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A role for protein kinase C in the attenuated response to 5-hydroxytryptamine in aortas from streptozotocin-diabetic rats

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Abstract

We investigated protein kinase C participation in the contractile response to 5-hydroxytryptamine (5-HT), and in the interaction between 5-HT and endothelin-1, in aortas from control and diabetic rats. Diabetic rats display attenuated reactivity to 5-HT (i.e., approximately 47% of control maximum). The protein kinase C inhibitor calphostin C (1 μM) significantly reduced responses to 5-HT only in aortas from control rats. In diabetic rats, maximum responses to 5-HT, in the presence of endothelin-1 (3 nM), were not significantly different to controls. The additional presence of calphostin C significantly reduced responses only in aortas from diabetic rats. These results may indicate an abnormality in the protein kinase C second messenger system during diabetes.

Keywords: Diabetes; 5-HT (5-hydroxytryptamine); Protein kinase C; Endothelin-1; Aorta

1. Introduction

Increased platelet aggregation is a feature of diabetes and, accordingly, plasma 5-HT levels (Barradas et al., 1988), plasma endothelin levels (Collier et al., 1992; Kawamura et al., 1992; Takahashi et al., 1990) and thromboxane A_2 levels (for a review see Hodgson et al., 1992) have been reported to be increased in diabetic humans. Our laboratory has previously reported that maximum responses to endothelin-1 (Fulton et al., 1991; Hodgson and King, 1992), mediated by ET_A receptors, and 5-HT (James et al., 1994; Sikorski et al., 1993), mediated by 5-HT_2 receptors, of aortic rings from streptozotocin-diabetic rats are attenuated compared to those obtained in control rats. However, reactivity to noradrenaline was unaltered (Fulton et al., 1991) indicating that these changes are not due to a non-specific effect of diabetes on the smooth muscle. We have also reported that endothelial-derived thromboxane A_2 appears to play a role in the potentiation of 5-HT responses by endothelin-1 in aortas from diabetic rats (James and Hodgson, 1995a). It is therefore possible that in vivo interactions between locally released endothelin-1, 5-HT and thromboxane A_2 play a role in the genesis of the vascular complications associated with diabetes.

The present study investigated whether protein kinase C activation contributes to the contractile response to 5-HT in aortas from control and diabetic rats. In addition, the role of protein kinase C activation in the interaction between 5-HT and endothelin-1 was examined.

2. Materials and methods

Male Wistar rats were treated with streptozotocin (60 mg/kg, i.v.) or vehicle (50 mM citrate buffer) under 4% halothane anaesthesia (O_2/N_2O, 2:1) as previously described (James and Hodgson, 1995a; James and Hodgson, 1995b). The animals were then housed in treatment pairs, being allowed free access to food and water at all times. Only rats displaying elevated blood glucose levels (16 mM, Ames Minilab 1) after two weeks were considered to be diabetic. Control rats had normal (4–9 mM) blood glucose levels over the two week period. After two weeks, a diabetic and control pair were killed and 5 mm rings cut from each descending thoracic aorta. Rings were placed under 10 g resting tension (Fulton et al., 1991), in 15 ml organ baths. Tissues were equilibrated for 1 h in Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, glucose 11.1. Physiological solutions

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Table 1
Body weights, blood glucose levels and aortic ring weights

	Body weight (g)		Blood glucose (mM)		Aortas (mg)
	Initial	Final	Initial	Final	
Control ($n = 20$)	338 ± 7	379 ± 7 [a]	6.1 ± 0.3	6.7 ± 0.3	1.78 ± 0.04
Diabetic ($n = 28$)	330 ± 4	299 ± 6 [a,b]	6.0 ± 0.3	23.3 ± 0.6 [a,b]	1.66 ± 0.03 [b]

Initial measurements were made at the time of streptozotocin or vehicle injection, and final measurements made 2 weeks later. [a] Significantly different from initial value in same treatment group, $P < 0.05$. [b] Significantly different from corresponding value in control group, $P < 0.05$.

were maintained at 37°C, and bubbled with carbogen (5% CO_2 in O_2). Contractions were recorded on a Grass polygraph (model 7D).

The presence of endothelium was confirmed by the response obtained to acetylcholine (10 μM) in rings precontracted with a submaximal concentration of phenylephrine (0.3 μM). A cumulative concentration-response curve to 5-HT (0.1 μM to 0.1 mM) was then obtained. 5-HT was added at intervals of between 3–10 min depending on the time taken for the previous response to reach a plateau. Where indicated responses to 5-HT were obtained in the presence of calphostin C (1 μM). At the concentration used, calphostin C has been shown to be an effective and selective inhibitor of protein kinase C having no significant effect on the contractile response to KCl (Shimamoto et al., 1992). Calphostin C was allowed to equilibrate for 45 min before the addition of 5-HT. In additional experiments, responses to 5-HT were obtained in the combined presence of a threshold concentration of endothelin-1 (3 nM) and calphostin C (1 μM). Only one curve was obtained in each preparation. Contractile responses were expressed as grams tension. After each experiment, aortic rings were oven dried at 50°C and tissue weights recorded.

