Biochimica et Biophysica Acta, 1143 (1993) 91–96

BBABIO 43831

The acute effects of the creatine analogue, β-guanidinopropionic acid, on cardiac energy metabolism and function

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(Received 5 October 1992)

Key words: β-Guanidinopropionic acid; Creatine; NMR, ^{31}P-; Creatine kinase; Cardiac energy metabolism; (Isolated perfused rat heart)

1. Perfusion of isolated rat hearts with 150 mM βGPA led to the linear accumulation of intracellular PβGPA (approx. 150 nmol/min per g (dry wt.)) after an initial lag period of 20 min. 2. This accumulation of intracellular PβGPA was accompanied by a decrease in PCr (30%) and an increase in total phosphagen content (20%). These results show that PCr was not equally replaced by PβGPA, but was degraded at the expense of βGPA phosphorylation to produce a net increase in cardiac phosphagen content. Correspondingly, total phosphate (the sum of PCr, PβGPA, P_i and ATP) was increased, indicating that there was no cellular necrosis and that the sarcolemma remained intact throughout the perfusion. 3. An increase in P_i and decrease in ATP also occurred concomitantly with PβGPA accumulation, indicating that ATP synthesis was not keeping up with demand. This may be due to the gradual replacement of PCr by the less efficient phosphagen, PβGPA, resulting in inadequate transduction of energy and hence an imbalance between energy demand and supply. However, the increased hyperosmolarity of the perfusate may be partly responsible for these effects on cardiac energy metabolism, as perfusion with 150 mM mannitol produced a similar decrease in ATP, but a smaller rise in P_i. 4. Perfusion with either 150 mM βGPA or mannitol led to a significant intracellular alkalosis (max. pH_i 7.3), which was reversed on returning to normal perfusate. In addition, both hyperosmolar perfusions led to a significant reduction in cardiac frequency (40 and 15%, respectively). However, only βGPA caused significant negative inotropism. The time-courses for the changes in cardiac frequency and pH_i did parallel the increase in PβGPA. This suggests that both hyperosmolarity and the production of PβGPA during βGPA perfusions determine the degree of negative chronotropism, but that hyperosmolarity alone causes alkalosis and βGPA phosphorylation, a decrease in developed tension. 5. When hearts, acutely loaded with PβGPA were perfused with control medium, the levels of ATP, PCr and PβGPA stabilised to produce a new steady state. There was no decrease in PβGPA concentration during this procedure, implying that βGPA efflux was negligible.

Introduction

Creatine (Cr) transport and handling are known to be perturbed in animal models of cardiac hypertrophy [1] and hypertension [2], as well as in the diseased human myocardium [3], such that the overall effect is a reduction in cardiac intracellular Cr. In order to understand the importance of Cr in cardiac energy metabolism, various Cr analogues, including β-guanidinopropionic acid (βGPA), have been used to reduce intracellular Cr content experimentally [4–7].

βGPA is a competitive inhibitor of Cr transport [8] and a poor substrate for creatine kinase [9]. Rats fed on a 1% (w/w) diet of βGPA for 6–10 weeks showed a 90% reduction in PCr in the heart, which was replaced with phosphorylated βGPA (PβGPA) [7]. Furthermore, this procedure produced a 250% increase in the calculated free [ADP], yet had no measurable effect on [P_i], [ATP], pH_i, O_2 consumption or cardiac performance of the heart at high or low work loads, implying that PCr cannot be an important intermediate in cardiac energy metabolism. However, recent work has shown that Cr depletion by βGPA feeding may lead to cardiac failure [10] or hypertrophy [11]. However, comparisons between these studies are complicated by differences in the diet and the age of the animals under investigation [12]. Overall previous work

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Abbreviations: βGPA, β-guanidinopropionic acid; PβGPA, phosphorylated form of βGPA; Cr, creatine; PCr, phosphocreatine

on the βGPA-fed rat has demonstrated that the importance of PCr and Cr in cardiac energy transduction remains unclear.

