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ORIGINAL ARTICLE

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Contribution of ATP-sensitive potassium channels to β -adrenoceptor-mediated responses

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Abstract In isolated hearts the inotropic response to the β -adrenoceptor agonist isoproterenol is known to be abolished after ischaemia and reperfusion. The aim of this study was to investigate whether at decreased glucose levels the β -adrenoceptor-mediated responses in vascular smooth muscle would be depressed, since low glucose conditions mimic the influence of ischaemia. Accordingly, we investigated the influence of low glucose levels in the extracellular space on the vasorelaxation induced by isoproterenol and salbutamol in rat isolated thoracic aortic ring preparations with an intact endothelium and we attempted to further analyze the underlying mechanisms. Therefore, forskolin, dibutyryl-cAMP and glibenclamide (an ATP-sensitive K^+ -channel blocker) were studied as well.

In a glucose-free medium the concentration-response curve for isoproterenol was shifted to the left compared to that obtained under normal glucose conditions. The maximal relaxation induced by isoproterenol was not affected by the absence of glucose. In contrast, the maximal relaxation induced by salbutamol in glucose-free medium decreased by 50% compared to that obtained under normal glucose conditions. Glibenclamide caused a concentration-dependent decrease of the maximum relaxation by isoproterenol in a glucose-free medium, but had no effect under normal glucose conditions. Glibenclamide did not influence the concentration-response curves for salbutamol, neither in the presence nor in the absence of glucose in the medium. The relaxation caused by forskolin and dibutyryl-cAMP was not influenced by glibenclamide in a medium devoid of glucose. In endothelium-denuded preparations glibenclamide did not affect isoproterenol-induced responses neither in the presence nor in the absence of glucose. It is concluded that β -adrenoceptor stimulation opens ATP-sensitive potassium channels under conditions of im-

paired ATP-metabolism by a cAMP-independent pathway, which needs an intact endothelium.

Key words β -adrenoceptor · ATP · Ischaemia · Vascular smooth muscle · Relaxation · ATP-sensitive potassium channels

Introduction

β -Adrenoceptor agonists are known to cause relaxation of vascular smooth muscle via an interaction with the β -receptor which activates a G-protein and, subsequently, adenyl cyclase which transforms intracellular ATP into adenosine 3'5'-cyclic mono-phosphate (cAMP). cAMP binds to, and activates, protein kinase A, which mediates relaxation of vascular smooth muscle by phosphorylating functionally relevant substrates, thus changing their activity. Specific proteins and processes that may be modulated by phosphorylation are Ca^{2+} -ATPase ($\uparrow$), Na^+/K^+ -ATPase ($\uparrow$), Ca^{2+} channels ($\downarrow$), Ca^{2+} -activated K^+ channel ($\uparrow$), phosphatidylinositol metabolism ($\downarrow$) and especially myosin light chain kinase ($\downarrow$) (Torphy 1994). This induces an efflux of Ca^{2+} which causes a decrease of the intracellular $[Ca^{2+}]$. It seems likely that this complex mechanism, where energy rich phosphates play a pivotal role, will be highly susceptible to ischaemia or other derangements of energy supply.

In isolated hearts, for instance, the well-known inotropic response to the β -adrenoceptor agonist isoproterenol proved abolished after ischaemia and reperfusion (Baumann et al. 1981; Van den Ende et al. 1991). Vleeming et al. (1989) also described a depressed dose-response curve for dobutamine in the isolated perfused rat heart.

Similar experiments concerning the influence of ischaemia on the vasorelaxant activity of β -adrenoceptor stimulants in vascular smooth muscle have not been performed. Ischaemia is characterized biochemically by a depletion of ATP-stores. Such a depletion can also be obtained by re-

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ducing the glucose concentration in the extracellular space and such experiments can indeed be performed in vascular smooth muscle preparations.

In order to study the influence of ATP-store depletion, thus mimicking the biochemical sequela of ischaemia, we investigated the influence of low glucose levels in the extracellular space on the vasorelaxation induced by β -adrenoceptor stimulation in rat isolated thoracic aortic ring preparations. Accordingly, the vasodilator responses of isoproterenol (β_1/β_2 -adrenoceptor agonist) and salbutamol (a selective β_2 -adrenoceptor agonist) were investigated in the presence and absence of glucose.

