British Journal of Pharmacology (2000) 130, 731–738

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Insulin modulation of intracellular free magnesium in heart: involvement of protein kinase C

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1 In the present study of rat heart using ^{31}P-nuclear magnetic resonance, we examined the interaction between β-adrenergic and insulin receptors in terms of the intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) regulation.

2 $[Mg^{2+}]_i$ was estimated from the separation of the chemical shifts of the α- and β-adenosine triphosphate (ATP) peaks, using the dissociation constant of MgATP 87 μM (established recently). In normal (phosphate-free Krebs-Henseleit) solution, $[Mg^{2+}]_i$ was approximately 1.02 mM.

3 Insulin at physiological and pathological concentrations increased $[Mg^{2+}]_i$ and contractility in a dose-dependent manner.

4 Insulin (more than 100 μu ml^{-1}) suppressed the decrease in $[Mg^{2+}]_i$ caused by isoprenaline (100 nM), and these effects of insulin on $[Mg^{2+}]_i$ and contractility were blocked by LY333531 (macrocyclic bis (indolyl) maleimide, 100 nM), a protein kinase C (PKC) inhibitor.

5 The isoprenaline-induced decrease in the concentrations of ATP ([ATP]) with insulin application was significantly smaller than that without insulin.

6 Insulin modulates $[Mg^{2+}]_i$ and haemodynamics, presumably *via* activation of PKC, thereby antagonizing the reduction of $[Mg^{2+}]_i$ induced by β-adrenoceptor stimulation.
British Journal of Pharmacology (2000) **130,** 731–738

Keywords: Insulin; magnesium; nuclear magnetic resonance; protein kinase C

Abbreviations: H-7, 1-(5-isoquinoline-sulphonyl)-2-methylpiperazine dihydrochloride; LY333531, macrocyclic bis (indolyl) maleimide; $[Mg^{2+}]_i$, intracellular free Mg^{2+} concentration

Introduction

Insulin elicits a remarkable array of biological responses. Insulin is also the primary hormone responsible for controlling the uptake, utilization, and storage of cellular nutrients. Insulin's anabolic actions include the stimulation of intracellular utilization and storage of glucose, amino acid, and fatty acid, while it inhibits catabolic processes, such as the breakdown of glycogen, fat, and protein *via* β-adrenoceptor stimulation (Davis & Granner, 1996).

In isolated cells and tissues, the application of insulin has been shown to alter the intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) (Barbagallo *et al*., 1993). A potential role of insulin as an endogenous regulating factor of Mg^{2+} homeostasis has also been indicated by the findings that alterations of blood and tissue Mg^{2+} levels are accompanied by decrease in insulin release from pancreatic β-cells and insulin resistance in other tissues and organs (White & Campbell, 1993).

In cardiac muscle, numerous intracellular mechanisms are known to be under the influence of the Mg^{2+} concentration (White & Hartzell, 1989) so that this parameter is of a great interest with regard to cardiovascular disease. For instance, hypomagnesemia seems to be linked with cardiac arrhythmia (Ebel & Günther, 1983). Further, anti-ischaemic and anti-arrhythmic effects of Mg^{2+} are well established: intravenous applications are often used as clinical therapy to suppress ventricular tachycardia (Shattock *et al*., 1987). On the other hand, epidemiological studies have suggested that Mg^{2+} deficiency is another important risk factor in ischaemic heart disease (Singh *et al*., 1996; White & Campbell, 1993). Recently, animal experiments have provided lines of evidence linking these two: β-adrenoceptor stimulation slowly decreases the intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$), (Nishimura *et al*., 1993; Watanabe *et al*., 1998) whereas it increases serum Mg^{2+} levels (Keenan *et al*., 1995).

In the present study of rat heart using ^{31}P-nuclear magnetic resonance (^{31}P-nmr), we thus examined the interaction between β-adrenergic and insulin receptors in terms of $[Mg^{2+}]_i$ regulation, and found that insulin attenuates isoprenaline-induced decrease in $[Mg^{2+}]_i$, presumably *via* activation of protein kinase C (PKC). This effect of insulin on $[Mg^{2+}]_i$ regulation could have clinical significance.

Methods

Preparation of isolated hearts and ^{31}P-nmr measurements

The methods employed for preparing isolated rat hearts and making ^{31}P-nmr measurements were essentially the same as previously described (Watanabe *et al*., 1998). Male Wistar rats weighing 350–400 g were pretreated with heparin (2000 units kg^{-1}, intraperitoneal) and anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, intraperitoneal). The animals were treated in accordance with the Animal Experimental Guide of Nagoya University School of Medicine. Their hearts were quickly excised and arrested in ice-cold saline. A cannula of 3.3 mm diameter was inserted into the aorta for retrograde perfusion, and a water-filled balloon was placed in the left ventricle for monitoring developed pressure. Each heart

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preparation was mounted in an nmr tube of 25 mm diameter, and perfused at a constant flow rate of 15–16 ml min^{-1} with a peristaltic pump.

