Endothelium-derived Relaxing Factor Inhibits Transport and Increases cGMP Content in Cultured Mouse Cortical Collecting Duct Cells

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Abstract

Stimulation of the release of endothelium-derived relaxing factor (EDRF) in the kidney has been shown to result in natriuresis without affecting glomerular filtration rate. This may be due to EDRF directly regulating solute transport in the cortical collecting duct (CCD). To test this hypothesis, we measured the effect of bradykinin (Bk) or acetylcholine (Ach) on short-circuit current (I_{sc}; a measure of active transport) in a CCD cell line (M-1), in the presence or absence of cow pulmonary artery endothelial (CPAE) cells. 10^{-9} M Bk or 10^{-7} M Ach had no effect on M-1 I_{sc} in which CPAE cells were absent. The addition of CPAE cells to M-1 cells also did not affect M-1 I_{sc}. On the other hand, when 10^{-9} M Bk or 10^{-7} M Ach were added to M-1 cells in the presence of CPAE cells, I_{sc} decreased from 43 ± 4.5 to 26 ± 4 and 64 ± 9 to 33 ± 4 $\mu A/cm^2$, respectively ($P < 0.001$). Nitroarginine (N-Arg, 10^{-4} M), a competitive inhibitor of EDRF production, blocked the inhibition in M-1 I_{sc} due to both agonists. Since cGMP is the second messenger of EDRF in vascular smooth muscle, we measured the effects of Bk on cGMP production in M-1 cells in the presence and absence of CPAE cells. Bk increased cGMP content in M-1 cells in the presence of CPAE cells from 33 ± 3.4 to 132 ± 11.7 fmol/10^6 M-1 cells ($P < 0.001$). When cultures of M-1 and CPAE cells were treated with N-Arg and challenged with Bk, Bk's effect on cGMP was partially blocked (61.4 ± 12 fmol/10^{-6} M-1 cells; NS). These data suggest that EDRF inhibits transport and increases cGMP content in M-1 cells. (*J. Clin. Invest.* 1992. 89:761–765.) Key words: endothelial cells • short-circuit current • potential difference

Introduction

Endothelium-dependent relaxation of blood vessels results from the release of a diffusible substance with a plasma half-life of < 30 s, termed endothelium-derived relaxing factor (EDRF).[1] Experimental evidence suggests that EDRF may be

1. *Abbreviations used in this paper:* Ach, acetylcholine; BK, bradykinin; CCD, cortical collecting duct; CPAE, cow pulmonary artery endothelial; EDRF, edothelium-derived relaxing factor; I_{sc}, short-circuit current; M-1, a CCD cell line; N-Arg, nitroarginine; PD, transepithelial potential difference.

either NO (1) or a nitrosothiol such as s-nitrosocysteine (2, 3). The synthesis of EDRF is thought to be from the amino acid L-arginine (4); and its release can be stimulated by a variety of agents, including bradykinin (Bk) and acetylcholine (Ach) (1, 5). L-N^G-nitroarginine (N-Arg), a guanidino-substituted L-arginine analogue, is a competitive inhibitor of EDRF synthesis (6). The mechanism of action of EDRF in vasorelaxation involves activation of soluble guanylate cyclase, which causes intracellular cGMP to increase (7, 8). Inhibition of soluble guanylate cyclase by methylene blue blocks EDRF-induced relaxation (9).

Intrarenal infusion of Bk or Ach, resulting in release of EDRF, has been shown to cause diuresis and natriuresis without affecting glomerular filtration rate (10, 11). This may be due to EDRF mediation of hemodynamic changes, a direct inhibition of tubular transport, or both. Although no direct action of EDRF inhibiting transport in individual nephron segments has been reported to date, it is an intriguing possibility since cGMP, which has been shown to act as EDRF's second messenger (7, 8), also inhibits transport in the collecting duct. Atrial natriuretic factor, which acts by increasing intracellular cGMP content (12), inhibits both sodium and water transport in the cortical and inner medullary collecting duct (13–15). Additionally, Zeidel and colleagues (14) have shown that sodium nitroprusside, an activator of soluble guanylate cyclase, inhibits sodium entry into inner medullary collecting duct cells. Therefore, EDRF may be capable of regulating transport in these segments by a mechanism involving cGMP.

