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The Inotropic Effect of 4-Aminopyridine and pH Changes in Rabbit Papillary Muscle

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Abstract—The effects of 4-aminopyridine (4AP), and pH changes have been examined on tension responses and cyclic nucleotide levels in rabbit isolated right ventricular papillary muscles. 4AP augmented papillary muscle contractions in a concentration-dependent manner. However, this positive inotropic action was largely due to an increase in extracellular pH produced by 4AP, rather than an intrinsic activity of the drug. Increases in extracellular pH (from 5 to 9) produced graded and reproducible increases in contractile force which were not blocked by propranolol (1×10^{-7}M) but were inhibited by verapamil in a concentration-dependent manner. The positive inotropic effects of Ca^{2+} were enhanced and depressed by alkaline and acidic pH, respectively. Neither 4AP nor alkaline pH significantly changed cyclic AMP concentration in rabbit papillary muscles. The cyclic GMP content, however, was increased by 4AP only and this effect was blocked by atropine. The results suggest that the positive inotropic effect associated with a rise in pH from neutrality may be due to facilitation of translocation of membrane Ca^{2+} and/or to increase the release of Ca^{2+} from sources within the cell. They also illustrate that a major component of the inotropic effect of 4AP is a result of an increase in extracellular pH.

4-Aminopyridine facilitates neuromuscular transmission by increasing the evoked release of acetylcholine (for review see Bowman & Savage 1981). At higher concentrations, it also exerts a direct stimulant effect on skeletal muscle (Khan & Edman 1979). According to Sobek (1970), Frank et al (1978) and Yanagisawa & Taira (1979), 4-aminopyridine also produces a positive inotropic action in isolated cardiac tissues. Although enhanced noradrenaline release may be partially responsible for this effect, a direct stimulatory effect on cardiac excitation-contraction coupling has also been proposed (Yanagisawa & Taira 1979). The experiments reported here, were intended to analyse the mechanisms involved in the positive inotropic effect of 4-aminopyridine. In initial experiments, we were unable to demonstrate more than a weak inotropic effect of 4-aminopyridine hydrochloride. However, a strong inotropic effect was produced by 4-aminopyridine base which is the form in which some other workers had used the compound. Since the base produces a substantial elevation of extracellular pH, the effects of pH itself on cardiac contractions were also examined.

Materials and Methods

Tension experiments

Papillary muscles, from the right ventricles of male New Zealand white rabbit hearts, were suspended in either normal Krebs Henseleit solution (KHS) (containing in mM: NaCl 118, KCl 4·7, $MgSO_4$ 1·2, KH_2PO_4 1·2, $CaCl_2$ 2·5, $NaHCO_3$ 25, glucose 11·7) or Tris-buffered KHS at 32°C and electrically evoked (1 Hz, 1 ms, supermaximal voltage) contractions recorded as described by Rodger & Shahid (1984). Preliminary experiments revealed that 4-aminopyridine (free base) produced precipitation and loss of calcium salts in normal-KHS. This could, however, be avoided by using Tris-buffered KHS containing (mM): Tris-HCl 25, NaCl 118, KCl 4·7, $MgSO_4$ 1·2, $CaCl_2$ 2·5, glucose 11·7 (pH = 7·2) bubbled with 100% oxygen. Alterations in extracellular pH were achieved by introducing solutions containing different ratios of Tris-base to hydrochloride, adjusted to give the required pH which was checked at the start and end of each experiment. Tris was used as buffering agent because the most interesting results were obtained at pH 8–9. Substitution of Tris with other buffering agents for experiments at pH below 7·5 did not significantly affect the results obtained. The protocol for experiments involving the construction of interval-force curves has been described by Rodger & Shahid (1984). Time-matched control experiments had shown that interval-force curves could be repeated readily in the same tissue without any significant alteration in the force-interval relationship. The interval-force relationship was not modified by propranolol (1×10^{-7} M) or 24 h pretreatment of muscles with reserpine (5 mg kg^{-1} i.p.), thus ruling out the involvement of noradrenaline release under these conditions. $CaCl_2$ concentration-effect curves were produced by lowering the extracellular Ca^{2+} to 0·25 mM, after the initial equilibration period, followed by cumulative additions of the cation. In experiments examining the effects of extracellular pH on the responses to Ca^{2+}, the concentration-effect relationship at the five different pH values was determined for each papillary muscle. This protocol provided a better test for detecting pH-related changes in Ca^{2+}-sensitivity and prevented misinterpretations due to variations in responsiveness of different muscle preparations. Time-matched controls had shown that there was no significant change in Ca^{2+}-induced responses over the experimental period.