An nmr spectrometer (JEOL GSX270W, Tokyo, Japan) was operated at 109.4 MHz for measurement of phosphorus compounds. Radiofrequency pulses corresponding to a 30° flip angle were repeated at 0.6 s intervals. Spectra were usually obtained by accumulating 2500 signals over 25 min. Under perfusion of phosphate-free Krebs-Henseleit (KH) solution, five major peaks were observed, assigned by their chemical shift to inorganic phosphate (P_i), phosphocreatine (PCr), and γ-, α- and β-ATP. The concentrations of the intracellular phosphorus compounds were estimated by integrating the spectral peaks and by correcting with their saturation factors (Nakayama *et al*., 1988).

Estimation of intracellular pH and free Mg^{2+}

pH_i was estimated from the chemical shift of the P_i peak, as previously described (Eisner *et al*., 1987; Moon & Richards, 1973). $[Mg^{2+}]_i$ was normally estimated from the separation of the observed α- and β-peaks of ATP ($\delta_{o(\alpha\text{-}\beta)}$), using the following equation (Gupta *et al*., 1984; Nakayama & Tomita, 1990):

$$[Mg^{2+}]_i = K_D^{MgATP}(\delta_{o(\alpha-\beta)} - \delta_{f(\alpha-\beta)})/(\delta_{b(\alpha-\beta)} - \delta_{o(\alpha-\beta)}),$$

where K_D is the dissociation constant of MgATP, and $\delta_{b(\alpha\text{-}\beta)}$ and $\delta_{f(\alpha\text{-}\beta)}$ are the resonance separations of the α- and β-peaks for Mg^{2+}-binding and metal-free ATP, respectively. The values used for $\delta_{b(\alpha\text{-}\beta)}$ and $\delta_{f(\alpha\text{-}\beta)}$ were 8.35 and 10.85 parts per million (obtained at 37°C, pH 7.2), respectively (Gupta *et al*., 1984). Recently, the K_D^{MgATP} value has been re-estimated to be approximately twice (87 μM at pH 7.2) (Zhang *et al*., 1997) that previously published. In the present study, we used the new K_D^{MgATP} value, thus estimated $[Mg^{2+}]_i$ proportionally increased. Also, the K_D^{MgATP} value was corrected with pH_i.

Solutions

The composition of phosphate-free, KH solution, employed as a normal solution, was as follows (mM): NaCl, 118; KCl, 5.9; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 12; Na_2EDTA, 0.5, (pH 7.4 at 37°C). The KH solution was prewarmed (to approximately 40°C) and was oxygenated with a mixture of 95% O_2 and 5% CO_2. The temperature of the preparation was set at 37°C.

Drugs

(±)-Isoprenaline hydrochloride and (±)-verapamil hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and 1-(5-isoquinoline-sulphonyl)-2-methylpiperazine dihydrochloride (H-7) from Seikagaku Co. (Tokyo, Japan). Macrocyclic bis (indolyl) maleimide mesylate (LY333531), a generous gift from Eli Lilly Co. (Indianapolis, IN, U.S.A.), was dissolved in dimethyl sulphoxide to give a stock solution. The half-maximal inhibitory concentrations (IC_{50}) of LY333531 are 4.7 nM for β_I- and 5.9 nM for β_{II}-isoforms of PKC. The IC_{50} values for other PKC isoforms are greater than 250 nM except PKC-η (IC_{50} = 52 nM) (Ishii *et al*., 1996). Thus, the concentration (100 nM) of LY333531 used in the present study was chosen to block both β-isoforms of PKC.

To rule out the effect of Mg^{2+}-buffering during application of insulin and/or isoprenaline, in the latter part of the present study, we used 25 μM verapamil, which caused cardiac arrest and protected myocardium from ATP depletion. In rat heart it has been shown that $[Mg^{2+}]_i$ regulation is partially affected by this drug only under Mg^{2+}-free conditions (Handy *et al*., 1996), which we did not employ.

Statistics

Numerical data are expressed as mean ± s.e.mean. Differences between groups in haemodynamics, pH_i and $[Mg^{2+}]_i$ were compared using ANOVA and Scheffé's test for multiple comparisons. A value of $P<0.05$ was considered statistically significant in all cases.

Results

Effects of insulin on $[Mg^{2+}]_i$

^{31}P-nmr measurements were performed for 200 min, after the heart preparations had been equilibrated with KH solution for approximately 30 min. Each spectrum was obtained by 25 min data accumulation, and $[Mg^{2+}]_i$ was estimated from the separation of the chemical shifts between the α- and β-ATP peaks, and expressed as values corrected for pH_i, estimated from the P_i peak. When isolated rat hearts were perfused with normal solution, $[Mg^{2+}]_i$ did not significantly change over 200 min. The control value for $[Mg^{2+}]_i$ was increased 2 fold compared to our previous estimation in line with the new K_D^{MgATP} value applied (Handy *et al*., 1996; Hongo *et al*., 1994).

Figure 1 shows the effects of insulin on $[Mg^{2+}]_i$ (a) and pH_i (b). After observing two control spectra (50 min), various concentrations of insulin were applied for 100 min (four spectra during insulin application). $[Mg^{2+}]_i$ estimated from the control spectra was 1.02 ± 0.02 mM (n = 5). No significant change was observed during application of 10 μu ml^{-1} insulin, while 100 and 1000 μu ml^{-1} insulin caused dose-dependent increase by 0.15 ± 0.02 (15%) and 0.21 ± 0.03 mM (21%), respectively, after 100 min. Subsequent to washout of insulin, $[Mg^{2+}]_i$ returned to the control level. pH_i was not significantly altered by insulin application.