To test these possibilities, we measured the effect of Bk or Ach on short-circuit current (I_{sc}), a measure of active transport, in a cortical collecting duct (CCD) cell line M-1, in the presence or absence of cow pulmonary artery endothelial (CPAE; CCL 209; American Type Culture Collection, Rockville, MD) cells. To determine whether an effect on I_{sc} was due to EDRF release by CPAE cells, we also measured the effect of Bk and Ach on M-1 I_{sc} in the presence of CPAE cells treated with N-Arg or methylene blue. Since cGMP can inhibit transport in the CCD, we measured the effects of Bk on cGMP production in M-1 cells in the presence and absence of CPAE cells, with and without N-Arg.

These experiments were performed with cultured cells because transport is easily measured, there are abundant cells for cGMP measurements, and hemodynamic effects of EDRF can be avoided. These studies suggest that EDRF decreases transport and elevates cGMP levels in cultured CCD cells.

Methods

Cell line. The M-1 cell line was originally developed from a mouse transgenic for the early region of simian virus 40, Tg(SV40E)Bri/7, and has been reported to retain many of the transport functions and hormone responsiveness of the CCD (16). M-1 cells were grown on perme-

able collagen membranes (Cellegen units; ICN Biochemicals, Cleveland, OH) and maintained on PC-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 5% fetal bovine serum and antibiotics. Monolayers from passages 7–13 were randomly selected and used in the following studies. M-1 cells demonstrate no significant difference in transport characteristics during these passages (16).

CPAE cell line. CPAE cells were grown on cross-linked collagen microcarriers (Ventrex Laboratories) and maintained on Dulbecco's modified essential medium supplemented with 20% fetal bovine serum. Confluent microcarriers were added to M-1 monolayers in a cell ratio of ~1:1 for transport studies and cGMP measurements.

Bioassay of EDRF production by CPAE cells. Left circumflex coronary arteries were removed from anesthetized mongrel dogs. Excised tissues were placed in cold Krebs-Ringer salt solution (mM: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 Ca-EDTA, and 11.1 glucose), cleaned of connective tissue, and cut into rings (5-mm length). The endothelium was removed by gently rolling the luminal surface with small forceps. The ring was mounted between two stirrups, one anchored to a steel plate, the other connected to a strain gauge transducer (model FT03C; Grass Instrument Co., Quincy, MA) for the recording of isometric force. The bioassay ring could move freely below two vertically mounted polypropylene chromatographic columns (i.d. 7 mm) maintained at 37°C by a water jacket. One column was filled with CPAE cells on microcarrier beads (endothelial superfusion) and the other with microcarrier beads without CPAE cells (direct superfusion). Each column was perfused at a constant flow (3 ml/min) by means of a roller pump, with the salt solution oxygenated with a 95% O_2 and 5% CO_2 gas mixture (pH 7.4, 37°C). Any difference in response between direct and CPAE superfusion indicates the presence of factor(s) released from the CPAE cells in the perfusate. Compounds injected above the column of microcarrier beads without CPAE cells interact only with the bioassay ring, whereas compounds injected above the column of microcarrier beads with cells interact with both the CPAE cells and the bioassay ring.

The bioassay ring was stretched under direct superfusion in a stepwise manner until the response to 20 mM KCl was optimal (average 8 g). The removal of the endothelium was demonstrated by the absence of relaxation to Bk (10^{-8} M) under direct superfusion, after precontraction with $PGF_{2\alpha}$ (10^{-5} M). All experiments were done in the presence of indomethacin (10^{-5} M) to block PG synthesis (see reference 17 for review of the method).