Cyclic nucleotide measurements

Papillary muscle cyclic nucleotide concentrations were measured as described by Rodger & Shahid (1984).

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Values are quoted as means ± s.e.m. (n) throughout. The significance of differences were calculated by Student's *t*-test for unpaired data.

Drugs

The drugs used were: 4-aminopyridine-base (Aldrich), 4-aminopyridine-HCl, (Pymadin, Gedeon-Richter), (±)-propranolol (Sigma), verapamil-HCl (Abbott), atropine sulphate (Koch-Light Labs.) and reserpine (BDH). All drug solutions were freshly prepared immediately before use. Other chemicals were of Analar grade.

Results

Effects on contractile force

Initial experiments examined the effects of 4-aminopyridine hydrochloride (4AP-HCl) on the electrically-evoked contractions of papillary muscles bathed in normal-KHS. The compound, however, only exerted a weak positive inotropic action producing a maximal 25% increase in contractile force, over the concentration range (0·8-26 mM) tested. This response was markedly smaller when compared with the results reported by other workers (Sobek 1970; Frank et al 1978; Yanagisawa & Taira 1979). In an attempt to further analyse this apparent discrepancy the effects of the free base of 4-aminopyridine (4AP-base), used by these workers, was examined. A modified KHS containing Tris was used in these experiments. Since Tris has been reported to inhibit smooth muscle contraction (Altura et al 1978), it was important to establish that it had no such effects on cardiac muscle. This was tested by comparing the interval-force relationship and sensitivity to extracellular calcium for papillary muscles bathed in normal- and Tris-KHS. In both cases the responses obtained in Tris-KHS were not significantly different from those obtained in normal KHS. Increasing the concentration of Tris (to 0·25 M) was also without any effect on developed tension.

4AP-base augmented papillary muscle contractions in a concentration-dependent manner (Fig. 1a). However, the positive inotropic effect was significantly smaller in normal KHS due to precipitation (Fig. 1b). In contrast, greater positive inotropic responses were obtained when 4AP-base was tested in Tris-KHS; there was no precipitation at high concentrations. In the latter experiments 4AP-base, at concentrations greater than 10^{-2} M, also produced contracture and subsequent 'systolic' arrest in most preparations (See Fig. 1a). These effects were readily reversed upon washout of the drug and contractions gradually returned to predrug size. The data in Fig. 1 indicate that 4AP-base produces its maximum effect at 1×10^{-2} M (145 ± 5% increases in tension). Beyond this concentration the positive inotropic response declines due to contracture. In parallel

