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The effect of gliclazide and glibenclamide on preconditioning of the human myocardium

Mahmoud Loubani[a], Alan Fowler[a], Nicholas B. Standen[b], Manuel Galiñanes[a,*]

[a]Department of Integrative Human Cardiovascular Physiology and Cardiac Surgery, University of Leicester, Glenfield Hospital, Leicester, LE3 9QP, UK
[b]Department of Cell Physiology and Pharmacology, University of Leicester, Glenfield Hospital, Leicester, UK

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Abstract

The cardioprotection of ischaemic preconditioning may be abolished in diabetic patients especially when some oral hypoglycaemics are used. The dose–response effect of gliclazide and glibenclamide on ischaemic preconditioning and the action of glibenclamide on signal transduction in human myocardium were investigated using right atrial appendages from cardiac surgery patients. Glibenclamide (0.1, 1, 3 and 10 μM) and gliclazide (1, 10, 30 and 100 μM) were added for 10 min prior to ischaemic preconditioning. The cardioprotection was abolished by glibenclamide at all concentrations and by gliclazide at supratherapeutic concentrations of 30 and 100 μM. Glibenclamide abolished the protective effect of mitoK_{ATP} channel opening but not that of protein kinase C (PKC) or p38 mitogen activated protein kinase (p38MAPK) activation. In conclusion, glibenclamide and gliclazide differential effects may be a result of differential sensitivities. Glibenclamide does not block protection conferred by either PKC or p38MAPK activation. These findings may have clinical implications in ischaemic heart disease.

Keywords: Gliclazide; Glibenclamide; Human myocardium; Preconditioning

1. Introduction

Sulphonylureas are widely used in the treatment of type 2 diabetes, acting to stimulate insulin secretion from pancreatic β-cells by closing their principal target in the cell membrane, the ATP-sensitive potassium (K_{ATP}) channel. Blockade of K_{ATP} channels results in the depolarisation of the cell membrane, thereby triggering the opening of voltage-gated Ca^{2+} channels and leads to the elevation of intracellular Ca^{2+} and the stimulation of insulin secretion (Ashcroft and Rorsman, 1989). However, K_{ATP} channels of differing subtypes are also expressed in both cardiac and vascular smooth muscle cells, and inhibition of these channels by sulphonylureas may be related to certain cardiovascular side effects of the same drugs (Brady and Terzic, 1998; Klamann et al., 2000). In the heart, there is extensive evidence that K_{ATP} channels are involved in the cardioprotection induced by ischaemic preconditioning, and the sulphonylurea glibenclamide is known to inhibit such protection (Cohen et al., 2000; Ghosh et al., 2000a).

The exact identity of the channels involved in cardioprotection and the mechanism by which this occurs has been the subject of much recent controversy. A considerable body of pharmacological evidence, based on the selectivity of the K_{ATP} channel opener diazoxide and the blocker 5-hydroxydecanoate (5-HD), led to suggestions that a mitochondrial K_{ATP} channel is involved in ischaemic preconditioning (Garlid et al., 1997; Ghosh et al., 2000b; Gross and Fryer, 1999) and has subsequently been demonstrated in the rat (Singh et al., 2003). However, the molecular composition of any such mitochondrial K_{ATP} channel remains unconfirmed in the human and the selectivity of these compounds has also been questioned

* Corresponding author. Tel.: +44 116 2563032; fax: +44 116 2502449.
E-mail address: mg50@le.ac.uk (M. Galiñanes).

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(D'hahan et al., 1999). In particular, diazoxide and 5-HD have recently been shown to exert effects on mitochondrial metabolism that appear unrelated to K_{ATP} channels, but which might account for their effects on cardioprotection (Hanley et al., 2002).