Electrophysiology. I_{sc} was measured in M-1 monolayers in an Ussing-type chamber as previously described (16). The entire chamber was placed in an incubator at 37°C and 5% CO_2 for the course of the experiment. The chamber contained four wells, filled with 0.9% saline, in which agar bridges and Ag/AgCl electrodes were positioned. The electrodes connected to a voltage clamp (DVC-1000; W-P Instruments, Inc., New Haven, CT). The voltage clamp was zeroed daily with 0.9% saline before the experiment. I_{sc} was measured for 2 s at 30-s intervals. Transepithelial potential difference (PD) was measured during the 30-s intervals, thereby maintaining the culture in a predominant open-circuit condition. Once baseline recordings were stable, CPAE cells on microcarriers were added to the lumen and the bath. Once I_{sc} was stable, Bk (10^{-9} M) or Ach (10^{-7} M) was added to both the lumen and bath. Recordings of I_{sc} and PD from M-1 cells alone, recordings of I_{sc} and PD from M-1 cells in the presence of CPAE cells, and recordings of I_{sc} and PD from M-1 cells in the presence of CPAE cells and Bk were sequentially taken from the same M-1 cultures. (Similar studies were repeated in which Bk was replaced by Ach.) Each M-1 culture dish was an n value of 1.

In a second set of experiments, CPAE cells were pretreated with 10^{-4} M N-Arg (Sigma Chemical Co., St. Louis, MO) for 15 min before their addition to the M-1 cultures. On addition of the CPAE cells to the M-1 cultures, N-Arg was added to both the lumen and the bath to maintain the concentration at 10^{-4} M. As above, recordings of I_{sc} and PD from M-1 cells alone, recordings of I_{sc} and PD from M-1 cells in the presence of N-Arg–treated CPAE cells, and recordings of I_{sc} and PD from M-1 cells in the presence of N-Arg–treated CPAE cells and Bk

were sequentially taken from the same M-1 cultures. (Similar studies were repeated in which Bk was replaced with Ach.) Each M-1 culture dish was an n value of 1.

In another set of experiments, 10^{-5} M methylene blue (Sigma Chemical Co.) was added to M-1 cultures. Once I_{sc} was stable, CPAE cells on microcarriers were added to the lumen and bath, followed by addition of Bk (10^{-9} M).

cGMP content. M-1 cells were grown to confluence on 31-mm cross-linked collagen membranes. Isobutyl methyl xanthine (1 mM) in HBSS solution containing 10 mM Hepes was added to M-1 cells, CPAE cells, and cultures of M-1 and CPAE cells, alone or with 10^{-9} M Bk. Samples were incubated for 20 min at 37°C. The medium was aspirated and the cells were lysed with 300 μl of 5% TCA. The TCA was collected and frozen at -70°C until the day of the assay. On the day of the assay, the acid was extracted from the samples with water-saturated ether. The samples were dried under N_2 gas at room temperature, reconstituted in buffer, and assayed for cGMP using a kit from Biomedical Technologies Inc., Stoughton, MA. A total of four assays was run, and samples were run in duplicate. The inter- and intraassay coefficients of variation were 12.2 and 6.5%, respectively. The detectable range was 4–400 fmol. To examine the recovery of cGMP during the extraction process, 150 fmol of cGMP in 300 μl of TCA was added to permeable collagen membranes (without cells); 94±2.5% of it was recovered. Recoveries were monitored in each experiment.

Statistics. Values are reported as the mean±SE. Individual culture dishes were considered as an n value of 1. Data from transport studies were evaluated with analysis of variance for repeat measures and multiple paired t tests with a Bonferroni adjustment to determine significant differences between each of the treatments. Data from cGMP measurements were evaluated using analysis of variance with three factors and multiple unpaired t tests with Bonferroni adjustment to determine significant differences between treatment groups. A ≥ 95% probability that the means were different was considered significant.

Results

Bioassay of EDRF production by CPAE cells. To demonstrate that our cultured CPAE cells release EDRF, we examined their ability to relax precontracted vascular rings (Fig. 1). Endothelial-denuded vascular rings from canine coronary arteries were superfused either directly or with a solution exposed to CPAE cells. 10^{-8} M Bk infused in the direct superfusion caused a small but insignificant contraction of the preconstricted rings. Bk infused in the endothelial superfusion produced a 100% relaxation of the preconstricted rings. These data demonstrate that our cultured CPAE cells produce and release a vasodilator substance (EDRF) in response to Bk.