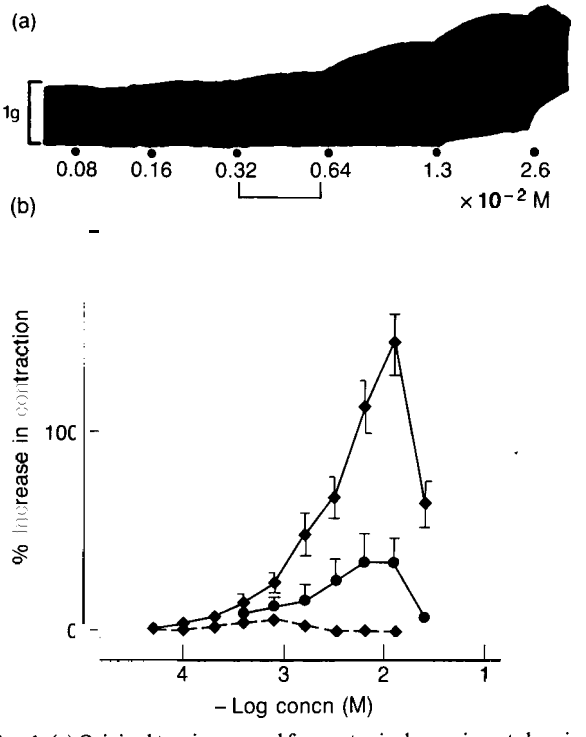

FIG. 1. (a) Original tension record from a typical experiment showing positive inotropic effect of 4-aminopyridine-base in a rabbit papillary muscle being stimulated at a frequency of 1Hz. Note the contracture produced after $2{\cdot}6 \times 10^{-2}$M—the bath pH at this point was 9·6. Time scale 5 min. (b) Mean concentration-effect curves for the positive inotropic action of 4-aminopyridine-base, in normal (●——●) or Tris-KHS (◆——◆), and 4-aminopyridine hydrochloride (◆- - - -◆) in rabbit papillary muscles (n = 4–6). The mean (± s.e.m.) basal tension for these experiments was 800 ± 150 mg.

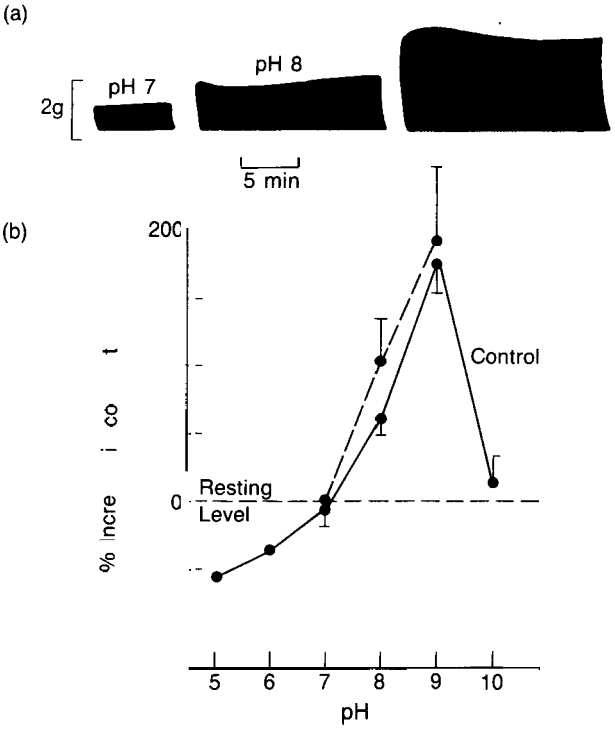

FIG. 2. (a) The positive inotropic effect of increased pH. The left-hand panel illustrates the normal contractile responses of a papillary muscle driven at 1 Hz in Tris-KHS at pH 7. The middle and right-hand panels illustrate the responses produced by the same muscle in Tris-KHS at pH 8·0 and pH 9·0, respectively. The tension (g) relating to these experiments was: 0·83 ± 0·8, 1·30 ± 0·10, 1·99 ± 0·13, respectively, for the pH values. (b) A graphic representation of the effects of Tris-KHS at different pH levels on the contractile responses of papillary muscles driven at 1 Hz. The broken line graph illustrates the responses of pH 7, 8 and 9 in the presence of propranolol (●- - -●; 1×10^{-7}M). The 'resting level' indicates the responses obtained at pH7. Each point is the mean ± s.e.m. of at least 8 preparations.

