Journal of Physiology (1992), **458**, *pp.* 289–306
With 11 *figures*
Printed in Great Britain

EFFECT OF CATECHOLAMINES ON INTRACELLULAR pH IN SHEEP CARDIAC PURKINJE FIBRES

BY HONGSHENG GUO, J. ANDREW WASSERSTROM AND JAMES E. ROSENTHAL

From the Reingold ECG Center (Division of Cardiology, Department of Medicine), Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611–3008, USA

(*Received 25 April 1991*)

SUMMARY

1. It has been reported that catecholamines affect intracellular pH (pH_i) in a number of tissues, generally by altering the kinetics of the Na^+–H^+ exchanger. We postulated that catecholamines might affect pH_i in cardiac tissue. We tested this in resting sheep cardiac Purkinje fibres by measuring transmembrane potential and pH_i with standard and H^+-sensitive microelectrodes.

2. Adrenaline and the β-adrenergic agonist isoprenaline, both $5{\cdot}0 \times 10^{-6}$ M, resulted in depolarization and intracellular acidification (adrenaline, $0{\cdot}03 \pm 0{\cdot}01$ pH units, $n = 8$, $P = 0{\cdot}005$; isoprenaline, $0{\cdot}08 \pm 0{\cdot}01$ pH units, $n = 17$, $P = 0{\cdot}0001$). The α-adrenergic agonist phenylephrine, at concentrations up to 200 μM, had no significant effect on membrane potential or pH_i.

3. Isoprenaline significantly attenuated the half-time ($t_{0{\cdot}5}$) for pH_i recovery from intracellular acidification induced via the NH_4Cl pulse technique. Isoprenaline also attenuated the hyperpolarization that is normally seen at the onset of pH_i recovery. Phenylephrine slightly reduced the $t_{0{\cdot}5}$ for recovery, although the reduction did not reach statistical significance.

4. Forskolin, $7{\cdot}5–10 \times 10^{-5}$ M, an agent that raises intracellular cyclic adenosine 3′,5′-monophosphate (cyclic AMP), also induced depolarization and acidification, similar to that induced by adrenaline and isoprenaline.

5. In the presence of the Na^+–H^+ exchange blocker 5-dimethyl amiloride, $2–6 \times 10^{-5}$ M, isoprenaline-induced acidification was blunted but not abolished. When administered in Na^+-free Tyrode solution, isoprenaline-induced acidification was also not abolished. Buffering power, tested using the NH_4Cl method, was not decreased by isoprenaline, but rather, was slightly increased. Reversal of H^+ driving force across the cell membrane from the normally inward direction to outward (achieved by increasing pH_o to 8·3–8·5 and depolarizing the membrane with 10 mM K^+ solutions) did not prevent intracellular acidification from occurring in the presence of isoprenaline. When glycolysis was inhibited by a 60 min exposure to glucose-free solution containing 5·5 mM 2-deoxyglucose, acidification by isoprenaline was nearly abolished.

6. We conclude that, in resting sheep Purkinje fibres, β- but not α-adrenergic

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stimulation results in intracellular acidification and depolarization, probably mediated via an increase in cyclic AMP. β- but not α-adrenergic stimulation slows the rate of recovery from intracellular acidification and blunts the hyperpolarization associated with this recovery.

7. The intracellular acidification appears to be due both to partial inhibition of Na^+–H^+ exchange and to stimulation of glycolysis by β-adrenergic agents.

INTRODUCTION

Multiple membrane actions have been ascribed to catecholamines. These include actions on transmembrane currents and on exchange mechanisms such as the Na^+–K^+ exchanger (Wasserstrom, Schwartz & Fozzard, 1982). Reports of effects by catecholamines on intracellular pH (pH_i) have been few and have generally focused on the effects of these agents on the Na^+–H^+ exchanger. This exchanger, under normal conditions, maintains pH_i by extruding intracellular H^+ in exchange for extracellular Na^+. Gesek & Strandhoy (1990) have shown in rat proximal nephron that both α_1- and α_2-adrenergic agonists can enhance Na^+–H^+ exchanger activity. α-Adrenergic agonists have been shown to stimulate Na^+–H^+ exchange in glioma cells (Isom, Cragoe, & Limbird, 1987), rat brown adipose tissue (Giovannini, Seydoux & Girardier, 1988) and in cultured rat vascular smooth muscle cells (Owen, 1986). β-Adrenergic agonists have also been shown to stimulate the Na^+–H^+ exchanger, at least transiently, in trout erythrocytes (Baroin, Garcia-Romeu, LaMarre & Motais, 1983).

Very little information is available regarding the effect of catecholamines on pH_i in cardiac tissue, and that which is available is controversial. Farrell & Milligan (1986) found that adrenaline had no effect on pH_i during extracellular acidosis in perfused trout heart. Data on mammalian heart are available only in abstract form. Breen & Pressler (1988) reported that α-adrenoceptor stimulation with a high concentration of phenylephrine resulted in minimal intracellular alkalinization and acceleration of recovery from an intracellular acid load in dog and sheep Purkinje fibres. The acceleration of recovery was blocked by amiloride, suggesting that the effect on pH_i was due to stimulation of Na^+–H^+ exchange. Similarly, stimulation of the Na^+–H^+ exchanger by α-adrenergic agonists has been reported by Wallert & Fröhlich (1989), using a pH-sensitive fluorescent dye in isolated ventricular myocytes. They interpreted the alkalinization as being due to a shift in the pH_i dependence of the exchanger. Gambassi, Spurgeon & Capogrossi, (1990) found that phenylephrine alkalinized unstimulated rat myocytes in bicarbonate-buffered solutions. The effect was antagonized by ethylisopropylamiloride, suggesting stimulation of the Na^+–H^+ exchanger. β-Adrenergic stimulation with isoprenaline had no effect on pH_i. Wu & Vaughan-Jones (1988) reported that, in sheep Purkinje fibres, agents that raise cyclic AMP, including isoprenaline, slightly inhibit the rate of pH_i recovery from intracellular acidification.

Thus, since only minimal information is available regarding the effect of catecholamines on pH_i in cardiac tissue, we undertook a study of these agents in sheep Purkinje fibres. We initially used adrenaline which, in the heart, activates both α- and β-adrenergic receptors (Lefkowitz, Hoffman & Taylor, 1990).

METHODS

Experimental preparation

Sheep hearts were obtained from a local slaughterhouse. The heart was removed as soon as possible after slaughter and quickly transferred to cold, modified Tyrode solution and transported to the laboratory. A free-running Purkinje fibre, dissected from the left ventricle, was pinned to the wax bottom of a 3·5 ml tissue bath, and continuously superfused with modified Tyrode solution at 37 °C. Flow rate was approximately 3 bath volumes min^{-1}.