Electrophysiology. To examine the effects of Bk alone on transport in M-1 cells, Bk was added to the bath and lumen of M-1 cultures in doses of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M (Fig. 2). Bk did not affect I_{sc} or PD at doses < 10^{-6} M. To ensure against a direct effect of Bk, a dose 1,000-fold less than that necessary to elicit a response was used.

The effects of Bk on I_{sc} and PD were measured in cultures of M-1 cells in the presence of CPAE cells. The addition of CPAE cells to M-1 cultures did not affect I_{sc} (Fig. 3). Baseline I_{sc} in M-1 monolayers was 47±4 μA/cm² before CPAE cell addition and 43±4.5 μA/cm² after addition. PD decreased slightly but significantly, from -20 ± 1 to -16 ± 2 mV ($P < 0.01$). The subsequent addition of Bk inhibited I_{sc} from 43±4.5 to 26±4 μA/cm², a 40±6% decrease ($P < 0.001$) (Fig. 3). PD decreased from -16 ± 2 to -9 ± 1 mV with Bk ($P < 0.001$). The addition of vehicle to cultures of M-1 and CPAE cells produced no significant effect on transport. These experiments were repeated us-
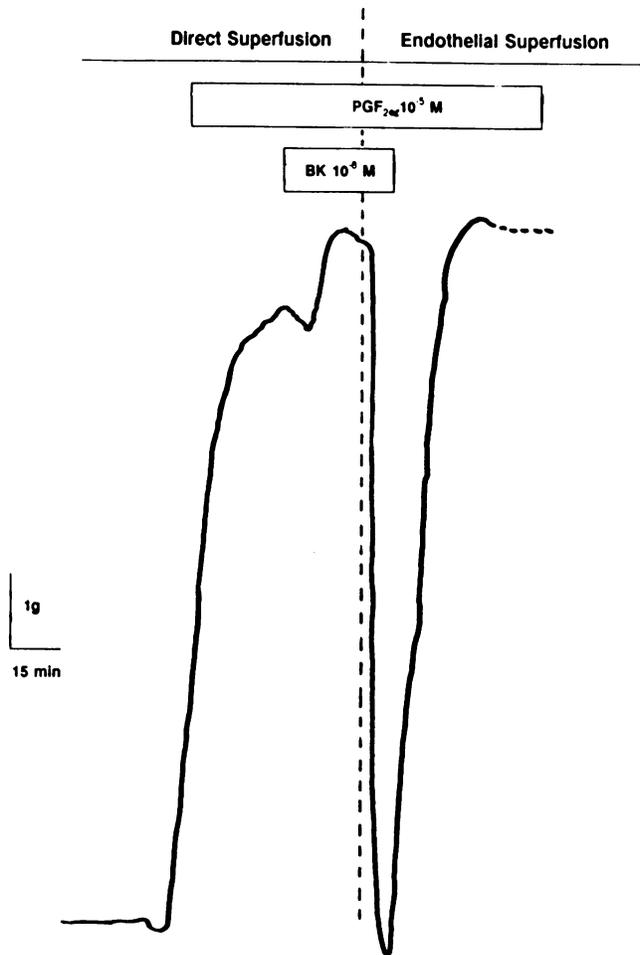
Figure 1. A representative isometric tension recording of the bioassay ring of canine coronary artery (without endothelium). Under direct superfusion the ring was contracted with $PGF_{2\alpha}$ and Bk produced a small contraction, confirming the absence of endothelium. When switching to CPAE superfusion, Bk produced a complete relaxation, confirming that the CPAE cells produce a vasodilator substance (EDRF).
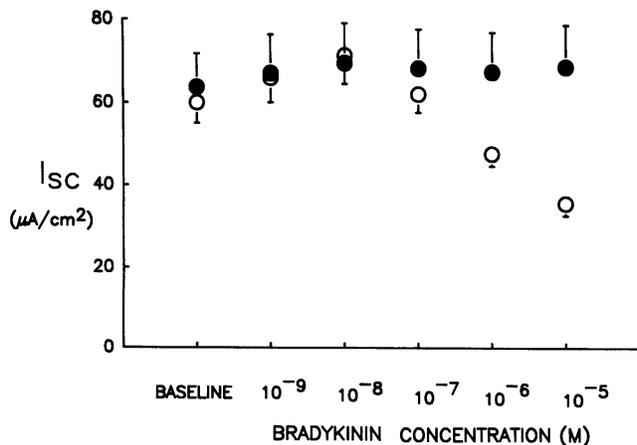

