Glycolytic pathway intermediates activate cardiac ryanodine receptors

Helen Kermode, Wei Mun Chan, Alan J. Williams, Rebecca Sitsapesan*

Cardiac Medicine, NHLI, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY, UK

Received 12 March 1998; revised version received 27 May 1998

Abstract During myocardial ischaemia and reperfusion, enhancement of glycolytic activity occurs and this may lead to fluctuating levels of glycolytic intermediates [1–3]. We demonstrate that sugar phosphate intermediates of glycolysis, particularly fructose-1,6-diphosphate (FDP; 100 μM–10 mM), can activate sheep cardiac ryanodine receptor (RyR) channels incorporated into bilayers (open probability (Po) increases up to approximately 0.6) and stimulate [^{3}H]ryanodine binding (>200%) to isolated cardiac sarcoplasmic reticulum (SR) membrane vesicles. The relative effectiveness of the sugar phosphates in stimulating [^{3}H]ryanodine binding and increasing the Po of the channels was FDP > glucose-1-phosphate (G-1-P) > fructose-6-phosphate (F-6-P) > glucose-6-phosphate (G-6-P). These novel properties of the sugar phosphate compounds indicate that changes in glycolytic flux may influence the release of SR Ca^{2+} by modulating RyR channel gating.

Key words: Ryanodine receptor; Ca^{2+}-release channel; Sarcoplasmic reticulum; Glycolysis; Ischemia; Fructose-1,6-diphosphate

1. Introduction

The cardiac ryanodine receptor (RyR)/Ca^{2+}-release channel is located in the junctional sarcoplasmic reticulum (SR) and is the pathway for Ca^{2+} release to the contractile proteins. It is of interest, therefore, that glycolytic enzymes have been demonstrated to be associated with the SR as this may lead to compartmentalisation of phosphorylated intermediates close to RyR channels [4]. Glycolytic metabolism increases during myocardial ischaemia and reperfusion and is critical for the functional recovery of the heart [1–3]. Oscillations in SR Ca^{2+} release have also been shown to be associated with alterations in glucose metabolism via glycolysis [5]. In addition it has been shown that accumulation of sugar phosphate intermediates of glycolysis is associated with Ca^{2+} overload in cardiac muscle [3]. As the levels of sugar phosphates may change in localised regions close to the SR, as has been reported for ATP [6], we have investigated if sugar phosphate intermediates of glycolysis can interact with and modulate the function of cardiac RyR. Our results demonstrate that sugar phosphates increase the open probability (Po) of the sheep cardiac RyR in the following order of potency: fructose-1,6-diphosphate (FDP) > glucose-1-phosphate (G-1-P) > fructose-6-phosphate (F-6-P) > glucose-6-phosphate (G-6-P) = inorganic phosphate (Pi) and raise the possibility that glycolytic intermediates may play a novel regulatory role in controling SR Ca^{2+} release under physiological and pathophysiological conditions.

*Corresponding author. Fax: (44) (171) 823 3392.
E-mail: r.sitsapesan@ic.ac.uk

2. Materials and methods

2.1. Preparation of junctional SR

SR membrane vesicles were prepared from sheep cardiac muscle as previously described by Sitsapesan et al. [7]. Heavy SR membrane vesicles were frozen rapidly in liquid nitrogen and stored at −80°C.

2.2. [^{3}H]Ryanodine binding

Membrane vesicles were diluted to 50–100 μg protein/ml and incubated at 37°C in 1 M KCl, 5 μM PMSF, 25 mM PIPES-KOH, 10 μM free Ca^{2+} buffered with 2-di(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA) and $CaCl_2$, pH 7.2, with 5 nM [^{3}H]ryanodine for 60 min. Following incubation, the samples were diluted with 5 ml of ice-cold buffer and filtered through Whatman GF-B filters. Filters were washed with a further 3×5-ml aliquots of buffer and counted in 10 ml aqueous counting scintillant the following day. Non-specific binding was determined from incubations to which 5 μM unlabelled ryanodine had been added. All incubations were performed in triplicate.

