

# Bradykinin Regulates the Expression of Kininogen Binding Sites on Endothelial Cells

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The vasoactive compound bradykinin (BK) is liberated by proteolytic cleavage from high molecular weight kininogen (HK) and low molecular weight kininogen (LK). Expression of kininogens on cell surface receptors may affect the delivery of BK at sites of inflammation. Therefore, we investigated whether BK itself alters the expression of binding sites for its parent molecules, HK and LK, on the surface of cultured human umbilical vein endothelial cells (HUVEC).  $^{125}\text{I}$ -LK and  $^{125}\text{I}$ -HK each bind to a single class of sites on HUVEC in reactions that are saturable, reversible, and zinc-dependent ( $B_{\text{max}} = 9.7 \pm 0.2 \times 10^5$  sites/cell;  $k_d = 43.3 \pm 8$  nmol/L;  $n = 5$  and  $B_{\text{max}} = 10.3 \pm 0.4 \times 10^5$  sites/cell;  $k_d = 40.3 \pm 0.9$  nmol/L;  $n = 3$  for LK and HK, respectively). HK and LK compete for the same binding site ( $K_i = 19.4 \pm 5$  nmol/L HK v  $^{125}\text{I}$ -LK;  $K_i = 24.5 \pm 4$  nmol/L LK v  $^{125}\text{I}$ -HK,  $n = 3$ ). Moreover, 50-fold molar excess light chain of HK inhibits  $^{125}\text{I}$ -LK binding 51% and 50-fold molar excess LK and the heavy chain of HK inhibit  $^{125}\text{I}$ -light chain of HK binding 92% and 76%, respectively. Preincubation of HUVEC with BK produces a transient, concentra-

tion-dependent increase in the binding of HK and LK, reaching a maximum 3 to 4 hours after addition of BK (46% increase over control for HK; 57% increase over control for LK;  $P < .005$  for each ligand). Des-Arg<sup>9</sup>-bradykinin, a B<sub>1</sub> receptor agonist, increases kininogen binding to the same extent as BK; the upregulation of kininogen binding sites by BK is partially blocked by a B<sub>1</sub> but not by a B<sub>2</sub> receptor antagonist. The protein kinase C inhibitors (PKC), sphingosine and H7, completely block the induction of HK receptors by BK. Phorbol 12-myristate 13-acetate (PMA), which also activates PKC, stimulates the binding of HK and the purified light chain of HK to HUVEC as well. However, unlike HK and its light chain, binding of LK and the heavy chain of HK are increased by PMA only in the presence of added calcium ion. These studies show that BK upregulates a common binding site for HK, LK, and each chain of HK on HUVEC. Induction of kininogen receptors on endothelial cells by BK may modulate the generation of this vasoactive compound at sites of vascular injury.

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**T**HE PLASMA kininogens are multidomain proteins produced by a single gene. The first identified function of the kininogens was as the parent molecules of bradykinin (BK).<sup>1-3</sup> The kininogens are now known to have additional functions. Both high and low molecular weight kininogen (HK, LK) share a common heavy chain that contains a cysteine protease inhibitory region located on domains 2 and 3.<sup>4</sup> Domain 3 of the common heavy chain of the plasma kininogens also contains a platelet and endothelial binding site, as well as a region that inhibits the binding of  $\alpha$ -thrombin to platelets.<sup>5,6</sup> The unique 56-Kd light chain of HK has additional functions, including binding sites for prekallikrein and factor XI on domain 6,<sup>7,8</sup> and a binding site for anionic surfaces rich in histidines, lysines, and glycines on domain 5.<sup>9</sup> The light chain of HK also contains a second region which

supports binding to platelets that is distinct from the cellular binding site on the heavy chain.<sup>10</sup>

Expression of binding sites for kininogen on cell surfaces may influence the rate of bradykinin delivery at sites of inflammation.<sup>10,11</sup> When HK binds to platelets and endothelial cells, the molecule is relatively protected from cleavage by plasma and tissue kallikrein, thereby delaying the liberation of BK.<sup>10</sup> This finding suggests that factors which stimulate the expression of HK receptors, including possibly the cleavage products of kininogen itself, may serve to downregulate the generation of BK. To test that hypothesis, we studied the effect of BK on the expression of kininogen receptors by human umbilical vein endothelial cells (HUVEC), cells shown previously to bind and synthesize HK.<sup>12,13</sup> These studies indicate that BK, a potent mediator of inflammation, stimulates the expression of kininogen receptors on the endothelial cell surface.