Figure 2. The effect of various concentrations of Bk on I_{sc}. A significant interaction effect ($P = 0.001$) was found between group and dosage ($n = 6$). ●, vehicle; o, bradykinin.
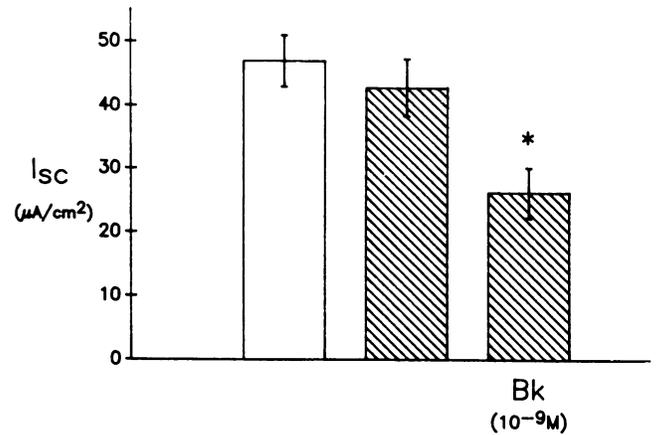

Figure 3. The effect of 10^{-9} M Bk on I_{sc} in the presence of CPAE cells. Control I_{sc} values were measured in M-1 monolayers alone. Cocultures consisted of M-1 monolayers and CPAE cells on microcarriers. Addition of CPAE cells produced no significant effect on I_{sc} in M-1 cells. Bk was added to the bath and lumen. Bk-stimulated release of EDRF decreased I_{sc} 40±6% in the cocultures. Vehicle addition had no significant effect on coculture I_{sc} ($n = 6$; *$P < 0.001$). □, M-1 cells; ▨, coculture.

ing 10^{-7}M Ach as the stimulatory agent rather than Bk. Addition of Ach to M-1 cells produced no significant effect on I_{sc} or PD. However, addition of Ach to M-1 cells in the presence of CPAE cells inhibited I_{sc} from 64±9 to 33±4 μA/cm², a 44% decrease, and PD from −42±3 to −28±3 mV, a 33% decrease.

Addition of N-Arg (10^{-4} M) alone to M-1 cultures had no significant effect on I_{sc} or PD. Addition of N-Arg–treated CPAE cells to M-1 cultures also produced no significant effect on I_{sc} (Fig. 4): 46±4 μA/cm² before CPAE cell addition and 42±5 μA/cm² afterwards. PD (−25.5±6 to −27±7.5 mV) did not significantly change. Fig. 4 also shows the effect of Bk on N-Arg–treated cultures of M-1 and CPAE cells. There was no
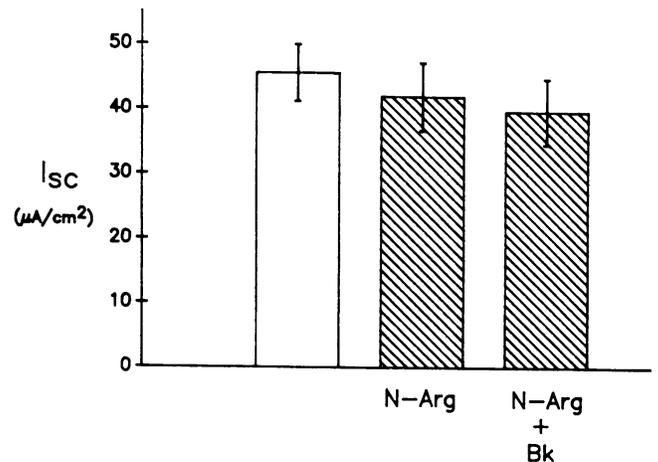