Interaction between insulin and a β-adrenoceptor agonist

In heart, the interaction between insulin- and β-adrenoceptor-induced Mg^{2+} homeostasis has not been examined, while in liver it has been shown that insulin blocks Mg^{2+} efflux *via* β-adrenoceptors (Keenan *et al*., 1996). Thus, we carried out such experiments in heart. Application of 100 nM isoprenaline alone significantly decreased $[Mg^{2+}]_i$ by 0.27 ± 0.03 mM and pH_i by 0.06 ± 0.02 units, after 100 min (n = 5, open circles in Figure 2a,b). These results are consistent with our previous measurements (Nishimura *et al*., 1993; Watanabe *et al*., 1998). On the other hand, during simultaneous applications of 100 nM isoprenaline and 100 μu ml^{-1} insulin, no significant decrease in $[Mg^{2+}]_i$ was observed after 100 min (n = 5, closed diamonds in Figure 2a). The decrease in pH_i induced by isoprenaline was not altered by the additional application of insulin (closed diamonds in Figure 2b).

Changes in pH_i and intracellular free Ca^{2+} ($[Ca^{2+}]_i$) are known to affect Mg^{2+} buffering (Freudenrich *et al*., 1992; Koss *et al*., 1993). Furthermore, isoprenaline significantly decreases the concentrations of ATP ([ATP]), which is also known as an important intracellular Mg^{2+} buffer. In the following experiments, the interaction between insulin and isoprenaline was, thus, examined in the presence of a Ca^{2+} channel blocker, verapamil. Application of verapamil (25 μM) caused cardiac arrest, and fully depressed left ventricular developed pressure

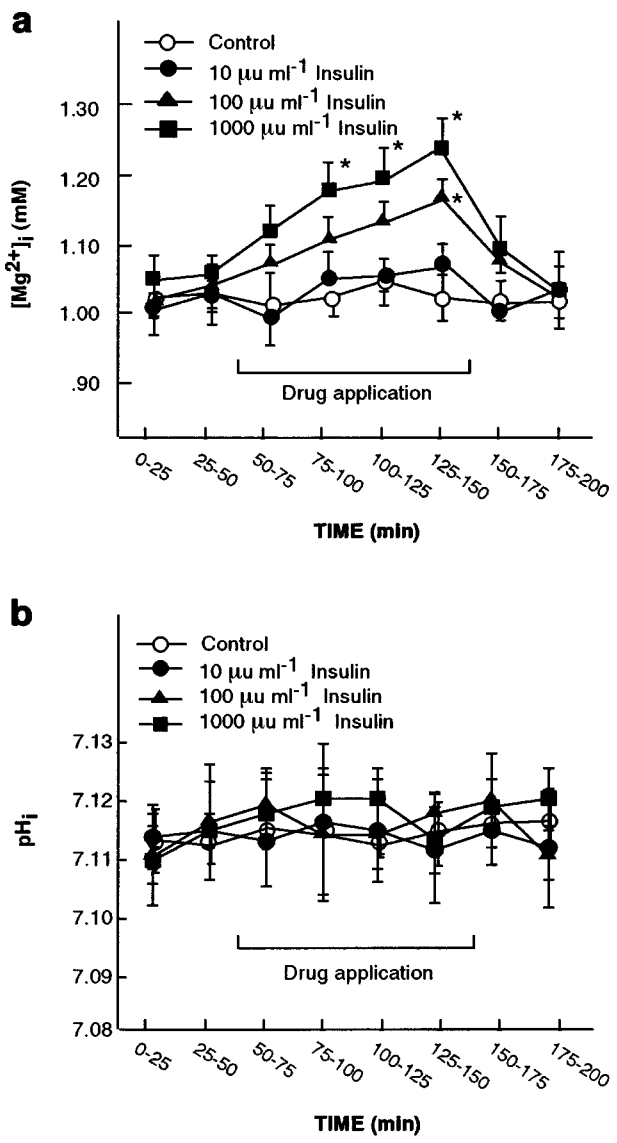

Figure 1 Time course of changes in intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) (a) and intracellular pH (pH_i) (b). Each point was obtained by accumulation of ^{31}P-nmr signals over 25 min. Various concentrations of insulin were applied for 100 min after two control spectra (values) were obtained with normal Krebs-Henseleit solution. Vertical bars are s.e. values. ($n=5$). $^*P<0.05$ vs the control group at the same time points.