We indeed found a clear influence of the presence/absence of glucose and we attempted to further analyze the underlying mechanisms. Accordingly, we used forskolin (adenylyl cyclase stimulator) and dibutyryl-cAMP itself, thus avoiding the direct influence of β -adrenoceptors.

Another important mechanism which might play a role in the β -adrenoceptor-mediated responses involves ATP-sensitive potassium (K_{ATP}) channels. These channels are modulated by changes in intracellular levels of ATP. The sulfonylurea glibenclamide, a widely used oral antidiabetic drug, is a potent blocker of the K_{ATP} channel(s) in the pancreatic β -cell (Petit and Loubatières-Mariani 1992) as well as in heart, skeletal muscle, brain and smooth muscle (Quast 1992; Quast and Cook 1989a,b), and a well known tool used for the pharmacological analysis of K_{ATP} channels. Accordingly, we also studied the influence of glibenclamide on β -adrenoceptor-induced vasorelaxation, in the presence and absence of extracellular glucose and in the presence and absence of the endothelium.

Materials and methods

Aortic ring preparations. Male Wistar rats of 225–250 g (Harlan Olac) were sacrificed by stunning and exsanguination. The thoracic aorta was carefully excised and placed in an oxygenated physiological salt solution (PSS) with or without 5.5 mM glucose at room temperature. The aortae were cleaned of superficial fat and loose connective tissue, care being taken not to stretch the vessels or to damage the endothelium. In some experiments the endothelium was destroyed by gently rubbing the intimal surface with a plastic rod. Aortic rings were cut in segments of approximately 3 mm long and mounted between two triangular stainless steel hooks in water-jacketed (37°C) organ baths of 5.0 ml capacity containing PSS of the following composition (mM): NaCl (136); KCl (2.5); $MgCl_2$ (0.5); $CaCl_2$ (1.8); NaH_2PO_4 (0.42); $NaHCO_3$ (11.9) and glucose (5.5). The composition of the glucose free PSS was the same, except that glucose was omitted.

The medium was continuously gassed with a mixture of 95% O_2 and 5% CO_2 and kept at a physiological pH (7.4). Isometric tension was measured via Kyowa force displacement transducers, connected to Kyowa amplifiers and a WKK recorder. The aortic rings were equilibrated in PSS for 60 min at a resting tension of 10.0 mN, which was kept constant throughout the experiment.

Experimental protocol. After the equilibration period the aortic rings were subjected for 5 min to a depolarizing PSS (containing 40 mM K^+), thus causing contraction of the vascular smooth muscle. In this high potassium solution 37.5 mM NaCl had been isotonicity replaced by KCl. With 20 min equilibration intervals this was followed by 5 min periods of vasoconstriction induced by the α_1 -adrenoceptor

agonist phenylephrine (PhE, 1 μ M), and subsequently again by exposure to high potassium PSS for 5 min.

Twenty minutes after the last potassium-induced contraction, the aortic rings were incubated for 1 h with 0, 0.1, 1 and 3 μ M glibenclamide, respectively. Subsequently, the aortic rings were exposed to 1 μ M PhE, which produced a nearly maximal response. After the contraction had reached its equilibrium, increasing doses of isoproterenol, salbutamol, forskolin and dibutyryl-cAMP were added to the bath in order to induce relaxation. Concentration-response curves from control experiments were compared to pretreated preparations by means of pD_2 -, E_{max} - and slope (Hill-coefficient) values. Concentration-response curves induced by isoproterenol were also constructed after incubation for 1 h with bisoprolol and ICI 118,551, respectively.

