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Effect of trypsinization in cell culture on bradykinin receptors in vascular endothelial cells

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One of many important roles of the naturally occurring nonapeptide, bradykinin (BK), is the modulation of vascular tone. A major cellular site for the action of bradykinin is on endothelial cells. The ability to culture endothelial cells has aided the elucidation of the cellular and molecular pharmacology of bradykinin [1–6]. Although the actions of bradykinin are clearly mediated via receptors, the characterization and quantification of endothelial bradykinin receptors by biochemical binding studies have been reported only recently [7]. During the course of these studies we observed that trypsin had profound effects on bradykinin receptors. This report describes in detail the effect of routine trypsinization in cell passaging upon BK receptors and its function in cultured endothelial cells.

Materials and Methods

Materials. Bovine pulmonary artery endothelial cells (BPAE) were obtained from the American Type Culture Collection (Rockville, MD). Bradykinin triacetate (BK), bovine serum albumin (BSA, globulin-free) and bacitracin were purchased from the Sigma Chemical Co. (St. Louis, MO). [2,3-*prolyl*-3,4-^{3}H(N)]Bradykinin (sp. act. = 61.7 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Trypsin–EDTA was prepared by the Medium Preparation Laboratory of Smith Kline & French Laboratories. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and related culture media were obtained from Gibco Laboratories (Grand Island, NY).

Cell culture. The cells (3×10^6) were grown in T-150 flasks containing 50 ml of DMEM supplemented with 20% (v/v) heat-inactivated FCS (DMEM-20% FCS) without the addition of antibiotics. They were incubated for 3–4 days at 37° under constant humidity (93%), 5% CO_2, and then harvested for experiment or passage. The cells were harvested either mechanically or enzymatically as follows.

Mechanical harvesting. The monolayer of the cells was scraped gently off the flasks, with a disposable cell scraper (Costar, Cambridge, MA), and pooled. The cell suspension was triturated gently using an automatic pipetter. Two aliquots were taken, spun down, and treated briefly with 0.05% (w/v) trypsin–0.25% (w/v) EDTA (trypsin–EDTA) to obtain a homogeneous suspension for cell counting and estimation of the number of population doubling (PD) [8].

Enzymatical harvesting (tryptin–EDTA treatment). The growth medium was first removed from the flask, followed by a 10-ml serum-free DMEM wash, and then 5 ml of prewarmed (37°) trypsin–EDTA was added to the monolayer of BPAE. The excess trypsin–EDTA was removed 5–7 sec later. The cells in the flask were allowed to stand at room temperature for an additional 30 sec, followed by a sharp lateral movement of the flask to dislodge cells from the flask. To the loosened cells 5 ml of DMEM–20% FCS was added quickly to inactivate any remaining trypsin. None of the treatments described above decreased the viability (>95% viable) of endothelial cells as determined by trypan blue dye exclusion.

[^{3}H]Bradykinin binding assay. BPAE cells between 3 and 6 passages (by mechanical or enzymatic harvesting) were obtained by scraping from culture flasks containing DMEM–20% FCS. Cells were washed twice with calcium-free, magnesium-free Dulbecco's phosphate-buffered saline (DPBS) solution. The washed cells were suspended in a small volume (2–3 ml) of ice-cold 25 mM potassium phosphate buffer, pH 6.5 (buffer), and homogenized at 0–4° with a Brinkmann polytron (model PT 10/35 control unit, PTA7 probe, Brinkmann Instruments Co., Westbury, NY), at setting 7. Three homogenations of 20 sec each were needed to obtain uniform homogenates containing less than 5% intact cells. The homogenates were then centrifuged at 50,000 *g* for 15 min at 4°. The precipitate was washed twice with intermediate rehomogenation in buffer. At this stage, no intact cells remained in the homogenates.