Figure 4. The effect of 10^{-9} M Bk on I_{sc} in the presence of N-Arg–treated CPAE cells. Control I_{sc} values were measured in M-1 monolayers alone. Cocultures consisted of M-1 monolayers and 10^{-4} M N-Arg–treated CPAE cells on microcarrier beads. Addition of N-Arg–treated CPAE cells produced no significant effect on I_{sc} in M-1 monolayers. Bk was added to the bath and lumen in the presence of N-Arg–treated CPAE cells and produced no significant effect on I_{sc} ($n = 6$). □, M-1 cells; ▨, coculture.
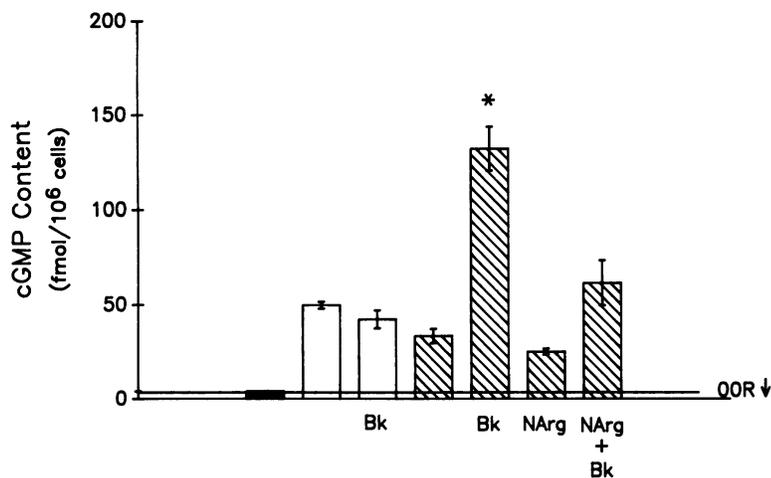
Figure 5. The effect of 10^{-9} M Bk on cGMP content in cocultures of M-1 and CPAE cells. OOR↓ denotes values below the 4-fmol limit of detectability. Bk was added to the bath and lumen and subsequently produced an ~ 300% increase in cGMP content in cocultures of M-1 and CPAE cells. cGMP content in N-Arg–treated cocultures challenged with Bk was 61.4 ± 12 fmol/10^6 M-1 cells, not significantly higher than coculture baseline content ($n = 5$–11; *$P < 0.001$). ■, media; □, M-1 cells; ▨, coculture.

significant change in I_{sc} (42 ± 5 to 40 ± 5 μA/cm²) or PD (-27 ± 7.5 to -22 ± 6 mV). Similarly, when Ach was given to N-Arg–treated cultures of M-1 and CPAE cells, I_{sc} (48 ± 3 to 46 ± 5 μA/cm²) and PD (-34 ± 6 to -32 ± 6 mV) did not change.

Addition of methylene blue (10^{-5} M) alone to M-1 cultures had no significant effect on I_{sc} (51 ± 2 to 54 ± 1 μA/cm²) or PD (-37 ± 2 to -39 ± 2 mV). Addition of Bk (10^{-9} M) to methylene blue–treated M-1 cells in the presence of CPAE cells also pro- duced no significant effect on I_{sc} (54 ± 0.5 to 56 ± 0.5 μA/cm²) or PD (-40 ± 1 to -39 ± 1 mV).

cGMP content. The cGMP contents of CPAE cells alone and those stimulated with Bk were below the limits of detectability (< 4 fmol). The cGMP content of the media was also below detectable limits (Fig. 5). The cGMP content of individual cultures of M-1 cells alone and those stimulated with Bk in the absence of CPAE cells was 49.7 ± 1.8 and 42.2 ± 4.7 fmol/10^6 cells, respectively. Cultures of M-1 and CPAE cells contained 33.3 ± 3.7 fmol of cGMP per million M-1 cells, which increased to 132.0 ± 11.7 fmol/10^6 M-1 cells with the addition of Bk (10^{-9} M). The content of cGMP was stimulated ~ 300% by Bk addition to cultures of M-1 and CPAE cells compared with unstimulated cultures. When cultures of M-1 and CPAE cells were treated with N-Arg and challenged with Bk, the cGMP content was 61.4 ± 12 fmol/10^6 M-1 cells, not significantly higher than baseline culture levels.