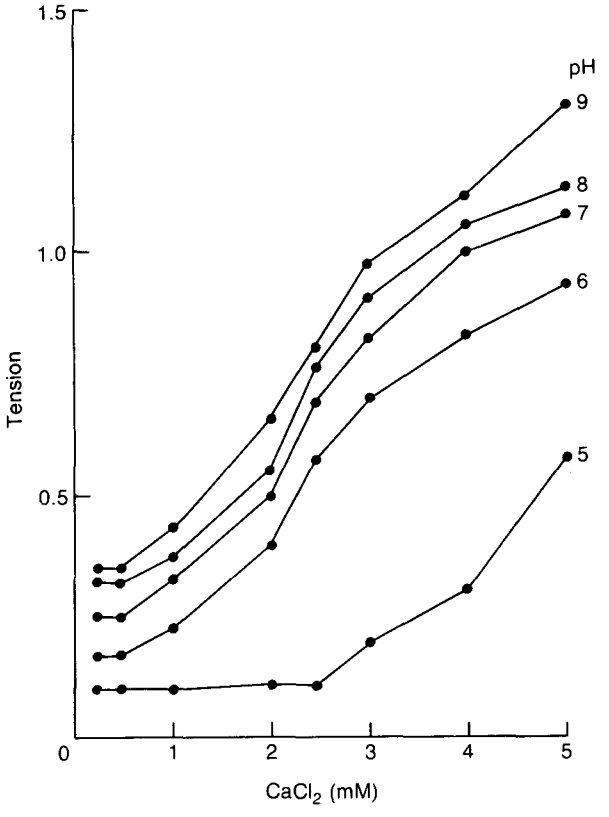

FIG. 3. The effects of extracellular pH on the positive inotropic action of $CaCl_2$ in electrically driven (1 Hz) rabbit papillary muscles. The results shown are from one typical experiment. The cumulative additions of $CaCl_2$ were started after a lapse of 15 min once the extracellular pH had been changed to the appropriate value. A 30–40 min period was allowed for equilibration between dose-response curves. Tension is in g.

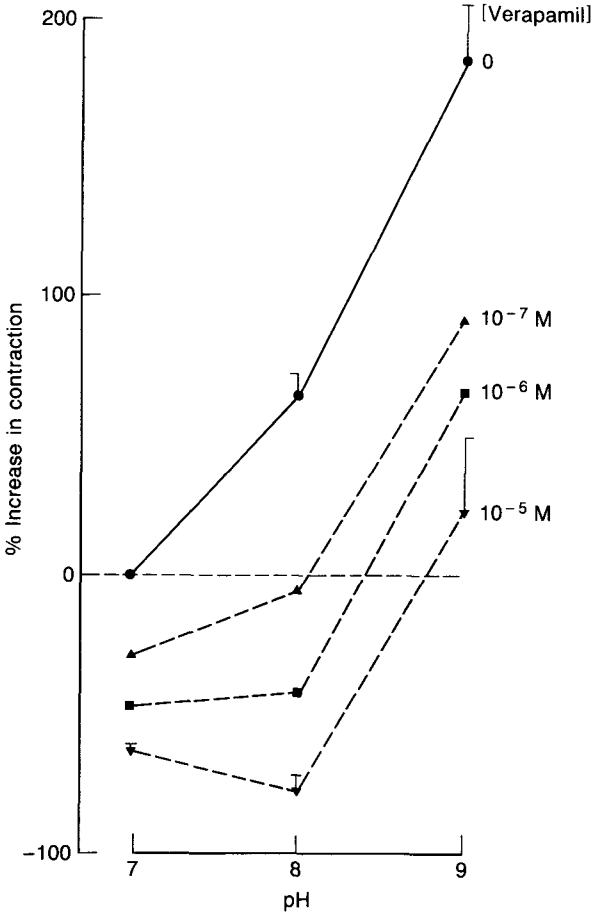

FIG. 4. The effects of verapamil on the positive inotropic action of alkaline pH. Verapamil was allowed to act for 15-20 min before the changes in extracellular pH.

with the increase in cardiac force, 4AP-base also elevated the extracellular pH from 7·2 to 9·6 at the highest concentration used. In normal-KHS the pH change was smaller, the increase being of approximately one pH unit. The effects of 4AP-HCl on papillary muscles bathed in Tris-KHS were also examined. The results are similar to these obtained from experiments in normal KHS and show that the hydrochloride salt, which had no effect on extracellular pH, was almost devoid of positive inotropic activity (Fig. 1b). Furthermore, there was no muscle contracture produced with high concentrations of 4AP-HCl, nor salt precipitation when tested in normal-KHS. Analysis of the positive inotropic action of 4AP-base at elevated extracellular pH (8 and 9) showed that the magnitude of the maximum response was reduced (to $75\% \pm 14$ and $23\% \pm 7$, respectively). These data suggest that the positive inotropic effects of 4AP-base are largely due to its ability to increase extracellular pH, rather than an intrinsic activity of the drug.