2.3. Single channel studies

Vesicles were fused with planar phosphatidylethanolamine lipid bilayers as previously described [7]. The vesicles fused in a fixed orientation such that the *cis* chamber corresponded to the cytosolic space and the *trans* chamber to the SR lumen. The *trans* chamber was held at ground and the *cis* chamber held at potentials relative to ground. Following fusion, the *cis* chamber was perfused with 250 mM *N*′-2-hydroxyethylpiperazine-*N*′-2-sulphonic acid (HEPES), 125 mM tris-(hydroxymethyl)-methylamime (Tris), 10 μM free Ca^{2+} buffered with EGTA and $CaCl_2$, pH 7.2, and the *trans* chamber was perfused with a solution containing 250 mM glutamic acid, 10 mM HEPES, pH 7.2, with $Ca(OH)_2$ (free [Ca^{2+}] approximately 50 mM). The experiments were performed at room temperature (23 ± 1°C).

The free [Ca^{2+}] and pH of the solutions used in the ryanodine binding and the single channel studies were measured at the appropriate temperature using a calcium electrode (Orion 93-20) and Ross-type pH electrode (Orion 81-55) as described previously in detail [7]. Additions of the sugar phosphates used in this study (≤ 20 mM) did not alter the free [Ca^{2+}] of the solutions.

The mean value ± standard error of the mean (S.E.M.) is given where $n \geq 4$. For $n = 3$, standard deviation (S.D.) is given. Individual n values were obtained from different membrane preparations. Student's *t*-test was used to compare the effect of a compound with the control. A P value < 0.05 was accepted as a statistically significant difference.

2.4. Data acquisition and analysis

Single-channel recordings were displayed on an oscilloscope and recorded on Digital Audio Tape (DAT). All steady-state recordings were carried out at 0 mV. Under these conditions, current flow through the cardiac ryanodine receptor was in the luminal to cytosolic direction. Current recordings were filtered at 1 kHz and digitised at 2 kHz. Channel open probability (Po) and the lifetimes of open and closed events were monitored by 50% threshold analysis. Channel Po values were obtained from three minutes of steady-state recording. Lifetime analysis was carried out only when a single channel incorporated into the bilayer. Events < 1 ms in duration were not fully resolved and were excluded from lifetime analysis. Lifetimes accumulated from approximately 3-min steady-state recordings were stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to a probability density function (pdf) by the method of maximum likelihood [8] according to the equation: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots\ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$ with areas a and time constants τ. A missed events correction was applied as described

PII: S0014-5793(98)00725-X

by Colquhoun and Sigworth [8]. A likelihood ratio test [9] was used to compare fits to up to four exponentials by testing twice the difference in $\log_e$ (likelihood) against the chi-squared distribution at the 1% level. Single-channel current amplitudes were measured from digitised data using manually controlled cursors.

2.5. Chemicals

[^{3}H]Ryanodine was purchased from New England Nuclear Ltd. Unlabelled ryanodine was purchased from Calbiochem (Nottingham, UK). All other chemicals were of AnalaR or best available grade from BDH (Poole, UK) or Sigma (Poole, UK). Aqueous counting scintillant was purchased from Amersham International (Amersham, UK).

3. Results

Fig. 1A illustrates the concentration dependence of the effects of the sugar phosphate intermediates of glycolysis, G-1-P, F-6-P, G-6-P and FDP on [^{3}H]ryanodine binding to the sheep cardiac heavy SR membrane fraction in the presence of 10 μM free Ca^{2+}. G-6-P had no significant effect on