## EXPERIMENTAL PROCEDURES

### Materials

Na  $^{125}\text{I}$  (50 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Iodogen (1,3,4,6-tetrachloro-3 $\alpha$ , 6 $\alpha$ -diphenylglycoluril) was obtained from Pierce Chemical Co (Rockford, IL). Dithiothreitol, high and low molecular mass standards for sodium dodecyl sulfate gel electrophoresis (SDS-PAGE), were purchased from BioRad (Richmond CA). Apiezon A oil was purchased from Biddle Instruments (Blue Bell, PA). Phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-didecanoate (P1), phorbol 12-myristate 13 acetate 4-O-methyl ether (P2), 4 $\alpha$  phorbol 12,13-didecanoate (P3), Mezerein, BK, Des-Arg<sup>9</sup>-bradykinin (D-BK1), Des-Arg<sup>9</sup>-[Leu<sup>8</sup>]-bradykinin (D-BK3), and sphingosine were purchased from Sigma Chemical Corp (St Louis, MO). HOE 140 (D-Arg-[Hyp<sup>3</sup>, Thi<sup>5</sup>, D-Tic, Oic<sup>8</sup>]-bradykinin) was generously provided by Klaus J. Wirth (Hoechst AG, Frankfurt, Germany). Enalapril was obtained from UpJohn (Kalamazoo MI).

### Plasma

Pooled normal plasmas were purchased from George King Biomedical, Inc (Overland Park, KS). Total kininogen-deficient

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plasma (plasma deficient in both HK and LK) was donated by the late Mayme Williams (Philadelphia, PA).

### Protein Purification

HK was purified using a modification<sup>14</sup> of the method of Kerbiriou and Griffin<sup>15</sup> by adding 0.2 mol/L  $\epsilon$ -aminocaproic acid to all buffers and 2 mmol/L diisopropylfluorophosphate to the pooled material before each step. After reduction with 2%  $\beta$ -mercaptoethanol, over 98% of the purified HK migrated as a single band having an apparent molecular mass of 120 Kd on a 7.5% polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE).<sup>16</sup> The procoagulant activity of the purified HK was assessed by a kaolin-based, activated partial thromboplastin time using total kininogen-deficient plasma as a substrate.<sup>10</sup> Purified HK had a specific activity of 12 to 20 U/mg.

LK was purified from normal plasma by affinity chromatography using carboxymethylpapain-Sepharose 4B followed by chromatography on DEAE-Sephadex (Pharmacia-LKB Biotechnology, Piscataway, NJ).<sup>10,17-19</sup> Diisopropylfluorophosphate (2 mmol/L) was added to the starting plasma and each protein fraction before every chromatographic step. All buffers also contained 3 mmol/L EDTA, 0.1 mol/L  $\epsilon$ -aminocaproic acid, and 1 mmol/L benzamidine. A single immunoprecipitin arc was produced when LK was incubated with an antiserum that recognizes both heavy and light chain of kininogen;<sup>20</sup> LK had no procoagulant activity. Residual HK contaminating the LK preparations was adsorbed using a suspension of 0.25% kaolin in 0.01 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.4 with constant mixing at 37°C for 15 minutes followed by centrifugation at 3,000g for 15 minutes to remove the kaolin.<sup>6</sup> Homogeneity of the LK preparation was assessed by SDS-PAGE. The purified LK migrated as a single chain molecule containing its BK moiety because addition of human urinary kallikrein liberated a 4-Kd peptide, seen on 13% SDS-PAGE, consistent with the known molecular weight of the LK light chain.