Drugs. L-phenylephrine hydrochloride, L-isoproterenol-D-bitartrate, salbutamol-base and dibutyryl-cyclic-AMP ($N^6,2'$ -O-Dibutyryladenosine-3':5'-cyclic monophosphate sodium) were obtained from Sigma (St Louis, Mo., USA). Bisoprolol fumarate was obtained from Merck (Amsterdam, The Netherlands) and ICI 118,551 from ICI Ltd (Macclesfield, Cheshire, UK). Forskolin was kindly donated by Hoechst (Amsterdam, The Netherlands). Glibenclamide was purchased from Hoechst AG (Frankfurt am Main, Germany). All drugs, except glibenclamide and forskolin, were dissolved in distilled water before being added to the PSS containing organ bath. Glibenclamide was dissolved in 100% DMSO and subsequently diluted to a stock-solution of 5×10^{-3} M, this stock-solution was diluted with 50% DMSO to the concentrations 5×10^{-4} M and 5×10^{-5} M, after which they were added to the organ bath. Forskolin was dissolved in 100% DMSO to a concentration of 10^{-2} M, and the solution was subsequently diluted with a mixture of propyleneglycol:DMSO (9:1) to a concentration of 5×10^{-3} M. Lower concentrations were obtained by appropriate dilution with distilled water.

Evaluation of data. Using a computer program (GraphPad, Institute for Scientific Information, USA), concentration-response curves based on the relationship

$$E = E_{max} \cdot A^P \cdot (A^P + EC_{50}^P)^{-1}$$

were fitted to log concentration-response data obtained in the individual experiments. In the equation, E is the response obtained at a given concentration A, E_{max} is the maximally attainable response, EC_{50} is the concentration required to induce the half maximal effect, and the exponent P describes the slope of the relationship (Hill-coefficient). The pD_2 is defined as the negative logarithm of the EC_{50} ($-\log [EC_{50}]$).

The parameters of the concentration-response curves of at least 6 individual experiments were averaged. Antagonist potency was expressed as pK_b values. The dissociation constant K_b was derived from the equation $\log K_b = \log [B] - \log [DR - 1]$, where B is the concentration of antagonist and DR is the agonist dose-ratio produced by the antagonist (Kenakin 1993). The data shown are means $\pm$ SEM. Statistical significance was established by means of an unpaired, double-sided Student's *t*-test. A *P*-value of less than 0.05 was considered significant.

Results

No differences in tonic contractions for phenylephrine were found under low glucose conditions compared to those obtained under control conditions. Under control conditions the absolute tension value induced by 10^{-6} M phenylephrine was 6.67 ± 0.18 mN ($n = 78$), under low glucose conditions this value was 6.68 ± 0.18 mN ($n = 110$).

In a glucose-free medium the concentration-response curve (CRC) for the relaxant effect of isoproterenol was

Fig. 1 Concentration-response curves for the dilator response curves of isoproterenol (A) and salbutamol (B) in isolated rat aortic ring preparations. The preparations were precontracted with 1 μ M phenylephrine. * means significantly different ($P < 0.05$) from normal conditions (5.5 mM glucose)

shifted to the left (pD_2 -value of 7.19 ± 0.08) compared to the CRC obtained under normal conditions (PSS with 5.5 mM glucose) (pD_2 -value of 6.67 ± 0.12) (Fig. 1A). Glucose did not influence the maximal relaxation (E_{max}) induced by isoproterenol. In contrast, salbutamol appeared to require the presence of glucose for a full vasodilator effect (Fig. 1B). In the PSS devoid of glucose the E_{max} for salbutamol decreased by 50% compared to the E_{max} in PSS with 5.5 mM glucose. The CRC for salbutamol was shifted rightward in a glucose-free medium, and the differences were significant at the concentration of 10^{-7} M and higher.

Figure 2A shows that in PSS with 5.5 mM glucose 10^{-6} M bisoprolol and 10^{-8} M ICI 118,551 significantly shifted the isoproterenol response curve to the right and reduced the extent of relaxation with pK_B values of 8.57 ± 0.01 for ICI 118,551 and 6.16 ± 0.71 for bisoprolol. Even in the PSS without glucose 10^{-8} M ICI 118,551 shifted the CRC for isoproterenol to the right (Fig. 2B). Additional 3×10^{-8} M bisoprolol showed no further rightward shift of the isoproterenol response curve in the presence of 10^{-8} M ICI 118,551.

