Alteration of Contractile Properties to Serotonin in Gastric Fundus Smooth Muscle Isolated from Streptozotocin (STZ)-Induced Diabetic Rats

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Abstract

Contractile responses to serotonin (5-HT) of fundic smooth muscle strips isolated from both control and streptozotocin (STZ)-induced diabetic rats were investigated. Contrary to carbachol (CCh) which causes contractile hyperreactivity in DM, 5-HT response tended to decrease in DM compared to that of the control. Pindolol (10^{-5} M) increased the value of EC_{50} of the concentration-response to 5-HT about 2.5 times in both the control and DM. After treatment with pindolol, the maximal tension to 5-HT in DM significantly decreased compared to that of the control. Pindolol showed no effect on the contractile response to CCh. Pindolol significantly inhibited the relaxation caused by isoproterenol in DM more than in the control. Mianserin (10^{-4} M) increased the EC_{50} of the response to 5-HT about 2-2.5 times in both groups, but did not cause a significant difference between the control and DM. The Ca^{2+}-induced contraction caused hyperreactivity in DM in the presence of 10^{-6} M CCh, but that in DM was not significantly different from the control in the presence of 10^{-4} M 5-HT. Pretreatment of phorbol 12-myristate 13-acetate (PMA, 10^{-5} M) significantly attenuated the response to 5-HT in the control, but not in DM. Results suggest that the contractile response to 5-HT in DM is related to the altered Ca^{2+} signal transduction system via disturbed protein kinase C (PKC) activity, and that there are alterations of receptor characteristics and of the density in 5-HT receptor subtypes, especially 5-HT_{1A}, during DM development.

Key words: 5-HT, Fundic smooth muscle, Diabetes, Protein kinase C

Introduction

Pathophysiological changes of the smooth muscle in diabetes mellitus (DM) including streptozotocin-induced diabetic rats have been reported to be alterations of the contractile responses of the vascular smooth muscle (White and Carrier, 1990; Abebe and Macleod, 1990), gastrointestinal disorders (Sakai et al., 1991; Soulie et al., 1992), functional bladder changes (Luheshi and Zar, 1991; Malmgren et al., 1992), and sexual dysfunction (Longhurst, 1991; Hassan et al., 1993). These functional disorders are generally considered to be due to auto-
nomic neuropathy. However, DM is a metabolic disease not only because of the neuronal effect but also because of the metabolic changes in the smooth muscle itself. We have already reported that the hyperreactive contraction induced by KCl and acetylcholine (Aihara and Sakai, 1989), and the reversal of the response to norepinephrine (NE) gastric fundus (Sakai et al., 1991) suggests that contractile hyperreactivity is related to the alteration of signal transduction (Sakai et al., 1994). Concerning signal transduction, previous studies suggested that DAG levels were elevated in various tissues in diabetics (Okumura et al., 1991). Protein kinase C (PKC) activity during hyperglycemia contributed to the development of diabetic complications (Abebe and Macleod, 1990; Sakai et al., 1994), and an increase in Ca²⁺ influx (Sakai and Kwan, 1993). Serotonin (5-HT), as a widely-existing substance in many tissues, is a potent contractile agonist of rat gastric fundus (Vane, 1957). Several studies have been done to identify the receptor responsible for the contraction of the gastric fundus smooth muscle, its associated transduction system (Cohen, et al., 1987, and Growcott et al., 1993) and receptor cloning (Foguet et al., 1992, Kursar et al., 1992). This novel 5-HT receptor has been renamed 5-HT₂B receptor under a cloning classification (Humphrey et al., 1993). The 5-HT₂B receptor was shown to stimulate production of inositol 1, 4, 5-trisphosphate in transformed cells (Wainscott et al., 1993) and also seems to be coupled directly or indirectly to a Gαz-like protein (Wang et al., 1993).

Although recent progress has been made in characterizing the molecular identity of the rat gastric fundus 5-HT contractile receptor, the signal transduction mechanism coupled to this receptor remains unclear. However, interaction of cholinergic agonists, including carbachol, with muscarinic receptors in various tissues was coupled to phosphatidylinositol hydrolysis (Best et al., 1985, Marc et al., 1986).

To gain insight into the mechanism of action for 5-HT in gastric fundus smooth muscle isolated from control and diabetic rats, we characterized and compared contractile responses induced by 5HT and CCh.

