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Role of Heterocellular Gap Junctional Communication in Endothelium-Dependent Smooth Muscle Hyperpolarization: Inhibition by a Connexin-Mimetic Peptide

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A synthetic connexin-mimetic peptide (Gap 27 peptide) was used to evaluate the contribution of gap junctional communication to smooth muscle responses mediated by the endothelium-dependent agonist acetylcholine (ACh) in rabbit mesenteric arteries. Hyperpolarizations and relaxations to 0.1 and 1 μM ACh observed in the presence of nitric oxide synthase and cyclooxygenase inhibition were markedly attenuated by the peptide at a concentration of 300 μM, whereas the hyperpolarizing response to levcromakalim, a K_{ATP} channel opener, was unaffected. The peptide also attenuated intercellular transfer of Lucifer yellow in confluent cultures of COS-7 cells, thus confirming its ability to modulate the permeability of gap junctions. The findings demonstrate that heterocellular gap junctional communication contributes to NO- and prostanoid-independent mechanisms of vasorelaxation that are widely attributed to an endothelium-derived hyperpolarizing factor. © 1999 Academic Press

Key Words: **gap junctions; endothelium-dependent hyperpolarization; acetylcholine; levcromakalim; Lucifer yellow.**

NO- and prostanoid-independent vascular relaxations are generally thought to be mediated by an endothelium-derived hyperpolarizing factor (EDHF) that diffuses from the endothelium, via the extracellular space, to cause hyperpolarization and vasodilatation by opening smooth muscle K^+channels (1–5). The existence of myoendothelial gap junctions in the vascular wall also suggests the possibility of direct electrical and chemical transfer of signals between the endothelium and adjacent smooth muscle cells. Indeed, recent studies indicate that heterocellular gap junctional communication plays a central role in NO- and prostanoid-independent relaxations induced by acetylcholine (ACh), adenosine triphosphate (ATP) and cyclopiazonic acid (CPA) in rabbit aorta, superior mesenteric and iliac arteries (6, 7).

In the present study, we have used intracellular recordings of smooth muscle cell membrane potential to study the effects of a short synthetic connexin-mimetic undecapeptide on the hyperpolarizing responses evoked by ACh in rabbit mesenteric arteries. This peptide possesses sequence homology with a region of the second extracellular loop (Gap 27) of connexin subtypes found in the vascular wall, and is highly effective in interrupting cooperative cell-cell interactions such as the synchronous beating of embryonic cardiomyocytes (8), rhythmic smooth muscle contractile activity (9) and endothelium-dependent relaxations (6). It is likely that the peptide prevents accretion of free connexon hemichannels present in the cell membrane into gap junctions and/or perturbs the connexin-connexin interactions that maintain channel integrity in already-formed gap junctions (9). To assess the effect of Gap 27 peptide on direct cell-cell coupling we studied dye transfer of Lucifer yellow CH (LY), a small fluorescent tracer with a charge of −2 and molecular weight of 457 Da (10–12) in cultured COS-7 cells, a monkey fibroblast cell line that endogenously expresses low levels of Cx43 (13, 14). To confirm the specificity of Gap 27 peptide against electrical events mediated via the endothelium, levcromakalim, which hyperpolarizes vascular

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smooth muscle directly through the activation of K_{ATP} channels, was used as a control.

MATERIALS AND METHODS

Dye transfer. COS-7 cells (ECACC, Wiltshire, UK) were seeded at a density of 1×10^6 cells per 60-mm^2 tissue culture dish in complete Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 $\mu g \cdot ml^{-1}$ penicillin/streptomycin/glutamine and 250 μgzmdml^{-1} amphotericin B (GibcoBRL). Cells were incubated at 37°C in 5% CO_2 until confluent monolayers formed, usually three days post seeding. The monolayers were washed twice with phosphate buffered saline (120 mM NaCl; 2.7 mM KCl; 10 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.4) and incubated in Leibowitz L-15 medium (supplemented as above) on a microscope stage at 37°C (Zeiss). Individual cells were injected with Lucifer yellow CH (LY) (Sigma) [5% (w/v) in 0.3 M LiCl]. Their ability to transfer tracer following incubation with Gap 27 peptide at concentrations of 300 and 500 μM for 60 min was quantified by a standardized cluster technique in which cells were returned to the incubator for 15 min before fixation in 4% paraformaldehyde (15). The number of injected cells transferring LY to >10 neighboring cells was determined by fluorescence microscopy using filter set 05 (395–440 nm/460–470 nm; Zeiss) on an Axiostat microscope (Zeiss).