The effects of extracellular pH on cardiac contraction was examined directly by using Tris-KHS of different pH values. Increasing the extracellular pH from 5 to 9 produced graded and reproducible increases in contractile force (Fig. 2), reaching a maximum at pH 9 ($176 \pm 23\%$). This inotropic effect, which was readily reversed to control levels on returning to pH 7 solution, was fast in onset and reached a peak within 15 min. The data showed that reduction in pH from 7·4 depressed contraction whilst the opposite effect was elicited with increases in pH (Fig. 2b). At pH 10 contracture occurred leading to a reduction in the size of the inotropic response. It is also clear from Fig. 2 that the positive inotropic effects of pH were not blocked by propranolol (1×10^{-7} M) thus excluding the involvement of β-adrenoceptors.

It is possible that changes in extracellular pH may modify contractility by altering the Ca^{2+} sensitivity of cardiac muscle. This was tested by examining the effects of extracellular pH on Ca^{2+}-induced positive inotropism. Fig. 3 shows that Ca^{2+} increased contractile force in a concentration-dependent manner in the pH range 5 to 9. However, the magnitude of the Ca^{2+}-induced responses was markedly affected by extracellular pH. The positive inotropic effects of Ca^{2+} were enhanced as the pH was elevated, thus the increases in tension were much greater at pH 9 when compared with the responses obtained at pH 5. The largest shift in the Ca^{2+} concentration-effect curve was observed when pH was increased from 5 to 6. The Ca^{2+}-antagonist, verapamil blocked the pH-induced positive inotropism in a concentration-dependent manner (Fig. 4). The Ca^{2+} channel blocking actions of verapamil, in cardiac muscle, are known to be dependent on the frequency of stimulation (Mannhold et al 1978; Chappell et al 1985). Thus the effects of