2.1. Drugs

The following drugs were used: acetylcholine chloride (Sigma), calphostin C (Sapphire Bioscience), dimethylsulphoxide (Sigma), endothelin-1 (Auspep), 5-hydroxytryptamine creatine sulphate (Sigma), phenylephrine hydrochloride (ICN Pharmaceutical), streptozotocin (Sigma).

Phenylephrine was dissolved in catecholamine diluent (0.312 g NaH_2PO_4 and 0.08 g ascorbic acid per litre of 0.9% (w/v) saline). Endothelin, acetylcholine and 5-HT were dissolved in distilled water. Calphostin C was dissolved in dimethylsulfoxide (DMSO). The final bath concentration of DMSO (i.e., 1%) had no significant effect on responses to 5-HT (data not shown).

2.2. Statistics

pEC_{50} values were determined from the E_{max} of each individual curve and the geometric mean (i.e., mean of the log values) determined. Aortic ring dry weights were analysed using a Student's *t*-test. Rat body weights, blood glucose levels, maximum responses and pEC_{50} values were analysed using a two-way analysis of variance (ANOVA) (Apple Macintosh, CLR ANOVA package) and, if significant, by one-way ANOVA and Tukey's test. Values shown are means ± S.E.M. In all cases, statistical significance is indicated by $P < 0.05$.

3. Results

Two weeks after streptozotocin treatment diabetic rats displayed significantly increased blood glucose levels com-

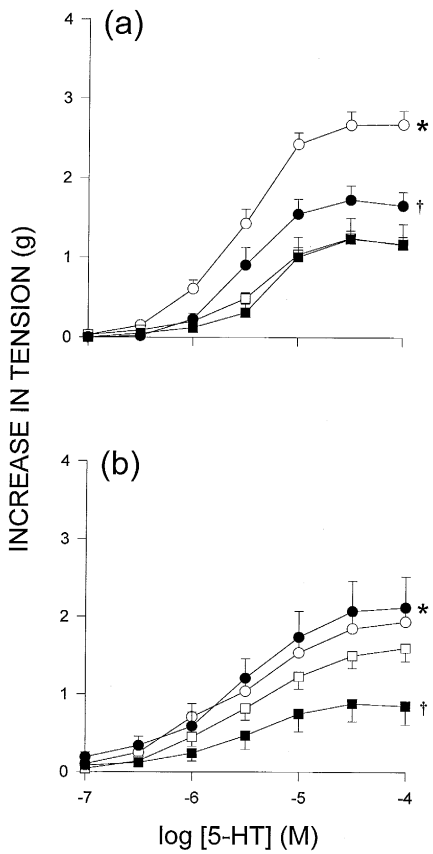

Fig. 1. Cumulative concentration–response curves to (a) 5-HT alone ($n = 11–14$, control, ○; diabetic, □), in the presence of calphostin C ($n = 5–6$; 1 μM; control, ●; diabetic, ■), (b) in the presence of endothelin-1 ($n = 11–12$, 3 nM, control, ○; diabetic, □) or in the combined presence of endothelin-1 (3 nM) and calphostin C (1 μM; $n = 5$; control, ●; diabetic, ■) of rat endothelium-intact aortae. * $P < 0.05$, significantly different to corresponding diabetic group, ANOVA. † $P < 0.05$, significantly different to corresponding group without calphostin C, ANOVA.

Table 2
pEC_{50} values for 5-HT of aortas from control and diabetic rats

	Control	Diabetic
5-HT	5.59 ± 0.07	5.45 ± 0.06
5-HT + calphostin C	5.50 ± 0.07	5.29 ± 0.05
5-HT + endothelin-1	5.80 ± 0.21	5.66 ± 0.18
5-HT + endothelin-1 + calphostin C	5.61 ± 0.10	5.51 ± 0.13

pared to their pretreatment values, and compared to vehicle-treated rats (Table 1; $P < 0.05$, ANOVA). Streptozotocin-induced diabetes was also associated with a significant reduction in body weight. After two weeks, body weights of diabetic rats were significantly lower than their corresponding pre-injection weights. Body weights of control rats significantly increased over the same time period (Table 1; $P < 0.05$, ANOVA). Dry weights of aortic rings from diabetic rats were significantly decreased compared to those of controls (Table 1; $P < 0.05$, *t*-test).