The binding assay was performed in 1.5-ml polypropylene Eppendorf microcentrifuge tubes (Brinkmann Instruments Co.) precoated with 0.1% BSA. The incubation mixture consisted of 0.1% BSA, 0.3 mM bacitracin, buffer, and [^{3}H]BK at various concentrations as described in the text, with and without unlabeled BK at 1000-fold excess of the highest ligand concentration used. The incubation was initiated by adding 50 μl of membrane preparation (100–200 μg) in a final volume of 0.25 ml. The assay was performed at 4° for 1 hr at which time binding was found to reach equilibrium. The reaction was stopped by adding 750 μl of cold PK buffer, and the tube was spun immediately in an Eppendorf microfuge (model 5415, Brinkmann Instruments Co.) at a maximum speed for 5 min. The medium was removed, and the pellet was washed once with 1 ml of cold PK buffer. The tip of the tube containing the pellet was cut into a glass liquid scintillation vial. The pellet was solubilized and counted for radioactivity. The results are presented as specific [^{3}H]BK binding which is defined as the difference between binding in the absence and presence of 1000-fold excess unlabeled BK. At a 5 nM concentration of [^{3}H]BK, specific binding of the radiolabeled ligand to BPAE cell membranes accounted for 55–65% of the total binding. The protein was measured by the method of Lowry *et al.* [9].

Bioassay of endothelium-derived relaxing factor (EDRF). BPAE cells were harvested by scraping and grown to confluence on Cytodex 3 microcarrier beads. The ability of the cells to release EDRF was measured by the method of Shikano and Berkowitz [10].

Results and Discussion

Pilot studies suggested that the use of trypsin to assist in the passage of cells via standard tissue culture techniques may have an effect on BK receptors. We therefore carried out systematic studies to address this question using direct binding of [^{3}H]BK and a functional assay of endothelial cells. Table 1A shows that treatment of the BPAE cell monolayer with trypsin-EDTA for 2–6 passages (chronic) did not affect significantly the rate of population doubling of the cells when compared with control cells passaged without trypsin; however, the binding of [^{3}H]BK (5 nM) to the membranes prepared from trypsinized cells was reduced significantly (43–70%). The chronic treatment with trypsin–EDTA did not affect overall growth pattern (e.g. population doubling rate and lifespan) of BPAE cells (data not shown). For the effect of a single (acute) treatment of BPAE cells with trypsin–EDTA on BK binding, the BPAE cells were subcultivated in flasks by dislodging cells mechanically and without trypsin for 3–6 passages. They were then separated into two sets of flasks. One set of cells was scraped and cell membranes were prepared (control). The other set of cells was treated briefly with trypsin–EDTA,

Table 1. Effect of trypsin–EDTA in cell passage (chronic) and cell harvesting (acute) on bradykinin binding to endothelial cell membranes

Experiment	Number of trypsin–EDTA exposures	PDs	Specific binding (fmol/mg)	% Suppression of binding
(A) Chronic effect				
1	None	22.7	86.9 ± 9.0	
	2	22.7	26.5 ± 6.2	70*
2	None	25.4	109.8 ± 7.8	
	3	25.3	41.0 ± 5.6	63*
3	None	27.0	99.5 ± 3.8	
	4	27.5	43.9 ± 11.0	56*
4	None	28.5	80.4 ± 0.3	
	6	27.3	46.0 ± 2.4	43†
(B) Acute effect				
1	None	27.2	88.6 ± 6.7 (4)	
	1		51.9 ± 3.7 (5)	41†
2	None	30.0	112.7 ± 12.6 (5)	
	1		72.7 ± 9.2 (5)	35‡
3	None	36.9	78.9 ± 3.2 (4)	
	1		78.8 ± 3.4 (4)	NS§
4	None	37.2	64.1 ± 4.8 (6)	
	1		63.8 ± 4.8 (6)	NS

A 5 nM concentration of [^{3}H]BK was used in the assay. Results (mean ± SEM) are values obtained from triplicate replications or the number of replications indicated in parentheses.

*–‡ Significantly different from control (Student's t-test): *$P < 0.01$, †$P < 0.001$, and ‡$P < 0.05$.