Solution

The composition of the modified Tyrode solution was (mM): NaCl, 135; $MgCl_2$, 0·5; NaH_2PO_4, 0·9; KCl, 6·0; $CaCl_2$, 1·8; dextrose, 5·5; HEPES, 5; NaOH, 5. The solution containing 20 mM NH_4Cl was made, without osmotic compensation, from a 1 M stock solution within 10 min of use. Solutions were gassed in the reservoir bottles with 100% O_2 and delivered to the tissue chamber via standard polyethylene tubing, slightly greater than 1 m in length. The pH of all solutions was approximately 7·4 and was regularly checked throughout the experiments.

Chemicals

Unless indicated otherwise, reagents were obtained from Sigma Chemical Company (St Louis, MO, USA) or Fluka Chemical Corporation (Ronkonkoma, New York). 5-Dimethyl amiloride (DMA) and 5-hexamethylene amiloride were obtained from Research Biochemicals Incorporated (Natick, MA, USA). The concentration of adrenaline, phenylephrine and isoprenaline was $5{\cdot}0 \times 10^{-6}$ M. To be sure that we were assessing pure α- or β-adrenergic effects, solutions that contained phenylephrine also contained the β-blocker metoprolol, $5{\cdot}0 \times 10^{-6}$ M; solutions that contained isoprenaline also contained the α-blocker prazosin $5{\cdot}0 \times 10^{-6}$ M. A small number of experiments early in the project, though, were performed without metoprolol or prazosin. Since their results did not differ, they were analysed together with those in which blockers were used. To avoid oxidation of catecholamines, solutions were prepared from the powdered reagent immediately before use.

Microelectrodes and recording apparatus

Conventional microelectrodes (resistance, 10–30 MΩ) were pulled from filamented, thick-walled borosilicate glass tubing (outer diameter 1·5 mm, World Precision Instruments, WPI) and filled with 3 M KCl. pH-sensitive microelectrodes (ISEs) were made using modifications of techniques described by Ammann, Lanter, Steiner, Schulthess, Shijo & Simon (1981) and Chao, Ammann, Oesch, Simon & Lang, (1988). In brief, they were pulled from unfilamented, thin-walled borosilicate glass tubing (o.d. 1·5 mm, WPI). Micropipettes were placed in an oven, covered with a glass beaker and dried at 150 °C for 30 min. After 50 μl of *N*-trimethylsilyldimethylamine (T7272 Sigma) was injected into the beaker, oven temperature was increased to 200 °C and the reagent was allowed to react with the glass surface for 30 min. Micropipettes were back-filled with 0·1 M NaCl with 0·1 M citrate buffer (pH 5·6 at 25 °C). The pipettes were then dipped for a few seconds in a resin containing H^+-sensing ionophore 'cocktail' (ETH 1907 or, in early experiments, tridodecyclamine, both from Fluka). A short column ($\sim$ 100 μm) of the resin was drawn into the pipette tip. Completed pH-ISEs were calibrated immediately before and after each experiment at 37 °C in calibrating solutions with a pH range of 6·40–7·85. Calibrating solutions were buffered with 5 mM PIPES or HEPES and contained (mM): KCl, 147–152; NaCl, 10; $MgCl_2$, 3; KOH, 2·5–7·5. The response to pH changes of acceptable electrodes ranged from 51–61 mV per pH unit. Data were discarded when recalibration following the experiment indicated that either the slope had deteriorated or that the calibration curve had shifted by more than 0·14 pH unit.

Impalements 0·3 to 1·2 mm apart were made with a conventional electrode and with a pH-ISE. Voltage signals were amplified by two WPI amplifiers (Models 750 and FD 223). Intracellular pH was determined as the difference in the voltage output of the two microelectrodes. Amplified signals were monitored on a Tektronix Model 5111 oscilloscope and on digital voltmeters; they were recorded on an FM tape recorder (Hewlett Packard Model 3964A) and a strip chart recorder (Gould Model 2600).

Measurement of intrinsic buffering power

Intrinsic buffering power, β, is defined as the physicochemical, non-CO_2-dependent buffering against fluctuations in $[H^+]_i$, thought to be provided by intracellular proteins (Roos & Boron, 1981; Vaughan-Jones & Wu, 1990). The value of β was calculated from the decrease in pH_i following removal of NH_4Cl. These measurements were performed in the presence of 5-dimethyl amiloride (DMA) in order to avoid overestimating β, because of the contribution to pH_i regulation by an active Na^+–H^+ exchanger (Vaughan-Jones & Wu, 1990). Minimum pH_i was taken as the point of intersection of the tangents to portions of the curves in Fig. 9 representing the most rapid pH_i drop after NH_4Cl removal and the initial 'plateau' of the recovery phase (i.e. when DMA was present). We calculated β using formulae delineated in Vaughan Jones & Wu (1990).

Experimental protocol and statistical analysis

Fibres were allowed to recover for at least one hour. They were used for experimentation only if the maximum diastolic potential of stimulated action potentials was negative to -70 mV, although in some cases, the resting membrane potential declined to levels somewhat positive to -70 mV during subsequent quiescence. For experiments in which the effects of catecholamines on membrane potential and pH_i was tested, quiescent fibres were exposed to catecholamine until potential and pH_i stabilized (approximately 10 min). Values for membrane potential and pH_i representing the peak change were compared to values immediately preceding the intervention, using Student's paired t tests. Membrane potential and pH_i were then allowed to recover to control values in drug-free solution before an additional agent was tested on a fibre. The order in which agents were tested was varied.

In experiments in which intracellular acidification was induced, fibres were exposed to NH_4Cl for 8–10 min, and allowed to recover from the resulting intracellular H^+ load. After membrane potential and pH_i had returned to baseline values, the fibre was exposed to a solution containing both NH_4Cl and catecholamine for 8–10 min, and then, during pH_i recovery, to a solution containing catecholamine alone. If a fibre was to be used to test an additional catecholamine, it was allowed to recover in drug-free Tyrode solution for at least 40 min. No more than two agents were tested in a single fibre, and the order of exposure was varied.

Data comparisons between control and experimental 'runs' were made using Student's paired t tests. In the pH_i recovery experiments, to compensate for the fact that up to two comparisons were made using the same control run, $P < 0.025$ was required to establish significance (Bonferonni's method; Ware, Mosteller & Ingelfinger, 1986).

Pooled data are reported as means ± S.E.M.