The depletion of the total creatine pool by βGPA through dietary regimes is a relatively slow (5–19 weeks) and variable process. Thus the possibility that some biochemical or physiological adaptation has occurred due to the changed intracellular energy status cannot be discounted. Indeed, recent work [11] showed that, on Cr depletion in the rat, there was a change in the isozymic expression of myosin from V1 to the slower forms, V2 and V3. This process was accompanied by global cardiac hypertrophy and a slowing of cross bridge cycling, which demonstrates that the heart had adapted by trying to improve the economy of cardiac contraction. In contrast, no change has been observed in ventricular myosin isozyme expression in βGPA-fed mice [13]. In this study, we have investigated the acute effects of βGPA perfusion and its phosphorylation on the energy metabolism and function of the isolated rat heart. This permitted a kinetic assessment of the response of the heart to the acute substitution of PCr with the much poorer phosphagen, PβGPA. Furthermore, this acute introduction of βGPA in preference to Cr into cardiac energy metabolism may help to elucidate the mechanism and control of Cr handling by the healthy and diseased heart. Moreover this type of acute experiment would avoid any long term adaptation of the heart, as seen in βGPA-fed rats, to the changed intracellular energy status.

A small part of the present work has been reported briefly as a meeting abstract [14].

Materials and Methods

Materials

All biochemicals and chemicals were of the highest grade commercially available and were purchased from Sigma Chemical Co. or BDH Chemicals Ltd. (Poole, Dorset, UK).

Heart perfusions

Hearts from male Wistar rats (300–400 g) were perfused in the Langendorff mode at a constant flow rate (12 ml/min) in a fluid-filled chamber. All hearts were perfused at 37°C with gassed (O_2/CO_2, 19:1