§ Not significant.

as described in Materials and Methods, and the cell membranes were prepared. Table 1B demonstrates that membranes from cells briefly exposed to trypsin–EDTA had lost BK binding ability by approximately 40% (experiments 1 and 2). The trypsin–EDTA effect was due to trypsin, since EDTA (0.25%) under similar conditions did not alter the ability of the membrane to bind BK (data not shown). The binding of BK became insensitive to acute trypsinization (experiments 3 and 4) when cells approached the end of the lifespan (PD = 37). Figure 1A illustrates the saturable, high-affinity binding of [^{3}H]BK to membranes prepared from cells passaged by the two techniques as described. The binding of [^{3}H]BK reached saturation at 10–20 nM in both preparations; however, the maximal binding was substantially less in the membrane prepared from cells passaged with trypsin–EDTA treatment. Scatchard analysis of the saturable binding (Fig. 1B) revealed equilibrium dissociation constants (K_d) of 1.9 and 1.9 nM and maximum binding (B_{max}) values of 122 and 61 fmol/mg protein for the control and trypsin–EDTA treated preparation

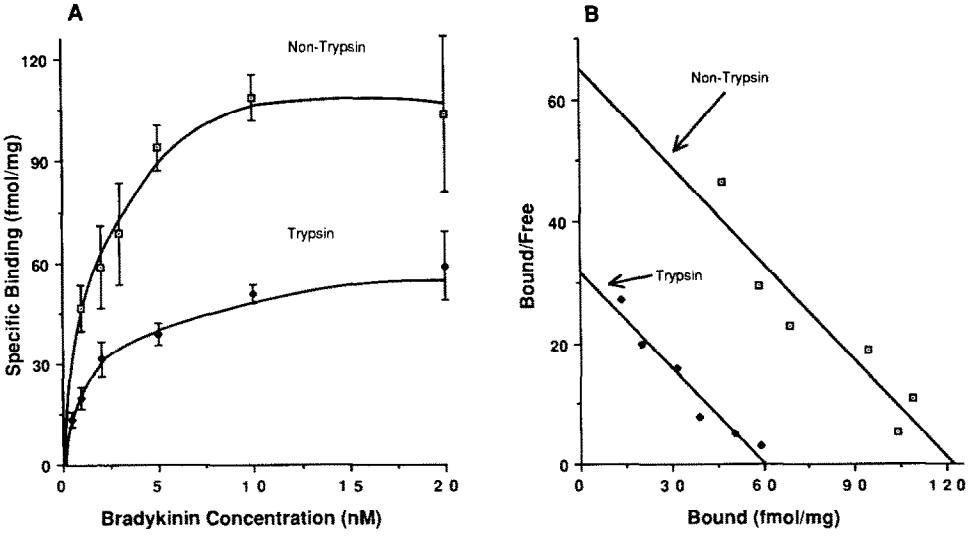

Fig. 1. Effect of trypsinization in cell passage on [^{3}H]BK binding to BPAE cell membranes. BPAE cells were subcultured with and without trypsin as described in Materials and Methods for 3–6 passages and were then harvested by scraping. The crude membranes prepared from these cells were used to test BK binding. (A) Saturation curve. (B) Scatchard plot. Key: (⊡—⊡) control (non-trypsin), and (●—●) trypsinization. Results are the means ± SEM of values obtained from four experiments.