RESULTS

Effect of catecholamines on membrane potential and pH_i: adrenaline

Figure 1 shows the results of representative experiments in which fibres were exposed to catecholamines. In Fig. 1*A*, a 10 min exposure to adrenaline resulted in a depolarization of 9 mV. It also resulted in an intracellular acidification of 0·06 pH units.

Figure 2*A* summarizes the results in eight experiments in which fibres were exposed to adrenaline. As shown in the top panel, adrenaline resulted in depolarization in all eight experiments (mean $8·8 \pm 1·0$ mV; $P = 0·0001$).

The bottom panel of Fig. 2*A* shows that adrenaline produced a small amount of intracellular acidification in seven out of eight experiments, and no change in pH_i in one. Overall, the mean acidification was small ($0·03 \pm 0·01$ pH units, $P = 0·005$).

Phenylephrine

We wished to determine whether the acidification and depolarization induced by adrenaline was due to its α- or β-adrenergic effects. We therefore tested the effects of the selective α- and β-adrenergic agonists phenylephrine and isoprenaline, re-

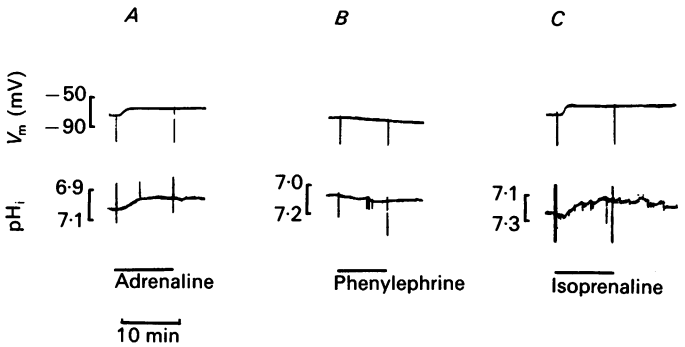

Fig. 1. Effect of adrenaline (*A*), phenylephrine (*B*) and isoprenaline (*C*) on membrane potential and pH_i in representative experiments. In each panel, the traces represent resting membrane potential, V_m (top) and pH_i, calculated as $V_H - V_m$ (bottom).

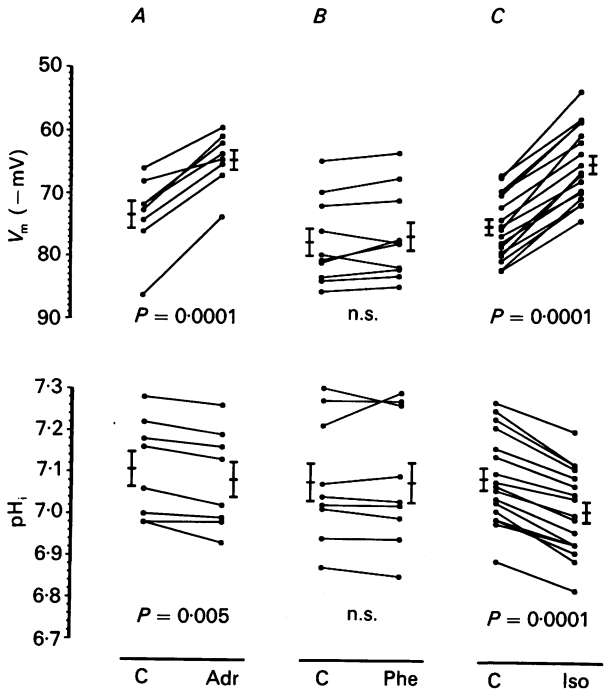

Fig. 2. Resting membrane potential and pH_i at control (indicated as C) and following adrenaline (Adr; *A*), phenylephrine (Phe; *B*) and isoprenaline (Iso; *C*). Bars indicate mean values ± S.E.M. for each data set. Following withdrawal of drug, V_m and pH_i recovered to control values, but these data are not shown to avoid cluttering the figure.

spectively. The results of a representative experiment in which phenylephrine was tested are shown in Fig. 1*B*. In this experiment, phenylephrine resulted in a slight hyperpolarization of 2 mV, and in a slight alkalinization of 0·02 pH units. Pooled results of the ten fibres exposed to phenylephrine (Fig. 2*B*), though, showed no consistent change in membrane potential or pH_i.

Similar experiments were performed using phenylephrine concentrations of 50, 100 ($n = 7$), and 200 ($n = 5$) μM. There was no significant effect on pH_i at any of these concentrations.

Isoprenaline

Tracings from a representative experiment in which a fibre was exposed to isoprenaline are shown in Fig. 1*C*. Isoprenaline depolarized the fibre by 12 mV, and

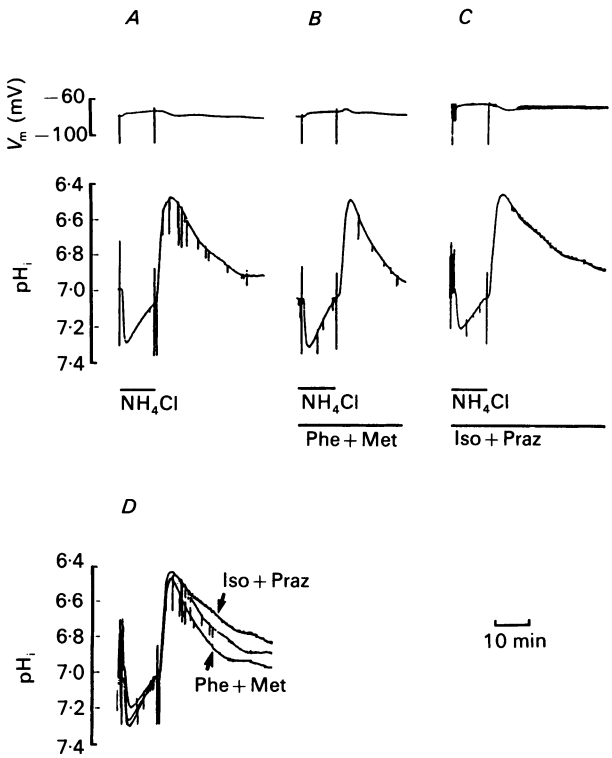

Fig. 3. Effects of phenylephrine (Phe) and isoprenaline (Iso) on recovery from intracellular acidification. The acidification was induced by withdrawal of NH_4Cl. *A* shows the record of a control run from an experiment in which both phenylephrine and isoprenaline were tested. *B* shows the effect of phenylephrine administered with metoprolol (Met) and *C* shows the effect of isoprenaline administered with prazosin (Praz). *D* represents the superimposition of the pH_i recordings of *B* and *C*.

produced an intracellular acidification of 0·11 pH units. Results from similar experiments are shown in Fig. 2*C*. Isoprenaline resulted in depolarization ($9·75 \pm 0·60$ mV; $P = 0·0001$) and in acidification ($0·08 \pm 0·01$ pH units; $P = 0·0001$) in all seventeen experiments.