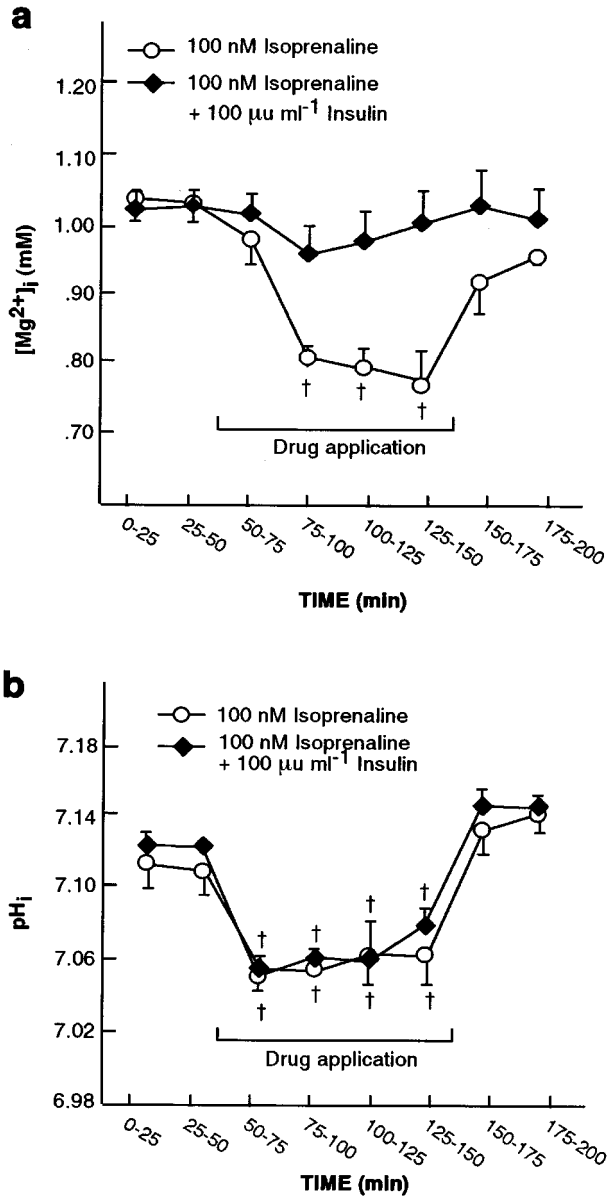

Figure 2 Effects of insulin on isoprenaline-induced changes in intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) (a) and intracellular pH (pH_i) (b). Isoprenaline (100 nM) was applied with or without insulin (100 μu ml^{-1}) for 100 min. Each time course was obtained with five hearts. Vertical bars are s.e. values. $\dagger P<0.05$ vs the basal value (perfusion with normal Krebs-Henseleit solution at 25–50 min).

(LVDP). Figure 3 shows the time course of changes in $[Mg^{2+}]_i$ and pH_i in such experiments. As observed in its absence (closed diamonds in Figure 2a), despite the presence of verapamil, applications of insulin (100 μu ml^{-1}) still prevented a decrease in $[Mg^{2+}]_i$ caused by isoprenaline (100 nM) application after 100 min ($n=5$, Figure 3a). On the other hand, pH_i did not significantly change throughout the experiments (Figure 3b). We have previously shown that isoprenaline significantly decreases $[Mg^{2+}]_i$ even in the presence of verapamil (Watanabe *et al*., 1998).

Effects of a protein kinase inhibitor

In many cells and tissues, it has been suggested that insulin causes diacylglycerol formation and PKC activation (Acevedo-Duncan *et al*., 1989; Bandyopadhyay *et al*., 1997; Egan *et al*., 1990; Hoffman *et al*., 1991). Furthermore, in the heart, activation of PKC suppresses Mg^{2+} efflux (Romani *et al*., 1992). Thus, we investigated whether PKC is involved in the interaction between insulin and β-adrenoceptor agonists with regard to $[Mg^{2+}]_i$ regulation in heart. No significant change in $[Mg^{2+}]_i$ was observed during application of 100 nM LY333531, which selectively inhibits the β-isoform of PKC. In the presence of 100 nM LY333531, application of insulin (1000 μu ml^{-1}) did not significantly alter $[Mg^{2+}]_i$, so that the gradual increase was blocked (data not shown). Figure 4 shows the effects of the PKC inhibitor on the interaction between insulin and a β-adrenoceptor agonist. When insulin (100 μu ml^{-1}) and isoprenaline (100 nM) were applied together with LY333531, $[Mg^{2+}]_i$ significantly decreased from 1.03 ± 0.02 mM to 0.78 ± 0.02 mM ($n=4$, Figure 4a), as was observed during isoprenaline application in the absence of insulin (open circles in Figure 2a). These results suggest that the β-isoform of PKC may play an important role in the effects of insulin on $[Mg^{2+}]_i$ regulation. When LY333531 was increased to 1000 nM, at which concentration PKC isoforms would be non-selectively blocked, the decrease in $[Mg^{2+}]_i$