Despite these considerations, the known specificity of sulphonylureas for their respective receptors (Gribble et al., 1998) may explain their differential effects on ischaemic preconditioning. This view is consistent with recent evidence demonstrating that, unlike glibenclamide, the sulphonylurea glimepiride does not abolish the protection of ischaemic preconditioning (Mocanu et al., 2001; Klepzig et al., 1999). Gliclazide, another sulphonylurea in wide clinical use, shows high selectivity for pancreatic over cardiac K_{ATP} channels (Lawrence et al., 2001). In the present study, we have therefore compared the dose-dependent effects of the second generation sulphonylurea, gliclazide with those of the classical sulphonylurea, glibenclamide on ischaemic preconditioning in a human tissue model. Both of these drugs are in extensive clinical use, which makes important to ascertain their possible adverse effects on myocardial cardioprotection.

Diabetes mellitus is a common disease in the general population and particularly in patients with ischaemic heart disease. It has been associated with increased morbidity and mortality in cardiac surgery (Fietsam et al., 1991). This may be due to the effects of diabetes on the various organs and vascular system. However, K_{ATP} channel blockade by sulphonylureas may also contribute to the poor outcome of diabetic patients subjected to myocardial ischaemia (Brady and Terzic, 1998; University Group Diabetes Program, 1976). We hypothesized that these clinical observations might correlate with the loss of cardioprotection in diabetic tissue per se. Indeed recent findings in the human atrial appendage model are consistent with the protective mechanism activated by either diazoxide or ischaemic preconditioning, possibly the mitoK_{ATP} channel or another mitochondrial mechanism, lying upstream of protein kinase C (PKC) and p38 mitogen activated protein kinase (MAPK) in the ischaemic preconditioning signal transduction pathway (Loubani and Galiñanes, 2002). Accordingly, the negative effect of sulphonylureas on protection by preconditioning might be offset by stimulation of factors downstream of mitochondria and K_{ATP} channels. Here we have therefore investigated whether the blockade by glibenclamide of cardioprotection by ischaemic preconditioning can be bypassed by stimulation of downstream signal transduction cascades.

2. Materials and methods

2.1. Experimental preparation

Experiments were performed on trabecular muscle sections obtained from the right atrial appendage of patients undergoing elective coronary artery bypass graft surgery or aortic valve replacement. We employed a cell necrosis model developed and characterised in our laboratory (Zhang et al., 2000). Donor patients were excluded if they had enlarged atria, diabetes mellitus, atrial arrhythmias, poor left ventricular function (ejection fraction < 30%), right ventricular failure or were receiving oral hypoglycaemic agents, opioid analgesia, K_{ATP} channel openers or catecholamines. Local ethical committee approval was obtained for the harvesting technique and the investigation conforms to the principles outlined in the Declaration of Helsinki. The specimens were collected in oxygenated Krebs Henseleit HEPES buffer (KHH) and sliced immediately at 4–5 °C. (Zhang et al., 2000) The tissue was placed with the epicardial surface face down onto a ground glass plate, lightly covered with a buffer-moistened glass microscope slide to prevent crushing artefact and was sliced freehand to a thickness of between 300 and 500 μm, using a surgical skin graft blade (Swann-Morton, UK). The specimen and the slide were moistened with ice-cold buffer throughout the procedure. Sections (weight 30–50 mg) were transferred to 25 ml Erlenmeyer flasks (Schott Glaswerk, Mainz, Germany) containing 10 ml of oxygenated KHH buffer and the flasks placed in a shaking water bath (100 cycles/min) at 37 °C. The incubation medium was oxygenated by a continuous flow of 95% O_2/5% CO_2 gas which was adjusted to maintain a PO_2 between 25 and 30 kPa and a CO_2 between 6 and 6.5 kPa (Zhang et al., 2000) and monitored using an automated blood gas analyser (model 855 Blood Gas System, Chiron Diagnostics). The pH of the incubation fluid was adjusted to between 7.36 and 7.45. For the induction of simulated ischaemia, the medium was bubbled with 95% N_2/5% CO_2 (pH 6.8–7.0) and D-glucose replaced with 2-deoxy-D-glucose (10 mM).