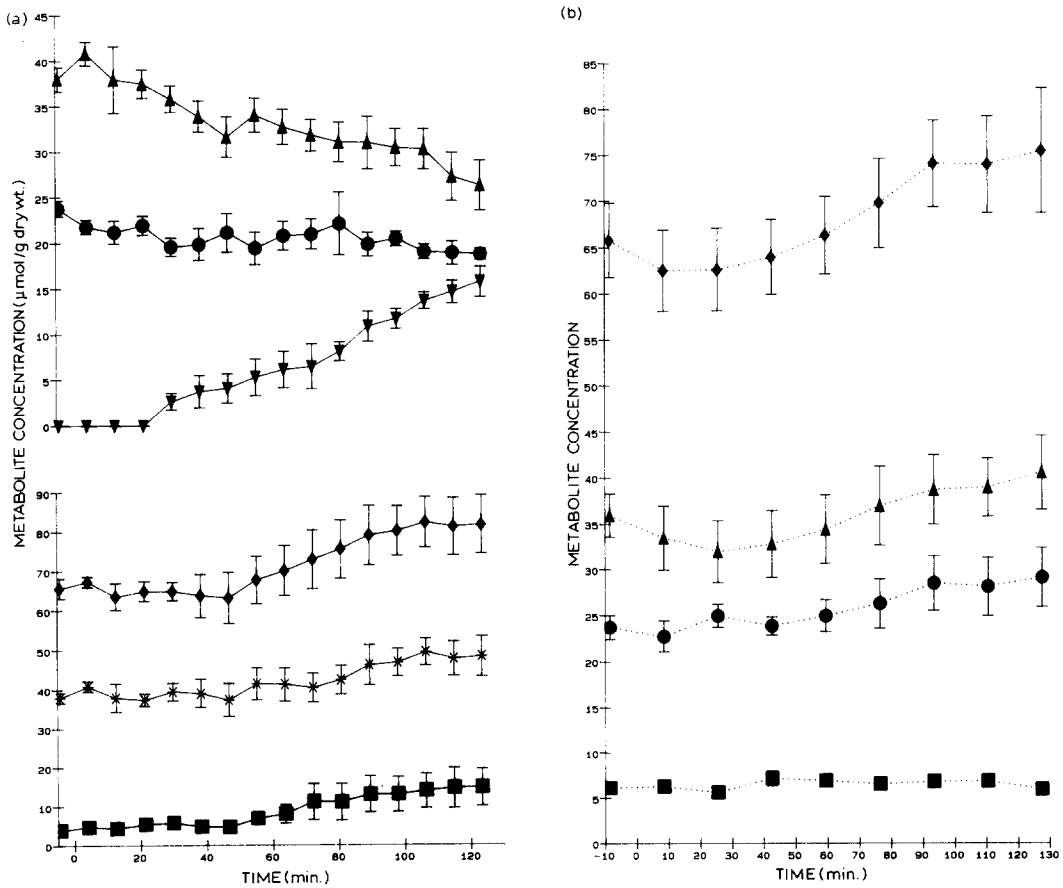

Fig. 1. The accumulation of PβGPA in the isolated rat heart perfused with βGPA and its effect on PCr and ATP. Hearts were perfused as described in the text in the (a) presence (———) and (b) absence (· · · · · ·) of 150 mM βGPA. PCr (▲), ATP (●), PβGPA (▼), P_i (■), phosphagen (Σ PCr and PβGPA) (*) and total phosphate (Σ PCr, P_i, ATP and PβGPA) (◆) are expressed as μmol/g (dry wt.). Points and error bars represent the means ± S.E. from at least five hearts and correspond to the mid-point of spectral collection times.

(v/v)) bicarbonate buffered medium (after Ref. 15), which contained 1.25 mM $CaCl_2$ and 11 mM glucose in the presence and absence of 150 mM βGPA [16]. Preliminary studies demonstrated that detection of PβGPA production by ^{31}P-NMR using this technique over a 2 h period required a concentration of βGPA in the perfusate in excess of the K_m for the Cr transporter. The minimum perfusate concentration of βGPA which led to detectable phosphorylation over the perfusion period was found to be 150 mM.

To investigate whether the effects of perfusing βGPA on cardiac metabolism and function were due to its effects on cardiac energy status or due to the hyperosmolarity of the βGPA perfusate, isolated rat hearts were perfused as before except that 150 mM D-mannitol was included instead of βGPA. This compound has no net charge, like βGPA at pH 7, it is not metabolised and is totally excluded from the intracellular space.

^{31}P-NMR

Following a stabilization period of about 20 min to allow time for optimization of the magnetic-field homogeneity in the sensitive volume of the NMR probe. Hearts were perfused for a further 20 min in control medium and then for 2 h in the absence or presence of 150 mM βGPA or 150 mM D-mannitol. This resulted in a total perfusion time of approx. 2.5 h.

^{31}P-NMR spectroscopy was performed in a wide-bore (72 mm) 7.03 T vertical superconducting magnet at a resonant frequency of 121.5 MHz using a custom built probe.

Phosphorus spectra were acquired by co-adding 256 or 512 free induction decays generated by a 60° pulse and interpulse delay of 2 s. The NMR-visible metabolite concentrations (PCr, PβGPA and P_i) throughout the time-course were calculated from the corresponding peak height, corrected for saturation, by comparison with the β-ATP signal measured in the last spectrum (see Ref. 11). This was quantified by determination of the ATP content of the corresponding heart freeze clamped after the last spectrum had been collected. Heart ATP content was determined by HPLC analyses [17,18] and extracted as previously described [19].

Intracellular pH (pH_i) was measured by the method of Moon and Richards [20] using the chemical shift of inorganic phosphate relative to PCr from ^{31}P-NMR spectra acquired during the perfusions.

Experiments parallel to those performed in the magnet were carried out on a bench-top perfusion rig under similar perfusion conditions. Cardiac function was monitored by measuring the developed isometric tension and frequency of the heart, as previously described by England [21].

Results and Discussion

Fig. 1a shows that perfusion of the isolated rat heart with 150 mM βGPA led to the formation of PβGPA and a concomitant 25–30% reduction in PCr after an initial lag period of 20 min. The rate of phosphorylation of βGPA in the heart was linear and was approx. 150 nmol/min per g (dry wt.). The phosphorylation of intracellular βGPA and depletion of PCr are shown as a stack plot of consecutive chronological ^{31}P-NMR spectra in Fig. 2. The total amount of phosphagen (the sum of PCr and PβGPA) in the perfused heart increased slightly on perfusion with βGPA (see Fig. 1a).

