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GUANOSINE 5'-O-(3-THIOTRIPHOSPHATE) CAUSES ENDOTHELIUM-DEPENDENT, PERTUSSIS TOXIN-SENSITIVE RELAXATIONS IN PORCINE CORONARY ARTERIES

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Summary. To determine whether direct stimulation of endothelial G-proteins causes relaxations of the underlying vascular smooth muscle, the effects of guanosine 5'-O-(3-thiotriphosphate) (GTPγS) and sodium fluoride were studied in porcine coronary arteries and endothelial cells. Isometric tension was measured in coronary rings contracted with prostaglandin $F_{2\alpha}$. GTPγS (in the presence of saponin) and sodium fluoride (in the presence of $AlCl_3$) relaxed rings with, but not those without endothelium. The responses were inhibited by nitro-L-arginine and pertussis toxin. In membrane fractions of coronary endothelial cells, GTPγS and sodium fluoride inhibited the ADP-ribosylation of G-proteins catalyzed with [^{32}P]-NAD and pertussis toxin. These data suggest that direct stimulation of G-proteins in endothelial cells by GTPγS and sodium fluoride causes a pertussis toxin-sensitive relaxation which may be attributed to the release of nitric oxide.

5-HT_1-serotonergic and α_2-adrenergic receptors, which mediate endothelium-dependent relaxation, are coupled to pertussis toxin-sensitive G-proteins in porcine and canine coronary arteries (1,2). Presumably this activation of the G-proteins is followed by the release of nitric oxide (1,3,4,5). This hypothesis is consistent with the observation that sodium fluoride, which can stimulate G-proteins directly (6), causes endothelium-dependent relaxations in coronary arteries (7,8). However, there is no direct evidence that sodium fluoride actually binds to G-proteins in coronary artery endothelial cells. If the activation of G-proteins in the endothelium causes coronary vasodilatation, guanosine 5'-O-(3-thiotriphosphate) (GTPγS), a hydrolysis-resistant analogue of GTP and a potent activator of G-proteins (9), should also cause endothelium-dependent relaxation in these arteries. The purpose of the present study was to examine whether or not GTPγS causes endothelium-dependent responses in the porcine coronary artery. In addition, the effects of GTPγS and sodium fluoride on pertussis toxin catalyzed ADP-ribosylation of G-proteins were studied in native porcine coronary endothelial cells, to determine whether these agents interact directly with endothelial G-proteins.

MATERIALS AND METHODS

Organ chamber studies. Left anterior descending coronary arteries were taken from porcine hearts obtained from a slaughterhouse. The arteries were rinsed in modified Krebs-Ringer bicarbonate solution [composition in mM; NaCl 118.3; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2;

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KH_2PO_4 1.2; $NaHCO_3$ 25; glucose 11.1; calcium-EDTA 0.026 (control solution)] and cleaned of connective tissue. The arteries were cut into rings (4 mm). In some rings, the endothelium was removed mechanically (1,2). The rings were suspended in organ chambers filled with control solution (aerated with 95 % O_2 and 5 % CO_2; pH 7.4, maintained at 37 °C). Isometric force was measured by a strain gauge transducer (Statham UC2, Los Angeles, CA). The rings were stretched to the optimal point of their active length-tension curve (6-8 g; based on preliminary experiments with 40 mM KCl). After one hour of equilibration, the rings were contracted with prostaglandin $F_{2\alpha}$ (2 x 10^{-6} M) and the response to bradykinin (10^{-8} M) was examined to confirm the absence or presence of endothelium. All experiments were performed in the presence of indomethacin (10^{-5} M) to prevent the formation of vasoactive prostanoids.

Relaxations to sodium fluoride. The rings were divided into four groups; preparations with endothelium incubated in (a): control solution, (b): in the presence of nitro-L-arginine (3 X 10^{-5} M, an inhibitor of nitric oxide synthase), (c): in the presence of pertussis toxin (100 ng/ml), and (d): rings without endothelium incubated in control solution. After 90 minutes of incubation, the rings were contracted with prostaglandin $F_{2\alpha}$ (2 x 10^{-6} M; a concentration causing 72 ± 5 % (n=6) of the maximal contraction with prostaglandin $F_{2\alpha}$) and the response to cumulative concentrations of sodium fluoride (10^{-3} - 10^{-2} M) was determined in the presence of $AlCl_3$ (10^{-5} M). Relaxations are expressed as percentage of the initial contraction to prostaglandin $F_{2\alpha}$.