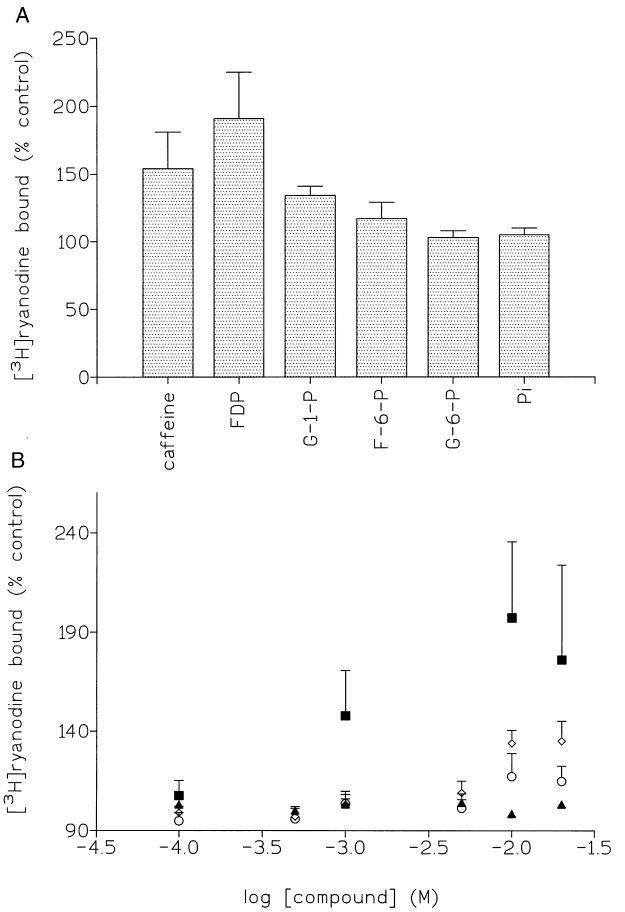

Fig. 1. A: The effects of FDP (squares), G-1-P (diamonds), F-6-P (circles) and G-6-P (triangles) on [^{3}H]ryanodine binding to heavy SR in the presence of 10 μM Ca^{2+}. The results are expressed as a percentage of the control binding at 10 μM cytosolic Ca^{2+} which was 2.16 ± 0.31 pmol [^{3}H]/mg protein (S.E.M.; $n = 5$). The error bars are S.D. ($n \geq 3$) for FDP and S.E.M. ($n = 5$) for G-1-P, G-6-P and F-6-P. B: Comparison of the maximal [^{3}H]ryanodine binding obtained with the sugar phosphates (at 10 mM) and Pi (50 mM) with that obtained in the presence of 40 mM caffeine. The results are expressed as a percentage of the control in 10 μM cytosolic Ca^{2+}. The error bars are S.D. ($n \geq 3$) for FDP and caffeine and S.E.M. ($n = 5$) for G-1-P, G-6-P, F-6-P and Pi. FDP significantly increased [^{3}H]ryanodine binding above control levels at 10 μM Ca^{2+} at 1, 10 and 20 mM ($P > 0.05$).