2.2. Solutions and chemicals

The incubation medium was prepared daily with de-ionized distilled water and contained (in mM): $NaCl_2$ (118), KCl (4.8), $NaHCO_3$ (27.2), $MgCl_2$ (1.2), KH_2PO_4 (1.0) $CaCl_2$ (1.25), D-glucose (10) and HEPES (20). As mentioned above, D-glucose was substituted with 2-deoxy-D-glucose (10 mM) during simulated ischaemia to maintain a constant osmolarity. All the chemicals were purchased from Sigma Chemicals. Gliclazide was provided by Technologie Servier (Orleans, France).

2.3. Experimental time course

All the muscle sections (between three and five per specimen) were equilibrated at 37 °C for a 30-min period. Some of the preparations were added to new flasks, which also contained 10 ml of oxygenated medium, for another 210 min (240 min total), to serve as time-matched aerobic controls. The remaining preparations were subjected to a 90-min period of simulated ischaemia at 37 °C as described above. Following this the muscle sections were reoxygenated for a further 120 min by incubation in 10 ml of oxygenated medium at 37 °C with 10 mM D-glucose added. At the end of the experimental protocols, aliquots of the incubation media used during the 120 min reoxygenation period were collected for the assessment of creatine kinase (CK) leakage while the tissue was taken for the assessment of cell viability (reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetriazolium bromide, MTT). The drugs under test were added for 10 min before the induction of ischaemic preconditioning or simulated ischaemia.

2.4. Study groups

There were 8 specimens per group, each from a different patient

Study 1: To investigate the dose-dependence of the effect of glibenclamide and gliclazide on ischaemic preconditioning. The following groups were studied: (i) time matched aerobic control, (ii) 90 min simulated ischaemia/120 min reoxygenation alone, (iii) ischaemic preconditioning with 5 min simulated ischaemia and 5 min reoxygenation prior to 90 min simulated ischaemia/120 min reoxygenation, (iv–vii) glibenclamide at various concentrations (0.1, 1, 3 and 10 μM) for 10 min prior to ischaemic preconditioning and (viii–xi) gliclazide at various concentrations (1, 10, 30 and 100 μM) for 10 min prior to ischaemic preconditioning.

Study 2: To investigate whether the abolition of preconditioning-induced protection by sulphonylureas can be offset by stimulation of the downstream signal transduction cascade. Glibenclamide was chosen for these studies because its properties are better characterised than those of gliclazide and because only a low dose was required. The following groups were studied: (i) time matched aerobic control, (ii) 90 min simulated ischaemia/120 min reoxygenation alone, (iii) ischaemic preconditioning with 5 min simulated ischaemia and 5 min reoxygenation prior to 90 min simulated ischaemia/120 min reoxygenation, (iv) diazoxide (100 μM) for 10 min prior to 90 min simulated ischaemia/120 min reoxygenation, (v) phorbol 12-myristate-13-acetate (PMA) (1 μM) for 10 min prior to 90 min simulated ischaemia/120 min reoxygenation, (vi) anisomycin (1 nM) for 10 min prior to 90 min simulated ischaemia/120 min reoxygenation, (vii) glibenclamide (1 μM) for 10 min prior to diazoxide (100 μM) administered for another 10 min and then followed by 90 min simulated ischaemia/120 min reoxygenation, (viii) glibenclamide (1 μM) for 10 min prior to PMA (1 μM) administered for another 10 min and then followed by SI/R, (ix) glibenclamide (1 μM) for 10 min prior to anisomycin (1 nM) administered for another 10 min and then followed by 90 min simulated ischaemia/120 min reoxygenation. The concentration of each of the agents used was chosen from previous dose–response studies from our laboratory.

2.5. Assessment of tissue injury and viability

Tissue injury was determined by measuring the leakage of CK into the incubation medium during the 120 min reoxygenation period. This was assayed by a kinetic ultraviolet method based on the formation of NAD (Sigma Catalogue No. DG147-K) and the results expressed as U/g wet wt.