Relaxations to GTPγS. The rings were divided into five groups. The first group consisted of rings with endothelium were incubated in control solution (a). In the other experiments (b, c, d, e), saponin (20 μg/ml) was added to the organ chambers to permit penetration of GTPγS through the cell membrane (10) twenty minutes before the addition of prostaglandin $F_{2\alpha}$;the four consisted of rings with endothelium incubated in either (b): control solution, (c): in the presence of nitro-L-arginine (3 X 10^{-5} M), or (d): in the presence of pertussis toxin (100 ng/ml), and (e): rings without endothelium incubated in control solution. After incubation for 90 minutes in total, all rings were contracted with prostaglandin $F_{2\alpha}$ (2 X 10^{-6} M), and responses to cumulative concentrations of GTPγS (10^{-6} - 3 X 10^{-4} M) were determined.

ADP-ribosylation of G-proteins with pertussis toxin. Porcine coronary arteries, opened longitudinally and pinned out flat, were rinsed with control solution. Endothelial cells were harvested by scraping the intimal surface of the arteries with a scalpel blade (10). The absence of contamination of the endothelial cells with smooth muscle cells was confirmed using a monoclonal antibody against α-smooth muscle actin and a Western blotting procedures. Freshly isolated porcine coronary artery smooth muscle cells were used as positive controls. The endothelial cells were collected in control solution and washed by centrifugation. After sonication for 30 seconds on ice (Artek, sonic dismembrator, model 300), homogenates were centrifuged at 13,600 x g for 10 minutes, and the pellet was resuspended in 10 mM Tris-HCl pH 7.6 containing 1 mM EDTA and 27% sucrose. Crude membrane fractions (10 μl containing 10 μg protein) were incubated for 10 minutes with either sodium fluoride (10^{-3} or 10^{-2} M; in the presence of 10^{-5} M $AlCl_3$) or GTPγS (10^{-5} or 10^{-4} M) prior to the ADP-ribosylation. The ADP-ribosylation reaction mixture contained 10 μl of 45 mM Tris-HCl pH 8.0, 3 mM EDTA, 30 mM thymidine, 3 mM ATP, 3 mM GDPβS, 6 mM dithiothreitol, 0.5 % Lubrol PX, and 0.05 % bovine serum albumin, 5 μl of pertussis toxin (90 μg/ml) activated by 50 mM dithiothreitol, 5 μl of 10^{-6} M [^{32}P]-NAD (5 X 10^6 cpm), and 10 μl of crude membrane fractions (11). The mixture (30 μl) was incubated for 30 minutes at 32 °C and stopped by addition of 80 μl of 2 X Laemmli's sample buffer containing 9 % β-mercaptoethanol and 4 mM unlabeled NAD. The ADP-ribosylated proteins were separated on a SDS-PAGE slab gel (10 % acrylamide/bis-acrylamide). Gels were stained with Coomassie blue prior to autoradiograph using Kodac X-Omat film.

Materials. Adenosine 5'-triphosphate sodium salt (ATP), bovine serum albumin, bradykinin, DL-dithiothreitol, guanosine 5'-triphosphate sodium salt (GTP), guanosine 5'-O-(2-thiodiphosphate) trilithium salt (GDPβS), guanosine 5'-O-(3-thiotriphosphate) tetralithium salt (GTPγS), β-nicotinamide adenine dinucleotide (NAD), indomethacin, pertussis toxin, monoclonal antibody against α-smooth muscle actin, saponin, thymidine were obtained from Sigma Chemical Co. (St. Louis, MO); aluminium chloride, nitro-L-arginine, sodium fluoride from Aldrich Chemical Co. (Milwaukee, WIS): Reagents for polyacrylamide gel electrophoresis were from BioRad (Richmond, CA); and prostaglandin $F_{2\alpha}$ from Upjohn (Kalamazoo, MI). ^{32}P-NAD was synthesized and provided by The Diabetes Center of Baylor College of Medicine (12).

Statistical analysis. Results in organ chamber studies are shown as means ± S.E.M., and n refers to the number of animals from which coronary rings were obtained. Statistical comparisons were performed by means of paired Student's *t*-test and an analysis of variance

(ANOVA) followed by Scheffe's test when more than two groups were compared. P values of less than 0.05 were considered to indicate statistically significant differences between groups.

RESULTS

Relaxations to sodium fluoride. In the four groups studied, the contractions to 2 x 10^{-6} M prostaglandin $F_{2\alpha}$ were not significantly different (control, 12.2 ± 1.7; in the presence of nitro-L-arginine, 14.1 ± 2.8; in the presence of pertussis toxin, 15.0 ± 2.5; without endothelium; 11.9 ± 2.9 g, n=5). Sodium fluoride caused concentration-dependent relaxations in control rings with endothelium. The relaxations were inhibited with nitro-L-arginine (3 X 10^{-5} M) and pertussis toxin (100 ng/ml) (Fig 1). In rings without endothelium sodium fluoride induced no relaxations but concentration-dependent contractions (Fig. 1).