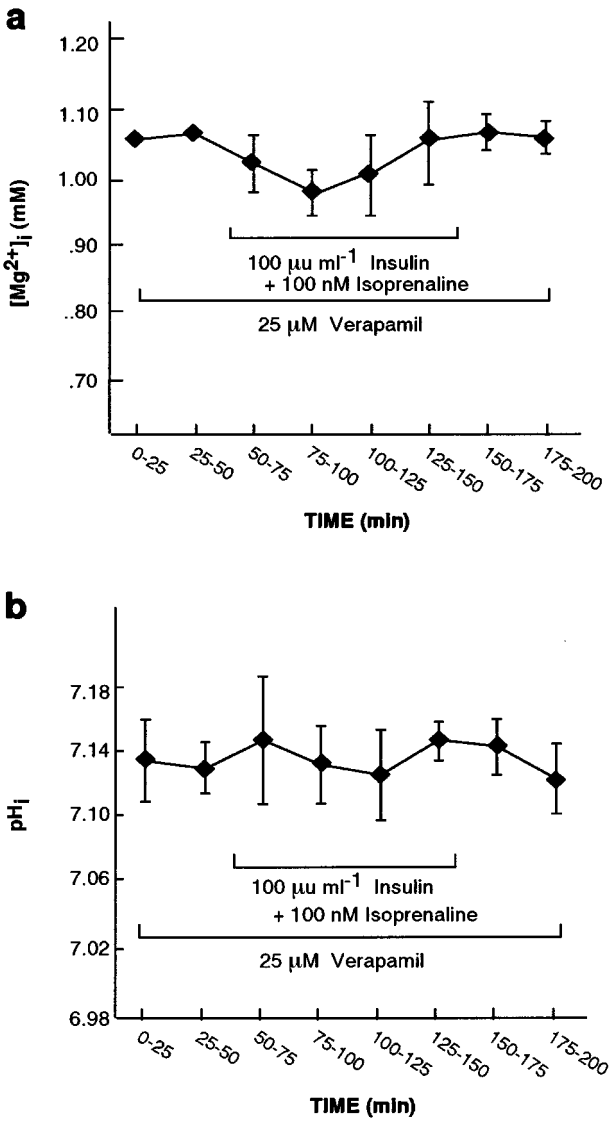

Figure 3 Changes in intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) (a) and intracellular pH (pH_i) (b) in the presence of verapamil. Isoprenaline (100 nM) and insulin (100 μu ml^{-1}) were simultaneously applied for 100 min in the presence of verapamil (25 μM) ($n=5$). Vertical bars are s.e. values.

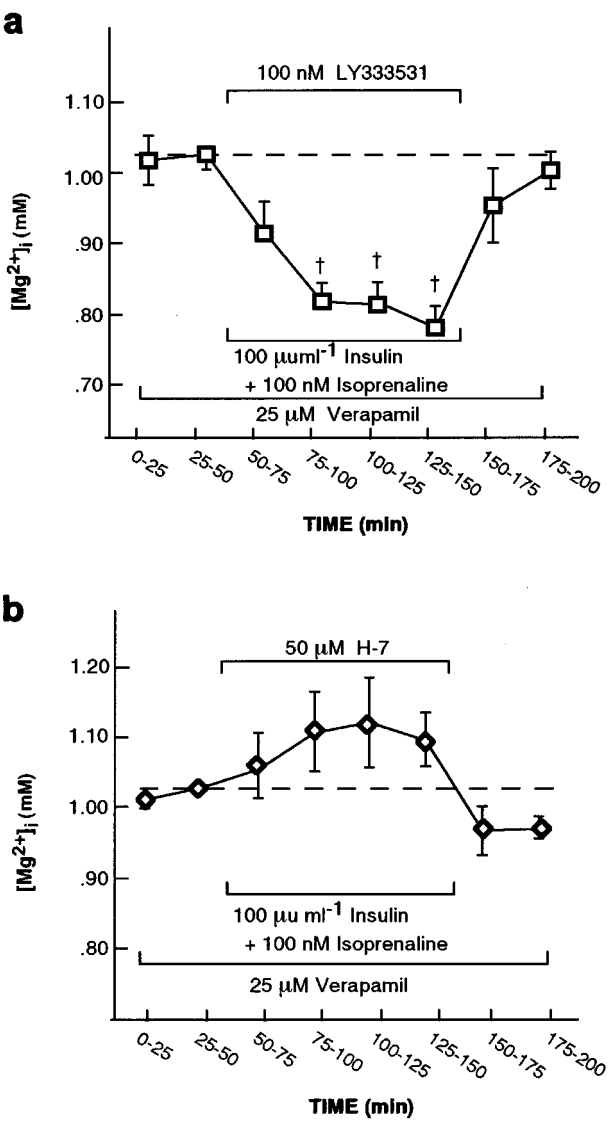

Figure 4 Effects of LY333531 (a) and H-7 (b) on intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$). Either LY333531 (100 nM, $n=4$) or H-7 (50 μM, $n=4$) were applied together with insulin (100 μu ml^{-1}) and isoprenaline (100 nM) for 100 min. †$P<0.05$ vs the basal value (perfusion with verapamil-containing solution at 25–50 min).

induced by isoprenaline (in the presence of insulin) was still observed. In these experiments verapamil was also present throughout.

High concentrations of H-7 non-specifically inhibit both A- and C-kinases (Hidaka *et al.*, 1984). When H-7 (50 μM) was employed instead of LY333531, simultaneous applications of insulin and isoprenaline again did not depress $[Mg^{2+}]_i$, increase being observed after 100 min (Figure 4b). In the light of the effects of LY333531 on $[Mg^{2+}]_i$, the results obtained in the presence of H-7 suggest that A-kinase is involved in the β-adrenoceptor agonist-induced decrease in $[Mg^{2+}]_i$ (also consistent with our previous deduction that cyclic AMP plays a central role) (Watanabe *et al.*, 1998).

