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# Relation between cytosolic free $Ca^{2+}$ concentration and the control of pyruvate dehydrogenase in isolated cardiac myocytes

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1. The proportion of pyruvate dehydrogenase existing in the active form ($PDH_A$) in suspensions of unstimulated cardiac myocytes oxidizing glucose is approx. 30%. 2. Depolarization of the cells with concentrations of $K^+$ above physiological values leads to an increase in the content of $PDH_A$. Overloading of the cells with $Na^+$ by treatment with veratridine and ouabain gives the same result. 3. Each of these interventions is shown in experiments with Quin 2-loaded myocytes to lead to an increase in cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_c$). 4. Treatment of the cells with Ruthenium Red, an inhibitor of $Ca^{2+}$ transport into mitochondria, largely prevents an increase in $PDH_A$ in response to addition of KCl or of veratridine plus ouabain. Ruthenium Red does not attentuate the increase in $[Ca^{2+}]_c$ that occurs under these conditions. 5. By contrast, treatment of the cells with ryanodine, an inhibitor of sarcoplasmic-reticulum $Ca^{2+}$ transport and therefore of contraction, does not diminish the response of $PDH_A$ content to agents which raise $[Ca^{2+}]_c$; nor does loading of the cells with the $Ca^{2+}$-chelating agent Quin 2, which also prevents contraction, at appropriate concentrations. 6. It is concluded that an increase in $[Ca^{2+}]_c$ causes an increase in $PDH_A$ content of cardiac myocytes independently of an increase in mechanical work. In the normal physiological situation the activation of dehydrogenases by $Ca^{2+}$ is thought to help to maintain the balance of energy supply and demand during periods of increased work-load, which are associated with an increased myoplasmic $[Ca^{2+}]_c$.

## INTRODUCTION

A role for $Ca^{2+}$ ions in activating the pyruvate dehydrogenase (PDH) complex has emerged quite clearly from studies both at the level of the purified enzyme and at the level of isolated intact mitochondria. Work with the enzyme established that $Ca^{2+}$ ions facilitate the binding of a phosphatase to the transacetylase core of the multienzyme complex, thereby promoting the removal of phosphoryl groups from sites on the $\alpha_1$ subunit of the pyruvate decarboxylase enzyme, and generating the active form of the enzyme complex ($PDH_A$) (Linn *et al.*, 1969*a*,*b*; Denton *et al.*, 1972; Pettit *et al.*, 1972; Randle *et al.*, 1974; Severson *et al.*, 1974; Yeaman *et al.*, 1978). Phosphorylation, with concomitant inactivation, is catalysed by a specific PDH kinase, and the amount of $PDH_A$ present under any condition depends on the relative balance of kinase and phosphatase activities. These relations have been reviewed by Reed (1981) and by Wieland (1983). Work with isolated mitochondria has demonstrated that increasing the free $Ca^{2+}$ concentration of the incubation medium from 0.1 to 1 $\mu$M, a plausible range for the cytosol of myocytes, results in an increased content of $PDH_A$ [Hansford & Cohen (1978); Denton *et al.* (1980); Hansford (1981); reviewed by Hansford (1985) and Denton & McCormack (1985)].

It has been more difficult to establish the role of $Ca^{2+}$ ions in modulating PDH interconversion in whole tissues or organs. Such a role would be expected in muscle, where an increased cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) is the signal for contraction (see Katz, 1970), and increased contractile work requires a matching increase in mitochondrial oxidative phosphorylation. Indeed, increased work performance in isolated hearts is known to result in an increased $PDH_A$ content (Illingworth & Mullings, 1976; Hiltunen & Hassinen, 1976; Reinauer & Muller-Ruchholtz, 1976; Pearce *et al.*, 1980; McCormack & Denton, 1981; Bunger *et al.*, 1982; Kobayashi & Neely, 1983*b*). However, the assignment of a role to $Ca^{2+}$ ions in mediating this change is not straightforward, as increased work is also associated with decreases in [ATP]/[ADP] and [NADH]/[$NAD^+$] ratios in heart muscle (Illingworth *et al.*, 1975; Reinauer & Muller-Ruchholtz, 1976), and these parameters also affect PDH interconversion (see reviews by Hansford, 1980; Reed, 1981; Wieland, 1983). McCormack & England (1983) addressed this point by using Ruthenium Red in studies of isolated perfused hearts: this inhibitor of $Ca^{2+}$ uptake into mitochondria (Moore, 1971) prevented positive inotropic agents from elevating $PDH_A$ content, though they did increase force production and the content of glycogen phosphorylase *a*, both of which are indirect indicators of a raised value of $[Ca^{2+}]_c$. This result was consistent with a requirement of $Ca^{2+}$ uptake into mitochondria to generate increased $PDH_A$ content, and indeed with a predominant role of $Ca^{2+}$ compared with the other effectors. A similar conclusion was reached by McCormack and Denton (1984) in studies in which the activation status of PDH was raised by perfusing hearts in the presence of adrenaline or an elevated perfusate $[Ca^{2+}]$: subsequent rapid isolation of

Abbreviations used: PDH, pyruvate dehydrogenase; $PDH_A$, its active form; $[Ca^{2+}]_c$, cytosolic free $Ca^{2+}$ concentration; DTPA, diethylenetriaminepenta-acetic anhydride.

mitochondria allowed the preservation of this increased $PDH_A$ content, but only if the mitochondria were allowed to retain an elevated Ca content.