The uptake of creatine by the heart and other tissues under normal physiological conditions is by active Na^+-linked transport [22,23] and is reported to have a K_m of 0.05 mM and V_{max} of 20 nmol/min per g (dry wt.) [1]. βGPA enters skeletal muscle and presumably cardiac tissue with similar kinetics to that of creatine, but with a higher K_m (0.2 mM) [24]. Thus, active transport of βGPA under the conditions used here would be expected to be fully saturated. Furthermore, the observed rate of βGPA phosphorylation is much greater (7–8 times) than the V_{max} for active Cr uptake, implying that the majority of βGPA enters the heart by passive diffusion across the sarcolemmal membrane. The observed lag period between the switch to βGPA perfusate and the appearance of PβGPA

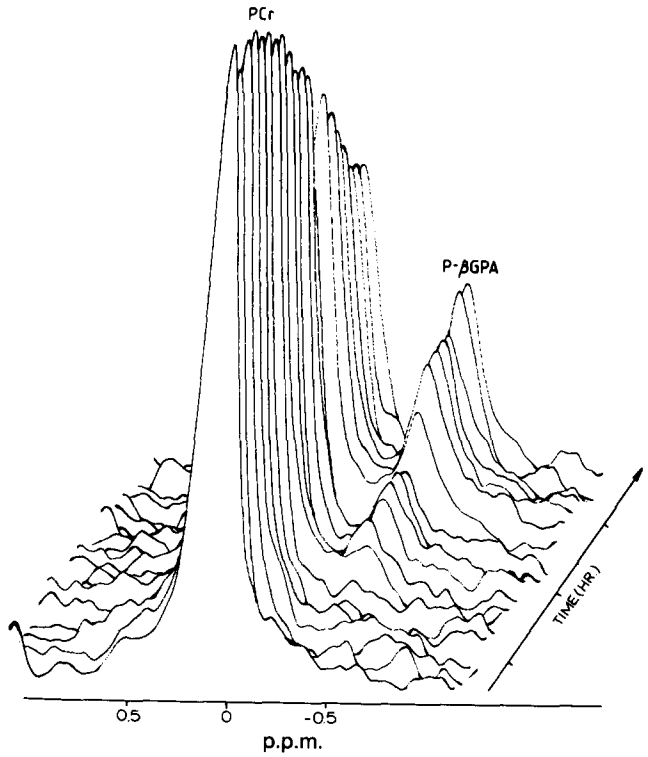

Fig. 2. A stack plot of ^{31}P-NMR spectra showing the accumulation of PβGPA in the isolated rat heart perfused with 150 mM βGPA for 2 h. Spectra were accumulated in 8.5 min time blocks using a 60° pulse and a 2 s interpulse delay over a 3 h time period, as described in Materials and Methods.

may be due to a combination of passive entry and slow accumulation of intracellular βGPA to a level at which βGPA competes with endogenous intracellular Cr for phosphorylation by creatine kinase.

These results show that PCr is not equally replaced by PβGPA, but is degraded at the expense of βGPA phosphorylation to produce a net increase in cardiac phosphagen content. The 20% increase in phosphagen content observed in these experiments is similar to that found in βGPA-fed animals, where the total amount of phosphagen was also greater (40–50%) than the concentration of PCr in equivalent control hearts [11]. This higher concentration of PβGPA than PCr may be explained by the different equilibrium constants for creatine kinase with PβGPA/βGPA as opposed to PCr/Cr. Furthermore, the decrease in ATP associated with PβGPA production (see Fig. 1a) has been previously observed in βGPA fed rat hearts [11], though in not all dietary studies (see Refs. 7, 10). It should be noted that the ratio of PCr: PβGPA is much higher (30–50-fold) in βGPA perfused hearts (approx. 1.7) compared to fed animals (approx. 0.04), making comparison of the two models difficult.

This decrease in PCr with βGPA perfusion was not due to hyperosmolarity of the perfusate or the actual perfusion itself, as perfusion with mannitol (see Fig. 5) or control medium (see Fig. 1b) had little effect on the concentration of PCr.