Figure 3B shows that in a glucose-free medium glibenclamide, an ATP-sensitive potassium (K_{ATP}) channel blocker, caused a concentration-dependent decrease of the E_{max} of isoproterenol ($86.3 \pm 6.4\%$, $73.0 \pm 5.5\%$, $55.7 \pm 5.8\%$ and $51.1 \pm 4.4\%$ for 0, 0.1, 1 and 3 μ M glibenclamide, respectively). This effect was blocked by 10^{-8} M ICI 118,551. However, under normal conditions (PSS with

Fig. 2 Concentration-response curves for the effect of 10^{-6} and 10^{-7} M bisoprolol (—) and 10^{-8} and 10^{-9} M ICI 118,551 (----) on the dilator response of isoproterenol in isolated rat thoracic aortic rings in PSS with 5.5 mM glucose (A). Concentration-response curve for the effect of 10^{-8} M ICI 118,551 on the dilator response of isoproterenol in isolated rat thoracic aortic rings in PSS devoid of glucose (B). The preparations were precontracted with 1 μ M phenylephrine

5.5 mM glucose) glibenclamide did not influence the isoproterenol-induced relaxation (E_{max} of $91.8 \pm 2.2\%$) of aortic ring preparations (Fig. 3A). Glibenclamide did not influence the pD_2 -values and the slopes of the CRC for isoproterenol, neither in the presence nor in the absence of glucose. Concomitantly, glibenclamide did not have any effect on the phenylephrine-induced tension under both conditions.

Glibenclamide did not influence the concentration-response curves for salbutamol, neither in the presence of 5.5 mM glucose containing PSS nor in a glucose-free medium (data not shown).

Similar experiments were performed with the adenylyl cyclase stimulator forskolin and with dibutyryl-cAMP. Both compounds induce vasodilation which is not mediated by β -adrenoceptor stimulation and therefore does not primarily involve K_{ATP} channels. Figure 4A shows the CRC for dibutyryl-cAMP in the presence and in the absence of glucose. No significant differences in both curves were observed. However, the CRC for forskolin in the absence of glucose (pD_2 -value of 6.57 ± 0.02) was significantly shifted to the right when compared with the CRC for forskolin in the presence of 5 mM glucose (pD_2 -value of 5.80 ± 0.11) (Fig. 3B). Forskolin and dibutyryl-cAMP

Fig. 3 Concentrations-response curves for the effect of 0, 0.1, 1 and 3 μM glibenclamide on the dilator response of isoproterenol in isolated rat thoracic aortic ring preparations in PSS with 5.5 mM glucose (A) and in PSS devoid of glucose (B). The preparations were precontracted with 1 μM phenylephrine

Fig. 4 Concentrations-response curves for the effect of the dilator response of dibutyryl-cAMP (A) and forskolin (B) in isolated rat thoracic aortic ring preparations in PSS with and in PSS devoid of glucose. The preparations were precontracted with 1 μM phenylephrine

Fig. 5 Concentrations-response curves for the effect of 0 (closed symbols) and 3 μM (open symbols) glibenclamide on the dilator response of isoproterenol in isolated rat endothelium-denuded thoracic aortic ring preparations in PSS with 5.5 mM glucose (—) and in PSS devoid of glucose (---). The preparations were precontracted with 1 μM phenylephrine

both caused a maximal relaxation by $100.0 \pm 0.0\%$ under both conditions. Glibenclamide did not influence the relaxation caused by forskolin and dibutyryl-cAMP (data not shown). In addition, neither the pD_2 -values nor the $E_{\max}$ nor the slopes of the CRCs with respect to the forskolin and dibutyryl-cAMP induced relaxation were influenced by glibenclamide neither in the presence of 5.5 mM glucose containing PSS nor in a glucose-free medium.