Tissue viability was assessed by the reduction of MTT to an insoluble blue formazan product at the end of the experimental period. The tissue was loaded into a Falcon conical tube (15 ml, Becton Dickinson Labware, New Jersey, USA) and 2 ml of phosphate buffer solution (0.05 M), containing MTT (1.25 mg/ml, 3 mM at final concentration), was added and then incubated for 30 min at 37 °C. Following this, the tissue was homogenized in 2 ml of dimethylsulfoxide (Homogenizer Ultra-Turrax T25, dispersing tool G8, IKA-Labortechnic, Staufen, Germany) at 9500 rpm for 1 min. The homogenate was then centrifuged at $1000 \times g$ for 10 min and 0.2 ml of the supernatant was dispensed into a 98-well flat-bottom microtiter plate (Nunc Brand Products, Denmark). After this, the absorbance of the blue formazan formed was measured on a plate reader (Benchmark, Bio-Rad Laboratories, California, USA) at 550 nm and the results expressed as mM/g wet wt.

2.6. Data analysis

Results were expressed as mean values ± S.E.M throughout. Statistical significance was tested using analysis of variance (ANOVA) followed by the Bonferroni post-hoc test, and $P < 0.05$ was considered statistically significant. Dose–response relations were fitted to the following equation using the least squares algorithm in Sigmaplot (Jandel Scientific).

$$y = 1 - \left[1 + (x/K_i)^H\right]^{-1} \quad (1)$$

where y is the fractional block of ischaemic preconditioning protection, x is the sulphonylurea concentration, K_i is the concentration for half inhibition, and H is the Hill coefficient.

Dose–response curves for gliclazide inhibition of protection as reflected by the increase in the in CK release following ischaemic preconditioning were constructed by calculating y, the fractional inhibition of ischaemic preconditioning in the presence of gliclazide as:

$$y = \{(CK_{\mathrm{Glic}} - CK_{\mathrm{IPC}})/(CK_{\mathrm{SIR}} - CK_{\mathrm{IPC}})\} \quad (2)$$

where CK_{SIR}, CK_{IPC}, and CK_{Glic} are the levels of CK release with 90 min simulated ischaemia/120 min reoxygenation alone, in the presence of ischaemic preconditioning, and with ischaemic preconditioning plus gliclazide at the given concentration, respectively. Similar curves were constructed for MTT reduction.

3. Results

All the specimens entering into the studies were included in the analysis. The demographic data of the patients included in the studies is shown in Table 1.

3.1. Effects of glibenclamide and gliclazide on ischaemic preconditioning

Fig. 1A and B show the effects of the sulphonylureas glibenclamide and gliclazide on ischaemic preconditioning as assessed by CK release and MTT reduction, respectively. Simulated ischaemia/reoxygenation increased CK release substantially (4.50 ± 0.47 fold) over that observed in aerobic control slices, indicative of increased tissue damage (Fig. 1A). Ischaemic preconditioning had a protective effect, so that simulated

Table 1
Demographic data of all the patients included in the studies

Study	Number of patients	Number of specimens	Male/ Female	Age
Study 1	17	88	11:6	62.4 ± 6.8
Study 2	14	72	10:4	63.3 ± 7.1