Following withdrawal of adrenaline and isoprenaline, both membrane potential and pH_i returned to control values, though recovery took as long as 30–60 min in some fibres.

To test whether the acidification and depolarization decayed over time, we performed four experiments in which we used longer exposures to isoprenaline,

ranging from 21 to 46 min (not illustrated). In each of these fibres, the depolarization and acidification induced by isoprenaline remained stable during the entire period of exposure.

Effect of catecholamines on the recovery from an intracellular H^+ load

The data presented thus far suggest that β-adrenoceptor stimulation induces intracellular acidification, while α-adrenoceptor stimulation has no effect on pH_i. To

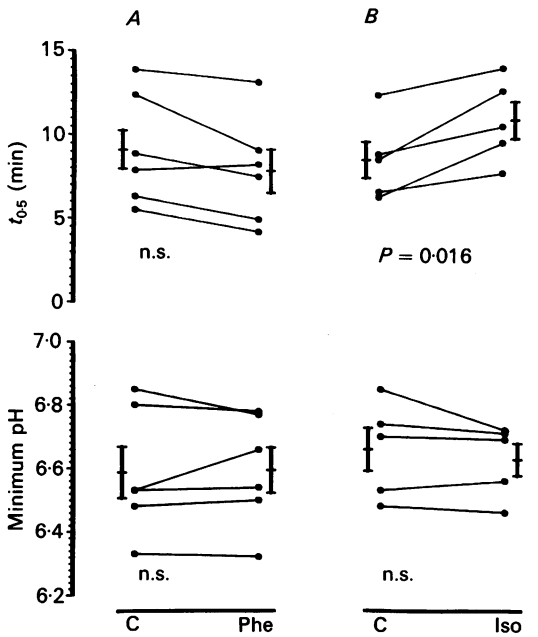

Fig. 4. Summary of pH_i recovery data. The top panel shows the half-time ($t_{0.5}$) for recovery from intracellular acidification. The bottom panel shows the minimum pH_i reached following withdrawal of NH_4Cl. In each panel, points on the left represent data for the control run (C), and points on the right for runs in which phenylephrine (Phe; *A*) or isoprenaline (Iso; *B*) were present. Bars indicate mean values ± S.E.M. for each data set.

test further whether the acidification by adrenaline is due to its β-adrenergic effect, we evaluated the effects of phenylephrine and isoprenaline on the recovery from an intracellular acid load, using the NH_4Cl pulse technique (Boron & De Weer, 1976; Roos & Boron, 1981). With this technique, application of NH_4Cl results in an initial intracellular alkalinization due to the entry of NH_3, which combines with H^+ to form NH_4^+. The pH_i then gradually decreases as NH_4^+ slowly enters the cell in exchange for NH_3, liberating H^+. When NH_4Cl is removed from the bath, a rapid intracellular acidification occurs as NH_3 exits, leaving behind H^+. pH_i then gradually recovers to its baseline value as H^+ is removed from the cell by the Na^+–H^+ exchanger.

Figure 3 shows the effect of phenylephrine and isoprenaline on recovery. Figure 3*A* shows the strip chart recording from a control run of a representative experiment in which both α- and β-adrenergic agonists were administered during separate runs. Panel *D*, in which the records from panels *B* and *C* have been superimposed, shows

that in this experiment, phenylephrine (administered with metoprolol, $5{\cdot}0 \times 10^{-6}$ M) increased the rate of recovery from an intracellular acidification. Isoprenaline (with prazosin, $5{\cdot}0 \times 10^{-6}$ M), on the other hand, decreased the rate of recovery.

The recovery data are summarized in Fig. 4. Figure 4*A* (top) shows that, with phenylephrine, the half-time for recovery ($t_{0{\cdot}5}$) was slightly reduced in five out of six

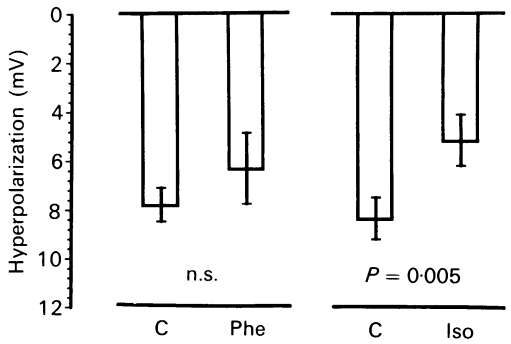

Fig. 5. Voltage changes following intracellular acidification. The mean voltage change (±S.E.M.) following withdrawal of NH_4Cl is shown for control runs (C) and for phenylephrine (Phe, left) and isoprenaline (Iso, right).

experiments. However, the mean reduction of $1{\cdot}3 \pm 0{\cdot}5$ min was small, and did not reach statistical significance.

Isoprenaline, on the other hand, significantly prolonged the $t_{0{\cdot}5}$ of recovery by $2{\cdot}3 \pm 0{\cdot}6$ min (panel *B*, top; $P = 0{\cdot}016$, $n = 5$).

Recovery rate might be affected by the amount of acidification achieved following imposition of the intracellular pH load, since the rate of Na^+–H^+ exchanger activation is related in large part to pH_i. As shown in the bottom panel of Fig. 4, none of the agents, when compared to control, significantly affected the amount of intracellular acidification.

Membrane potential during pH_i recovery

Following withdrawal of NH_4Cl, membrane potential undergoes a fairly abrupt hyperpolarization, often to a membrane potential more negative than that at control (Piwnica-Worms, Jacob, Shigeto, Horres & Lieberman, 1986). Such hyperpolarization is apparent in the control run shown in Fig. 3*A*. The mean hyperpolarization following NH_4Cl withdrawal is shown in Fig. 5. Compared to the control, isoprenaline ($n = 8$) significantly attenuated the hyperpolarization. Phenylephrine ($n = 8$) had no significant effect on this hyperpolarization.