Though an important study, the work by McCormack & England (1983) lacked a direct measure of $[Ca^{2+}]_c$, and it was considered worthwhile to perform analogous studies in suspensions of isolated cardiac myocytes, in which it is possible to monitor $[Ca^{2+}]_c$ continuously by using the fluorescent $Ca^{2+}$-chelating agents Quin 2 and Fura 2 (Tsien, 1980, 1981; Grynkiewicz *et al.*, 1985). This paper reports the results of such studies, including parallel measurements of $PDH_A$ and $[Ca^{2+}]_c$ in response to a number of interventions which increase cellular Ca. Further, it exploits the intracellular chelation of $Ca^{2+}$ by Quin 2 as a means to dissociate increases in $[Ca^{2+}]_c$ from increased work-performance, and thus to assess the relative significance of $[Ca^{2+}]_c$, [NADH]/[$NAD^+$] and [ATP]/[ADP] ratios as control parameters. Use of the compound ryanodine, which inhibits sarcoplasmic-reticulum $Ca^{2+}$ release (Sutko *et al.*, 1979; Sutko & Willerson, 1980; Sutko & Kenyon, 1983), also allows such a distinction to be made.

## EXPERIMENTAL

### Preparation of cardiac myocytes

Hearts were disaggregated into isolated myocytes by perfusion with collagenase-containing medium, followed by chopping with scissors. The heart was removed rapidly from a 300–400 g male Sprague–Dawley rat, cannulated via the aorta and perfused with 20 ml of medium comprising 116 mM-NaCl, 5.4 mM-KCl, 26 mM-$NaHCO_3$, 1 mM-$NaH_2PO_4$, 1 mM-$MgSO_4$ and 5.6 mM-D-glucose ('preparation medium') which had been equilibrated with an atmosphere of $O_2/CO_2$ (19:1). The perfusion fluid was maintained at 37 °C. After this initial wash-out phase, a recirculating perfusion was begun with 100 ml of preparation medium to which had been added 100 mg of collagenase (type 1 from Cooper) and $CaCl_2$ to give 50 $\mu$M. Perfusion was ended after 30 min, at which point the heart was palpably soft. The ventricle tissue was then chopped with scissors for 3 min in 15 ml of collagenase-containing medium: the resulting crude suspension was filtered through nylon gauze to remove pieces of tissue, and the cells were allowed to settle under gravity for 6 min. The supernatant was removed by aspiration and the pellet of cells was resuspended in 20 ml of preparation medium containing additional $CaCl_2$ (final concn. 0.25 mM). The cells were again allowed to sediment under gravity for 6 min, and the pellet was then resuspended in 8–20 ml of preparation medium which had been supplemented with 5 mg of extensively dialysed bovine serum albumin (Fraction V, from Sigma)/ml, 10 mM-Hepes/NaOH, pH 7.4, 1 mM-$CaCl_2$ and D-glucose to give 20 mM ('incubation medium'). This medium was used for all of the studies reported in this paper.

The resulting cell suspensions contained approx. 70–80% viable cells, as judged by a rod-shaped morphology, and were maintained for up to 4 h at 37 °C. Portions (2 ml) were shaken gently, with an orbital motion, in plastic scintillation vials, under $O_2/CO_2$ (19:1).

### Measurement of $PDH_A$

Portions of myocyte suspensions (75 $\mu$l) were removed and added to 250 $\mu$l portions of an ice-cold 'quenching' solution designed to prevent further interconversion of PDH. This solution comprised 0.05 M-Hepes/KOH, pH 7.1, 0.1% Triton X-100, 3 mM-EDTA ($K^+$ salt), 25 mM-NaF, 1 mM-potassium dichloroacetate, 1 mM-dithiothreitol, 20 $\mu$M-leupeptin and 0.4 mM-phenylmethanesulphonyl fluoride. The active form of the enzyme was then assayed spectrophometrically at 25 °C by measuring formation of NADH from pyruvate and $NAD^+$ generated by lactate dehydrogenase: this assay has the advantage of being applicable to crude tissue samples. Details of the assay are given in Hansford & Castro (1985). The only modification in the present work was that all cell samples were exposed to 'quenching' solution for 4 min and were then clarified before assay by a 30 s centrifugation in an Eppendorf micro-centrifuge.

Protocols based on micro-centrifugation followed by the freezing of the cell pellet in liquid $N_2$ proved incapable of preventing further PDH interconversion, and thus were found not to be useful.