Fig. 1a also demonstrates that the amount of P_i and total phosphate increased and ATP decreased on perfusion with βGPA. In contrast, hearts perfused with control medium (see Fig.1 (b)) showed very little change in ATP, total phosphate, P_i or pH_i during the 2 h perfusion period. The changes in P_i and ATP produced on perfusion with βGPA indicate that the ATP production is not keeping up with demand. This may be due to the replacement of PCr by the inefficient phosphagen, PβGPA, leading to a inability to maintain adequate energy transduction and a disruption of the balance between energy demand and supply. However, the increased hyperosmolarity of the perfusate may be partly responsible for these effects on cardiac energy metabolism, as perfusion with mannitol (see Fig. 5) produced a similar decrease in ATP, but a smaller rise in P_i. The reasons for these hyperosmolar effects probably involve cell dehydration leading to changes in the concentration of intracellular ions [25,26], ultimately affecting cardiac energy metabolism.

The content of total phosphate in the perfused hearts changed slightly, though not significantly, in control and mannitol perfusions, but increased markedly on perfusion with βGPA (see Figs. 1 and 5) due to the net increase in phosphagen. Thus perfusion with hyperosmolar perfusate does not lead to sarcolemmal damage during the perfusion and consequently no phosphate is lost.

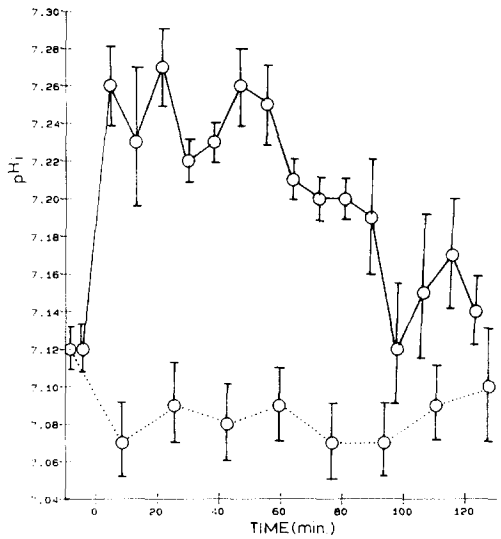

Fig. 3. The effect on pH_i of perfusing an isolated rat heart with βGPA. Isolated rat hearts were perfused as described in text, in the presence (———) and absence (· · · · · ·) of 150 mM βGPA. Points and error bars represent the means ± S.E. from at least three hearts and correspond to the mid-point of the spectral collection times.

In addition, perfusion with βGPA led to a significant alkalosis (maximum of pH 7.3) (see Fig. 3). However, the time-course for this alkalosis during βGPA perfusion followed the switch to the hyperosmolar βGPA solution rather than the formation of PβGPA. Perfusion of hearts with hyperosmolar mannitol also led to an more sustained increase in pH_i with a similar time-course (see Fig. 3). Further evidence that the changes in pH_i are solely due to hyperosmolarity of the βGPA perfusate is provided in Fig. 6, which shows that on returning to control perfusate from hyperosmolar βGPA, the pH_i also returns to control pH_i (approx. 7.1).

As well as altering the hearts energy metabolism, perfusion with βGPA also affected cardiac function (see Fig. 4). Perfusion of βGPA led to a significant reduction in cardiac frequency (approx. 40%) and developed tension (approx. 10%) compared with that produced during control perfusion. However, the time-course for this negative chronotropic effect during βGPA perfusion followed the switch to the hyperosmolar βGPA solution and not to the formation of PβGPA. Also, perfusions with hyperosmolar mannitol led to a decrease in cardiac frequency, which was smaller (15%) than that shown with just βGPA (40%) of the same osmolarity. However, perfusion with βGPA alone caused a decrease in developed tension with a time-course similar to that of the accumulation of PβGPA.

The reasons for hyperosmolarity perturbing cardiac pH_i may be due to the onset of tissue dehydration, which may increase intracellular Na^+ concentration, and to compensate for this, Na^+/H^+ antiporter activity would increase, so elevating pH_i; this would explain

the observed alkalosis. It can be concluded that the reasons for hyperosmolarity inducing intracellular alkalosis in the heart are unclear, but must be connected with various changes brought about by cell dehydration [27].