The light chain of HK (LC) and the heavy chain of HK (HC) were prepared from purified HK by cleavage with human urinary kallikrein, generously provided by Dr Fu-Kuen Lin (AMGEN, Inc, Thousand Oaks, CA). The tissue kallikrein was used at a 1/100 molar ratio (33  $\mu$ g tissue kallikrein in 33  $\mu$ L was added to 10 mg HK in 2 mL 10 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.4 for 16 hours at 37°C). The fully cleaved HK was then reduced with 50 mmol/L DTT followed by alkylation in the dark with 120 mmol/L iodoacetamide. The cleaved, reduced, and alkylated HK was then applied to an SP Sephadex column in 0.01 mol/L sodium acetate, 0.08 mol/L NaCl, pH 5.4. The HC of HK was not adsorbed to the cation exchanger, while the LC was eluted with a linear NaCl gradient from 0.08 mol/L to 0.5 mol/L. The LC of HK migrated as two bands at 56 and 46 Kd on 7.5% SDS-PAGE. The purified LC of HK had a specific activity of 8 U/mg. On immunoblot, the purified LC was detected by a monoclonal antibody (MoAb) to the LC of HK; an MoAb to the HC of the kininogens did not recognize the purified LC.<sup>21</sup> The HC product of HK migrated as a single band at 64 Kd on 7.5% SDS-PAGE. It had no procoagulant activity and was detected by an MoAb to the HC of the kininogens but not by one directed to the LC.<sup>21</sup> The immunoblot assay we used can detect 5 ng/mL kininogen fragment; this value corresponds to a concentration of the LC and HC of HK of 90 pmol/L and 78 pmol/L, respectively. Thus, the preparations of LC and HC are estimated to contain less than 2% of other chain.

### Iodination of Proteins

Purified HK was radiolabeled with Na<sup>125</sup>I using Iodogen by the method of Fraker and Speck<sup>22</sup> under conditions previously described.<sup>5,10</sup> The specific radioactivity of the protein varied from 6 to 9  $\mu$ Ci/ $\mu$ g such that greater than 34% to 50% of the HK molecules contained an iodine molecule. The radiolabeled protein was greater

**Table 1. Specificity of Radiolabeled Kininogens and Their Subunits' Binding to HUVEC**

Radioligand	Protein Competitor	% Inhibition of Binding* (mean $\pm$ SEM)	Ki (nmol/L)†
<sup>125</sup> I-LK	None	0	—
	No Zn <sup>2+</sup>	93 $\pm$ 2	—
	HK	99 $\pm$ 5	19 $\pm$ 5
	LK	100	13 $\pm$ 7
	LC	51 $\pm$ 7	—
<sup>125</sup> I-HK	None	0	—
	No Zn <sup>2+</sup>	96 $\pm$ 5	—
	HK	100	18 $\pm$ 6
	LK	103 $\pm$ 4	24 $\pm$ 4
	LC	79†	—
<sup>125</sup> I-LC	None	0	—
	HK	94†	—
	LK	92†	—
	LC	100	—
	HC	76†	—

\* HUVEC grown to confluence in 96-well microtiter plates were washed with HEPES-Tyrode's buffer and incubated with 8.3 nmol/L <sup>125</sup>I-HK, 10 nmol/L <sup>125</sup>I-LK, or 5.0 nmol/L <sup>125</sup>I-LC for 1 hour at 4°C in the presence of 50  $\mu$ mol/L Zn<sup>2+</sup> and the indicated competitor proteins added at 50-fold molar excess. The cells were then washed, and the cell-associated radioactivity was measured. Data are expressed as percentage of binding measured in the presence of 50-fold molar excess of unlabeled ligand. The values represent the mean  $\pm$  SEM of three independent experiments unless indicated otherwise.

† The Ki of the radiolabeled kininogen by the unlabeled kininogens was determined by the procedure of Muller.<sup>26</sup>

‡ Values represent the mean of two experiments with nearly identical results.