Complete conversion of PDH into $PDH_A$ was achieved by incubating suspensions of myocytes (4–5 mg of protein/ml) with 10 $\mu$g gramicidin/ml for 10 min. This treatment causes a large increase in $[Ca^{2+}]_c$ (see below), as the ionophore collapses the $Na^+$ electrochemical gradient and allows entry of $Ca^{2+}$ by both voltage-dependent $Ca^{2+}$ channels and by $Na^+/Ca^{2+}$ exchange. Higher concentrations of gramicidin, or combination with proton ionophore uncoupling agents, gave no further increase in $PDH_A$. Values for total PDH are slightly lower than those found for intact hearts, though part of the difference is due to the higher assay temperatures used in other studies. Thus Hiltunen & Hassinen (1976) reported 23 nmol/min per mg of protein, at 30 °C: Whitehouse *et al.* (1974) 19–32 nmol/min per mg dry wt., at 30 °C, and Kobayashi & Neely (1983*a*) 16–24 nmol/min per mg dry wt., at 27 °C.

### Monitoring $[Ca^{2+}]_c$ by use of Quin 2 and Fura 2

Cells were loaded with the fluorescent $Ca^{2+}$-chelating agents Quin 2 and Fura 2 by incubation for 30 min at 37 °C in incubation medium to which the acetoxymethyl (AM) ester of Quin 2 or Fura 2 had been added. Normally 2 $\mu$M-Fura 2/AM was used, whereas a range of Quin 2/AM concentrations of 5–100 $\mu$M was used, depending on the intracellular $Ca^{2+}$ buffering desired. Cell protein concentration was in the range 3–4 mg/ml. Loading of the cells was terminated by centrifugation at 50 *g* for 2 min, followed by resuspension in 2 ml of incubation medium. An additional washing step seemed to make little difference to the fraction of the $Ca^{2+}$-indicator which behaved as 'extracellular'.

Fluorescence of the cell suspension was monitored with a Farrand $A_2$ fluorimeter, fitted with a temperature-controlled chamber, and magnetic stirring. The temperature was 37 °C and a gas phase of $O_2/CO_2$ (19:1) was maintained in the chamber. Excitation was at 333 nm, with a Hg lamp and a narrow band-pass interference filter: emission of wavelengths greater than 480 nm was collected, by using a sharp-cut yellow filter (Farrand no. 3-71). Changes in fluorescence of cells not loaded with $Ca^{2+}$-indicator were extremely small under these conditions, being not more than 4% of those changes obtained with cell suspensions loaded with 10 $\mu$M-Quin 2/AM. If digitonin was used to equilibrate intra- and extra-cellular dye (see below), instead of

Triton X-100, changes in fluorescence of unloaded cells were even less. Cell protein was determined by the biuret reaction.

### Materials

Quin 2/AM, Quin 2, Fura 2/AM and ionomycin were from Calbiochem; veratridine and DTPA were from Sigma Chemical Co.; Ruthenium Red was from Polysciences; digitonin was from Fisher Scientific Co., and recrystallized twice and dissolved in ethanol; ouabain was from Boehringer Mannheim Corp. Ryanodine was a gift from Dr. E. Lakatta of this Institute: forskolin was a gift from Dr. H. Metzger, of Hoechst. All other reagents were of the highest grade available commercially.

## RESULTS AND DISCUSSION

### Effect of plasma-membrane depolarization on the $PDH_A$ content of suspensions of cardiac myocytes

Raising the concentration of KCl in the cell incubation medium, a procedure which gives a graded depolarization of the plasma membrane and raises $[Ca^{2+}]_c$ (Powell *et al.*, 1984; Sheu *et al.*, 1986; and see below), gives rise to an increase in $PDH_A$ content (Table 1*a*). This is not affected by prior exposure of the cells to the $\beta$-adrenergic agonist isoprenaline. It is, however, largely prevented by the $Ca^{2+}$-channel blocker verapamil, when KCl concentration is 45 mM (results not shown). In these experiments, samples were taken 10 min after the addition of $K^+$-containing medium, a time at which the system is close to achieving a new steady state (Fig. 1*a*).

Other protocols that were used to increase $PDH_A$ content included exposure to ouabain, which causes the cells to become loaded with $Na^+$ through inhibition of the $Na^+$- and $K^+$-dependent ATPase (Escueta & Appel, 1969), and to veratridine, which potentiates the opening of $Na^+$ channels (Ohta *et al.*, 1973), with a similar result. A consequence of this is plasma-membrane depolarization and loading of the myocytes with $Ca^{2+}$. Table 1(*d*) shows that, in each case, $PDH_A$ content was elevated, with 25 $\mu$M-veratridine having a larger effect than 5 $\mu$M. Fig. 1(*b*) presents the time course of the response to ouabain in a single, typical, preparation of cells: the control curve of Fig. 1(*c*) shows the more rapid response to addition of both ouabain and 25 $\mu$M-veratridine.

The impact of the inhibitor Ruthenium Red on these relations was of major interest, in view of its site of action at the level of mitochondrial $Ca^{2+}$ uptake (Moore, 1971; Vasington *et al.*, 1972). Table 1(*b*) shows that, whereas Ruthenium Red had no effect on the $PDH_A$ content of suspensions of resting cells (i.e. in medium containing 5 mM-$K^+$), it almost abolished the response of $PDH_A$ content to raised concentrations of $K^+$. Similarly, Ruthenium Red prevented the increase in $PDH_A$ caused by veratridine plus ouabain (Table 1*d*).