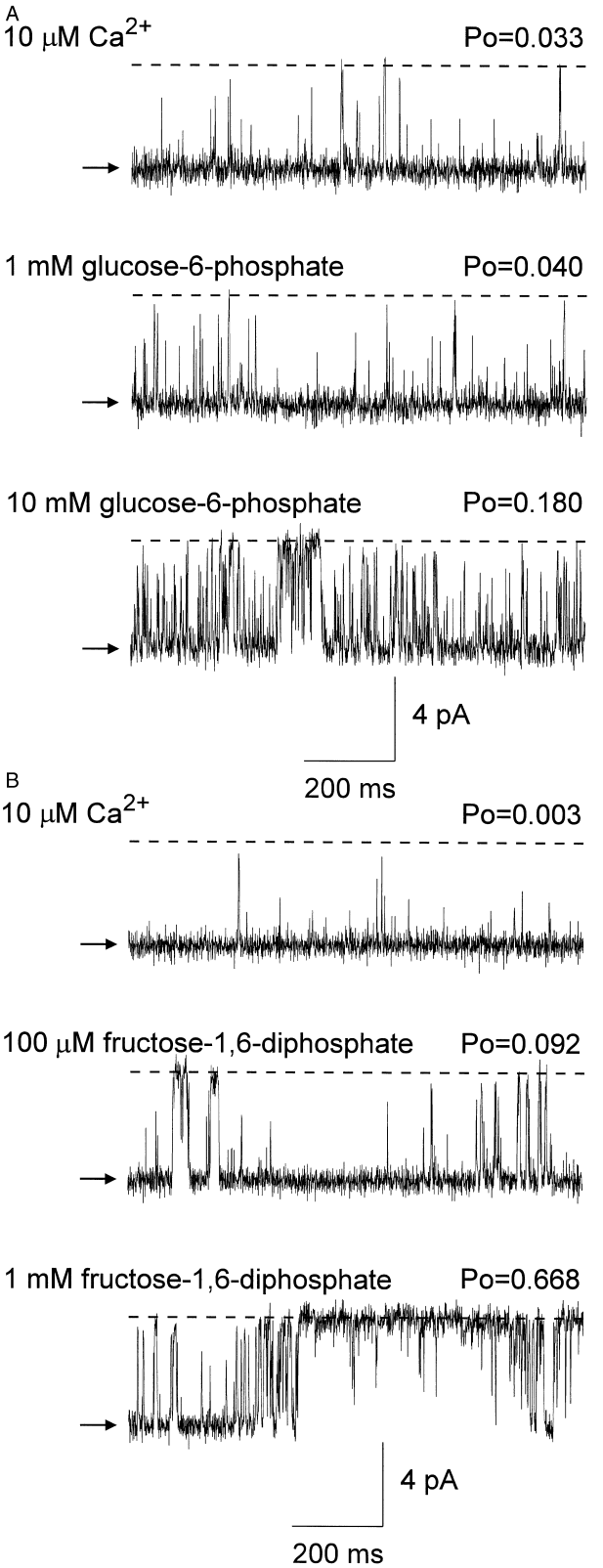

Fig. 2. Effects of sugar phosphates on the gating of sheep cardiac RyR incorporated into bilayers. A: The effects of 1 and 10 mM G-6-P on the gating of a representative single channel. The Po values are indicated above each trace. The arrows indicate the zero current level and the dotted lines indicate the fully open channel level. B: The effects of 100 μM and 1 mM FDP on the gating of a typical single channel.

[^{3}H]ryanodine binding at concentrations up to 20 mM. F-6-P and G-1-P stimulated binding at concentrations of 10 mM and above but only FDP caused any increase in binding at 1 mM. 1 mM, 10 mM and 20 mM FDP increased binding by 144 ± 21%, 191 ± 34% and 171 ± 0.45% (S.D.; $n \geq 3$) of the control binding (2.16 ± 0.31 pmol [^{3}H]/mg protein (S.E.M.; $n = 5$)) in the presence of 10 μM free Ca^{2+}. Evidence suggests that ryanodine binds only to open conformation(s) of the RyR channel and therefore stimulation of ryanodine binding is thought to indicate an increase in the Po of the channels. On this basis our results suggest that sugar phosphates, particularly FDP, activate RyR. In order to understand how sugar phosphate activation of RyR compares with other activators of the channel we have measured the increase in [^{3}H]ryanodine binding in the presence of inorganic phosphate (Pi, 50 mM) and high levels of caffeine (40 mM) under identical ionic conditions (Fig. 1B). We have previously demonstrated that Pi can increase the Po of cardiac RyR incorporated into bilayers [10]. Pi does not interact with the ATP sites on cardiac RyR [10] and it has been suggested that Pi may bind to a specific site on skeletal RyR [11]. The actions of Pi on the gating of RyR are interesting because it has no effect in the absence of activating levels of cytosolic Ca^{2+} [10] and even in the presence of Ca^{2+} cannot fully activate the channels. Analysis of the gating of the channels indicates that Pi-activation of cardiac RyR can be explained purely by sensitisation to cytosolic Ca^{2+} [10]. In contrast, although low [caffeine] may sensitise the channel to Ca^{2+}, high [caffeine] does not activate RyR by sensitisation to Ca^{2+}. Rather, caffeine (and caffeine analogues) and Ca^{2+} act synergistically to induce different gating behaviour and can fully activate the channels [12,13]. Fig. 1B compares the maximum level of binding obtained with G-1-P, F-6-P, G-6-P and FDP (10 mM) in the presence of 10 μM Ca^{2+} with that observed under identical conditions with caffeine (40 mM) and Pi (50 mM). Pi, which can activate sheep cardiac RyR in bilayers [10], did not cause a significant increase in binding above that observed in the presence of 10 μM Ca^{2+} alone. This result was not totally unexpected as 50 mM Pi was previously shown to only increase Po from approximately 0.01 to approximately 0.15 in the presence of 10 μM cytosolic Ca^{2+} [10]. This result confirms other reports that [^{3}H]ryanodine binding is not as sensitive as the single channel studies in detecting small changes in Po (≤ 0.1 to ≤ 0.3) [14]. 10 mM G-6-P had no significant effect on [^{3}H]ryanodine binding which was 103 ± 5% ($n = 3$) of the control binding. 10 mM F-6-P or G-1-P increased binding to 117 ± 12% (S.E.M.; $n = 5$) and 134 ± 7% (S.E.M.; $n = 5$) of the control, respectively. The figure illustrates that neither compound was as effective as caffeine or FDP at stimulating [^{3}H]ryanodine binding. These results suggest that whereas G-6-P may have little effect on the gating of sheep cardiac RyR, FDP would be expected to be an effective activator of the channel. We therefore investigated the effects of the four sugar phosphates on the single channel properties of single RyR channels incorporated into bilayers.