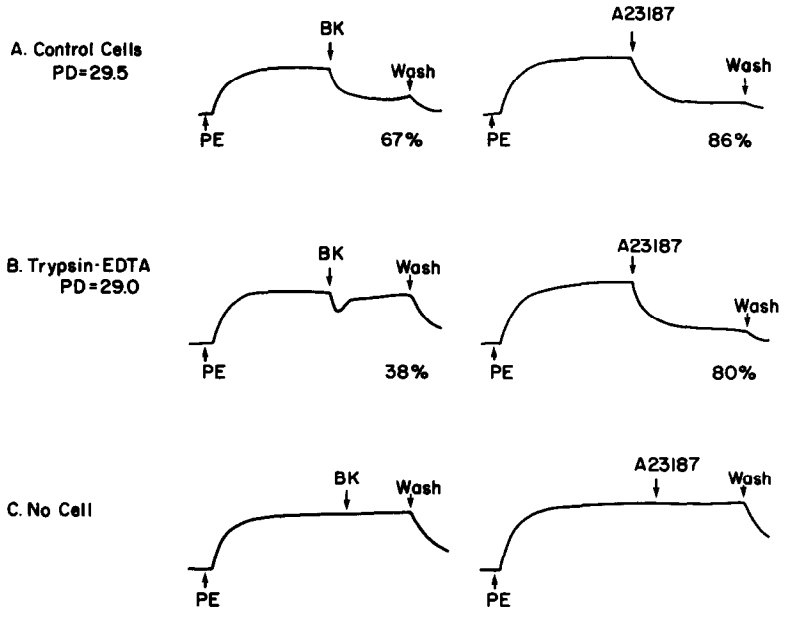

Fig. 2. Effect of trypsinization in cell passage on the ability of BPAE cells to release EDRF. BPAE cells were subcultured with and without trypsinization for 6 passages and harvested by scraping. Cells were then seeded and grown on Cytodex-3 microcarrier beads for 4 days. Cells (3×10^6) on the beads were superfused with BK (10^{-7} M) or A23187 (10^{-6} M) in a chromatography column to release EDRF as described in Materials and Methods.

respectively. Thus, trypsinization reduced the number of binding sites but not the affinity of the binding.

The inhibitory effect of trypsinization of cells during passage on [^{3}H]BK binding can be reversed by further subculturing of cells without trypsin–EDTA for a minimum of 2 passages or by seeding the cells at lower concentrations and allowing them to grow in the flask for at least 8 PDs (unpublished data).

To determine if the decrease in BK receptors was of pharmacological or functional significance, we made use of the observations that endothelial cells release EDRF in response to BK [10]. Figure 2 illustrates a typical tracing of the contraction of rabbit aorta [by phenylephrine (PE)] and the relaxation in response to EDRF released by maximal relaxing concentrations of BK (1×10^{-7} M) or A23187 (1×10^{-6} M). At comparable PDs, cells previously subcultured six times in trypsin–EDTA (Fig. 2B) released less EDRF compared to non-trypsinized control (38 vs 67%) in response to BK. The EDRF release response to the calcium ionophore A23187 was not altered significantly (86 vs 80%). As a control, the superfusion of the bioassay system without endothelial cells with BK (10^{-7} M) or A23187 (10^{-6} M) did not cause relaxation of assay tissues (Fig. 2C).

Trypsinization is a commonly used technique to dislodge cells from culture flasks in cell culture. It has been shown that a brief trypsinization in routine tissue culture of endothelial cells could cause the loss of membrane lipid from the cells [11] and inhibit angiotensin converting enzyme [12]. The direct effect of trypsinization in cell passage on receptors was not studied. In the present studies we monitored carefully the age (expressed as number of population doublings) or decline in successive subculturing (expressed as passages) of the cells. As cells got higher PDs, trypsin seemed to have less of an effect on BK receptor binding (Table 1B). These results clearly demonstrate the need to control carefully for the number of PDs or passages of cultured endothelial cells in pharmacological experiments. The effect of trypsin to decrease BK receptor binding could be due to proteolysis and/or lysis of membrane lipids. The latter effect must be considered since Kirkpatrick *et al.* [11] showed that trypsinization of monolayer-cultured endothelial cells in cell harvesting released a large amount of phospholipids. We have also observed that pretreatment of membranes with either phospholipase A_2 or phospholipase C significantly lowered BK binding (unpublished data).

In summary, we have demonstrated that trypsinization in tissue culture significantly decreased the number of BK receptor sites and inhibited the EDRF release from the cells in response to BK. The mechanism(s) of the trypsin effect on BK receptors and whether trypsin also affects other receptors on cell membrane of BPAE cells remain to be studied.