Haemodynamic effects of insulin

Table 1 shows data for heart rate (HR) and LVDP. 10 μu ml^{-1} insulin only slightly increased HR and LVDP (no statistical significance). When insulin was increased to 100 and 1000 μu ml^{-1}, both HR and LVDP were increased in a dose-dependent manner. These positive chronotropic and inotropic effects of insulin (as well as effects on $[Mg^{2+}]_i$) were completely blocked by LY333531, a PKC inhibitor, but not by a β-adrenoceptor antagonist, propranolol (10 μM).

As shown previously, isoprenaline application significantly increased both HR and LVDP. Although insulin prevented the decrease of $[Mg^{2+}]_i$ caused by isoprenaline, the haemodynamic effects of the latter were not suppressed by additional application of insulin and rather were enhanced with a greater concentration (100–1000 μu ml^{-1}).

When isoprenaline was applied in the absence of insulin, four out of nine hearts showed sudden fall of LVDP, presumably due to ventricular fibrillation (VF). The HR and LVDP were therefore estimated with the remaining five hearts. VF was not observed in the five hearts to which isoprenaline and insulin were simultaneously applied.

Phosphorus compounds

Table 2 summarizes data for [ATP], the concentrations of PCr ([PCr]) and Pi ([Pi]) during applications of insulin and isoprenaline. Application of isoprenaline (100 nM) over

Table 1 Haemodynamic effects of insulin

	Normal solution *T25-50 min*	*Drug application* *T75-100 min*	*T125-150 min*	*Washout* *T175-200 min*
Control ($n=5$)				
HR (b.p.m.)	263±5	263±5	261±5	262±5
LVDP (mmHg)	154±4	155±6	153±5	156±5
10 μu ml^{-1} Insulin ($n=5$)				
HR (b.p.m.)	255±6	267±5	261±8	258±4
LVDP (mmHg)	157±7	166±9	163±6	154±5
100 μu ml^{-1} Insulin ($n=5$)				
HR (b.p.m.)	261±3	285±6*	281±5*	265±4
LVDP (mmHg)	150±5	176±7*	175±7*	153±6
1000 μu ml^{-1} Insulin ($n=5$)				
HR (b.p.m.)	258±5	297±7*	296±8*	252±6
LVDP (mmHg)	155±6	188±10*	185±7*	144±7
100 nM Isoprenaline ($n=5$)				
HR (b.p.m.)	261±4	308±5*	313±6*	259±5
LVDP (mmHg)	154±6	203±9*	195±7*	131±4*
10 μu ml^{-1} Insulin + 100 nM Isoprenaline ($n=5$)				
HR (b.p.m.)	264±4	319±6*	322±7*	264±3
LVDP (mmHg)	153±5	209±5*	206±8*	121±7*
100 μu ml^{-1} Insulin + 100 nM Isoprenaline ($n=5$)				
HR (b.p.m.)	257±4	333±8*†	329±5*†	268±7
LVDP (mmHg)	148±6	230±7*†	228±7*†	131±9*
1000 μu ml^{-1} Insulin + 100 nM Isoprenaline ($n=5$)				
HR (b.p.m.)	255±4	345±7*†	352±5*†	247±9
LVDP (mmHg)	152±4	242±5*†	245±8*†	118±8*
100 nM LY333531 ($n=4$)				
HR (b.p.m.)	252±5	237±9	243±3	246±2
LVDP (mmHg)	151±6	149±6	150±4	137±5
100 μu ml^{-1} Insulin + 100 nM LY333531 ($n=4$)				
HR (b.p.m.)	256±3	239±9	244±5	241±5
LVDP (mmHg)	158±4	159±6	152±6	138±9
100 μu ml^{-1} Insulin + 100 nM Isoprenaline + 100 nM LY333531 ($n=4$)				
HR (b.p.m.)	256±5	311±9*	315±3*	245±7
LVDP (mmHg)	155±4	209±6*	200±3*	127±5*

LVDP, left ventricular developed pressure; HR, heart rate. Values are mean±s.e.mean. *$P<0.05$ vs the control value (perfusion with normal solution at the same time point). †$P<0.05$ vs the basal value (perfusion with isoprenaline-containing solution at the same time point).

100 min decreased [ATP] and [PCr] to 61 and 85%, respectively, of the initial values, while [P_i] was correspondingly increased. Subsequent washout of isoprenaline restored [PCr], but [ATP] further decreased. Insulin (100 μu ml^{-1}) decreased [ATP] only to 93% of the control values after 100 min, while [PCr] was increased. This preservation of high-energy phosphates, despite the positive chronotropic and inotropic actions, is presumably due to facilitated carbohydrate metabolism.

When isoprenaline (100 nM) and insulin (100 μu ml^{-1}) were simultaneously applied, [ATP] significantly decreased to 75% of the initial value after 100 min. This was prevented by prior application of verapamil (25 μM). The isoprenaline-induced decrease in [ATP] with insulin application was significantly smaller than that without insulin.