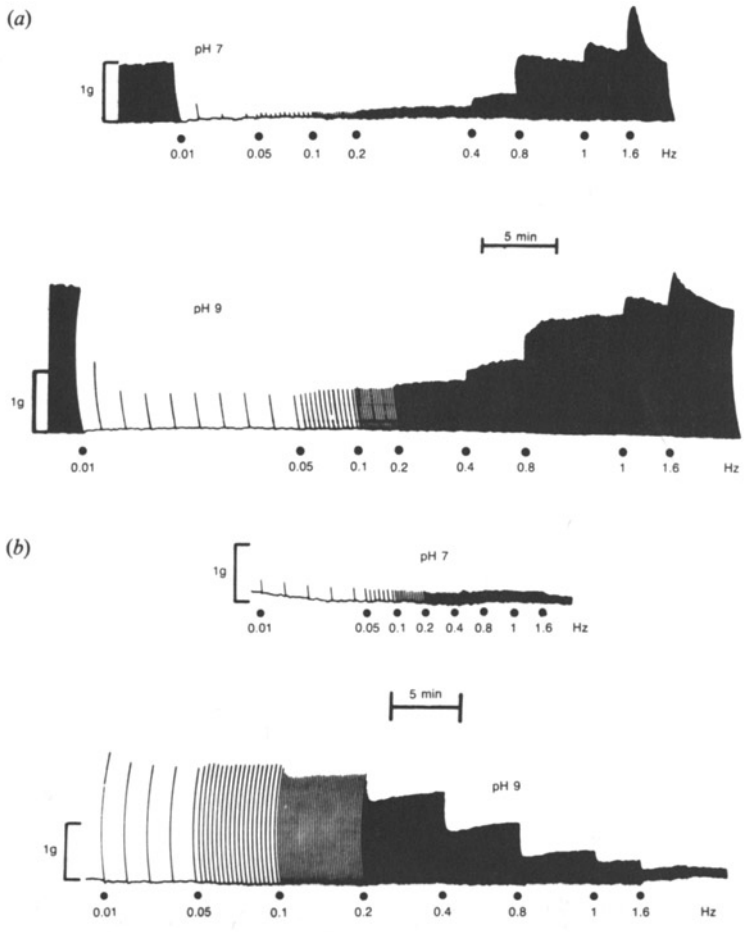

FIG. 5 (a) Typical original tension records illustrating the interval-force relationship at pH 7 (top panel) and pH 9 (bottom panel) for the same rabbit papillary muscle. The effects of changes in extracellular pH were allowed to stabilize (15–20 min) before construction of interval-force curve. (b). Original tension records illustrating the interval-force patterns elicited in the same papillary muscle in the presence of verapamil (1×10^{-5} M) at pH 7 (top panel) and pH 9 (bottom panel). Note the absence of the positive 'staircase' at pH 7 and the inverted (negative 'staircase') pattern produced at pH 9. In these experiments the protocol consisted of changing extracellular pH and allowing 15–20 min for action before the addition of verapamil. The Ca^{2+} antagonist was permitted to act for 15–20 min before the commencement of an interval-force curve.

stimulation frequency on pH-induced positive inotropism in the absence and presence of the Ca^{2+} antagonist were also examined. The original tension records shown in Fig. 5a illustrate that alkaline pH enhances developed tension at all stimulation frequencies tested, producing an upward shift in the interval-force curve (Fig. 6). Furthermore, these positive inotropic responses were proportionally greater at low frequencies of stimulation (0·1-0·2 Hz). In agreement with published data, verapamil (10^{-5} M) produced a frequency-dependent negative inotropic effect, such that contractions at low frequencies were relatively unaltered compared to those at higher frequencies, which were markedly depressed (Fig. 5). The effects of verapamil on the force-frequency curve at alkaline pH were complex as illustrated by the original tension records shown in Fig. 5b. In the presence of verapamil, increasing extracellular pH enhances contraction at all frequencies of stimulation tested. However, the augmentation at lower frequencies was much greater than obtained at high stimulation rates. Thus verapamil potentiated the positive inotropic effect of pH9 at 0·01 Hz whilst inhibiting the responses at 1 Hz. This dual action of verapamil is clearly shown in Fig. 6 as it causes an inversion of the normal force-interval relationship for papillary muscles.

Effects on cyclic nucleotide levels

Many of the biochemical reactions controlling the levels of intracellular metabolites, which participate in excitation-

Table 1. Effects of pH, 4-aminopyridine, on cyclic nucleotide levels in isolated electrically driven (1 Hz) papillary muscles (n = 4-5). Each point is the mean ± s.e. mean **, $P < 0{\cdot}01$ versus pH 7 control values.

Treatment	Concentration (M)	cAMP (pmol (mg tissue)$^{-1}$)	cGMP (pmol (mg tissue)$^{-1}$)
pH7 (control)	—	0·71 ± 0·04	0·031 ± 0·004
pH8	—	0·72 ± 0·03	0·024 ± 0·005
pH9	—	0·66 ± 0·05	0·031 ± 0·006
4AP-base	1×10^{-3}	0·86 ± 0·18	0·064 ± 0·019
	1×10^{-2}	0·95 ± 0·12	0·069 ± 0·02
4AP-HCl	1×10^{-3}	0·72 ± 0·18	0·050 ± 0·018
	1×10^{-2}	0·68 ± 0·09	0·074 ± 0·007**
4AP-HCl + atropine (10^{-6} M)	1×10^{-2}	0·75 ± 0·08	0·033 ± 0·006