Relaxations to GTPγS. Saponin did not significantly alter the resting tension of the rings (data not shown). There was no significant difference in contraction to prostaglandin $F_{2\alpha}$ in the rings of the five groups studied (in the absence of saponin, 11.8 ± 1.6; control treatment in the presence of saponin, 14.6 ± 2.5; in the presence of nitro-L-arginine and saponin, 17.0 ± 2.5; in the presence of pertussis toxin and saponin, 12.0 ± 1.0; after endothelium-removal and in the presence of saponin, 17.4 ± 2.0, n=7). GTPγS caused concentration-dependent relaxations in rings with endothelium treated with saponin (20μg/ml), but did not cause significant changes in tension in the absence of saponin (Fig. 2). The incubation of rings with nitro-L-arginine (3 x 10^{-5} M) and pertussis toxin (100 ng/ml) inhibited the relaxations to GTPγS in the presence of saponin. In rings without endothelium GTPγS did not cause relaxation in the presence of saponin (Fig. 2).

ADP-ribosylation of G-proteins. Analysis of incubations of the crude membrane fractions of endothelial cells with pertussis toxin and [^{32}p]-NAD on SDS-PAGE revealed a band at 40-41

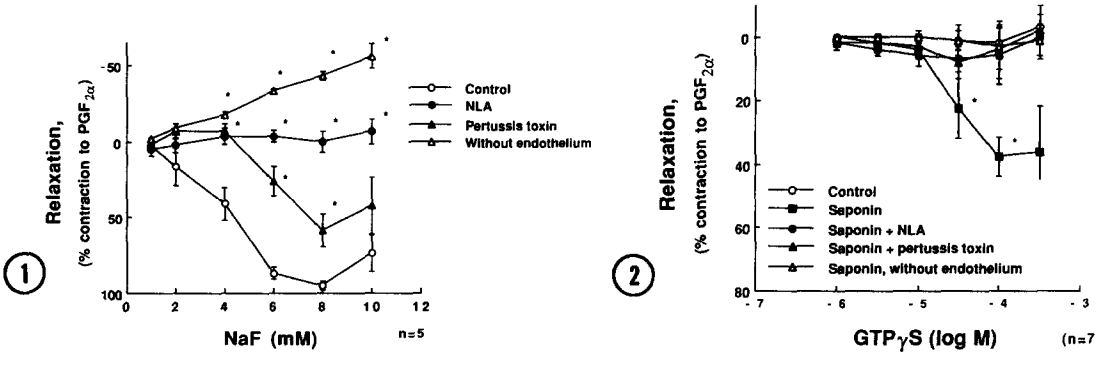

Fig.1. Relaxations to sodium fluoride (NaF) in rings of porcine coronary arteries with endothelium during contractions to prostaglandin $F_{2\alpha}$ (2 x 10^{-6}M). Nitro-L-arginine (NLA: 3 x 10^{-5} M) and pertussis toxin (100 ng/ml) inhibited the relaxations to sodium fluoride. Sodium fluoride contracted the rings without endothelium. Data are shown as means ± S.E.M. (n=5) and expressed as percent relaxation of the contractions to prostaglandin $F_{2\alpha}$. Asterisks denote significant differences from control ($P < 0.05$).

Fig. 2. Relaxations to GTPγS in the rings of porcine coronary arteries with endothelium during contractions to prostaglandin $F_{2\alpha}$(2 x 10^{-6} M). GTPγS did not change the tension in control solution, but caused relaxations in the presence of saponin (20 μg/ml). Nitro-L-arginine (NLA: 3 x 10^{-5} M) and pertussis toxin (100 ng/ml) inhibited the relaxations to GTPγS. In the rings without endothelium GTPγS did not induce relaxation in the presence of saponin. Data are shown as means ± S.E.M.(n=7) and expressed as percent relaxation of the contractions to prostaglandin $F_{2\alpha}$. Asterisks denote significant differences from "control" ($P < 0.05$).