Discussion

In the present study of isolated rat hearts using ^{31}P-nmr, insulin increased [$Mg^{2+}]_i$ in a dose-dependent manner (Figure 1a), and also blocked the isoprenaline-induced decrease in [$Mg^{2+}]_i$ (Figures 2a and 3a). Furthermore, insulin itself demonstrated positive chronotropic and inotropic effects, although its depletion of high-energy phosphates was significantly less than with isoprenaline.

It is well known that insulin activates PKC (Acevedo-Duncan *et al*., 1989; Bandyopadhyay *et al*., 1997; Egan *et al*., 1990; Hoffman *et al*., 1991). In the present study, a PKC inhibitor, LY333531, blocked positive haemodynamic effects and increases in [$Mg^{2+}]_i$ induced by insulin. These results suggest that the effects of insulin on contractility and [$Mg^{2+}]_i$ regulation in heart are brought about through a PKC pathway. On the other hand, isoprenaline application causes positive chronotropy and inotropy in heart, whereas [$Mg^{2+}]_i$ decreases during its application. It is noteworthy that while insulin and β-adrenoceptor agonists enhance cardiac contractility, respectively *via* PKC and protein kinase A pathways, these latter seem to possess opposite effects on [$Mg^{2+}]_i$ regulation.

It is now known that PKC is actually a family of related kinases, with specific tissue distributions, regulation, and substrate specificity. At the 100 nM concentration used in the present study, LY333531 selectively blocks β- and η-isoforms of PKC (Ishii *et al*., 1996). On the other hand, in several cell lines it has been shown that insulin potentiates the membrane

Table 2 Effcts of various drugs on levels of phosphorus compounds

	Normal solution T25-50 min	*Drug application T75-100 min*	*Washout T25-50 min*
Control ($n=5$)			
[ATP]	1.00	0.90±0.02	0.85±0.03
[PCr]	2.35±0.20	2.45±0.05	2.43±0.08
[P_i]	0.81±0.13	0.75±0.04	0.70±0.04
100 μu ml^{-1} Insulin ($n=5$)			
[ATP]	1.00	0.93±0.02	0.92±0.03
[PCr]	2.60±0.24	3.08±0.05*	2.84±0.05*
[P_i]	0.88±0.15	0.56±0.02*	0.51±0.02*
100 nM Isoprenaline ($n=5$)			
[ATP]	1.00	0.61±0.03*	0.57±0.03*
[PCr]	2.64±0.23	2.22±0.08*	3.21±0.13*
[P_i]	0.91±0.13	1.43±0.06*	0.72±0.07
100 μu ml^{-1} Insulin + 100 nM Isoprenaline ($n=5$)			
[ATP]	1.00	0.75±0.02*†	0.71±0.02*†
[PCr]	2.61±0.19	2.66±0.05†	2.93±0.11*
[P_i]	0.89±0.12	0.94±0.06*†	0.68±0.03
100 μu ml^{-1} Insulin + 100 nM Isoprenaline + 25 μM Verapamil ($n=4$)			
[ATP]	1.00	0.87±0.03	0.89±0.04
[PCr]	2.74±0.19	2.36±0.08	2.30±0.11
[P_i]	0.41±0.08	0.28±0.02*	0.25±0.02*

[ATP], [PCr] and [P_i] indicate the concentrations of ATP, phosphocreatine and inorganic phosphate, respectively, expressed as arbitrary units (initial [ATP] = 1 unit). [ATP] was estimated by intergrating the β-peaks of ATP. Values are mean±s.e.mean. *$P<0.05$ vs the basal value (perfusion with normal solution at the same time point). †$P<0.05$ vs the basal value (perfusion with isoprenaline-containing solution at the same time point).

PKC activity through translocation of PKC isoforms, with activation of PKC-β as a common observation (Bandyopadhyay *et al*., 1997; Standaert *et al*., 1996). Thus, it seems likely that PKC-β played a key role in the effects of insulin on both contractility and $[Mg^{2+}]_i$ regulation observed in the present study. In the presence of verapamil, a Ca^{2+} channel blocker, insulin application again increased $[Mg^{2+}]_i$. PKC-β is known as a classical, Ca^{2+}-sensitive PKC. Presumably, the resting $[Ca^{2+}]_i$ level in heart is sufficient for the Ca^{2+}-sensitive PKC to modulate $[Mg^{2+}]_i$ regulation even in the presence of a Ca^{2+} channel blocker (Takai *et al*., 1979).

Mg^{2+}-buffering is an important factor in regulating $[Mg^{2+}]_i$. In cardiac myocytes, changes in pH_i and $[Ca^{2+}]_i$ have been reported to affect intracellular Mg^{2+}-buffering capacity (Freudenrich *et al*., 1992; Koss *et al*., 1993). Also, ATP, the chemical shift of which is used to estimate $[Mg^{2+}]_i$, acts as an important Mg^{2+}-buffer. In the present study, insulin altered neither pH_i nor contractility (cardiac arrest) in the presence of verapamil. Furthermore, during application and washout of insulin, [ATP] slowly but consistently decreased, while $[Mg^{2+}]_i$ was increased by insulin application, and was reversed by its washout. These results suggest that intracellular Mg^{2+}-buffering is not a major factor in the modulation of $[Mg^{2+}]_i$ caused by insulin.