Role of cyclic AMP

Isoprenaline and other β-adrenergic agonists initiate a series of steps that result in an increase in the intracellular concentration of cyclic AMP (Lefkowitz *et al.* 1990). Alternatively, some β-adrenergic actions might be mediated directly by the stimulatory guanine nucleotide binding proteins (G proteins), G_s. Such an action has been demonstrated in that G_s appears to mediate directly the stimulation of Ca^{2+} channels (Imoto, Yatani, Reeves, Codina, Birnbaumer & Brown, 1988; Yatani,

Codina, Imoto, Reeves, Birnbaumer & Brown 1987). To test whether the intracellular acidification occurs via the usual increase in cyclic AMP or whether it results from a direct action of G proteins, we used the diterpene, forskolin $7{\cdot}5–10 \times 10^{-5}$ M. Forskolin is an agent that activates the catalytic subunit of adenylate cyclase

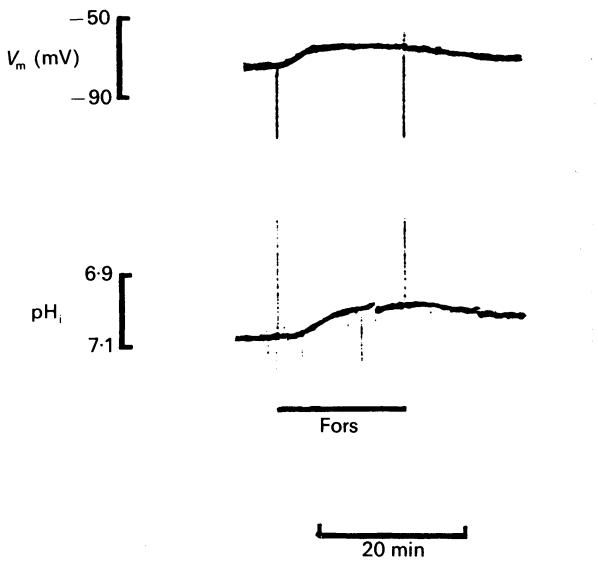

Fig. 6. Effect of forskolin (Fors) on resting membrane potential (top) and pH_i (bottom) in a representative experiment.

directly, without requiring the presence of G proteins (Seamon, Padgett & Daly, 1981; Seamon & Daly, 1986), thereby raising cyclic AMP. Figure 6 shows the effects of forskolin in a representative experiment. Forskolin caused intracellular acidification of 0·14 pH units and depolarized the membrane by 7·5 mV. These changes and their time course were similar to those produced by isoprenaline. Membrane potential and pH_i returned to control values following washout. Similar results occurred in a total of three experiments.

Mechanism of acidification by isoprenaline: role of Na^+–H^+ exchange

To test whether the intracellular acidification by isoprenaline results from inhibition of the Na^+–H^+ exchanger, 5-dimethyl amiloride (DMA), a potent inhibitor of the exchanger (Kleyman & Cragoe, 1988), was used in two series of experiments.

Figure 7*A* shows the results of a representative experiment. Under control condition, an exposure to isoprenaline for 10 min produced an immediate intracellular acidification of 0·11 pH unit. After 60 min of washout, the same fibre was treated with DMA (2×10^{-5} M), which induced a small intracellular acidification (0·06 pH unit). After pH_i had stabilized, exposure for 10 min to isoprenaline was repeated in the presence of DMA, and again induced acidification. However, compared to control, the decline in pH_i occurred more slowly, and was attenuated (0·075 pH unit). In the presence of DMA, $2–6 \times 10^{-5}$ M, the acidification induced by isoprenaline was attenuated in seven of eight fibres tested, and was unchanged in one

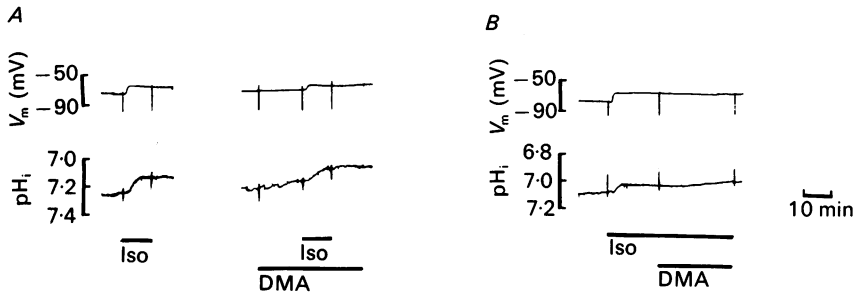

Fig. 7. Effect of 5-dimethyl amiloride (DMA) on the isoprenaline (Iso) induced acidification. *A* shows the control exposure to isoprenaline. Following recovery to approximately control pH_i, the same fibre was exposed to isoprenaline in the presence of DMA (right panel). In *B*, DMA was administered in a fibre that had been pretreated with isoprenaline.

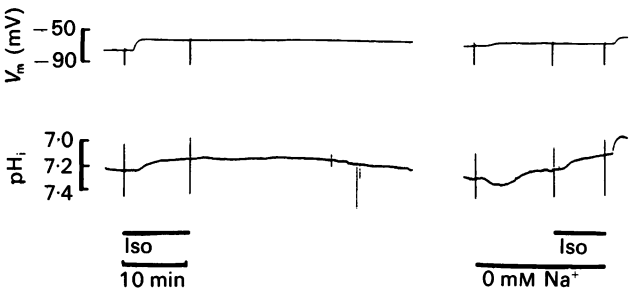

Fig. 8. Effect of isoprenaline under Na^+-free conditions. Comparison of isoprenaline (Iso) administration in normal Tyrode solution (left panel) and in Na^+-free solution (right panel). Tetramethylammonium was the Na^+ substitute.

fibre (mean acidification with and without DMA present, $0{\cdot}09 \pm 0{\cdot}01$ and $0{\cdot}06 \pm 0{\cdot}01$ respectively; $P = 0{\cdot}009$). Similar results (i.e. attenuation but not elimination of acidification by isoprenaline) occurred with DMA concentrations as high as 1×10^{-4} M, and with another potent amiloride derivative, 5-hexamethylene amiloride, 2×10^{-6} M.

A second series of experiments was performed based on the rationale that, if isoprenaline inactivated the Na^+–H^+ exchanger, then addition of DMA should not result in further intracellular acidification. The fibres were exposed to isoprenaline for 17–46 min. After pH_i stabilized at the acidified level, DMA (2×10^{-5} M) was added. A delayed, additional acidification occurred in all four fibres tested. Figure 7*B* shows the results of a representative experiment. Exposure to isoprenaline for 17 min decreased pH_i by 0·06 units. Addition of DMA produced additional acidification of 0·03 pH units (measured at 27 min of DMA exposure), suggesting that the exchanger had not been completely inactivated by isoprenaline.

