation to elicit action potentials. Since elevating a^i_{Na} by other means can increase twitch tension dramatically (Lee, Kang, Sokol & Lee, 1980; Eisner *et al.* 1981, 1984), it seems reasonable to conclude that a rise in a^i_{Na} is contributing to the rate-dependent positive force staircase although, clearly, other factors will also be involved. One obvious additional factor in many of the present experiments will be a change in membrane potential (see e.g. Fig. 1) since most experiments were not performed under voltage clamp. It is well documented that changes in both diastolic potential and action potential duration can affect tension (Fozzard & Hellam, 1968; see also Chapman, 1979).

Conclusion

Following an increase in the frequency of contraction of a Purkinje fibre, pH_i decreases in a rate-dependent manner. The fall in pH_i has a significant inhibitory effect on contraction and this inhibitory effect becomes increasingly important at higher frequencies. In the presence of CO_2–HCO_3^--buffered solutions, the rate-dependent fall in pH_i is smaller but will still exert an important attenuating influence on force. The force–frequency relationship of this tissue is thus shaped, in part, by changes in pH_i. It is possible, therefore, that rate-dependent changes of intracellular pH provide important modulation of normal cardiac contractility.

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