Electrophysiology and mechanical responses. Male New Zealand White rabbits (2.0–2.5 kg) were anaesthetized with sodium pentobarbitone (60 mg $\cdot$ kg^{-1}, iv) and killed by rapid exsanguination. Segments (2 mm in length) of third-order branches of the superior mesenteric artery (D_{100} 373 ± 21 mm; n = 9) were removed and mounted under normalized tension in a Mulvany-Halpern myograph for recording of smooth muscle membrane potential as previously described (16). The bathing solution consisted of Holmans buffer (composition in mM: 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, 10 sucrose) containing 2.8 μM indomethacin (Sigma) and 100 μM N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), and was continuously gassed with 95% O_2:5% CO_2 at 37°C. Acetylcholine (ACh, Sigma) and levcromakalim (LK, SmithKline Beecham) were pipetted into the 10 ml bath under static conditions, and washout was achieved by reintroducing superfusion flow. Arteries were incubated in the presence of Gap 27 peptide for 10 min before reassessing the hyperpolarization responses to ACh and LK.

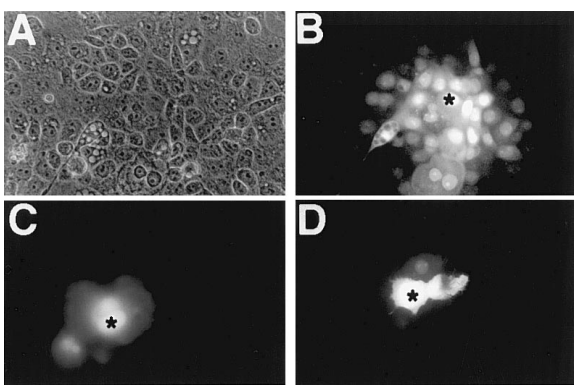

FIG. 1. Representative photographs illustrating Lucifer yellow dye transfer in COS-7 cells. (A) Typical phase view showing confluence. Note heterogeneity in the size of the cells. (B) Lucifer yellow dye transfer in control cells showing efficient gap junctional communication. (C, D) Dye transfer was reduced following preincubation with 300 and 500 μM Gap 27 peptide, respectively. Magnification ×40; * denotes the injected cell.

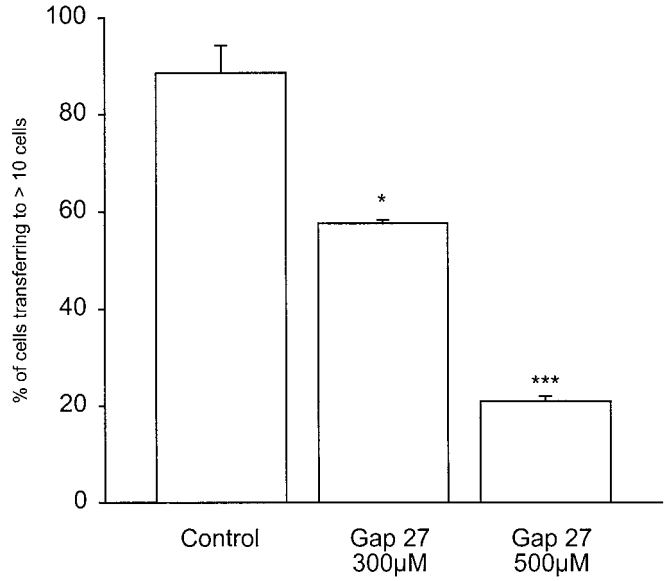

FIG. 2. Histogram showing the effects of Gap 27 peptide on Lucifer yellow dye transfer in COS-7 cells. Preincubation with the peptide caused concentration-dependent reductions in the number of cells expressing dye transfer to >10 neighboring cells following microinjection. *$P < 0.05$, ***$P < 0.005$ compared to control.

Rings of superior mesenteric artery (2–3 mm wide) were also dissected and suspended in oxygenated 3 ml tissue baths containing Holmans buffer at 37°C as previously described (6, 9). The preparations were contracted with 1 μM phenylephrine (PE) and cumulative concentration-relaxation curves to acetylcholine (ACh) constructed in the presence of 300 μM L-NAME plus 10 μM indomethacin under control conditions and after incubation for 30 min with 300 μM Gap 27 peptide. Gap 27 peptide (amino acid sequence SRPTEKTIFII) was synthesized by Severn Biotech, Kidderminster, UK; purity was >95%.