Finally, the role of the Na^+–H^+ exchanger was tested by exposing the fibre to isoprenaline in solutions in which sodium was replaced by tetramethylammonium. Under these conditions, any acidification induced by isoprenaline cannot be due to inhibition of the Na^+–H^+ exchanger. An example is shown in Fig. 8. Exposure to Na^+-free solution resulted in transient alkalinization followed by acidification. Since

contracture and loss of impalement generally occurred before acidification was completed, we had to administer isoprenaline when pH_i was still declining. Therefore, quantitative comparison of the amount of acidification between Na^+-containing and Na^+-free conditions was not possible. Nonetheless, isoprenaline clearly potentiated

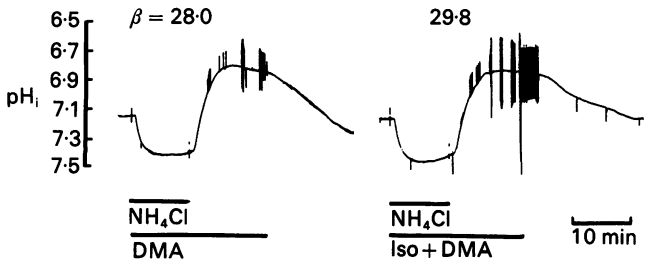

Fig. 9. Effect of isoprenaline on buffering power. Buffering power, β, was determined from the drop in pH_i following withdrawal of NH_4Cl in the absence (left panel) and presence (right panel) of isoprenaline (Iso). Experiments were performed in the presence of 5-dimethyl amiloride (DMA). The method of determining the minimum pH_i is described in the Methods section of the text.

the rate of acidification. The isoprenaline-induced depolarization was completely abolished under Na^+-free conditions. Similar results occurred in all six fibres tested in Na^+-free solutions.

The results of the experiments with DMA suggest that isoprenaline reduces Na^+–H^+ exchange. However, since acidification occurred even in the presence of potent inactivators of the exchanger, and in the absence of external Na^+, Na^+–H^+ exchanger inhibition cannot be the sole mechanism for the acidification. We therefore tested the possibility that some other mechanism might contribute to an intracellular accumulation of H^+. Such accumulation might occur because of a decreased buffering power, an increased permeability to H^+, or an increased production of H^+.

Effect of isoprenaline on buffering power

Figure 9 shows the results of a representative experiment in which the buffering power, β, was determined. As indicated in the Methods section, these experiments were performed in the presence of DMA. The value of β was not decreased, as would be expected if an alteration in β were responsible for the isoprenaline-induced decrease in pH_i. Rather, it was slightly increased from 28·0 (control) to 29·8 mM $pH^{-1}\,l^{-1}$ (isoprenaline). Isoprenaline resulted in a small increase in β in five of the six fibres tested (mean increase, $4{\cdot}13 \pm 1{\cdot}54$ mM $pH^{-1}\,l^{-1}$; mean pH_i at which β was determined, measured at 50% acid loading: control, $6{\cdot}85 \pm 0{\cdot}11$; isoprenaline, $6{\cdot}69 \pm 0{\cdot}16$).

Effect of isoprenaline on membrane permeability to protons

β-Adrenergic stimulation might decrease pH_i by increasing membrane permeability to protons. Assuming an average control pH_i in our experiments of 7·1, the equilibrium potential for H^+ was approximately $-18{\cdot}5$ mV, making the driving force for H^+ inward at normal resting membrane potentials. Thus, intracellular

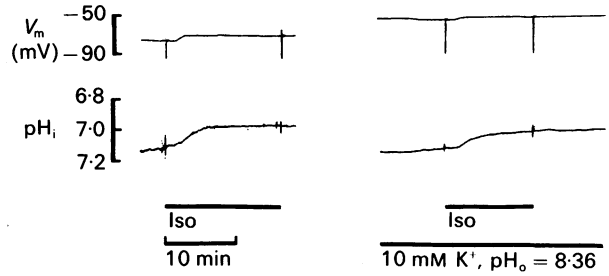

Fig. 10. Effect of reversing the driving force for H^+ on isoprenaline (Iso)-induced acidification. The panel on the left shows the usual acidification following isoprenaline administration. The panel on the right shows, in the same fibre, that isoprenaline induced a decrease in pH_i even following reversal of the normally inward driving force for H^+ to outward. The direction of the driving force was reversed by increasing pH_o to 8·36 and by increasing $[K^+]_o$ to 10 mM (in order to depolarize the fibre).

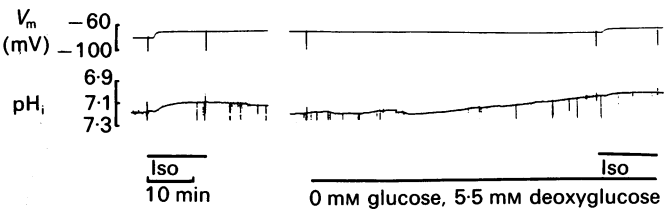

Fig. 11. Effect of inhibiting glycolysis. The panel on the left shows the control exposure to isoprenaline (Iso). The panel on the right shows the effect of exposing the fibre to isoprenaline in Tyrode solution in which glucose was replaced with 2-deoxyglucose.

acidification would occur if isoprenaline increased permeability to H^+. To test this, we reversed the driving force for H^+. Under these conditions, an increase in membrane permeability would be expected to result in alkalinization. Figure 10, left panel, shows the usual acidification in response to isoprenaline. In the panel on the right, pH_o was increased with NaOH to 8·36 and the membrane potential was depolarized by increasing $[K^+]_o$ to 10 mM. In this experiment, the equilibrium potential for H^+ under those conditions was approximately −77·5 mV and the driving force on H^+ ions was +27·5 mV (outward). Isoprenaline none the less induced intracellular acidification. Similar results occurred in five experiments in this series. Thus, isoprenaline-induced intracellular acidification is not due to an increase in permeability to H^+.

Effect of isoprenaline on the metabolic production of protons

Catecholamines might increase the production of H_i^+ by stimulating glycolysis. Glycolysis was inhibited using a method described by Allen & Orchard (1983) in order to reduce the metabolic production of proton. Figure 11 shows a representative experiment in which this was tested. In normal Tyrode solution (left panel), isoprenaline resulted in acidification of 0·08 pH units and in membrane depolarization of 9·8 mV. After pH_i and membrane potential returned to their baseline values, glycolysis was inhibited by exposing the fibre for 60 min to a solution in which dextrose was replaced by 2-deoxyglucose (DOG) at 5·5 mM (right panel), resulting in

intracellular acidification. Acidification by isoprenaline under those conditions was virtually abolished, although 5·3 mV depolarization was still observed. All four experiments in this series had similar results, as did two experiments performed in Purkinje fibres from cow hearts.