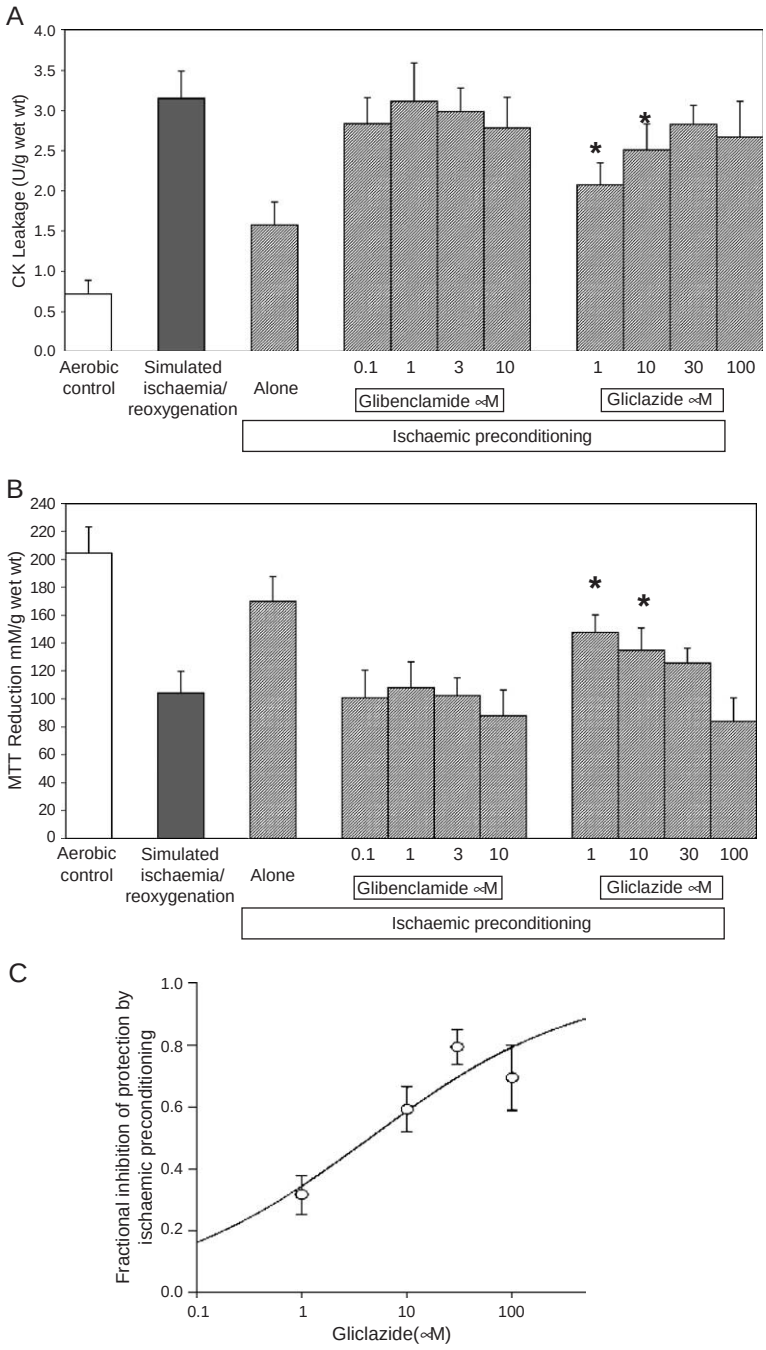

Fig. 1. (A) CK leakage into the media during the 120 min reoxygenation period. (B) MTT reduction by the slices at the end of the reoxygenation period. Human atrial myocardium was subjected to the protocols in Study 1 to investigate the dose–response of glibenclamide and gliclazide on ischaemic preconditioning. In each case the bars show mean (± S.E.M) values for $n=8$ preparations (*$P<0.05$ versus simulated ischaemia/reoxygenation alone). (C) Dose–response curve for the effect of gliclazide on protection of CK release by ischaemic preconditioning. Points show fractional inhibition calculated using Eq. (2) and the curve is drawn to Eq. (1) with K_i, the concentration for half-inhibition=4.5 μM and H, the Hill coefficient=0.431.

ischaemia/reoxygenation increased CK release by only 2.23±0.23-fold over the aerobic control under these conditions ($P<0.001$ compared to simulated ischaemia/reoxygenation alone). Glibenclamide abolished protection by ischaemic preconditioning at all concentrations used (0.1, 1, 3, and 10 μM).