These results strongly suggest that it is necessary for the increase in $[Ca^{2+}]_c$ that occurs in response to elevated $[K^+]$ and to veratridine plus ouabain, and which is documented below, to be translated into the mitochondria for an increase in $PDH_A$ to occur. This conclusion is in agreement with that reached by McCormack & England (1983) on the basis of experiments with intact perfused hearts.

**Table 1. Effect of Ruthenium Red and ryanodine on the increase in $PDH_A$ content induced by KCl and by veratridine plus ouabain**

Suspensions of rat cardiac myocytes in incubation medium were sampled for $PDH_A$ content as described in the Experimental section. The basal medium contained 5 mM-KCl: higher concentrations of $K^+$ were achieved by mixing volumes of an isosmotic medium in which $K^+$ replaced $Na^+$ ions. Sampling was 10 min after the addition to the suspension of $K^+$-containing medium or of veratridine plus ouabain, as appropriate. Where indicated, isoprenaline, Ruthenium Red and ryanodine were added 3 min before the $K^+$-containing medium or veratridine, to give concentrations of 1 $\mu$M, 12 $\mu$M and 1 $\mu$M respectively. Data are presented as means ± S.E.M., with the numbers of preparations in parentheses. Values of $PDH_A$ are significantly lower in the presence of Ruthenium Red than in otherwise identical incubations omitting Ruthenium Red: †$P < 0.05$; *$P < 0.005$; **$P < 0.001$.

| | Condition | $PDH_A$ (% of total) |
|---|---|---|
| (*a*) | 5 mM-KCl | 31.7 ± 1.4 (32) |
| | 20 mM-KCl | 39.5 ± 0.6 (4) |
| | 40 mM-KCl | 49.6 ± 4.6 (6) |
| | 55 mM-KCl | 49.3 ± 3.6 (8) |
| | 80 mM-KCl | 60.6 ± 3.9 (11) |
| | 5 mM-KCl + isoprenaline | 32.3 ± 3.5 (8) |
| | 40 mM-KCl + isoprenaline | 47.5 ± 2.9 (8) |
| | 80 mM-KCl + isoprenaline | 58.3 ± 3.7 (8) |
| (*b*) | 5 mM-KCl + Ruthenium Red | 31.5 ± 2.7 (3) |
| | 40 mM-KCl + Ruthenium Red | 38.7 ± 2.3 (3) |
| | 55 mM-KCl + Ruthenium Red | 35.2 ± 3.0 (5)† |
| | 80 mM-KCl + Ruthenium Red | 37.8 ± 3.3 (7)* |
| (*c*) | 20 mM-KCl + ryanodine | 39.8 ± 4.1 (3) |
| | 40 mM-KCl + ryanodine | 56.0 ± 6.9 (8) |
| | 55 mM-KCl + ryanodine | 62.3 ± 7.0 (8) |
| | 80 mM-KCl + ryanodine | 72.7 ± 9.2 (8) |
| (*d*) | 5 $\mu$M-Veratridine | 37.6 ± 4.5 (6) |
| | 25 $\mu$M-Veratridine | 51.0 ± 1.9 (10) |
| | 25 $\mu$M-Veratridine + 0.2 mM-ouabain | 57.6 ± 3.9 (8) |
| | 0.2 mM-Ouabain | 45.6 ± 5.0 (5) |
| | 25 $\mu$M-Veratridine + 0.2 mM-ouabain + Ruthenium Red | 35.8 ± 2.0 (10)** |
| (*e*) | 25 $\mu$M-Veratridine + 0.2 mM-ouabain + ryanodine | 52.6 ± 4.1 (3) |

### $PDH_A$ content of cardiac myocytes under depolarizing conditions not allowing mechanical activity

In contrast with the effect of Ruthenium Red the inhibitor ryanodine, which inhibits $Ca^{2+}$ release from the sarcoplasmic reticulum (Sutko *et al.*, 1979; Sutko & Willerson, 1980; Sutko & Kenyon, 1983) and thus inhibits contraction, was found not to attenuate the increase in $PDH_A$ content induced by either KCl (Table 1*c*) or by combination of veratridine plus ouabain (Table 1*e*). Examination of the cells under the microscope confirmed that they remained totally quiescent during these manoeuvres.

Further, loading of the myocytes with the $Ca^{2+}$-chelating agent Quin 2 (to 0.5–0.7 nmol/mg of protein) resulted in suspensions of cells which exhibited none of