DISCUSSION

The results show that, in quiescent sheep Purkinje fibres, catecholamines cause depolarization and intracellular acidification, mediated via β-adrenergic receptors. The β-adrenergic agonist isoprenaline slows the recovery from an intracellular acid load. The α-adrenergic agonist phenylephrine has no effect on membrane potential or pH_i in resting fibres but may slightly enhance the rate of recovery from an intracellular acidification.

The intracellular acidification by β-adrenergic agonists appears to be mediated by an increase in cyclic AMP, rather than by some other mechanism, such as a direct effect by the stimulatory G protein, G_s. It was attenuated, but not eliminated, by interventions that inhibit the Na^+–H^+ exchanger and virtually abolished by inhibition of glycolysis with DOG. These results suggest that β-adrenergic stimulation both inhibits the Na^+–H^+ exchanger and increases metabolic production of H^+.

Comparison with other studies

Intracellular acidification has not previously been described as an effect of catecholamines in cardiac tissue. However, a small inhibition of the rate of pH_i recovery from an internal acid load by isoprenaline and by other agents that raise cyclic AMP was reported by Wu & Vaughan-Jones (1988). Gambassi *et al.* (1990), on the other hand, using the fluorescent probe SNARF-1, found no effect of 0·05 μM isoprenaline on pH_i in quiescent rat ventricular myocytes. The reason for the difference in their findings is not apparent to us. Possibilities include the species difference, the 100-fold lower concentration of isoprenaline that they used and the fact that cells were bathed in bicarbonate rather than HEPES-buffered solutions

In the same abstract, the authors also report that α-adrenergic stimulation by phenylephrine, 50 μM, increased pH_i by approximately 0·05 pH unit. As with isoprenaline, the difference in species or in experimental conditions may have produced the different outcome. Similarly, Breen & Pressler (1988), using a very high concentration of phenylephrine, 100 μM, found minimal alkalinization (0·03 pH units) in four dog and sheep Purkinje fibres. After 60–90 min of exposure, phenylephrine 'seemed to accelerate pH_i recovery from NH_4Cl-induced acidosis.' They do not state whether these effects were statistically significant. Alkalinization was also found by Wallert & Fröhlich (1989) in isolated ventricular myocytes with 6-fluoronoradrenaline, with UK 14304 (α_1- and α_2-adrenergic agonists, respectively), with the phorbol ester, phorbol 12-myristate 13-acetate and with ATP. However, since information was not provided regarding the species used, the concentration of the drugs, or the amount of pH_i change, the data are difficult to evaluate.

Thus, in all studies, the change in pH_i induced by α-adrenergic stimulation was small, and high concentrations of agonist were used. Although we did not note a

change in resting pH_i, our results are consistent with those of Breen & Pressler (1988) in that we observed a small, consistent (though not statistically significant) acceleration in the rate of pH_i recovery following intracellular acidification.

Our finding suggesting that the acidification is mediated by cyclic AMP agrees with findings, reported in abstract form by Wu & Vaughan-Jones (1988) and by Wu (1990), that in sheep Purkinje fibres cyclic AMP, isoprenaline, and the phosphodiesterase inhibitor 3-isobutyl-l-methylxanthine (IBMX) all reduce resting pH_i. IBMX, forskolin and theophylline all reduced net acid efflux and decreased the rate of rise of intracellular Na^+ activity after an NH_4Cl-induced intracellular acidification, suggesting that elevation of cyclic AMP inhibits the Na^+–H^+ exchanger.

β-Adrenergic agonists have been reported to cause hyperpolarization of the resting membrane potential in cardiac tissues of many species. This hyperpolarization is thought to be due to stimulation of the Na^+–H^+ exchanger (Vassalle & Barnabei, 1971; Wasserstrom *et al.* 1982). In our experiments, on the other hand, exposure to adrenaline and isoprenaline consistently resulted in depolarization. This anomalous effect of β-adrenergic agonists in resting sheep Purkinje fibres has previously been reported, and is thought to be due to activation of an inward sodium current (Terris, Wasserstrom & Fozzard, 1986; Egan, Noble, Noble, Powell & Twist, 1987; Egan, Noble, Noble, Powell, Twist & Yamaoka, 1988; Chae, Wang, Gong & Lee, 1990). In our experiments, the elimination of the depolarization under Na^+-free conditions supports this conclusion.

Variability in control values

The considerable variability in pre-drug membrane potential and pH_i may reflect the fact that the time from slaughter to the time of extraction of the hearts fluctuated somewhat, a factor over which we had no control. Thus, despite the requirement that all fibres had to have a stimulated action potential with a maximum diastolic potential negative to -70 mV, the condition of the fibres may have been somewhat inconstant. However, the changes in membrane potential and pH_i that we report were independent of the control values.

Possible mechanisms for the results

Several possible explanations can be invoked for the acidification and for the slowing of recovery from an acid load. These include: (1) direct or indirect inhibition of the Na^+–H^+ exchanger; (2) change in intracellular intrinsic buffering power; (3) increased membrane permeability to H^+; and (4) increased intracellular production of H^+. Each of these possibilities will be considered.

(1) The intracellular acidification induced by β-adrenergic stimulation, and the slowing of recovery following intracellular H^+ loading raise the possibility that the Na^+–H^+ exchanger was inhibited by β-adrenergic agonists. In our experiments, the inhibition of acidification by DMA supports this possibility. The observation that catecholamines blunted the hyperpolarization following removal of NH_4Cl also supports the possibility of such inhibition. This hyperpolarization is thought to occur as a result of activation of the Na^+–H^+ exchanger by the abrupt decrease in pH_i (Piwnica-Worms *et al.* 1986). H^+ is extruded in exchange for Na^+. The increase in $[Na^+]_i$ activates the electrogenic Na^+–H^+ exchanger, thereby hyperpolarizing the

membrane potential. Blunting of this hyperpolarization, therefore, would be expected when Na^+–H^+ exchange is inhibited. It would also occur if the Na^+–H^+ exchanger were inhibited. This is unlikely, though, since β-adrenergic agonists are thought to stimulate rather than inhibit the Na^+–H^+ exchanger (Vassalle & Barnabei, 1971; Wasserstrom *et al.* 1982).

Inhibition of Na^+–H^+ exchange is supported by the observation by Wu (1990) that the phosphodiesterase inhibitor IBMX decreased acid efflux following H^+-loading and decreased Na^+ influx.