By contrast, gliclazide at 1 μM did not significantly reduce protection by ischaemic preconditioning. At 10 μM protection was reduced, but not abolished ($P<0.05$ compared to simulated ischaemia/reoxygenation alone). However, the protective effect of ischaemic preconditioning was lost at the higher concentrations of gliclazide tested (30 and 100 μM). Fig. 1C shows the dose–response curve for the inhibition of ischaemic preconditioning protection by gliclazide calculated according to Eq. (2). The fitted line gives a gliclazide concentration for half-inhibition of 4.5 μM.

Fig. 1B shows that the results obtained from measurements of MTT reduction were essentially consistent with those described above for CK release. Simulated ischaemia/reoxygenation reduced MTT reduction from that observed in aerobic controls, consistent

with reduced tissue viability, and ischaemic preconditioning had a protective effect, increasing MTT reduction above the level seen with simulated ischaemia/reoxygenation alone. As for CK release, glibenclamide abolished protection by ischaemic preconditioning at all concentrations tested, while gliclazide at 1 μM did not significantly reduce protection. At 10 μM protection was reduced, but not abolished, and the protective effect of ischaemic preconditioning was lost at 30 and 100 μM gliclazide (Fig. 1C). Fitting the dose–response curve (not shown) gave a gliclazide concentration for half-inhibition of 4.8 μM, very close to the value obtained for CK release.

3.2. Effect of stimulation of the downstream transduction cascade of preconditioning in the presence of glibenclamide

Fig. 2A and B show the effect of glibenclamide on protection induced by stimulation of the preconditioning pathway at various stages. As previously reported by our laboratory (Loubani and Galiñanes, 2002), diazoxide (100 μM), PMA (1 μM) or anisomycin (1 nM) resulted in an equivalent reduction in CK leakage and preservation of MTT to that induced by ischaemic preconditioning itself. Interestingly, however, whilst the protection induced by diazoxide was abolished in the presence of glibenclamide, the protection obtained by PKC activation with PMA or by p38MAPK/JNK activation with anisomycin remained unaffected.

4. Discussion

Our results show differences between the sulphonylureas glibenclamide and gliclazide in their effect on ischaemic preconditioning. Although glibenclamide abolished the protective effect of preconditioning even at 0.1 μM, gliclazide did not block ischaemic preconditioning at 1 or 10 μM. We also show that glibenclamide prevents preconditioning by diazoxide which is thought to have a mitochondrial action, possibly by opening mitoK_{ATP} channels. However glibenclamide does not block the protective effect of activation of PKC or p38MAPK/JNK.