Endothelium-intact aortic rings relaxed completely to methacholine (10^{-5} M), while in isolated endothelium denuded aortic ring preparations no relaxant response to methacholine was observed. Glibenclamide neither influenced the isoproterenol response in PSS with 5.5 mM glucose (pD_2 -value of 7.21 ± 0.11 and 6.80 ± 0.25 for 0 and 3 μM glibenclamide, respectively) nor in PSS without glucose (pD_2 -value of 7.51 ± 0.09 and 7.87 ± 0.13 for 0 and 3 μM glibenclamide, respectively) in endothelium denuded aortic rings (Fig. 5). Removal of glucose only influenced the CRC of isoproterenol in the presence of 3 μM glibenclamide. The pD_2 -value of the CRC of isoproterenol in PSS without glucose was significantly increased compared to the pD_2 -value in PSS with 5.5 mM glucose. No significant differences were observed in Hill-coefficients and $E_{\max}$ -values.

Discussion

The omission of glucose in the medium as in our experiments is known to reduce the cellular concentration of ATP and hence mimicks some of the biochemical effects secondary to ischaemia (Cole 1993).

In preparations with an intact endothelium, the vasorelaxant effects of isoproterenol and salbutamol were influenced by a glucose-free medium differently: the response to isoproterenol was shifted to the right, whereas the dilator response to salbutamol was blunted. Isoproterenol is a non-selective $\beta_1 + \beta_2$ -adrenoceptor agonist, whereas salbutamol is a selective stimulant of β_2 -adrenoceptors. Since

no selective β_1 -adrenoceptor agonists are as yet available the process of β_1 -induced vasodilation had to be studied in this indirect manner. To provide information whether β_1 -adrenoceptors were present in the thoracic aorta we inhibited the isoproterenol induced relaxations with the β_1 -antagonist bisoprolol and the β_2 -antagonist ICI 118,551. Surprisingly, these antagonists acted as non-competitive antagonists, whereas they block the positive chronotropic and isotropic effects in the heart in a competitive manner (Brodde 1991). The reason for this difference is unknown. The pK_B value for ICI 118,551 reflects the known affinity of this compound for β_2 -receptors. The high concentrations of bisoprolol required to inhibit isoproterenol-induced vasorelaxation exclude a contribution of β_1 -receptors to this effect and also reflect inhibition of β_2 -receptors (Brodde 1991). Hence, these studies show that rat thoracic aorta (with intact endothelium) possesses functional β_2 - but not β_1 -receptors. In pig basilar artery a β_1/β_2 ratio of approximately 65:35 was found (Miyamoto et al. 1993) and in endothelial cells of bovine aorta 13% of the β -adrenoceptors belong to the β_1 -subtype (Zink et al. 1993). In isolated rat cerebral arteries Hempelmann and Ziegler (1993) found that the isoproterenol-induced relaxation is mediated by different β -adrenoceptors.

Salbutamol-mediated vasodilation obviously requires energy rich phosphates and for that matter the presence of extracellular glucose. From these data with salbutamol we may not conclude that β_2 -adrenoceptors are not involved in the isoproterenol effect in the absence of glucose, because the data with ICI 118,551 say the contrary (Fig. 2). It is also possible that salbutamol is not a full β_2 -adrenoceptor agonist in the isolated rat aortic ring preparation or even atypical β -adrenoceptors may be involved. The responses to forskolin proved to be more sensitive in PSS with 5.5 mM glucose when compared with those obtained in a glucose-free medium. No differences were observed for the E_{max} of dibutyryl-cAMP and forskolin in PSS containing 0 mM glucose when compared with those obtained under control conditions (PSS with 5.5 mM glucose).

ATP-sensitive potassium channels appear not to be involved in β_2 -adrenoceptor-mediated vasodilation, since glibenclamide did not inhibit the relaxant effect of salbutamol, neither in the presence nor in the absence of glucose in the medium. In contrast, the leftward shift of the concentration-response curve for isoproterenol in a glucose-free medium when compared to the curve obtained under normal conditions may be explained by a hyperpolarizing potassium current. Potassium channels which are regulated by changes in intracellular levels of ATP ($[ATP]_i$) have been identified in pancreatic β -cells (Cook and Hales 1984), cardiac cells (Noma 1983) and in vascular smooth muscle cells (Standen et al. 1989). In contrast to pancreatic β -cells, ATP-sensitive potassium (K_{ATP}) channels in cardiac and vascular smooth muscle are closed under resting physiological conditions and the channels will open in response to a fall in $[ATP]_i$ (Standen et al. 1989; Longmore et al. 1990). This effect can be reversed, at least partially, by glibenclamide (Longmore et al. 1990; Noack et al. 1992; Allard and Lazdunski 1993).