**Materials and Methods**

*General procedures*

In 8-week-old male Wistar rats weighing 220-260 g, streptozotocin (STZ; 60 mg/kg, i.v.) in a citrate buffer was injected to induce diabetes. The control were injected with a vehicle. Six weeks after injection, animals were sacrificed by decapitation. Blood samples of 21 rats each were taken for glucose testing (108.3 ± 4.1 mg/dl in control, 445.1 ± 22.5 mg/dl in DM, respectively); the stomach fundus was dissected, opened along the lesser curvature, and after the content was washed away, put into a petri dish containing aerated modified Krebs’ solution. The mucosa was removed carefully with small scissors and the circular strips were made (2-3 mm wide, 10-15 mm long). The above processes were undertaken at room temperature.

The strips were set up at 37°C in a 10 ml organ bath containing Krebs’ solution aerated with 95% O₂ and 5% CO₂. Isometric tension was recorded after equilibration for 60 min under 1.0 g load. The Krebs’ solution contained the following compositions (mM): NaCl, 118; KCl, 5.8; CaCl₂, 2.5; MgCl₂, 1.2; NaH₂PO₄, 1.4; NaHCO₃, 21.4; glucose, 11.5.
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Before exposure to 5-HT and CCh, muscle strips were usually stimulated by 100 mM KCl to stabilize the sets, and by 60 mM KCl to check sensitivity and make a reference parameter for following agonists’ responses. During the experiment, the strips were washed every 15 min except during drug treatment. Cumulative contractile concentration-response curves to 5-HT and CCh were obtained by a stepwise increase in concentration. After the control responses, tissues were washed 4 times for 60 min equilibration and incubated with the antagonist for 10-15 min. Responses to agonists were then repeated in the presence of the antagonist. For each concentration of 5-HT, CCh remained in contact with the tissue for approximately 3 min before the addition of the next concentration. Contractile responses were expressed as an absolute number of contractile force per 100 mg wet tissue.

To eliminate the possible cholinergic, neuronal, prostglandin, adrenergic effects, atropine (10⁻⁵ M), tetrodotoxin (10⁻⁷ M), indomethacin (10⁻⁵ M) and guanethidine (10⁻⁵ M) were used throughout the experiment; however, atropine was not added before CCh treatment. Time-dependent changes of 60 min intervals were checked using a vehicle instead of an antagonist; no tachyphylaxis or desensitization was found (5-HT, n=30).

5-HT or CCh responses were checked in normal Krebs’ solution beforehand in a Ca²⁺ free condition. The preparations were washed with Ca²⁺ free Krebs’ solution 4 times, incubated with 0.1 mM EGTA for 10 min and then washed again. 5-HT (10⁻⁶ M) caused 20-30% contraction in normal Krebs’ solution, but CCh (10⁻⁶ M) caused 70-80% contraction. After washout, these responses almost disappeared. Subsequently, in the presence of 10⁻⁶ M 5-HT or CCh, Ca²⁺ was cumulatively added reaching a concentration level of 2.5 mM, the concentration of normal Krebs’ solution.

Statistical analysis

All values are expressed as means±S.E.M. The student t-test (paired and unpaired, two tails) was used to compare the results. A p value less than 0.05 was considered to be significant.

Results

Effects of pindolol on the 5-HT or CCh concentration responses and isoproterenol-induced relaxation

Pretreatment with 10⁻⁵ M pindolol for 15 minutes increased the value of EC₅₀ of the 5-HT response around 2.5 times in both groups, from (6.7±0.7)×10⁻⁸ M to (23.8±9.2)×10⁻⁸ M in the control, and from (10.3±2.0)×10⁻⁸ M to (26.6±5.5)×10⁻⁸ M in DM as shown in Fig. 1-left. The value of EC₅₀ was calculated as the concentration required to produce a response in 50% of the maximum tension at 10⁻⁶ M concentration in each preparation. After treatment with pindolol, the maximum tension to 5-HT decreased significantly in DM compared to the control. However, the contractile response to CCh increased significantly in DM; this effect was not influenced by pindolol as shown in Fig. 1-right. Pindolol, at a concentration of 10⁻⁶ M, did not inhibit the 5-HT response nor even increase this response slightly (data not shown), so we used pindolol at the concentration of 10⁻⁵ M.
Fig. 1. Effect of pindolol on the concentration-response curves produced by 5-HT or by CCh in gastric fundus smooth muscle isolated from the controls and DM rats. Values are mean ± SE. (n=6-8). Significantly different from control in the presence of 10⁻⁵ M pindolol (*p<0.05; **p<0.01).