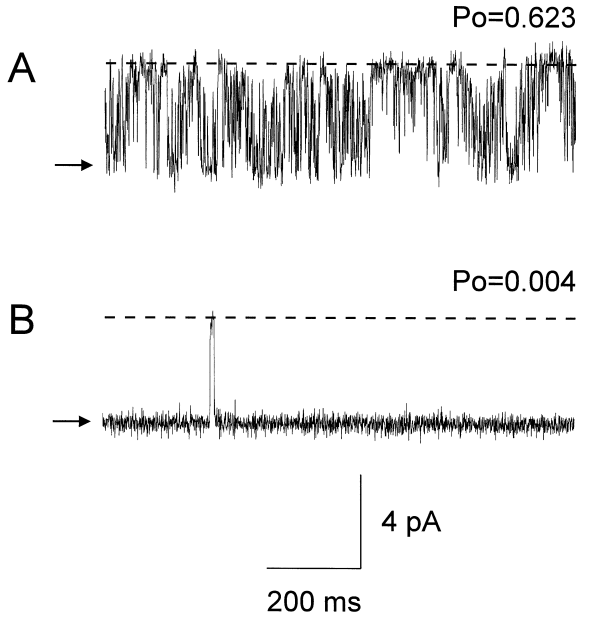

Fig. 3. The effects of 1 mM FDP on current fluctuations through a representative single RyR in the presence of 10 μM free cytosolic Ca^{2+} (A) or approximately 100 pM Ca^{2+} (after addition of 12 mM EGTA to the *cis* chamber) (B). The Po values are indicated above each trace. The arrows indicate the zero current level and the dotted lines indicate the fully open channel level.

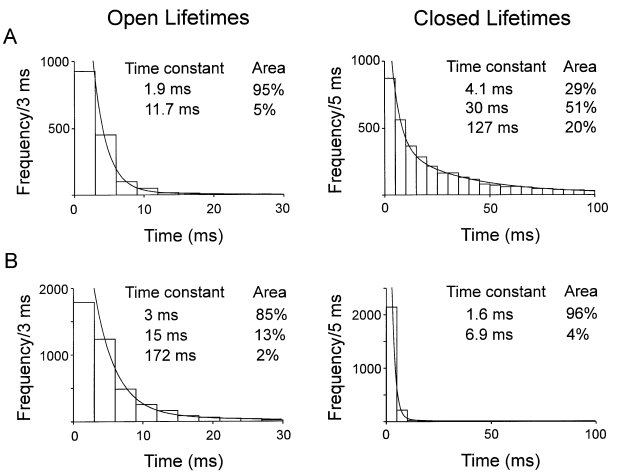

Fig. 4. The open and closed lifetime distributions and pdfs for a representative channel activated by 10 μM cytosolic Ca^{2+} alone, Po = 0.024 (A) and after addition of 1 mM FDP, Po = 0.582 (B). The time constants and percentage areas from the pdfs are shown. Lifetimes were determined and pdfs fitted as described in Section 2.