In guinea-pig and bovine trachea, isoproterenol and salbutamol cause hyperpolarization (Cook et al. 1993) which is accompanied by an increase in the efflux of $^{86}Rb^+$ and of $^{42}K^+$ efflux (Quast and Cook 1989b; Chiu et al. 1993), thus indicating the opening of membrane potassium channels. Glibenclamide was described to block in an apparently competitive manner both the in-vitro vasorelaxation and the increase in ^{86}Rb efflux due to cromakalim and diazoxide as well as the in-vivo vasodilator activity of these two potassium channel openers (Quast and Cook 1989a). Randall and McCulloch (1995) observed that K_{ATP} channels are involved in isoproterenol-mediated vasorelaxation in the rat isolated mesenteric arterial bed. In in vivo studies with rat basilar artery (Kitazono et al. 1993) and in dog coronary artery (Narishige et al. 1994) glibenclamide inhibited the β_1 -adrenoceptor induced vasodilation. However, in canine saphenous vein smooth muscle only β_2 - and not β_1 -adrenoceptors appear to be coupled to K_{ATP} channel activation (Nakashima and Vanhoutte 1995).

In our experiments glibenclamide did not influence the vasodilation induced by β -adrenoceptor stimulation by isoproterenol in the presence of glucose. It has been described previously, on the basis of electrophysiological studies, that the removal of the substrate required for ATP production results in the appearance of a glibenclamide-sensitive potassium-current (Noack et al. 1992). Accordingly, the inhibition of ATP generation in coronary arteries caused a glibenclamide-sensitive dilatation and hyperpolarization (Von Beckerath et al. 1991). Also in our experiments we observed that in a glucose-free medium the enhanced sensitivity to the β (isoproterenol)-receptor induced vasodilation was blunted by glibenclamide. This finding would indicate that the isoproterenol-induced relaxation may open glibenclamide-sensitive potassium channels under conditions of impaired ATP-generation. Neither the vasorelaxant effect of forskolin nor that of dibutyryl-cAMP was influenced by glibenclamide, irrespective of the presence or absence of glucose. Hence, there may be species and tissue differences with respect to the mechanism of coupling between β -adrenoceptors and K_{ATP} -channels.

Recent studies have shown that β -adrenoceptor responses in rat isolated thoracic aortic rings may act via an endothelium-dependent pathway (Gray and Marshall 1992; Wang et al. 1993). However, results about the influence of the endothelium in β -adrenoceptor induced vasodilation are rather conflicting (Bea et al. 1994). In our study we observed that removing of the endothelium did not influence the isoproterenol induced relaxations, therefore no endothelium-dependent pathway was involved in the β -adrenergic response. However, under low glucose conditions glibenclamide was not able to inhibit the isoproterenol induced vasodilation in isolated endothelium denuded rat thoracic aorta. Binding studies showed that glibenclamide, up to 100 μM , did not influence the binding of $[^3H]$ -dihydroalprenolol to the β -adrenoceptor in rat cerebral cortex (Randall and McCulloch 1995).

The precise mechanism underlying the coupling between receptor and channel activation cannot be elucidated

in detail from the data obtained in the present study, although it is clear that it is independent of adenylate cyclase or cAMP. It may be possible that β -adrenoceptor coupling to K_{ATP} channels is mediated via G-proteins, although, we proved in the present study that the endothelium plays a crucial role. The K_{ATP} channel opening induced by β -adrenoceptors may be counteracted by various factors from the endothelium [such as the endothelium-dependent hyperpolarizing factor (EDHF) or even the endothelium-dependent contracting factor (EDCF)].

Therefore, we may conclude that the opening of K_{ATP} channels as a result of β -adrenoceptor stimulation by isoproterenol under glucose-free condition appears to be endothelium-dependent. It seems likely that the process of K_{ATP} channel opening in a glucose-free medium occurs at the level of the β -adrenoceptor or the G-protein, that is via pathways which are independent of adenylate cyclase of cAMP.

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