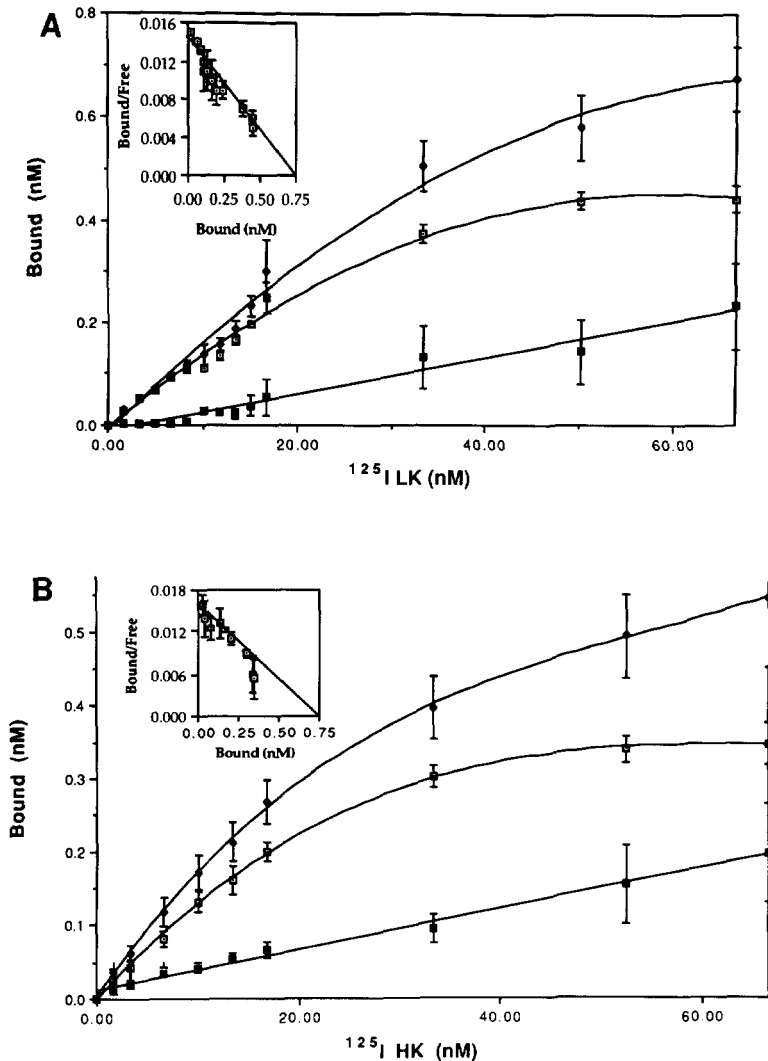
than 95% trichloroacetic acid-precipitable and retained more than 95% of its antigenicity and procoagulant activity. LK, the light chain of HK, and the heavy chain of HK were radioiodinated using the same procedure. The specific radioactivity was between 7 and 11  $\mu$ Ci/ $\mu$ g. Greater than 95% of the radioactivity was precipitated by 10% trichloroacetic acid, and greater than 18% of the protein molecules were iodinated. The protein concentrations of each preparation of <sup>125</sup>I-LK, -HK, -LC, and -HC was determined by radial immunodiffusion using a polyclonal antisera directed to both the HCs and LCs of the plasma kininogens as previously reported.<sup>5</sup> <sup>125</sup>I-HK and -LC were also quantified by their coagulant activity. Radiolabeled HK and unlabeled HK bound to HUVEC with equal affinity.

### Endothelial Cell Culture

Cultures of HUVEC were prepared and characterized using established methods described previously.<sup>23</sup> The cells were passaged two to four times in Medium 199 (GIBCO, Grand Island NY) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, VA), penicillin-streptomycin (GIBCO), and endothelial cell growth factor<sup>24</sup> and grown to confluence on fibronectin coated 96-well microtiter plates (Flow Laboratories). Cell counts were performed on completion of the binding experiments to ensure uniformity of cell numbers per well in control and treated cells.

### Cell Stimulation

Confluent monolayers of HUVEC were incubated with PMA (10 nmol/L) or BK (100 nmol/L) for various periods of time before mea-



**Fig 1.** Concentration-dependent binding of  $^{125}\text{I}$ -LK and  $^{125}\text{I}$ -HK to HUVEC. (A) HUVEC were incubated with increasing concentrations of  $^{125}\text{I}$ -LK in HEPES-Tyrode's buffer containing  $\text{Zn}^{2+}$  for 60 minutes. Binding in the absence (total binding,  $\blacklozenge$ ) or presence of a 50-fold molar excess of unlabeled LK (nonspecific binding,  $\blacksquare$ ) was determined. Specific binding ( $\square$ ) was calculated by subtracting nonspecific binding from total binding. (Insert) The specific binding data from (A) were fit on a bound/free versus bound plot according to the method of Scatchard.<sup>25</sup> The data shown are the mean  $\pm$  SEM of five experiments. (B) HUVEC were incubated with increasing concentrations of  $^{125}\text{I}$ -HK in HEPES-Tyrode's buffer containing  $\text{Zn}^{2+}$  for 60 minutes. Total binding, nonspecific binding ( $\blacksquare$ ), and specific binding ( $\square$ ) was determined as above. (Insert) The specific binding data from (B) were fit on a bound/free versus bound plot according to the method of Scatchard.<sup>25</sup> The data shown are the mean  $\pm$  SEM of three experiments.