We have previously reported that 5-HT (0.1 μM to 0.1 mM) produces concentration-dependent contractile responses which are significantly attenuated in endothelium-intact aortas from diabetic rats compared to controls ($P < 0.05$, ANOVA James et al., 1994; Fig. 1a). In the presence of calphostin C (1 μM), maximum responses to 5-HT of aortas from control rats were significantly reduced compared to those obtained in the absence of calphostin C ($P < 0.05$, ANOVA). However, maximum responses of aortas from diabetic rats were not significantly different to those obtained in the absence of calphostin C. Therefore, in the presence of calphostin C, maximum responses to 5-HT of aortas from diabetic rats were no longer significantly different to those obtained from controls (Fig. 1a).

We have also reported that in the presence of a threshold concentration of endothelin-1 (3 nM; control 0.80 ± 0.13 g; diabetic 0.74 ± 0.10 g) maximum responses to 5-HT were no longer significantly different between aortas from control and diabetic rats (James and Hodgson, 1995a; Fig. 1b, Table 2). In the presence of calphostin C (1 μM), endothelin-1 (3 nM) produced small contractile responses in aortas from control (0.82 ± 0.15 g) and diabetic (0.63 ± 0.09 g) rats. Maximum responses to 5-HT in the presence of endothelin-1 and calphostin C of aortas from diabetic rats were significantly reduced compared to responses obtained in the absence of calphostin C (Fig. 1b, $P < 0.05$, ANOVA). However, maximum responses of aortas from control rats were not significantly different to those obtained in the absence of calphostin C (Fig. 1b).

4. Discussion

In the present study, we provide evidence that the attenuated reactivity observed to 5-HT in aortas from diabetic rats may be due to reduced activation of protein kinase C. This is based on the finding that the protein kinase C inhibitor, calphostin C, attenuated contractile responses to 5-HT in aortas from control rats but had no effect on responses of aortas from diabetic rats. Therefore, it is likely that the full expression of the contractile response to 5-HT requires activation of a number of components, and that protein kinase C participation is suppressed during diabetes. However, in aortas from diabetic rats, the additional presence of endothelin-1 results in protein kinase C activation and, subsequently, potentiation.

5-HT (Nakaki et al., 1985) and endothelin-1 (Highsmith et al., 1992) produce their contractile effects on rat aortas by similar mechanisms, i.e., the stimulation of phospholipase C, and a subsequent increase in inositol-1,4,5-triphosphate and diacylglycerol levels. In turn, diacylglycerol stimulates protein kinase C. Nakayama et al. (1991) have shown that the potentiating effect of endothelin-1 on 5-HT responses occurred without a significant increase in intracellular Ca^{2+}. These authors demonstrated that endothelin-induced potentiation was qualitatively similar to that induced by the phorbol ester 12-deoxyphorbol 13-isobutylate (a protein kinase C activator). They suggested that the combined stimulation of 5-HT and endothelin-1 receptors may enhance intracellular signalling systems more effectively and produce a full activation of the contractile machinery by activating protein kinase C. In the present study, calphostin C prevented the augmentation of contractile responses to 5-HT, by endothelin-1, of aortas from diabetic animals such that the difference between responses of aortas from control and diabetic rats was once again observed. This suggests that, during diabetes, the combination of endothelin-1 and 5-HT activates protein kinase C whereas 5-HT alone does not. However, prolonged activation of protein kinase C may lead to the activation of negative feedback pathways which result in the inactivation of cell-surface receptors, the inhibition of hydrolysis of phosphatidyl 4,5 bisphosphate and activation of Ca^{2+} pumps which reduce intracellular Ca^{2+} (Nishizuka, 1984). Activation of this negative feedback pathway, by the interaction of endothelin-1 and 5-HT, may explain the inhibition of responses to 5-HT in aortas from control rats. Indeed, this negative feedback pathway has been suggested to be the mechanism responsible for the endothelin-1-induced inhibition of 5-HT responses previously observed by other workers (Nakayama et al., 1991; Wong-Dusting et al., 1991). Activation of this pathway, and the subsequent inhibitory effect on protein kinase C, would also explain the lack of effect of calphostin C on responses to 5-HT obtained in the presence of endothelin-1 in aortas from control rats. However, this negative feedback pathway does not appear to have been activated in aortas from diabetic rats as, in these tissues, endothelin-1 potentiatied 5-HT responses. In turn, this potentiation, presumably due to the activation of protein kinase C, was inhibited by calphostin C. This result further implicates an abnormality in this second messenger pathway, in vascular smooth muscle, during diabetes.

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