Statistical analysis. Physiological responses under the different experimental conditions were compared using the paired or unpaired Student's t test as appropriate. LY dye transfer was assessed by ANOVA. $P < 0.05$ was considered significant.

RESULTS

Effects of Gap 27 peptide on dye transfer. Individual cells within confluent COS-7 monolayers injected with LY dye demonstrated rapid spread of the tracer to neighboring cells, with 88.7 ± 5.7% of injected cells transferring the dye to >10 neighboring cells (n = 71 injected cells; Figs. 1 and 2). Following incubation of cells with Gap 27 peptide at 300 μM only 57.7 ± 0.7% of injected cells exhibited transfer of LY to >10 neighboring cells (n = 23 injected cells, $P < 0.05$). A further reduction to 21.0 ± 1.0% transfer between injected cells after incubation with 500 μM Gap 27 peptide cells was observed (n = 19 injected cells, $P < 0.005$).

Electrophysiology and mechanical responses. The resting membrane potential of mesenteric arterial smooth muscle cells was −59.5 ± 2.4 mV (n = 9 cells from

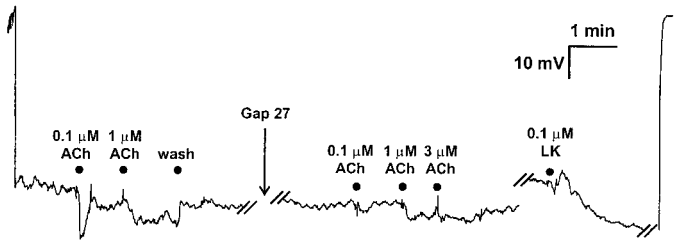

FIG. 3. Representative traces obtained before and after addition of Gap 27 peptide (Gap 27, 300 μM) illustrating attenuation of the hyperpolarization evoked by acetylcholine (ACh). Levcromakalim (LK) was added after reversal of the ACh hyperpolarization to demonstrate the ability of the smooth muscle cells to hyperpolarize in the presence of Gap 27 peptide. Membrane potential was recorded using an intracellular microelectrode.

nine tissues) in the absence, and -60.6 ± 1.5 mV ($n = 5$) in the presence of Gap 27 peptide (no significant difference). After addition of ACh to the bath, there was often a large, transient hyperpolarization which was attributable to incomplete initial mixing although after approximately 30 s the membrane potential stabilized (Fig. 3). The steady-state values after addition of 0.1 and 1 μM ACh were attenuated 86.0 $\pm$ 14.7% and 68.0 $\pm$ 15.5% ($n = 5$ in each case) by 300 μM Gap 27 peptide, respectively, whereas the hyperpolarization evoked by 0.1 μM levcromakalim was unaffected (Figs. 3 and 4a). In parallel studies, steady state relaxations to 0.1 μM and 1 μM ACh were attenuated 61.4 $\pm$ 4.2% and 39.1 $\pm$ 4.8% by 300 μM Gap 27 peptide ($n = 5$; Fig. 4b).

DISCUSSION

The present study provides the first direct electrophysiological evidence that smooth muscle hyperpolarization mediated by the endothelium-dependent vasodilator ACh involves heterocellular gap junctional communication. Under resting conditions, hyperpolarization was blocked by more than 50% in the presence of 300 μM Gap 27 peptide, and in preparations preconstricted with PE, the repolarization-associated relaxation to ACh was also blocked to an equivalent extent by the connexin-mimetic peptide. By contrast, hyperpolarizing responses to levcromakalim were unaffected. In parallel studies dye transfer in confluent COS-7 cells was attenuated by ~35% following incubation with Gap 27 peptide at a concentration of 300 μM, and by ~75% at a concentration of 500 μM. Taken together, these findings indicate that the mechanism underlying EDHF-type responses in rabbit mesenteric arteries involves gap junctional intercellular communication.

Agonists which are believed to stimulate the release of EDHF also have a direct hyperpolarizing effect on endothelial cells, which is secondary to an increase in endothelial cell intracellular [Ca^{2+}] (17, 18). In rat hepatic artery, there is evidence that K^+ ions released from the endothelium can diffuse extracellularly to hyperpolarize adjacent smooth muscle cells, but this mechanism does not fully explain the smooth muscle hyperpolarization response to ACh in mesenteric arteries (19). It is therefore possible that the signal that passes from the endothelium through gap junctions to adjacent smooth muscle cells to mediate NO-independent relaxation involves the transfer of electrical current as well as the diffusion of a low molecular weight EDHF. In many preparations, including rat mesenteric arteries, the repolarization and vasorelaxant response to acetylcholine is abolished by the combined application of the K^+ channel blockers charybdotoxin and apamin (4, 20). The primary hyperpolarization appears to occur in endothelial cells as apamin-sensitive K^+channels are absent in rabbit mesenteric artery smooth muscle cells (21) and in intact rat hepatic artery, endothelial cell hyperpolarization is blocked by charybdotoxin plus apamin (19).