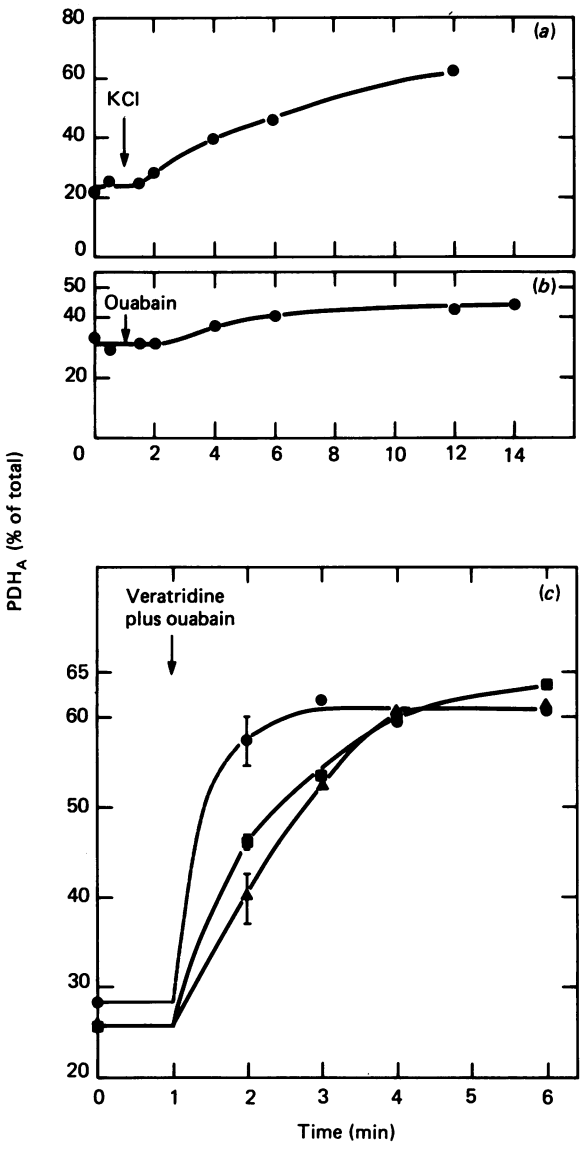


**Fig. 1. Time-course of the response of myocyte PDH$_A$ content to cell depolarization**

(*a*), (*b*), Portions (1 ml) of myocyte suspensions (approx. 4 mg of protein) were incubated and sampled as described in the Experimental section. At the point marked 'KCl', a 1 ml portion of an incubation medium in which NaCl was replaced by KCl was added, giving a final $K^+$ concentration of 80 mM. In (*b*), ouabain was added to give a final concentration of 0.2 mM. (*c*) Myocytes (1 ml portions containing 3–5 mg of protein) were loaded with Quin 2 by a 30 min incubation at 37 °C with 50 $\mu$M- (■) or 100 $\mu$M- (▲) Quin 2/AM (●, control cells). At the point indicated, 25 nmol of veratridine and 0.2 $\mu$mol of ouabain were added. Results are presented as the percentage of total PDH (PDH$_{TOTAL}$) in the active form (PDH$_A$). PDH$_{TOTAL}$ was found to be $11.8 \pm 1.4$ (3) nmol/min per mg of protein, measured at 25 °C. In (*c*), results were obtained with five (control and 50 $\mu$M-Quin 2/AM) and three (100 $\mu$M-Quin 2/AM) batches of cells: the error bars present the S.E.M., and the differences 1 min after stimulation are all significant ($P < 0.05$). Three cell preparations loaded with 7.5 $\mu$M-Quin 2/AM gave results which could be superimposed on the results from unloaded cells (not shown).

**Table 2. Effect of addition of KCl and of veratridine plus ouabain on the PDH$_A$ content of Quin 2-loaded cardiac myocytes**

The experiment was conducted as described for Table 1, except that the myocytes were loaded with Quin 2 by a prior 30 min incubation with 7.5 $\mu$M-Quin 2/AM, as described in the Experimental section. The data are derived from the numbers of experiments indicated, but only three preparations of cells were used.

| Condition | PDH$_A$ (% of total) |
|---|---|
| 5 mM-KCl | $34.2 \pm 4.6$ (6) |
| 20 mM-KCl | 41.5 (2) |
| 40 mM-KCl | $45.3 \pm 3.1$ (4) |
| 55 mM-KCl | $44.7 \pm 5.1$ (5) |
| 80 mM-KCl | $57.3 \pm 5.5$ (3) |
| 25 $\mu$M-Veratridine + 0.2 mM-ouabain | $62.5 \pm 2.8$ (4) |

the spontaneous contractile waves, which are normal when these cells are incubated in media containing 1 mM-$CaCl_2$, and which increase in frequency with increasing KCl concentration: the increase in PDH$_A$ caused by KCl or by veratridine plus ouabain was, however, undiminished (Table 2; cf. Table 1). The only exception to this finding of quiescence was a re-appearance of waves in some Quin 2-loaded cells which were treated with both 80 mM-KCl and 1 $\mu$M-isoprenaline.

Ruthenium Red (12 $\mu$M) had no effect on the frequency of waves induced by $K^+$ concentrations from 5 to 80 mM, despite a report that it is capable of inhibiting $Ca^{2+}$ transport by the sarcoplasmic reticulum of lysed myocytes (Altschuld *et al.*, 1985). These observations strongly suggest that an increased generation of ADP by actomyosin ATPase is not a necessary part of the mechanism whereby KCl-induced depolarization causes an increase in PDH$_A$ content in cardiac myocytes.