Discussion

Our study demonstrated that Bk or Ach addition to M-1 cells, in the presence of CPAE cells, decreased transport and increased cGMP content. Inhibition of transport was blocked by N-Arg, a competitive inhibitor of EDRF synthesis (6), and methylene blue, an inhibitor of soluble guanylate cyclase (9). Stimulation of cGMP content in M-1 cells was also partially blocked by N-Arg.

EDRF is a mediator of vascular tone and therefore participates in the regulation of peripheral resistance and, consequently, blood pressure. EDRF may also regulate blood pressure via effects on fluid and solute excretion in the kidney. It has been shown that stimulation of EDRF release in the kidney induces a natriuresis and diuresis without affecting glomerular filtration rate (10, 11). This would suggest that EDRF either has direct renal tubular effects on sodium reabsorption or causes hemodynamic effects that alter sodium reabsorption.

We know of no studies that directly address these possibilities and believe the present study provides the first evidence that EDRF has an effect on transport in cultured CCD cells.

The labile nature of EDRF raises the question of whether it remains intact long enough to diffuse from the endothelium to the CCD in vivo to affect transport. However, it can easily be demonstrated that EDRF can reach the collecting duct before it is degraded. Assuming that the distance EDRF must diffuse to reach the CCD can be represented by the average distance from the afferent arteriole to the CCD, 0.1 mm (18),[2] and that the diffusivity of EDRF can be approximated by that of a structurally very similar molecule, CO,[3] 2×10^{-5} cm²/s, then the time necessary for 50% of the EDRF produced by the afferent arteriole to diffuse 0.1 mm would be 5 s. This value is much less than the 30-s half-life of EDRF.

The inhibition of transport observed in this study is likely to be due to EDRF rather than to the agonists used to release EDRF for a number of reasons. First, we used two different agents, Bk and Ach, and the results were similar. Second, the doses of these agents used to release EDRF on their own had no effect on I_{sc}. Finally, the inhibition could be prevented by a competitive inhibitor of EDRF synthesis, as well as by an inhibitor of soluble guanylate cyclase.

It has been shown previously that atrial natriuretic peptide, which acts via cGMP, inhibits transport in the collecting duct (21) and that EDRF acts via cGMP in vascular smooth muscle (7, 8); therefore, it seemed likely that cGMP was the second messenger that induced inhibition of transport in the present experiments. This is supported by the observation that measured cGMP content increased 300% when EDRF release was stimulated in cultures of M-1 and CPAE cells and was blocked by the addition of N-Arg.

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2. This distance assumes that there is only a single source of EDRF from the afferent arteriole. Clearly there would be several afferent arterioles surrounding a collecting duct. Additionally, it is likely that there are other sources of EDRF besides the afferent arteriole (19), such as the renal capillaries and larger renal vessels (20).

3. The diffusivity of EDRF is likely to be very similar to CO, since this parameter is primarily determined by the square root of the molecular weight of a compound and to some extent the structure. The molecular weight of CO differs from NO by only 6% and the structures are analogous.

Endothelial cells may produce other substances besides EDRF that could inhibit I_{sc}, such as endothelin (22); however, since N-Arg and methylene blue blocked the inhibition of transport caused by Bk or Ach in the presence of CPAE cells, this would suggest that the inhibition was specifically due to the release of EDRF. Furthermore, cGMP has been proposed to be the second messenger of EDRF in smooth muscle (7, 8); therefore, the observation that cGMP was stimulated by the addition of Bk in cultures of M-1 and CPAE cells further supports the contention that EDRF is involved.

The M-1 cell line possesses many antigenic and differentiated transport properties of the CCD epithelium (16); therefore, the EDRF-stimulated inhibition of transport observed in the M-1 cells is probably also expressed in vivo. It is true, however, that phenomena that occur in cultured cells provide only indirect evidence that the indicated response is indeed physiological; thus, further studies are required in order to determine whether EDRF has direct tubular effects in vivo.

In conclusion, EDRF release from CPAE cells decreased transport in M-1 CCD cells and increased cGMP levels. This decrease in transport may account for the renal effects evoked by EDRF in in vivo studies.

Acknowledgments

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