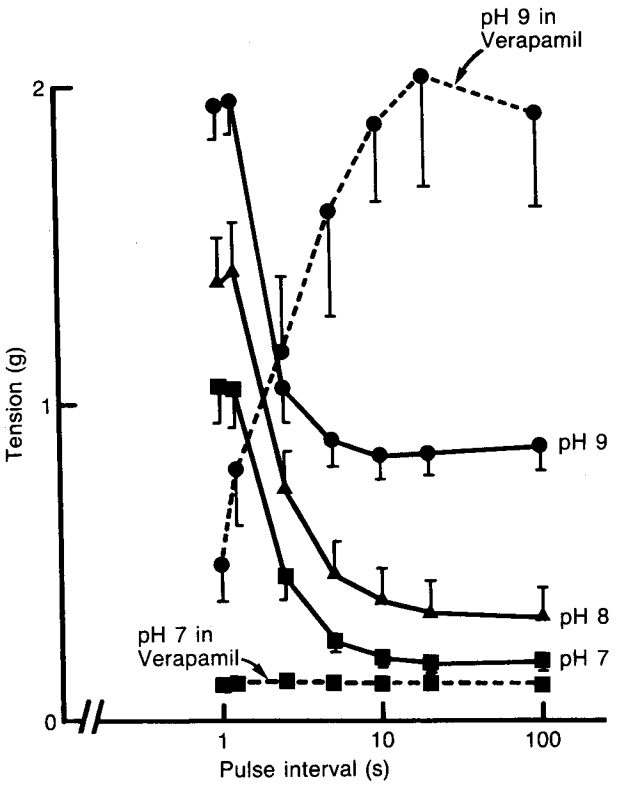

FIG. 6. Graph illustrating the mean interval-force relationship curves at pH 7, 8 and 9 in the absence and presence of verapamil (1×10^{-5} M). Each point is the mean ± s.e.m. of at least 10 experiments.

contraction coupling, are regulated by enzymes and are pH sensitive. It is conceivable that the cardiac effects of changes in extracellular pH may be mediated by an effect on intracellular metabolites, such as cyclic nucleotides, which are known to regulate contractility. Table 1 shows the results for the effects of extracellular pH changes on cyclic nucleotide levels. Increasing the extracellular pH from 7 to 9 had no significant effect on either cyclic(c)AMP or cyclic(c)GMP levels in papillary muscles. The data in Table 1 also show that 4-aminopyridine, whilst having no significant effect on cAMP, produced an increase in cGMP level, although statistical significance was only achieved with the higher concentration of 4AP-HCl. This latter effect was partially blocked by atropine (1×10^{-6} M) indicating the possible involvement of acetylcholine released from parasympathetic nerve terminal. Cholinomimetic agents have been shown to increase cGMP levels in rabbit papillary muscles (Dickson et al 1987).

Discussion

The results suggest that the positive inotropic effect of 4AP-base is largely a consequence of an increase in extracellular pH produced by the drug. Three main observations support this conclusion. The effects of 4AP-base are poor in normal-KHS where an increase in pH causes precipitation of Ca salts; elevating extracellular pH attenuates the positive inotropic action of 4AP-base and most importantly 4AP-HCl was almost inactive. Thus, as the ability of 4AP-base to increase pH is reduced or eliminated so is the positive inotropic action. The similarities in time course of action between, and the development of contracture caused by, 4AP-base and alkaline pH are also indicative of a common mechanism of action. The large changes in pH produced by 4AP-base are not totally surprising when it is realized that it has a pK_a value of 9·8 and a 0·5 M solution has a pH value of approximately 11 (Uges & Huizinga 1981). The observation that 4AP was almost devoid of direct positive inotropic activity is supported by the work of Bowman et al (1981). Those workers showed that the cardiac effects of 4AP in anaesthetized cats and dogs were largely due to facilitation of autonomic transmission. pH-Induced effects in these experiments would not be detectable due to the high buffering capacity of blood. There are a number of reports (Yanagisawa & Taira 1979; Glover 1981; Wollmer et al 1981; Furukawa et al 1985) which suggest that 4AP exerts a direct effect on cardiac muscle contraction and are consequently at variance with the results reported here. Although the exact reasons underlying this discrepancy are presently unclear, some of these studies demonstrated the involvement of noradrenaline release induced by 4AP and that the direct positive inotropic effects constituted a minor component of the overall response. Wollmer et al (1981) examined the effects of 4AP on reserpinized rabbit papillary muscles and could only demonstrate a rather variable and small positive inotropic response.