during the binding of  $^{125}\text{I}$ -HK (8.3 nmol/L),  $^{125}\text{I}$ -LK (10 nmol/L),  $^{125}\text{I}$ -LC (5 nmol/L), or  $^{125}\text{I}$ -HC (5 nmol/L). In some experiments, HUVEC were incubated with various concentrations of PMA or BK for 3 hours before measuring binding of  $^{125}\text{I}$ -HK or -LK. To determine whether B1 or B2 receptors were involved in the BK-induced increase in binding, Des-Arg<sup>9</sup>-bradykinin, a B1 receptor agonist (D-BK1), D-Arg-[Hyp<sup>3</sup>, Thi<sup>5</sup>, D-Tic<sup>7</sup>, Oic<sup>8</sup>]bradykinin, a B2 receptor antagonist (HOE 140), or Des-Arg<sup>9</sup>-[Leu<sup>8</sup>]bradykinin, a B1 receptor antagonist (D-BK3) were used either alone or together with BK (10 nmol/L), and the binding of  $^{125}\text{I}$ -HK was measured after an additional 3-hour incubation. In other experiments, HUVEC were preincubated for 1 hour with control media, media containing H7 (Seikagaku America Inc, St Petersburg, FL), or HA1004 (Seikagaku America Inc) or sphingosine (Sigma) before the addition of PMA (10 nmol/L) to assess the effect of inhibiting protein kinase C (PKC). Finally, in some experiments binding of HK to control HUVECs were compared to cells preincubated with the angiotensin converting enzyme inhibitor, enalapril (1  $\mu\text{mol/L}$ ) after both sets of cells were stimulated with various concentrations of bradykinin.

#### Binding of Radiolabeled Proteins to Endothelial Cells

HUVEC were cooled to  $4^\circ\text{C}$  for 30 minutes and then washed three times in HEPES-Tyrode's buffer (0.135 mol/L NaCl, 2.7 mmol/L KCl,

11.9 mmol/L  $\text{NaHCO}_3$ , 0.36 mmol/L  $\text{NaH}_2\text{PO}_4$ , 14.7 mmol/L HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] containing 1 mg/mL bovine serum albumin, 3.5 mg/mL dextrose pH 7.35, and 50  $\mu\text{mol/L}$   $\text{ZnCl}_2$ ). After washing, HUVEC were incubated with  $^{125}\text{I}$ -kininogens (8.3 nmol/L for HK, 10 nmol/L for LK, 5 nmol/L for the light chain of HK, or 5 nmol/L for the heavy chain of HK unless otherwise noted) in HEPES-Tyrode's buffer at  $4^\circ\text{C}$  for 60 minutes to achieve equilibrium.<sup>12</sup> The cells were washed five times with the same buffer, removed from the plate with 1 N NaOH, and the cell-associated radioactivity was determined in a Beckman Gamma 8000/counter (Beckman Instruments, Inc, Fullerton, CA). Nonspecific binding, unless stated otherwise, was determined by performing binding experiments in the presence of 50-fold molar excess unlabeled protein. Specific binding was determined by subtracting the total binding from the nonspecific binding. The equilibrium dissociation constant (kd) and maximum number of binding sites by cell (Bmax) were determined by Scatchard analysis.<sup>25</sup> The ability of unlabeled kininogens to inhibit the binding of radiolabeled kininogen ( $K_i$ s) were calculated using the formula of Muller<sup>26</sup> as previously reported.<sup>5,6,10,12</sup>

#### Statistical Analysis

All results are given as means  $\pm$  SEM. The statistical analysis was also performed by the Student's *t*-test. A *P* value  $<.05$  was considered significant.