None of the sugar phosphates caused any observable change in the conductance of the channel even when concentrations up to 20 mM were added to the cytosolic side of the bilayer (results not shown). Fig. 2A illustrates the effects of G-6-P on the gating of a typical RyR. At concentrations up to 1 mM, G-6-P had no significant effect on Po. Po was 0.036 ± 0.01 (S.E.M.; $n = 6$) in the presence of 10 μM Ca^{2+} only and 0.051 ± 0.012 (S.E.M.; $n = 6$) after the addition of 1 mM G-6-P. At 10 mM G-6-P, Po was 0.135 ± 0.032 (S.E.M.; $n = 4$). F-6-P (10 mM) and G-1-P (10 mM) were slightly more effective activators of the channel and increased Po to 0.310 ± 0.092 (S.E.M.; $n = 4$) and 0.490 ± 0.125 (S.D.; $n = 3$) respectively. FDP was the most effective and potent of the sugar phosphates and was able to induce significant increases in Po even at micromolar levels. This can be observed in Fig. 2B. Po increased from 0.040 ± 0.022 (S.E.M.; $n = 5$) in the presence of 10 μM Ca^{2+} alone to 0.262 ± 0.100 (S.E.M.; $n = 4$) and 0.540 ± 0.066 (S.E.M.; $n = 4$) after the addition of

100 μM and 1 mM FDP, respectively, to the cytosolic chamber. Higher [FDP] did not cause any further significant increases in Po. At 10 mM FDP, Po was 0.608 ± 0.055 (S.D.; $n = 3$) and therefore FDP cannot fully open the sheep cardiac RyR when the cytosolic free [Ca^{2+}] is maintained at 10 μM. The FDP-induced increase in Po could be reversed by perfusing away the FDP (results not shown). The ability of FDP to activate the channel was highly dependent on the presence of activating levels of cytosolic Ca^{2+}. Fig. 3 illustrates the effect of reducing the cytosolic [Ca^{2+}] from 10 μM to approximately 100 pM (by addition of 12 mM EGTA) on the gating of a single channel activated by 1 mM FDP. Although occasional openings were observed, Po was effectively 0 ($n = 4$).

Inspection of the single channel traces in Fig. 2B illustrates that FDP induces long open events which do not occur when the channel is activated solely by Ca^{2+}. The mean open time increased from 0.84 ± 0.43 ms in the presence of 10 μM cytosolic Ca^{2+} alone to 3.31 ± 1.22 ms (S.D.; $n = 3$) after addition of 1 mM FDP. An even greater effect on the mean closed time was observed which decreased from 104 ± 80 ms to 1.96 ± 1.02 ms (S.D.; $n = 3$). Lifetime analysis (see Fig. 4) confirms that the mechanism by which FDP increases Po is by increasing both the duration and the frequency of channel opening. Fig. 4 shows that the proportion of long open events is increased in the presence of 1 mM FDP and an extra open state of long duration can be observed. In contrast, the third long closed state can no longer be resolved after the addition of 1 mM FDP and 96% of the closings are < 2 ms.