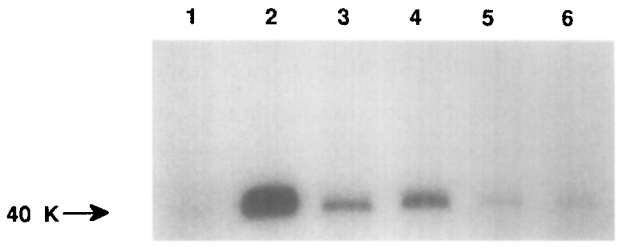

Fig.3. Inhibition by sodium fluoride and GTPγS of pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of G-proteins. Crude membrane fractions obtained from native porcine coronary endothelium were incubated with the reaction mixture in the absence (lane 1) or presence (lane 2) of pertussis toxin. Each sample was electrophoresed on a 10% SDS-polyacrylamide gel followed by autoradiograph. Treatment of membrane fractions with sodium fluoride (10^{-2} M; lane 3, 10^{-3} M; lane 4) or GTPγS (10^{-4} M; lane 5, 10^{-5} M; lane 6) diminished the intensity of the 40-41 kDa band. The apparent molecular weight is indicated.

kDa (Fig. 3). In the absence of pertussis toxin no band was detected. Treatment of the fractions with sodium fluoride (10^{-3} and 10^{-2} M) and GTPγS (10^{-5} and 10^{-4} M) inhibited the pertussis toxin-catalyzed ADP-ribosylation of G proteins (Fig. 3).

DISCUSSION

The results of the present study are consistent with earlier observations that sodium fluoride causes endothelium-dependent, pertussis toxin-sensitive relaxations in porcine coronary arteries (7,8). Relaxations induced by sodium fluoride, in the presence of $AlCl_3$, may be due to the production of prostacyclin by the endothelium, mediated by pertussis toxin-insensitive G-proteins (13). However, this is an unlikely explanation for the endothelium-dependent relaxations observed in the present study since all experiments were performed in the presence of indomethacin. Furthermore, the effect of nitro-L-arginine, an inhibitor of nitric oxide synthase (14), on the fluoride-induced relaxation suggests an involvement of nitric oxide in the process. Membrane-associated G-proteins obviously were not accessible to GTPγS, unless the preparations were permeabilized using saponin (15). There was no impairment of signal transduction at the coccentration of saponin used (16, 17). Although treatment with saponin may affect vascular responsiveness by altering Ca^{2+} exchanges (18), the contractions of the coronary rings to prostaglandin $F_{2\alpha}$ were not different in the absence and presence of the detergent. The interpretation of the present results requires caution concerning the specificity of the action of GTPγS, because the relaxation induced by GTPγS may be mediated by its action on purinoceptors on endothelial and smooth muscle cells (19, 20, 21, 22). However, in the present experiments, GTPγS induced relaxations in the presence but not in the absence of saponin,which rules out any involvement of purinoceptors. Furthermore, GTP (10^{-6} - 10^{-3} M) did not cause relaxation of the porcine coronary rings with endothelium (data not shown). Alternatively, GTPγS may have caused relaxation by direct opening of G-proteins coupled K^+ channels (23). However, this possibility is ruled out because no relaxation was observed in the presence of nitro-L-arginine, or in rings without endothelium. Hence, the present findings that GTPγS causes endothelium-dependent, pertussis toxin and nitro-L-arginine-sensitive relaxations, suggest that the relaxation evoked by GTPγS is related to the stimulation of G-proteins and the production of nitric oxide in

endothelial cells. These relaxations evoked by GTPγS in the porcine coronary arteries were similar to those induced by sodium fluoride. GTPγS can enhance contractions of vascular smooth muscle by increasing the Ca^{2+} sensitivity of the contractile proteins, or modulating the gating of calcium channels, which may impact on the stimulation of G-proteins (18, 24). However, in the present study, GTPγS did not augment contractions in rings contracted with a submaximal concentration of prostaglandin $F_{2\alpha}$.

The molecular weight of proteins (40-41 kDa) detected in SDS-PAGE/autoradiograph are consistent with that of the α subunit of pertussis toxin-sensitive G_i and/or G_o proteins (25). Incubation with GTPγS and sodium fluoride inhibited the [^{32}P] ADP-ribosylation of the α subunit of G-proteins catalyzed by pertussis toxin in the crude membrane fractions of native endothelial cells, as GTPγS does in purified native Gi-type proteins (26). These results strongly suggest that GTPγS and sodium fluoride act directly on G-proteins of the endothelial cells of the porcine coronary artery. Though GTPγS was more potent inhibitor of the ADP-ribosylation of G-proteins than sodium fluoride in the crude membrane fractions, the relaxation induced by GTPγS was smaller than that to sodium fluoride in the intact preparations. This apparent, qualitative, discrepancy between the two experimental conditions may be due to the limited permeability of cell membranes of the ring preparations to GTPγS, even in the presence of saponin.

In conclusion, GTPγS and sodium fluoride cause endothelium-dependent relaxations in porcine coronary arteries. The relaxation probably is mediated by the direct activation of G-proteins, which can be ADP-ribosylated by pertussis toxin in the endothelial cells.

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