Independent evidence in favour of this conclusion comes from the very minor degree of nicotinamide nucleotide oxidation which occurs on treatment of myocyte suspensions with KCl (results not shown). Fluorescence measurements, reflecting mainly mitochondrial nucleotide, give at most a 6% change, in the direction of oxidation, when this is compared with the total span obtained with uncoupling agent and rotenone (added separately). Owing to the near-equilibrium nature of mitochondrial [NADH]/[$NAD^+$] and cellular [ATP]/[ADP] × [$P_i$] ratios (see, e.g., Holian *et al.*, 1977), this strongly suggests that changes in [ATP]/[ADP] ratio are not a factor in the response of PDH interconversion to depolarization by 45 mM-KCl.

The Quin-2 loading studies described above all used 7.5 $\mu$M-Quin 2/AM and a protein concentration in the range 3–4 mg/ml, and generated cells containing 0.5–0.7 nmol of Quin 2/mg of protein. Use of higher concentrations of the Quin 2/AM ester in the loading protocol (50–100 $\mu$M) gave suspensions of cells in which the PDH$_A$ content responded less rapidly to depolarization, but in which the same steady-state value was achieved. This is shown in Fig. 1(*c*), for depolarization by veratridine plus ouabain. This result also emphasizes the importance of changes in $[Ca^{2+}]_c$ as a signal to enzyme interconversion.

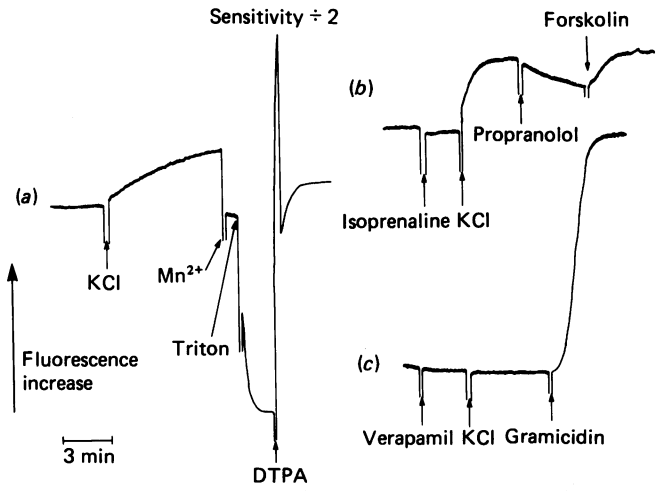


**Fig. 2. Effect of raising $[K^+]$ on the fluorescence of cardiac myocytes loaded with Quin 2**

Portions (2 ml) of myocyte suspension containing 3 mg of protein/ml were loaded with Quin 2 by incubation with 50 $\mu$M-Quin 2/AM, as described in the Experimental section. The fluorescence of Quin 2 was monitored as described in the Experimental section. In (*a*), KCl was added to 30 mM, $MnCl_2$ to 0.1 mM, Triton X-100 to 0.1/% and DTPA to 1 mM. In (*b*), isoprenaline was added to 1 $\mu$M, KCl to 30 mM, DL-propranolol to 1 $\mu$M and forskolin to 1 $\mu$M. In (*c*), verapamil was added to 1 $\mu$M, KCl to 30 mM and gramicidin to 1 $\mu$g/ml.

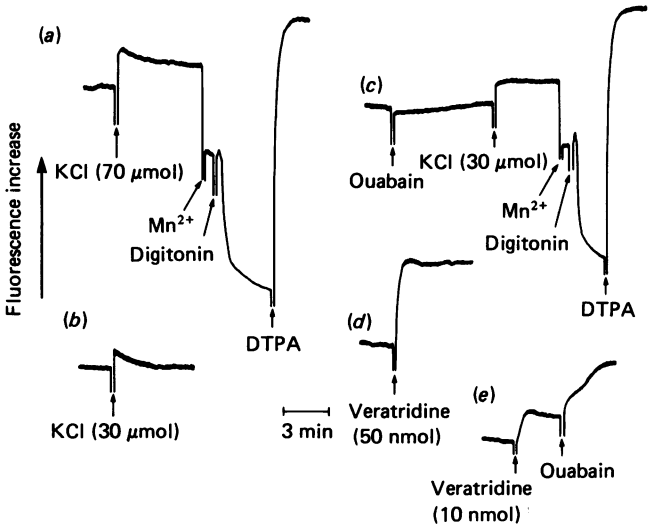


**Fig. 3. Effect of cell depolarization on the fluorescence of cardiac myocytes 'lightly' loaded with Quin 2**

Portions (2 ml) of myocytes, containing approx. 3.5 mg of protein/ml, were incubated in the presence of 7.5 $\mu$M-Quin 2/AM for 30 min at 37 °C. This gave cells containing 0.42 nmol of Quin 2/mg of protein, on the basis of $F_{max.}$ generated by ionomycin, or 0.73 nmol/mg of protein, on the basis of $F_{max.}$ generated by digitonin and DTPA. Where indicated, digitonin was added to 5 $\mu$M, $MnCl_2$ to 0.1 mM, ouabain to 0.2 mM and DTPA to 1 mM. Other additions are as shown.