4. Discussion

Our [^{3}H]ryanodine binding and single channel studies show that the gating of cardiac ryanodine receptors can be modulated by certain sugar phosphates which are intermediates in the glycolytic pathway. This novel aspect of sugar phosphate action is important in the light of reports that glycolytic enzymes are associated with the SR [6] and that glycolytic inhibition causes Ca^{2+} overload when sugar phosphate intermediates of glycolysis accumulate in the myocardium [3]. Phosphofructokinase has been identified as a glycolytic rate limiting enzyme, stimulation of which leads to reduced F-6-P levels and increased production of FDP [15,16]. It is well known that phosphofructokinase is stimulated by ADP, AMP and Pi but is inhibited by high levels of ATP and creatine phosphate [15]. Under the anaerobic conditions of ischaemia, increased levels of activators and lower levels of inhibitors accelerate phosphofructokinase leading to increased production of FDP. Maintained ischaemia leads to inhibition of glyceraldehyde-phosphate dehydrogenase due to high levels of NADH and allows the accumulation of FDP, glyceraldehyde-3-P and dihydroxyacetone-P [15]. Interestingly, in our experiments FDP is much more potent and effective at stimulating [^{3}H]ryanodine binding and increasing the Po of channels reconstituted into planar phospholipid bilayers than G-1-P, F-6-P and G-6-P. Micromolar levels of G-6-P, F-6-P and FDP have been measured intracellularly assuming that they are contained within a single compartment in the cell [16]. FDP activates the cardiac RyR at micromolar concentrations and therefore could play a role as regulator of channel gating, particularly under conditions of ischaemia. If the enzymes of glycolysis are located at the SR then the levels of sugar phosphates in the immediate locality of RyR may fluctuate more than the levels in the bulk cytosol during myocardial ischaemia. Whether sugar phosphate intermediates of glycolysis do modulate the gating of RyR in situ will depend on the levels of other RyR activators and the site(s) of action of the sugar phosphates. We are not yet certain whether FDP binds to a novel site on the cardiac RyR or if it binds to the adenine nucleotide binding sites or even to the same site as Pi (if indeed Pi binds to a specific site as suggested by Fruen et al. for the skeletal isoform of RyR [11]). Our experiments demonstrate that the different sugar phosphates exhibit different levels of effectiveness at increasing the Po of cardiac RyR. This, coupled to the observation that FDP is effective at micromolar concentrations indicates that the sugar phosphates may bind to a specific binding site on the cytosolic face of RyR. If FDP and other sugar phosphates act via a novel site then it would be expected that they would potentiate the effects of ligands acting at other sites. If FDP exerts its effects via, for example, the adenine nucleotide binding sites, then it would have to compete for the site with ATP and other adenine nucleotides and nucleosides that are present physiologically. Under conditions where glycolysis is inhibited, levels of ATP may be lowered and levels of AMP, ADP, Pi and adenosine will rise. The fluctuating levels of the different adenine nucleotides and nucleosides would, in this case, be expected to dictate the magnitude of the effect that FDP and other sugar phosphates will have. The ability of the sugar phosphate intermediates of glycolysis to bind to and modulate the gating of sheep cardiac RyR suggests that changes in glycolytic flux in cardiac cells could give rise to changes in Ca^{2+} homeostasis as a result of the interaction of these compounds with the RyR channels.

Acknowledgements: We are grateful to the British Heart Foundation for support.

References

[1] Mallet, R.T., Hartman, D.A. and Bunger, R. (1990) Eur. J. Biochem. 188, 481–493.
[2] Bunger, R., Mallet, R.T. and Hartman, D.A. (1989) Eur. J. Biochem. 180, 221–233.
[3] Kusuoka, H. and Marban, E. (1994) J. Clin. Invest. 93, 1216–1223.
[4] Xu, K.Y., Zweier, J.L. and Becker, L.C. (1995) Circ. Res. 77, 88–97.
[5] O'Rourke, B., Ramza, B.M. and Marban, E. (1994) Science 265, 962–966.
[6] Han, J.-W., Thieleczek, R., Varsanyi, M. and Heilmeyer Jr., L.M.B. (1992) Biochemistry 31, 377–384.
[7] Sitsapesan, R., Montgomery, R.A.P., MacLeod, K.T. and Williams, A.J. (1991) J. Physiol. 434, 469–488.
[8] Colquhoun, D. and Sigworth, F.J. (1983) in: Single-Channel Recording (Sakmann, B. and Neher, E., Eds.) pp. 191–263, Plenum, New York, NY.
[9] Blatz, A.L. and Magleby, K.L. (1986) Biophys. J. 49, 967–980.
[10] Kermode, H., Williams, A.J. and Sitsapesan, R. (1998) Biophys. J. 74, 1296–1304.
[11] Fruen, B.R., Kane, P.K., Mickelson, J.R. and Louis, C.F. (1996) Biophys. J. 71, 2522–2530.
[12] Sitsapesan, R. and Williams, A.J. (1990) J. Physiol. 423, 425–439.
[13] Williams, A.J. and Holmberg, S.R.M. (1990) J. Membr. Biol. 115, 167–178.
[14] Holmberg, S.R.M. and Williams, A.J. (1990) Biochim. Biophys. Acta 1022, 187–193.
[15] Neely, J.R. and Morgan, H.E. (1974) Annu. Rev. Physiol. 36, 413–459.
[16] Stryer L. (1995) Biochemistry, pp. 483-508. W.H. Freeman, New York, NY.