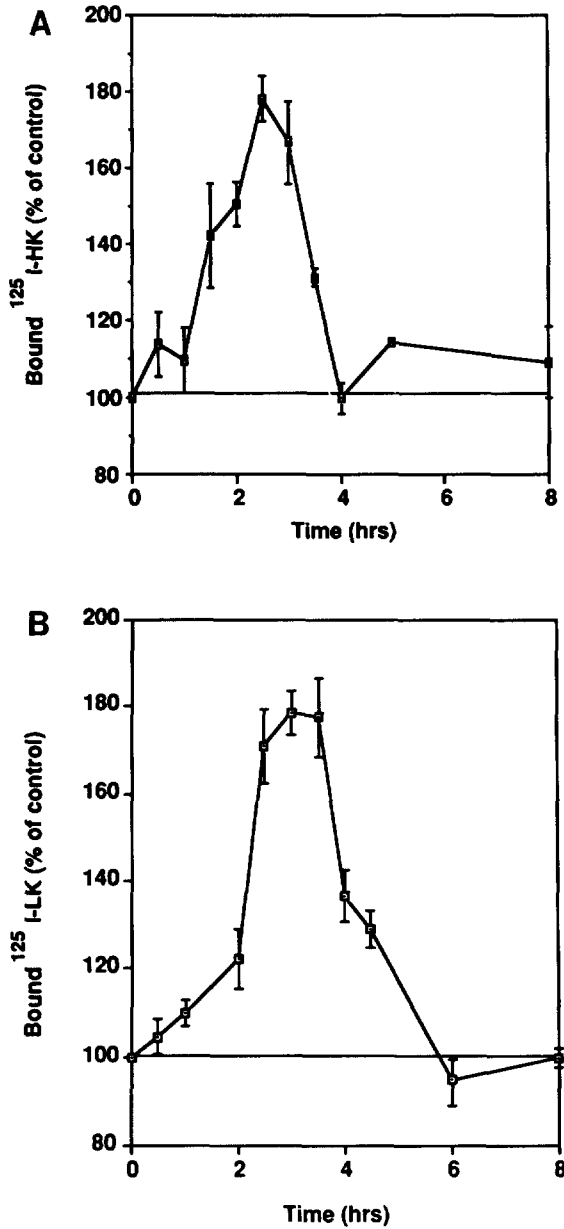


Fig 2. Time course of the induction of HK and LK binding sites by BK. (A) HUVEC were incubated with media in the absence or presence of BK (100 nmol/L) for various periods of time and the specific binding of  $^{125}\text{I}$ -HK (8.3 nmol/L) determined. Each point in the figure represents the mean  $\pm$  SEM of the values derived from four experiments. (B) HUVEC were incubated for the indicated times in the absence or presence of 100 nmol/L BK and the specific binding of  $^{125}\text{I}$ -LK (10 nmol/L) was determined. The values shown are the mean  $\pm$  SEM of three experiments.

## RESULTS

### Binding of LK and HK to Unstimulated HUVEC

We first determined whether unstimulated HUVEC express unoccupied binding sites for LK similar to those reported for HK.<sup>12,13</sup> Binding of  $^{125}\text{I}$ -LK to HUVEC was inhibited completely by 50-fold molar excess unlabeled LK (Table 1). Spe-

cific binding of LK to HUVEC required  $\text{Zn}^{2+}$  as observed previously with platelets.<sup>5</sup> Nonspecific binding of  $^{125}\text{I}$ -LK to HUVEC in the absence of zinc was equivalent to binding in the presence of zinc and 50-fold molar excess unlabeled LK (Table 1). Specific binding of  $^{125}\text{I}$ -LK to HUVEC was completely reversed within 15 minutes at  $4^\circ\text{C}$  when excess unlabeled ligand was added within 2 hours after addition of radiolabeled ligand (data not shown). Specific binding of  $^{125}\text{I}$ -LK reached saturation at a ligand concentration of 30 to 40 nmol/L (Fig 1A). At equilibrium, LK bound to a single class of sites ( $B_{\text{max}} = 9.7 \pm 0.2 \times 10^5$  sites/cell;  $k_d = 43.3 \pm 8$  nmol/L;  $n = 5$ ) (insert, Fig 1A). Binding of  $^{125}\text{I}$ -LK and binding of  $^{125}\text{I}$ -HK to unstimulated HUVEC performed in parallel gave similar results (Fig 1, A and B). As previously reported, specific binding of  $^{125}\text{I}$ -HK to unstimulated HUVEC required zinc. At equilibrium, HUVEC also expressed a single class of binding sites for  $^{125}\text{I}$ -HK ( $B_{\text{max}} = 10.3 \pm 0.4 \times 10^5$  sites/cell;  $k_d = 40.3 \pm 0.9$  nmol/L;  $n = 3$ ) (insert Fig 1B), values in close agreement with those previously reported ( $B_{\text{max}} = 9.3 \pm 2.0 \times 10^5$  sites/cell,  $k_d = 53 \pm 13$  nmol/L).<sup>11,12</sup>