Dye transfer experiments have previously demonstrated unidirectional coupling from the endothelium

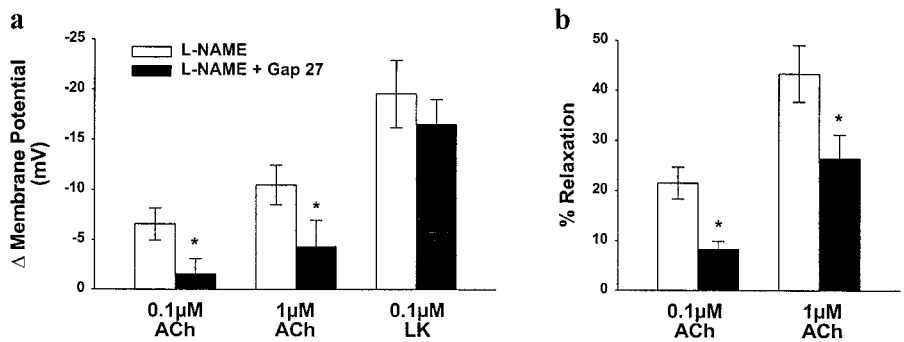

FIG. 4. (a) Summary of the effects of Gap 27 peptide on hyperpolarizations mediated by acetylcholine (ACh) and levcromakalim (LK). Values are means of 5 (ACh) and 3 (LK) experiments with SEM shown by vertical lines. *$P < 0.05$ compared to control values. (b) Effect of 300 μM Gap 27 peptide on relaxations mediated by 0.1 μM ACh and 1 μM ACh. All values are means of five experiments with SEM shown by vertical lines. *$P < 0.05$ compared to control values.

to smooth muscle (22). Conversely, electrophysiological experiments have provided clear evidence for electrical coupling from smooth muscle to endothelial cells (23–25), but this has been less consistently demonstrated in the reverse direction (26). Similarly, there is evidence for heterocellular transfer of a Ca^{2+} signal from smooth muscle to endothelium (27), but signaling in the reverse direction could not account for endothelium-dependent hyperpolarization unless highly localized membrane-associated rises in smooth muscle [Ca^{2+}] were able to activate Ca^{2+}-sensitive K^+channels. These diverse observations suggest that the inhibition of Lucifer yellow dye transfer by Gap 27 peptide demonstrated in the present experiments cannot be equated absolutely with changes in electrical communication. Indeed, electrostatic field effects associated with charged amino acids present within or near the central pore of connexins can differentially regulate electrical conductance and ion or dye selectivity in a connexin-specific fashion (28). Certain agonists may also cause electrical conductance and dye permeability to vary in an inverse fashion (29).

In the context of heterocellular endothelial-smooth muscle communication, the precise nature of the gap junctions participating in functional responses is unknown, but could involve connexins 37, 40, and 43 and both homotypic and heterotypic channels. In the case of heterotypic Cx 37/Cx 43 channels, conductance is increased when the Cx 37 side is more positive (i.e.) when there is cation flow from Cx 37 to Cx 43, thus suggesting that the charges lining the entrance of the mouth of the Cx 37 pore are negative and that the cation concentration is locally increased in this region (30). This could confer rectifying properties and polarity in the direction of information transfer. Gap 27 peptide would nevertheless be expected to affect the conductance and permeability of all such junctions as it contains a highly-conserved amino acid sequence corresponding to that found in the exofacial loops of connexins 37, 40, and 43 (SRPTEK).

In summary, electrophysiological studies in rabbit mesenteric arteries have demonstrated that Gap 27 peptide markedly reduces the ability of the endothelium to cause smooth muscle hyperpolarization, at a concentration that also inhibits dye transfer via gap junctions. We have previously shown that the peptide does not affect intrinsic smooth muscle tone or relaxation to exogeneous nitrovasodilators, and that its action is readily reversible, thus excluding non-specific effects on smooth muscle and endothelial cells (6, 9). In the present study its effects on electrical responses were shown to be specific to endothelium-dependent mechanisms of hyperpolarization. Such connexin-mimetic peptides may therefore gain widespread use in the study of gap junction function in the vascular system.

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