It is likely that inhibition of the Na^+–H^+ exchanger by β-adrenergic agonists is direct (occurring for example by decreasing its affinity for H^+), rather than reflecting an indirect effect resulting from a catecholamine-induced increase in intracellular Na^+. Catecholamines have been shown to elevate $[Na^+]_i$ by approximately 0·8 mM (Chae *et al.* 1990; noradrenaline 10^{-6} M in quiescent sheep Purkinje fibres). Given the $[Na^+]_o$ in our solutions of 141 mM, and assuming an intracellular Na^+ activity (a^i_{Na}) of 8 mM (Ellis & MacLeod, 1985; Chae *et al.* 1990), a 0·8 mM increase in intracellular Na^+ would decrease the reversal potential for Na^+, E_{Na}, from 77 to 74 mV. With a mean resting pH_i in our fibres of 7·1, the reversal potential for hydrogen, E_H, was −18·5 mV. Since net extrusion of H^+ can occur as long as $E_{Na} > E_H$ (Ellis & MacLeod, 1985) it is unlikely that this small change in E_{Na}:E_H would significantly affect Na^+–H^+ exchanger activity. Important in that regard is the observation that, when the Na^+ gradient is varied by altering $[Na^+]_o$, large reductions of $[Na^+]_o$ are required to affect the Na^+–H^+ exchanger. Ellis & MacLeod (1985) found that $[Na^+]_o$ had to be reduced to 8 mM before the rate of pH_i recovery was inhibited by 50%.

(2) The increase in intracellular Na^+ might affect pH_i by reducing intrinsic buffering power, β – the physicochemical, non-CO_2-dependent buffering against fluctuations in $[H^+]_i$, thought to be provided by intracellular proteins (Roos & Boron, 1981; Vaughan-Jones & Wu, 1990). The increase in $[Na^+]_i$ produced by β-adrenergic stimulation would be expected to suppress the Na^+–Ca^{2+} exchanger, resulting in an increase in $[Ca^{2+}]_i$. H^+ and Ca^{2+} are thought to compete for the same intracellular buffering sites (Bers & Ellis, 1982; Vaughan-Jones, Lederer & Eisner, 1983). Therefore, an increase in $[Ca^{2+}]_i$ might reduce β and, consequently, pH_i. Alternatively, β-adrenergic agonists might *decrease* $[Ca^{2+}]_i$ because of stimulation of sarcolemmal Ca^{2+}-ATPase, and increased uptake of Ca^{2+} by the sarcoplasmic reticulum (Lindemann, Jones, Hathaway, Henry & Watanabe, 1983). This would result in an increased β and pH_i.

In our experiments, isoprenaline induced a small *increase* in β. Such an increase is consistent with measurements of cytosolic calcium in resting cardiac myocytes using fluorescent indicators, that have shown it to be decreased following β-adrenergic stimulation (Sheu, Sharma & Korth, 1987; Danziger, Sakai, Lakatta & Hansford, 1990). A decrease in $[Ca^{2+}]_i$ and a consequent increase in β would have the opposite effect on pH_i to that which we report.

A second possible explanation for the small increase in β that we observed is that the mean pH_i at which β was determined was, as expected, slightly more acidic in the presence of isoprenaline. β increases in a linear fashion as pH_i decreases (Vaughan-Jones & Wu, 1990).

(3) Our results rule out a simple catecholamine-induced increase in membrane

permeability to H^+ as an explanation for the decrease in pH_i, since isoprenaline induced acidification even when the driving force for H^+ was reversed from its normally inward direction to outward.

(4) Catecholamines might increase the production of H^+ by stimulating intracellular metabolic processes. It has long been known that β-adrenergic stimulation increases the breakdown of glycogen. Moreover, this enhanced glycogenolysis is thought to occur as the result of cyclic AMP-dependent phosphorylation of phosphorylase *b* kinase to the activated phosphorylase *a* kinase (Dobson, 1981). The glucose-1-phosphate accumulating as a result of the enhanced glycogenolysis serves as a substrate for glycolysis, with accumulation of lactic acid and a fall in pH_i. In our experiments, this fall in pH_i was prevented when glycolysis had been inhibited with DOG.

Acidification following isoprenaline administration was quite rapid. It was generally complete within 5 min of the onset of catecholamine delivery into the tissue bath. Such a time course is more consistent with an effect on metabolism than on Na^+–H^+ exchange. Cyclic AMP response to catecholamines generally reaches a peak in less than 30 s, and peak inotropic response is reached within 2 min (Venter, 1981). The effect of inhibiting the Na^+–H^+ exchanger at resting pH_i, on the other hand, would be expected to occur more slowly. Following complete inhibition of the Na^+–H^+ exchanger with amiloride or its analogues, acidification is modest, and quite gradual (see, for example, Fig. 7 in the present manuscript, or Fig. 4*A* in Ellis & Macleod, 1985, where acidification has not yet reached a peak at the end of the approximately 19 min of exposure to 1 mM amiloride).

It has recently been suggested that DOG may inhibit the Na^+–H^+ exchanger (Weissberg, Little, Cragoe & Bobik, 1989; Vaughan-Jones & Wu, 1988). It might be argued that in our experiments the elimination of catecholamine-induced acidification by DOG was due to its effect on the exchanger. This seems unlikely, since other effective methods of inhibiting the Na^+–H^+, such as superfusion with DMA, a member of a class of the most potent known Na^+–H^+ exchange inhibitors, or Na^+-free solutions, failed to eliminate the acidification.

Conclusions

Our findings indicate that in resting sheep Purkinje fibres β-adrenergic stimulation induces intracellular acidification due primarily to an increased production of acid by the glycolytic pathway. Given the known actions of β-agonists, this is probably a consequence of their stimulation of glycogenolysis. However, acid production solely by the glycolytic pathway fails to explain several observations. (1) Amiloride derivatives attenuated isoprenaline-induced acidification, but failed to eliminate it; (2) the initial hyperpolarization following removal of NH_4Cl was attenuated by isoprenaline; (3) in the experiments reported by Wu (1990), the attenuation of pH_i recovery was accompanied by a parallel decrease in the rate of rise of intracellular Na^+ activity. Thus, it is probable that an inhibitory effect on the Na^+–H^+ exchanger also plays a role in the acidification by β-adrenergic agonists. As we have argued, though, the rapidity of the acidification makes it likely that the metabolic effect plays a greater role in mediating the decrease in pH_i than does the effect on the exchanger.

We thank Drs Peggy L. Barrington and Robert E. Ten Eick for their helpful criticisms, and Ms Erin Schlosser for her help in preparing the manuscript. Dr Guo's research was supported by a fellowship award from the Feinberg Cardiovascular Research Institute, Northwestern University Medical School. Additional support for this project came from the Reingold Estate, the Brinton Trust, the Cooley Charitable Trust and USPHS HL-30724 (J.A.W.). Drs Rosenthal and Wasserstrom are members of the Feinberg Cardiovascular Research Institute, Northwestern University Medical School.

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