Fig. 2. Effect of pindolol on the concentration-response curves of isoproterenol in gastric fundus smooth muscle contracted with 10⁻⁶ M CCh. Values are expressed as percentage of maximal relaxation and mean ± SE. (n=4-6). Significantly different from control in the absence of pindolol (*p<0.05). Significantly different from control in the presence of 10⁻⁵ M pindolol (**p<0.01).

Isoproterenol-induced relaxation of the CCh contraction was significantly less in DM than that in the control. Pretreatment with 10⁻⁶ M pindolol attenuated the isoproterenol-induced relaxation of contraction induced by CCh, which was more significant in DM than in the control as shown in Fig. 2.

Effect of mianserin on the 5-HT concentration-response

Figure 3 shows the effect of mianserin on the contractile responses to 5-HT. Pretreatment with 10⁻⁵ M mianserin, increased EC₅₀ of the 5-HT response about 2-2.5 times in both groups from (3.5±1.1)×10⁻⁸ M to (7.3±0.4)×10⁻⁸ M in the control and from (8.7±0.5)×10⁻⁸ M to (23.3±8.1)×10⁻⁸ M in DM, but no significant difference was observed between the control and DM. Methysergide, at a concentration of 10⁻⁵ M, blocked 5-HT response completely, and at 10⁻⁷ M, reduced the 5-HT response by 70% (data not shown).
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Fig. 3. Effect of mianserin on the concentration-response curves produced by 5-HT in gastric fundus smooth muscle isolated from the control and DM rats. Values are mean±SE. (n=4–6).

Ca²⁺ concentration dependency in the presence of 5HT or CCh

Figure 4 shows the Ca²⁺ concentration dependency of the contractile responses of fundus smooth muscle in the presence of 10⁻⁶ M 5-HT or 10⁻⁶ M CCh. As shown in Fig. 4, the Ca²⁺ concentration-dependent contractile responses tended to be lower than those of the control, but not significantly different in the presence of 10⁻⁶ M 5-HT. The contractile response was significantly greater in DM than that in the control in the presence of 10⁻⁶ M CCh. In some preparations in DM, a lower Ca²⁺ concentration caused a transient phasic contraction while its tone increased slowly in the presence of CCh.

Effect of phorbol ester on the contractile response to 5-HT

Although phorbol 12-myristate 13-acetate (PMA) itself induced a slightly increased tone in most of DM (16 out of 20) but about half of the control (11 out of 20), the concentration-response

Fig. 4. Concentration-response curves produced by Ca²⁺ in gastric fundus smooth muscle from the control and DM rats in the presence of 10⁻⁶ M 5-HT or 10⁻⁶ M CCh. Before the addition of 5-HT or CCh, preparations were treated with Ca²⁺-free Krebs' solution containing 0.1 mM EGTA for 15 min. Values are mean±SE. (n=6). Significantly different from control (*<0.05; **p<0.01).
to 5-HT was attenuated at a higher concentration in the control but it was not affected in DM, after pretreatment with $10^{-5}$ M PMA as shown in Fig. 5. After PMA treatment, contractile responses to 5-HT often increased a long-lasting tone even if the tissues were washed continuously. This contraction was almost completely inhibited by $10^{-6}$ M nifedipine.

**Discussion**

Diabetic development induced by streptozotocin is accompanied by various changes in the innervation of the enteric nervous system (Belai, *et al.*, 1987). Therefore, the present work was carried out under the presence of indomethacin, guanethidine, tetrodotoxin. In case of 5-HT, atropine was added to focus on the receptors in the smooth muscle itself.

Although 5-HT is a potent contractile agonist to rat gastric fundus, contrary to CCh, its contractile response in DM tended to be decreased. Consistent with the contractile response, the Ca$^{2+}$ induced response had a tendency to decrease in the presence of 5-HT, whereas the response increased significantly in the presence of CCh (Figs. 1 and 4). Smooth muscle contractile responses, induced by various agonists, are known to be associated with an increased intracellular Ca$^{2+}$ concentration mediated by Ca$^{2+}$ influx and release of Ca$^{2+}$ from intracellular store sites (Bolton, 1979; Van Breemen *et al.*, 1986). The Ca$^{2+}$ influx through both muscarinic-receptor operated and voltage-dependent Ca$^{2+}$ channels (VDC) may be responsible for the hyperreactivity of ACh and KCl in DM (Aihara and Sakai, 1989). The contribution of extracellular Ca$^{2+}$ to an overall 5-HT contractile response in the rat gastric fundus smooth muscle can be accounted for predominantly by Ca$^{2+}$ influx through the receptor operated Ca$^{2+}$ channels (ROC) and VDC. It is possible that the tendency of the decrease in contractile responses to 5-HT may be partially due to a low Ca$^{2+}$ influx through the ROC and/or VDC.