Previous work using cardiac muscle strips showed that extracellular hyperosmolarity induced by similar concentrations of mannitol and βGPA depressed cardiac contractility, but was dependent on the level of perfusate Ca^{2+}. These negative inotropic and chronotropic effects are probably due to a reduction in the intrinsic speed of muscle contraction caused by cell dehydration [25,26]. It can be concluded that the effects of βGPA on cardiac frequency are partly due to the hyperosmolarity of the perfusate, as mannitol had similar effects to βGPA, and partly to the introduction of PβGPA, which produced a greater decrease in cardiac frequency. The decrease in developed tension associated with βGPA perfusion may be linked to increase in P_i, which would lead to a desensitisation of the myofibrils for Ca^{2+} and reduction in the force generated [28].

The extent of the maintenance of PβGPA in the heart in the absence of extracellular βGPA is illustrated in Fig. 6. The preservation of a constant level of

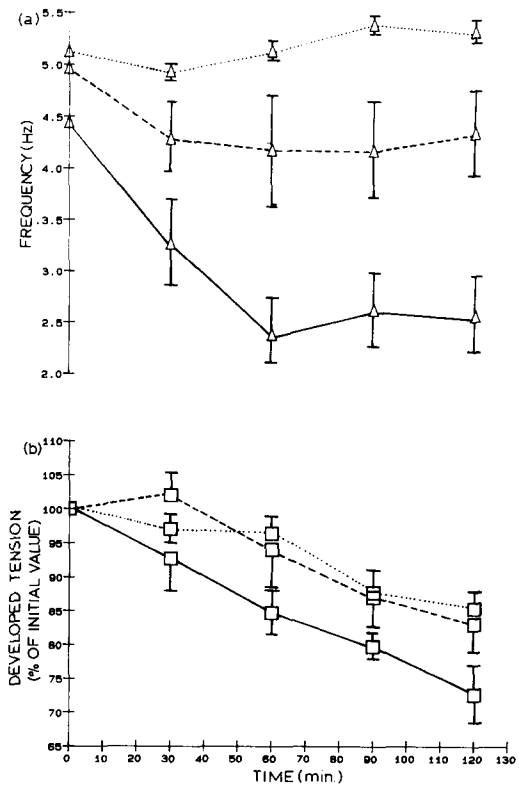

Fig. 4. The effect of perfusing βGPA and Mannitol on the (a) cardiac frequency and (b) relative developed tension of the isolated rat heart. Hearts were perfused as described in Materials and Methods in the absence (· · · · · ·) or presence of 150 mM βGPA (———) or 150 mM D-mannitol (– – –) under conditions analogous to those for the NMR experiments (see Fig. 1).

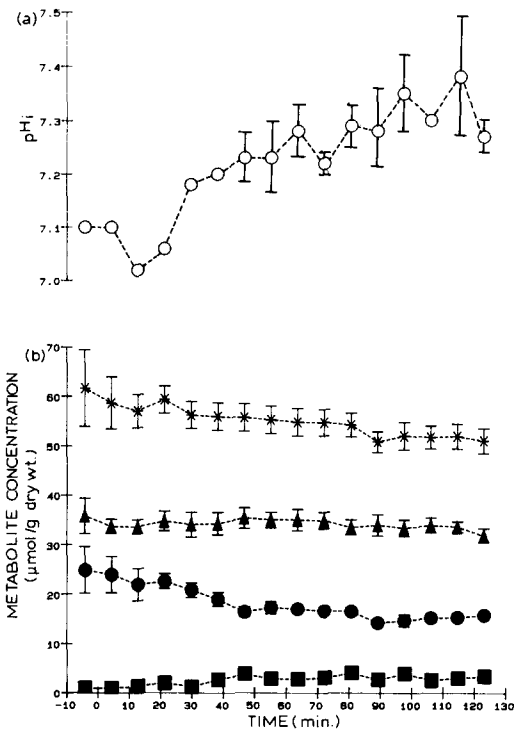

Fig. 5. The effects of perfusing mannitol on (a) the pH_i and (b) levels of PCr and ATP in the isolated rat heart. Hearts were perfused, as described in the text in the presence of 150 mM D-mannitol. PCr (▲), ATP (●), P_i (■) and total phosphate (*) are expressed as μmol/g (dry wt.). Points and error bars represent the means ± S.E. from at least five hearts and correspond to the mid-points of spectral collection times. pH_i readings without error bars represent means of two determinations, as not all the hearts at each time point exhibited a P_i peak and so pH_i could not always be calculated.