Taken together, these results suggested that HK and LK may recognize the same binding sites on unstimulated HUVEC, similar to what has been observed on human platelets.<sup>10</sup> This hypothesis gained additional support from the observation that each unlabeled kininogen inhibited the binding of the other radiolabeled kininogen to HUVEC (Table 1) with similar affinity ( $K_i = 19.4 \pm 5$  nmol/L for HK inhibition of  $^{125}\text{I}$ -LK binding  $\nu$   $K_i = 24.5 \pm 4$  nmol/L for the inhibition of  $^{125}\text{I}$ -HK binding by LK,  $n = 3$ ). Further, isolated light and heavy chains of HK (LC and HC) inhibited the binding of the complementary chain as well as both intact LK and HK (Table 1). LC inhibited binding of HK and LK by 79% and 51%, while HC inhibited the binding of HK, LK, and LC by 95%, 107%, and 76%, respectively.

### Binding of HK and LK to HUVEC Stimulated With BK

It has been reported previously that BK is liberated more slowly from cell-associated HK than from fluid-phase HK.<sup>10,11</sup>

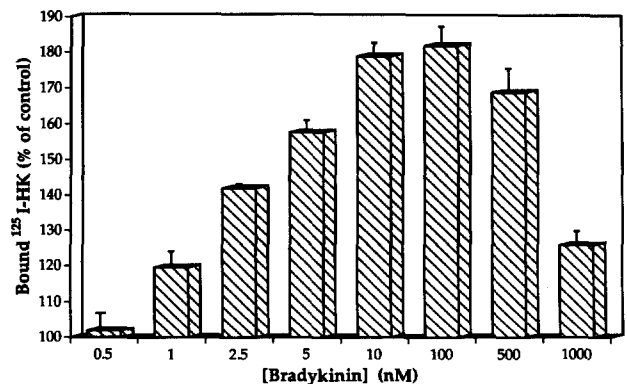
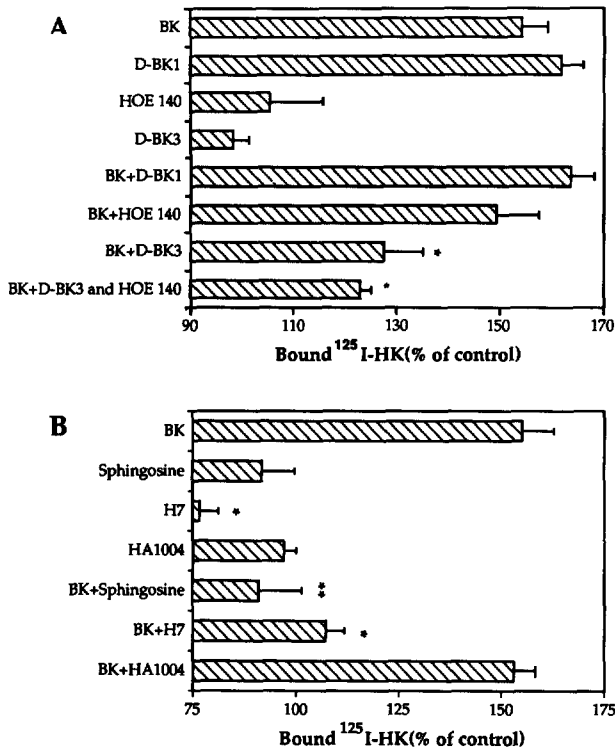


Fig 3. Concentration dependence of the induction of HK binding sites by BK. HUVEC were incubated with BK in concentrations from 0.1 nmol/L to 1 mol/L for 3 hours at  $37^\circ\text{C}$ . Specific binding of  $^{125}\text{I}$ -HK (8.3 nmol/L) was determined. Each bar represents the mean  $\pm$  SEM of three experiments.