### Monitoring of $[Ca^{2+}]_c$ in myocyte suspensions by Quin 2 fluorescence

The fluorescent $Ca^{2+}$-chelating agents introduced by Tsien (1980, 1981) and Tsien *et al.* (1982) allow the continuous monitoring of $[Ca^{2+}]_c$ in suspensions of cells. Fig. 2 shows results of experiments performed with cells 'heavily' loaded with Quin 2, by exposure to 50 $\mu$M-Quin 2/AM, at 3 mg of protein/ml, and containing 4.05 nmol of Quin 2/mg of protein. These results show that increasing the $K^+$ concentration of the medium from 5 to 30 mM results in an increase in $[Ca^{2+}]_c$ which is nearing completion at 10 min (*a*), is made much more rapid by prior exposure of the cells to 1 $\mu$M-isoprenaline (*b*), and is totally inhibited by blockade of $Ca^{2+}$ channels with verapamil (*c*). An increase in $[Ca^{2+}]_c$ owing to KCl addition has also been shown in studies of myocytes by Powell *et al.* (1984) using Quin 2, an activation by isoprenaline in studies by Thomas *et al.* (1986) and an inhibition by $Ca^{2+}$-channel blockers in studies by Powell *et al.* (1984), Sheu *et al.* (1986) and Thomas *et al.* (1986). Fig. 2(*c*) also shows that gramicidin, which collapses the plasma-membrane $Na^+$ electrochemical gradient, gives a massive increase in $[Ca^{2+}]_c$. As verapamil is present, the mechanism is presumably reversal of the plasma-membrane $Na^+/Ca^{2+}$ exchange. Gramicidin treatment was used to generate maximal values of $PDH_A$ in this study (see the Experimental section). Fig. 2 serves to define the nature of the KCl-induced increase in $[Ca^{2+}]_c$, which is an important tool in the enzyme studies in this paper: further, activation of $Ca^{2+}$ entry by $\beta$-adrenergic stimulation is more evident in experiments of this type, i.e. with 'heavily' loaded cells. However, a protocol involving loading of cells with 7.5 $\mu$M-Quin 2/AM (Fig. 3) relates more closely to the conditions of the enzyme studies of the present paper.

Fig. 3 shows that with these 'lightly' loaded cells an increase in $K^+$ concentration from 5 to 40 mM gives a large, rapid, increase in $[Ca^{2+}]_c$ (Fig. 3*a*), whereas 20 mM-$K^+$ gives a smaller increase (Fig. 3*b*). Addition of ouabain (0.2 mM) causes a very slow increase in $[Ca^{2+}]_c$: this is somewhat variable between cell preparations, with a suggestion that preparations of higher integrity (a greater percentage of rod-shaped cells) show a lesser response to ouabain. Veratridine (25 $\mu$M) gives a large increase in $[Ca^{2+}]_c$, approximately saturating the Quin 2 (Fig. 3*d*), whereas 5 $\mu$M-veratridine gives a submaximal response, allowing an additive effect of ouabain (Fig. 3*e*).

It is apparent that there is a general relationship between the degree of increase in $[Ca^{2+}]_c$ in Fig. 3 and the degree of activation of PDH shown in Tables 1 and 2. One possible anomaly is a finding of a raised $PDH_A$ content in response to 20 mM-$K^+$ (Table 1), whereas $[Ca^{2+}]_c$ has essentially returned to basal values after 10 min (Fig. 3*b*). A possible explanation is that mitochondria release $Ca^{2+}$ slowly, and that inactivation of PDH may therefore be delayed. Another anomaly is that $\beta$-adrenergic activation clearly enhances $Ca^{2+}$ uptake on voltage-dependent channels (Figs. 2*a* and 2*b*), but does not cause a further increase in $PDH_A$ content (Table 1). This comparison may not be appropriate, however, as the measurement of $PDH_A$ content was made only at 10 min, at which point $[Ca^{2+}]_c$ in treated and untreated cells seems similar; the $\beta$-adrenergic activation affects mainly the rate of rise of $[Ca^{2+}]_c$.

Addition of Ruthenium Red 3 min before KCl in experiments of the type shown in Fig. 3 resulted in a response of $[Ca^{2+}]_c$ which was undiminished ($102 \pm 2$% of control; $n = 3$), when a correction was made for

quenching by the Ruthenium Red (results not shown). Correction was on the basis of the fluorescence span $F_{max.} - F_{min.}$, and this was diminished to $62 \pm 2\%$ ($n = 3$) of the control values by the presence of the inhibitor. The response of $[Ca^{2+}]_c$ to the addition of veratridine plus ouabain was also unaffected by the presence of Ruthenium Red, when the same allowance was made for quenching (results not shown). Thus the severe attenuation by Ruthenium Red of the response of $PDH_A$ content to cell depolarization (Table 1) cannot be ascribed to inhibition of the entry of $Ca^{2+}$ into the cell.