PβGPA in the perfused rat heart in the absence of 150 mM extracellular βGPA suggests that the concentration of intracellular βGPA must also be constant. Thus efflux of βGPA via its concentration gradient must be negligible [8]. This is in agreement with hearts from βGPA-fed rats in which the amount of PβGPA did not decrease with perfusion time (see Ref. 7). Also, since the net phosphorylation of βGPA stopped immediately the perfusate was changed to control, it would appear that the rate of passive diffusion of βGPA into the heart is the rate limiting step in the production of PβGPA. Otherwise intracellular βGPA would continue to be phosphorylated until a new equilibrium had been reached. The results also show that the maintenance of intracellular PβGPA is not dependent on a large extracellular βGPA concentration.

The present work has demonstrated that βGPA enters the heart, mainly by passive diffusion, where it is phosphorylated by creatine kinase. This method of incorporating PβGPA into cardiac energy metabolism is similar to dietary regimes in that PCr and ATP consequently decrease, but different in respect of time-course, biochemical adaptation and the extent of PCr depletion with PβGPA. This perfusion technique

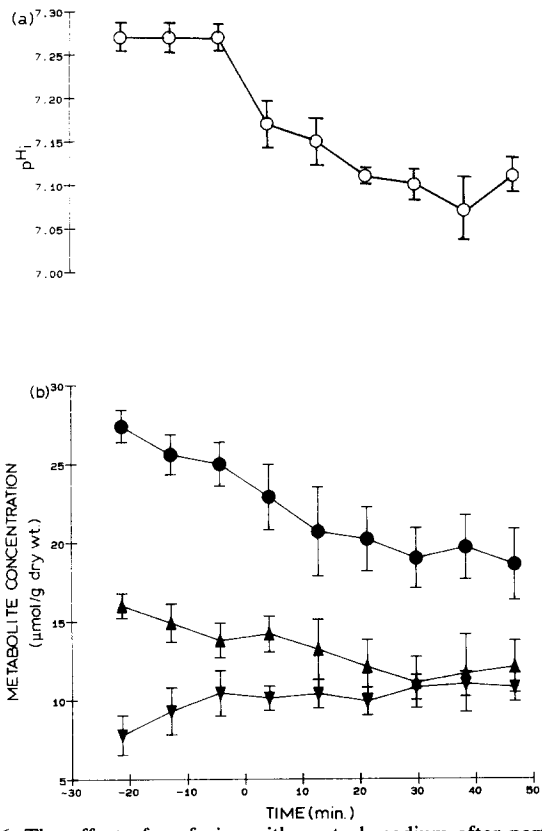

Fig. 6. The effect of perfusing with control medium after perfusing with βGPA on (a) the pH_i and (b) the levels of PβGPA, PCr and ATP in the isolated rat heart. Hearts were perfused as described in text in the presence of 150 mM βGPA ($t < 0$) for approx. 2 h and then perfused for 1 h from time zero in its absence ($t > 0$). PCr (●), ATP (▲) and PβGPA (▼) are expressed in μmol/g (dry wt.). Points and error bars represent means ± S.E. for at least five hearts and correspond to the middle of the spectral collection times.

provides a useful, non-invasive means of studying the time-course and effects of acute PCr replacement on cardiac energy metabolism, though is complicated by hyperosmolar perfusate effects on the heart. Furthermore, these investigations suggest that the heart is unable to adjust to such a rapid partial substitution of PCr by the much poorer phosphagen, PβGPA and that a gradual, long term adaptation to this replacement process (see Refs. 7, 11) is required to produce a heart capable of handling PβGPA. The nature of this adaptation may involve changes in myosin isozyme expression [11], but may ultimately lead to cardiac failure and hypertrophy [10,11], though this is debatable [7].

Acknowledgements

We thank the Medical Research Council of Great Britain, the British Heart Foundation and I.C.I. Pharmaceuticals Ltd. for their financial support. We acknowledge the help of Mark Field.

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