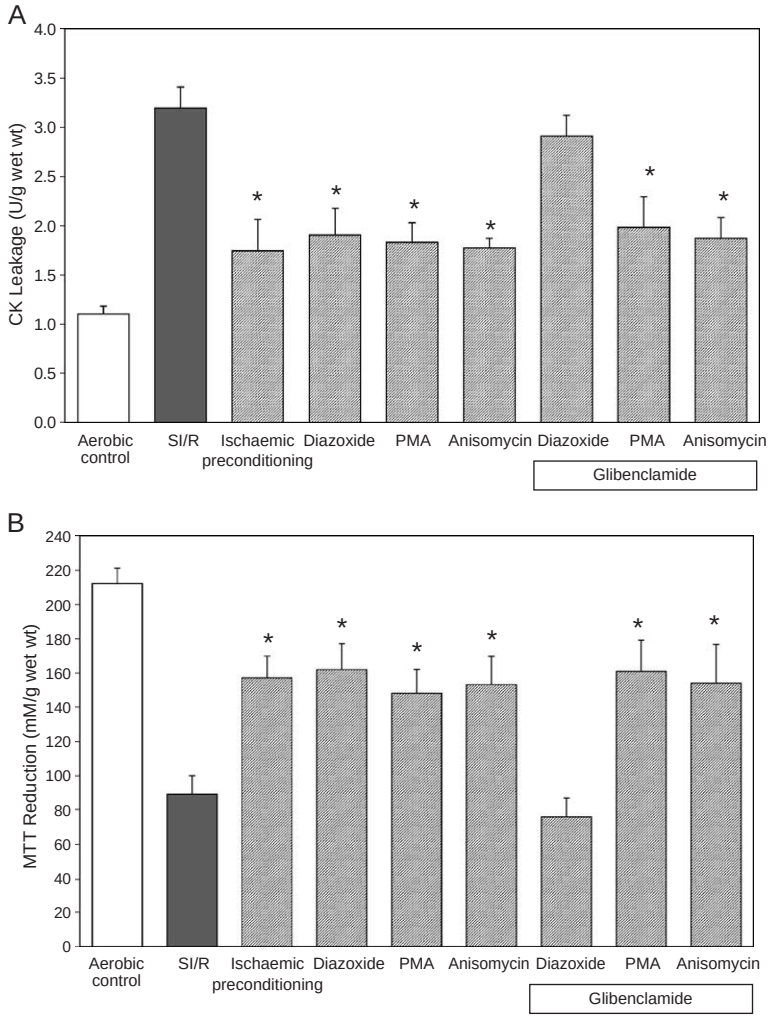

Fig. 2. (A) CK leakage into the media during the 120 min reoxygenation period, (B) MTT reduction by the slices at the end of the reoxygenation period in human atrial myocardium subjected to the protocols in Study 2 to investigate the effect of glibenclamide on the signal transduction mechanism of preconditioning. Data are expressed as mean (± S.E.M) for $n=8$. *$P<0.05$ versus simulated ischaemia/reoxygenation alone. Diazoxide 100 μM; PMA 1 μM; anisomycin 1 nM; glibenclamide 1 μM.

These results have potentially important clinical implications for the cardioprotection of diabetic patients with ischaemic heart disease and also provide important insights into the signal transduction mechanism of preconditioning.

4.1. Sulphonylureas and preconditioning

K_{ATP} channels have been demonstrated to be involved in the signal transduction mechanism of both ischaemic and pharmacological preconditioning (Cohen et al., 2000; Ghosh et al., 2000b; Gross and Fryer, 1999; Speechly-Dick et al., 1995). The failure of gliclazide at lower doses to abolish the cardioprotective effect of ischaemic preconditioning may possibly be explained by sequence specific differences in the K_{ATP} channels. The K_{ATP} channel is an octameric complex of two different protein subunits: an inwardly-rectifying K-channel, Kir6.2 or Kir6.1, and a sulphonylurea receptor, SU receptor (Aguilar-Bryan et al., 1998; Inagaki et al., 1996). The former acts as an ATP-sensitive K-channel pore while SU receptor is a channel regulator that endows Kir6.2 with sensitivity to drugs such as the inhibitory sulphonylureas and to K-channel openers. K_{ATP} channels in different tissues are composed of different Kir and SU receptor subunits. The different types of SU receptor subunit endow the K_{ATP} channels with different sensitivities to various drugs (Gribble et al., 1998). Thus cloned K_{ATP} channels containing SU receptor 1, the isoform expressed in the pancreas, are blocked by gliclazide with high affinity, whereas channels with the cardiac isoform SU receptor 2A are not (Gribble and Ashcroft, 1999). Similarly, in native tissues gliclazide shows > 100-fold selectivity for K_{ATP} channels of native β-cells over sarcolemmal K_{ATP} channels of rat cardiac ventricular myocytes. By contrast glibenclamide shows similar potency in both tissues (Lawrence et al., 2001). Our present results suggest a different affinity of these two sulphonylureas in human cardiac tissue. Recent work (Park et al., 2004) suggests that SU receptor 1 (pancreatic) has two binding sites, a benzamido site and a sulphonylurea site, whereas SU receptor 2 (cardiac) has only the benzamido binding site. Glibenclamide has both moieties, whereas gliclazide has only the sulphonylurea moiety and so does not block cardiac channels with high affinity. The doses of glibenclamide and gliclazide were selected to be equipotent in stimulation of insulin secretion, but only supratherapeutic doses of gliclazide blocked preconditioning while all doses of glibenclamide abolished the cardioprotection.