The most interesting observation of this study is that increases in extracellular pH from 7 to 9 produced marked positive inotropic responses in rabbit papillary muscles. To our knowledge these effects, and their dependence on frequency of stimulation as well as sensitivity to Ca^{2+}-channel blockers, have not been previously reported. Changes in extracellular pH may modify myocardial activity either by acting at the level of the plasmalemma and/or indirectly by affecting cytosolic processes through altering intracellular pH. The finding that the positive inotropic effects of pH were blocked by verapamil, in a concentration-dependent manner, suggests modification of Ca^{2+} slow channel activity. A detailed analysis of the effects of extracellular pH on cardiac Ca^{2+} channel kinetics was made by Prod'hom et al (1987); elevating extracellular pH (up to 9) increased the open time of the so called L-type Ca^{2+} channels whereas acidic pH reduced the single channel currents. Wada & Goto (1975),Kohlhardt et al (1976) and Irisawa & Sato (1986) have also shown that acidic pH decreases the Ca^{2+} inward current. Prod'hom et al (1987) proposed that protonation of the Ca^{2+}-channel may produce a conformational change in the channel proteins leading to reduced conduction. It is, therefore, likely that alkaline pH promotes Ca^{2+} entry during the cardiac action potential which would lead to a greater Ca^{2+} release from the sarcoplasmic reticulum and positive inotropy.

Alkaline pH-evoked enhancement of papillary muscle contractions was proportionally greater at low frequencies of stimulation, a response suggesting the involvement of an intracellular mechanism. This is further supported by the observation that verapamil did not inhibit the positive inotropic effect of alkaline pH at low stimulation frequencies. The frequency or use-dependent action of verapamil (McCans et al 1974; Mannhold et al 1978; Ferry et al 1985)

has been attributed to the preferential binding of verapamil to the inactivated state of the Ca^{2+}-channels (Chappell et al 1985). This mode of the Ca^{2+} channel is prevalent at high frequencies of stimulation, consequently a greater negative inotropic response is observed. Furthermore, if an intracellular source of Ca^{2+} was predominantly involved in tension generation at low frequencies of stimulation, verapamil would be rendered relatively ineffective. This view is supported by the observation that removal of extracellular Ca^{2+} markedly diminishes contractile force at high frequencies of stimulation whereas contractions at low frequencies are relatively unaffected (unpublished results). Changes in extracellular pH produce similar, but smaller, changes in cytosolic pH in mammalian cardiac cells (Ellis & Thomas 1976). Thus the effects of H^+ ions on the activities of organelles, such as the sarcoplasmic reticulum and mitochondria, or on the Ca^{2+}-sensitivity of contractile proteins, could be relevant to understanding the mechanism of pH-induced inotropism. Donaldson et al (1981) and Ricciardi et al (1986) have shown that acidosis produces a rightward shift in the force-pCa curves in permeabilized guinea-pig ventricular muscles, thus supporting the concept that protons can displace troponin-bound calcium. If this is the case then at alkaline pH values the balance of competition for binding to troponin would be in favour of Ca^{2+} and thus stimulating actin-myosin interaction.

The cyclic nucleotides data indicate that large changes in extracellular pH produced no significant alteration in the net concentrations of cAMP and cGMP. These results thus exclude the involvement of these metabolites in the cardiac actions of pH changes. The lack of effect of 4AP-base on cAMP also supports the pH data and the suggestion of non-involvement of noradrenaline release from sympathetic nerve terminals.

In conclusion, these results show that a rise in pH from neutrality, which is associated with positive inotropic responses, may act to facilitate translocation of membrane Ca^{2+} and/or to increase the release of Ca^{2+} from sources within the cell. They also illustrate the importance of controlling pH changes in isolated cardiac muscle and suggest that a significant component of the inotropic effect of 4-aminopyridine is a result of an increase in extracellular pH.

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