Previously, we reported a transient rise of $[Mg^{2+}]_i$ during the first period (0–25 min) of isoprenaline application (Nishimura *et al*., 1993). This result is consistent with an nmr measurement of $[Mg^{2+}]_i$ reported by another group who applied isoprenaline for a short period (20 min) (Headrick & Willis, 1989). The transient rise of $[Mg^{2+}]_i$ seen in the previous study could be interpreted by Mg^{2+} release from mitochondria (Romani *et al*., 1993) and/or breakdown of ATP (Headrick & Willis, 1989). Also, sympathomimetic drugs appear to potentiate Mg^{2+} efflux (Günther & Vormann, 1992; Romani & Scarpa, 1990). $[Mg^{2+}]_i$ is determined by the balance of Mg^{2+} fluxes. Thus, the discrepancy in the early change in $[Mg^{2+}]_i$ between previous (transient rise: Nishimura *et al*., 1993) and our recent results (small decrease: Watanabe *et al*., 1998) could be explained by different experimental procedures (e.g. the dose of drugs, the use of verapamil and the diameter of cannula), which would alter the subtle balance of Mg^{2+} influx, efflux and release from ATP during the early period of isoprenaline application. Indeed, even in our recent experiments, small increases in $[Mg^{2+}]_i$ were sometimes observed during the first period (0–25 min) of isoprenaline application, although the statistics (the average value) only showed decreases in $[Mg^{2+}]_i$. Since Mg^{2+} is considered as a chronic regulator of cellular functions (Grubbs & Maguire, 1987), slow $[Mg^{2+}]_i$ changes appear more important in physiological and clinical aspects. The emphasis of the present study was thus placed on the slow regulation of $[Mg^{2+}]_i$.

Recently, several groups have reported that administration of glucose and insulin with potassium (GIK), in addition to thrombolysis therapy, may significantly reduce mortality with acute myocardial infarction (AMI) (Apstein & Taegtmeyer, 1997; Díaz *et al*., 1998; Fath-Ordoubadi & Beatt, 1997). The efficacy of GIK treatment has been considered due to metabolic support *via* facilitated glycolysis. In the present study, we showed that insulin markedly enhanced cardiac contractility, although decreases of high-energy phosphates were much less than with isoprenaline. These results support the concept of metabolic protection for GIK therapy of AMI.

Mg^{2+}-deficiency has been reported to correlate with ischaemic heart disease (Ebel & Günther, 1983). Conversely, anti-ischaemic and anti-arrhythmic effects of Mg^{2+} are established (Shattock *et al*., 1987). Previously, we provided lines of evidence that $[Mg^{2+}]_i$ decreases with application of catecholamine (Nishimura *et al*., 1993; Watanabe *et al*., 1998), increase in which is known to raise the degree of severity in AMI (Ceremuzynski, 1981). In the present study insulin application suppressed isoprenaline-induced decrease in $[Mg^{2+}]_i$. Furthermore, when isoprenaline was applied in the absence of insulin, some of the heart preparations showed a sudden fall of LVDP to the basal level. This fatal pump failure was presumably caused by VF. Although the present experiments do not indicate a direct correlation between

$[Mg^{2+}]_i$ and VF, it is noteworthy that such a sudden LVDP fall was not observed during simultaneous applications of isoprenaline and insulin where $[Mg^{2+}]_i$ did not decrease. In the light of the present results, GIK treatment in AMI may be considered appropriate with respect to $[Mg^{2+}]_i$ regulation as well as metabolic support. Conversely, diabetes mellitus is a critical complication for AMI due to reduction of insulin secretion and/or peripheral insulin resistance in addition to organic changes in cardiovascular system frequently accompanied by this disease.

In heart, positive inotropic effects of catecholamine are separable into α_1- and β-adrenoceptor actions. Nevertheless, in the present study we used isoprenaline as a sympathomimetic drug for the following reasons: (1) β-adrenoceptors are predominant in heart; (2) previously we found that $[Mg^{2+}]_i$ is decreased by β-adrenoceptor stimulation through the cyclic AMP pathway and anti-arrhythmic effects of β-adrenoceptor antagonists are well established (Hoffman & Lefkowitz, 1996); and (3) it is known that α_1-adrenoceptor stimulation leads to activation of PKC. Therefore, in order to assess the interaction between PKC and PKA pathways, sympathomimetic drugs with substantial α_1-action would introduce confusion when used together with insulin, which also activates PKC. However, investigations of α_1-adrenoceptor stimulation in heart allowing identification of PKC isoforms would provide a more precise understanding of clinical aspects of insulin effects.

In conclusion, the results presented here indicate that insulin increases $[Mg^{2+}]_i$ in a dose-dependent manner, and antagonizes β-adrenoceptor stimulation-induced decrease in the rat heart. Also, insulin itself enhances cardiac contractility, despite relatively good preservation of high-energy phosphates. These effects of insulin appear to be caused by activation of PKC (presumably the β-isoform). Furthermore, in ischaemic heart disease insulin may be an important factor in terms of $[Mg^{2+}]_i$ regulation as well as energy metabolism.

We thank Dr D. Kirk Ways (Eli Lilly Co., Indianapolis, IN, U.S.A.) for kindly providing LY333531.

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(Received January 11, 2000
Accepted March 13, 2000)