4.2. Sequence of the signal transduction of preconditioning

The elucidation of the factors involved in the signal transduction pathway of preconditioning has been the subject of intense investigation and although the participation of factors such as PKC (Cohen et al., 2000; Speechly-Dick et al., 1995; Mitchell et al., 1995) and K_{ATP} channels (Gross and Fryer, 1999; Speechly-Dick et al., 1995; Gross and Auchampach, 1992) is well established, their relevant sequence of activation remains controversial. Evidence from our laboratory using human tissue (Loubani and Galiñanes, 2002) as well as in the rabbit (Pain et al., 2000) and rat (Wang et al., 1999) suggests that PKC is in fact downstream of K_{ATP} channels, or at any rate of the stage in the protective pathway which is triggered by diazoxide, while PKC also appears to be upstream of p38MAPK in human and rabbit (Loubani and Galiñanes, 2002; Pain et al., 2000). Our present findings are consistent with this sequence, since the K_{ATP} channel blocker glibenclamide did not prevent cardioprotection by direct pharmacological activation of PKC or p38MAPK.

4.3. Clinical implications

The findings from our studies obviously have implications for diabetic patients on sulphonylureas since our results imply that their myocardium can still benefit from the cardioprotective effect of preconditioning provided the sulphonylurea used does not block ischaemic preconditioning at therapeutic doses. In terms of gliclazide, plasma levels have been reported to vary between 2.6 and 8 μg/ml or (8–23 μM) (Maeda et al., 1981; Oida et al., 1985; Palmer and Brogden, 1993). Not all the drug is free in solution, however, and it has been estimated that 95% of the drug will bind to proteins (Campbell et al., 1991). From these data we estimate peak free gliclazide to be no higher than 0.4–1.15 μM. If the isolated atrial tissue model we have used corresponds to events that occur in the intact heart in vivo, such concentrations should have minimal effects on preconditioning, and of course the average concentration will in any case be considerably lower than the peak values. Overall, this suggests that gliclazide can be used in patients with ischaemic heart disease without abolishing cardioprotection. On the other hand all the tested doses for glibenclamide (49.4–4940 ng/ml) (Matsuda et al., 1983; Wan Mohamad et al., 2000) are within the therapeutic dose, which varies between 40 and 300 ng/ml, and therefore glibenclamide would prevent the cardioprotective effect of preconditioning in the clinical setting. It should be emphasized, however, that diabetes per se may be an additional cause for the failure to precondition the myocardium, as previously shown by our laboratory (Ghosh et al., 2001), and therefore under some circumstances both diabetes and sulphonylureas could contribute to the abolition of cardioprotection.

Another important implication of the current studies is that it may still be possible to induce the cardioprotective effect of ischaemic preconditioning in the presence of drug-induced mitochondrion-based dysfunction, such as blockade of K_{ATP} channels. This would be delivered by manipulation of the signal transduction pathway further downstream. PMA and anisomycin cannot be used in vivo, but it is possible that further research in this area could lead to

improved agents that target downstream pathways for cardioprotection.

4.4. Limitations of the study

The limited amount of human atrial tissue, which makes the results of the current studies more relevant, placed restrictions on the extent of doses that we were able to test in Study 1 and the use of both sulphonylureas in Study 2. However, The doses used were equipotent and within the therapeutic ranges of both drugs. Furthermore glibenclamide was chosen for investigation in Study 2 due to its familiar properties and the small dose required to block preconditioning. Also the use of a limited number of doses may limit the ability of accurately calculating IC_{50} but these results at least provide us with an indication of the concentration dependence effect of gliclazide.

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