Although it is tempting to calculate absolute values of $[Ca^{2+}]_c$ from experiments of the type shown in Figs. 2 and 3, as was originally done by Tsien *et al.* (1982) for lymphocytes, it is necessary to be cautious. In essence, such calculations depend on knowledge of the percentage saturation of the dye, and of the $K_d$ of the Quin-2/$Ca^{2+}$ chelate. Percentage saturation is derived from fluorescence at the value of $[Ca^{2+}]_c$ which is being determined ($F$), fluorescence corresponding to saturation of the Quin 2 with $Ca^{2+}$ ($F_{max.}$) and fluorescence corresponding to Quin 2 in the complete absence of $Ca^{2+}$ ($F_{min.}$). We have used ionomycin, gramicidin, digitonin or Triton X-100 to achieve $F_{max.}$, in the presence of 1 mM extracellular $Ca^{2+}$; the same reagents may be used to generate $F_{min.}$ in the presence of $Mn^{2+}$, which completely quenches the fluoresence of Quin 2 (Hesketh *et al.*, 1983). The transition-metal-specific chelating agent DTPA can be used after $Mn^{2+}$, to yield $F_{max.}$ again (Fig. 2). The need for caution arises because some of the dye seems not to be in the cytosol, and the analysis above is relevant only for a single cytosolic pool. Thus addition of Triton X-100 to 0.05% after $MnCl_2$ gives a lower value for '$F_{min.}$' than does 5 $\mu$M-digitonin. The difference increases with increasing concentration of Quin 2/AM used to load the cells. Similarly, Triton X-100 gives a higher value for '$F_{max.}$' than does 5 $\mu$M-digitonin, or the ionophore ionomycin, added in the presence of 1 mM-$Ca^{2+}$ in the medium (results not shown). Again, the disparity is greater for the 'heavily' loaded cells.

It seems likely that Triton X-100 is giving access to a pool that does not react rapidly in the presence of ionomycin or digitonin. Unfortunately, neither ionomycin nor digitonin is ideal, either. Ionomycin does not allow the attainment of $F_{min.}$ values within a reasonable time, and digitonin gives a biphasic response, with the second phase tending toward the value achieved in the presence of Triton X-100. Thus one can calculate a wide range of values of $[Ca^{2+}]_c$, depending on the method of calibration. Nevertheless, the relative magnitude of changes in $[Ca^{2+}]_c$ affecting PDH interconversion can be derived more convincingly. Experiments with cell suspensions loaded with 1 $\mu$M-Fura 2/AM confirm the shape of the changes shown in Fig. 3, suggesting that the $Ca^{2+}$ buffering provided by Quin 2 does not dominate the cell's intrinsic $Ca^{2+}$ buffering in the protocol of Fig. 3. The studies using Fura 2 show a greater component of extracellular dye and greater apparent 'compartmentation' effects than did Quin 2 studies of the type shown in Fig. 3, and are therefore not presented.

### General discussion

There have been no previous measurements of $PDH_A$ in suspensions of cardiac myocytes, though there have been studies in which flux through PDH has been measured (Long *et al.*, 1980; Montini *et al.*, 1981). The present report establishes the feasibility of measuring $PDH_A$ in suspensions of myocytes and shows that several conditions that raise $[Ca^{2+}]_c$ in these cells also raise the content of $PDH_A$. The fact that $Ca^{2+}$ is an important signal in controlling in the interconversion process emerges from the inhibitory effect of Ruthenium Red, in confirmation of the work of McCormack & England (1983). Ruthenium Red is likely to be an extremely poor penetrant of the plasma membrane of myocytes, owing to its highly charged nature (Luft, 1971). However, the clear impact of this compound on PDH interconversion (McCormack & England, 1983; the present work), despites its lack of effect on entry of $Ca^{2+}$ at the level of the plasma membrane (the present work), is hard to explain, other than by invoking a direct effect at the level of mitochondrial $Ca^{2+}$ transport. The failure of Ruthenium Red to diminish contractile waves suggests, however, that the intracellular concentration of the inhibitor is not sufficiently high to inhibit sarcoplasmic-reticulum $Ca^{2+}$ transport, something that is seen with myocytes permeabilized with digitonin (Altschuld *et al.*, 1985).

The role of the $Ca^{2+}$ ion as a signal also emerges quite clearly from experiments with cells 'lightly' loaded with Quin 2, which show responses of $[Ca^{2+}]_c$ (Fig. 3) and $PDH_A$ (Table 2) to membrane depolarization, but which are mechanically totally quiescent. Presumably, 'spikes' of $[Ca^{2+}]_c$ in excess of 1 $\mu$M are needed for contraction, in keeping with the systolic $[Ca^{2+}]_c$ value measured with aequorin (see Blinks *et al.*, 1982). The intracellular Quin 2 could buffer out such transients, while allowing steady-state values of $[Ca^{2+}]_c$ in excess of 0.5 $\mu$M, which are effective in activating PDH in experiments with isolated mitochondria (Denton *et al.*, 1980; Hansford, 1981). The present experiments do not rule out an important control role for [ATP]/[ADP] and [NADH]/[$NAD^+$] ratios under more physiological conditions (i.e. contraction with a frequency around 5 Hz). Indeed, the KCl protocol perhaps tends to emphasize the role of $Ca^{2+}$, by activating voltage-dependent $Ca^{2+}$ channels, but arresting contraction.

Finally, it became very apparent during the course of this study that preparations of cardiac myocytes vary in the percentage of $PDH_A$ at rest, and in the degree of stimulation afforded by KCl. They also vary in the fraction of cells which wave, and in the frequency of waving. Clearly, care is necessary in the extrapolation to the intact heart of biochemical findings made with isolated myocytes.

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