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Decreased selectivity of vasoactive intestinal peptide receptors by GTP

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High-affinity receptors for vasoactive intestinal peptide (VIP) bind peptides structurally homologous to VIP with relatively low affinity [1–3]. These peptides include growth hormone releasing factor (GRF), peptide histidine isoleucine (PHI) and secretin. Guanosine triphosphate (GTP) and its analogs inhibit VIP-receptor binding and potentiate cyclic AMP synthesis in response to VIP [1, 4, 5], suggesting a role for GTP-binding regulatory protein(s) (G-protein) in VIP-receptor regulation. We observed previously that detergent-induced disaggregation of a GTP-sensitive, VIP-preferring receptor complex solubilized from guinea pig lung membranes produces a binding species that is GTP insensitive and GRF preferring [6]. This observation suggested that a G-protein may influence the binding selectivity of VIP receptors. However, detergents interact intimately with integral membrane proteins [7] and may alter the binding characteristics of receptors. To eliminate the possible confounding influence of the detergent, we have measured the effect of GTP on the binding selectivity of VIP receptors present in intact lung membranes.

Materials and Methods

[^{125}I]*VIP*. Pure porcine VIP (provided by Dr. S. I. Said, Illinois University, College of Medicine) was labeled with [^{125}I] and purified according to Ref. 8, except that the iodination was for 30 sec with 25 μg chloramine-T, and the purification of [^{125}I]VIP was by reverse phase HPLC on a single Novapak-C18 column (Waters, Milford, MA) to a specific activity of 2000 Ci/mmol [8], a value close to the maximum specific activity of monoiodinated [^{125}I]VIP. This preparation was used routinely as tracer in the receptor binding studies. For comparison, some binding assays were also performed using [(^{125}I)-Tyr^{10}]VIP purchased from Amersham, Arlington Heights, IL.

Lung membranes. Freshly dissected guinea pig lungs were perfused (8 ml/min) with about 75 ml Krebs buffer, pH 7.4, until the tissue became near-white in color [9]. The lungs were homogenized twice (30 sec, 10,000 rpm; Biohomogenizer model 133/1281-0 Biospec Products, Bartlesville, OK) on ice in 5 vol. of 10 mM Trizma–HCl, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA (sodium salt), 100 units aprotinin/ml, 100 μM phenylmethylsulfonyl fluoride, and 5 μM pepstatin A (Sigma Chemical Co., St. Louis, MO). The homogenate was strained through six layers of medical gauze and centrifuged (30,000 *g*, 30 min, 4°), and the pellet was resuspended to 10–15 mg protein/ml in homogenization buffer, assayed according to Ref. 10.

[^{125}I]*VIP receptor binding*. The membranes (50 μg protein in 50 μl) were incubated (30 min, 23°) with [^{125}I]VIP (100 μl, 60–80 pM) in the absence and presence of increasing concentrations of unlabeled porcine VIP or rat GRF(1–44) (50 μl; Peninsula Laboratories, Belmont, CA) in polypropylene microfuge tubes previously treated (10 min) with 0.5 ml of assay buffer (100 mM Trizma–HCl, pH 7.4, containing 5 mM $MgCl_2$ and 1% bovine serum albumin). Termination was by addition of 0.5 ml of assay buffer, centrifugation (12,000 *g*, 3 min), and aspiration of the supernatant fractions. Radioactivity in the pellets was determined by gamma spectrometry (Beckman, model 5500). Saturable binding (a term synonymous with "specific binding" in this paper) was the radioactivity displaced by a 1 μM concentration of unlabeled VIP. Time course experiments (not shown) indicated that steady-state saturable binding of [^{125}I]VIP was achieved under these conditions. To interfere with G-protein : receptor coupling, GTP or its nonhydrolyzable analog, guanyl-5′-yl imidodiphosphate (GppNHp) (Sigma), was included in the assay mixture. K_d and B_{max} for VIP and GRF were computed using the computer programs EBDA and LIGAND (Elsevier Biosoft, Cambridge, U.K.) run on an IBM-AT computer [11]. K_d for [^{125}I]VIP, required for computation of K_d for GRF, was determined by assaying saturable binding at increasing [^{125}I]VIP concentrations (5–500 pM). Hill slopes close to unity (0.95–1.002) and linear Scatchard plots suggested that the binding of [^{125}I]VIP was predominantly by a single population of receptors with K_d values of 0.38