Although $\beta$-adrenergic antagonist pindolol is also a weak antagonist of 5-HT$_{1A}$/5-HT$_{1B}$ (Choppin and O’Connor, 1995), compared to its effect on the CCh contraction, the inhibitory
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effect on 5-HT can be clearly observed; moreover, the 5-HT response in DM appeared to be more effective to pindolol than to the control (Fig. 1). The contraction induced by 5-HT in DM was significantly less than that in the control after treatment with pindolol, which suggests: 1) a decreased number of 5-HT receptors in DM, 2) alteration of 5-HT subtype receptors in DM, and 3) an interaction between β-receptor and 5-HT receptors.

[125I]-Iodocyanopindolol binding to the gastric fundus membrane from DM was significantly less than that of the controls, which indicated that the number of β-receptor in DM decreased (Sakai, et al., 1991). 5-HT1A and β2-adrenergic receptors are both guanine nucleotide-binding protein-coupled receptors with the putative seven transmembrane topology (Guan et al., 1992). Choudhary et al. (1992) reported the terminal transmembrane domains of the structure of 5-HT receptor cDNAs for the following reasons: 1) adrenergic receptors directly implicate transmembranes VI-VII in antagonist pharmacology, 2) asparagine in transmembrane VII was essential for binding a variety of β-adrenergic antagonists, 3) asparagine in transmembrane VII is critical for the binding of pindolol to 5-HT1A receptors. These domains may be related to the fact that the relaxation induced by isoproterenol was significantly greater in DM than in the control after treatment with pindolol. However, pretreatment with mianserin did not cause a significant difference even if the sensitivity of the concentration-response to 5-HT tended to decrease in DM as shown in Fig. 3.

Pretreatment with PMA attenuated the response to 5-HT in the control, which suggests that PKC-mediated phosphorylation of the fundal G protein may interrupt the 5-HT receptor-mediated function (Wang, et al., 1993). Our previous report suggested that PMA, at a concentration of 10^-5 M, could induce contraction in most diabetic fundic strips but in few control strips, and that PKC activity in cytosol is significantly increased in DM compared to the control (Sakai, et al., 1994). The 5-HT2B receptor can couple to PI hydrolysis, a primary mechanism by which the G protein coupled receptor can stimulate smooth muscle contraction. Therefore, our results suggest that the response to 5-HT is related to alterations of characterization in 5-HT subtypes in DM.

Ryanodine completely blocked caffeine-evoked contractile responses and inhibited the contraction to CCh, establishing that ryanodine was acting on the Ca2+ release channel of the SR to inhibit agonist responses that depend upon intracellular Ca2+ release in the rat gastric fundus (Cox and Cohen, 1995). Ryanodine (4 μM) caused contraction in the rat gastric fundus smooth muscle, and the amplitude of this contraction was similar (4.4 g/100 mg n=6) in both the control and DM. The ryanodine sensitive Ca2+ store site may not be related to our results. A metabolite of NAD+, cyclic ADP-ribose, has been identified as a novel second messenger which can release Ca2+ from intracellular stores via a mechanism independent of IP3 and at a site with which ryanodine interacts (Galione, 1992). Thus, it is apparent that PI turnover is not the only signaling mechanism which mobilizes intracellular Ca stores, and the 5-HT2B receptor may be coupled to a novel mechanism to mobilize intracellular Ca2+.

Pretreatment with PMA failed to attenuate the response to 5-HT in DM. This confirms that PKC activity in DM has been disturbed, which may be related to PKC activity to stimulate the Ca2+ channel by positive feedback.

Therefore, the results suggest that the attenuated contractile response of 5-HT in diabetic
rat fundic strips is related to the altered Ca$^{2+}$ signaling system via disturbed PKC activity. The discriminated inhibitory effects of pindolol suggest that alterations of receptor characteristics and the density of 5-HT receptor subtypes, 5-HT$_{2B}$ and/or 5-HT$_{2C}$, especially the 5-HT$_{1A}$ receptor plays an important role during DM development.

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