**Fig 4.** Effect of BK agonists and antagonists and inhibitors of PKC on HK binding. (A) HUVEC were incubated for 3 hours with BK (10 nmol/L), D-BK1 for Des-Arg<sup>9</sup>-bradykinin (10 nmol/L), HOE 140 for D-Arg [Hyp<sup>3</sup>, Thi<sup>5</sup>, D-Tic, Oic<sup>8</sup>]-bradykinin (1  $\mu$ mol/L), D-BK3 for Des-Arg<sup>9</sup>, [Leu<sup>8</sup>]-bradykinin (1  $\mu$ mol/L), or a combination of BK and these BK analogues. The specific binding of <sup>125</sup>I-HK (8.3 nmol/L) was determined. The results shown are the mean  $\pm$  SEM of three experiments. (B) HUVEC were incubated for 3 hours with BK (100 nmol/L) alone, sphingosine (1  $\mu$ mol/L), H7 (10  $\mu$ mol/L), or HA1004 (40  $\mu$ mol/L). Costimulation was performed with BK (100 nmol/L) and each of these inhibitors. The results shown are the mean  $\pm$  SEM of three experiments. (\*) and (\*\*) denote results statistically different ( $P < .05$  or  $P < .005$ , respectively) from control.

Therefore, we investigated the possibility that BK may regulate the expression of its endothelial cell binding sites for its parent proteins, HK and LK.

**Binding of HK.** BK caused a time- and concentration-dependent increase in the binding of HK to HUVEC. Increased binding of <sup>125</sup>I-HK to HUVEC, which was first observed 60 to 90 minutes after the addition of BK (100 nmol/L) (Fig 2A), reached a peak between 2.5 and 3 hours after stimulation (178.1%  $\pm$  6.0% binding to stimulated cells  $\nu$  unstimulated cells [ $P < .005$ ],  $n = 3$ ), after which binding returned to basal levels by 4 hours. Increased <sup>125</sup>I-HK binding was detectable at BK concentrations as low as 1 nmol/L (Fig 3). HK binding continued to increase with addition of higher concentrations of BK, reaching a maximum at BK concentrations between 10 and 100 nmol/L; addition of BK at higher concentrations caused cell loss from the microtiter plate resulting in apparent decreased binding (Fig 3). When HUVEC had been stimulated with 100 nmol/L BK for 3 hours, the number of binding sites for HK increased 45.8%  $\pm$  1.7% over control levels ( $B_{max} = 14.9 \pm 0.5 \times 10^5$  sites/cell;  $P < .005$

$\nu$  control;  $n = 3$ ) with no change in receptor affinity ( $k_d = 42.3 \pm 3.2$  nmol/L). When HUVEC were preincubated with the angiotensin converting enzyme inhibitor, enalapril, the minimal concentration of BK necessary to upregulate kininogen binding sites was decreased 100-fold to 10 pmol/L (data not shown). These data also suggest that BK, rather than its proteolyzed fragments, upregulate kininogen expression on HUVEC.

**Binding of LK.** BK also stimulated the binding of LK to HUVEC. The increase in LK binding after addition of BK followed a similar time course as with HK. The peak increase (178.5%  $\pm$  5% binding to BK stimulated HUVEC,  $n = 3$ ,  $P < .025$   $\nu$  unstimulated cells) occurred 3 to 4 hours after the addition of BK. Binding of LK, like HK, returned to control levels by 6 hours (Fig 2B). Scatchard analysis of LK binding to HUVEC stimulated by BK for 3 hours indicated that there was a 57.2%  $\pm$  4% increase in the total number of LK binding sites over control cells ( $15.2 \pm 0.15 \times 10^5$  sites/cell;  $P < .005$   $\nu$  control;  $n = 4$ ), comparable to that seen with HK, with no change in receptor affinity ( $k_d = 39.4 \pm 6$  nmol/L).

**Mechanism of induction of HK receptors by BK.** We next investigated whether the induction of HK binding sites by BK was mediated through B<sub>1</sub> or B<sub>2</sub> receptors. HK binding to HUVEC was stimulated to the same extent by 10 nmol/L BK or the B<sub>1</sub> receptor agonist, Des-Arg<sup>9</sup>-bradykinin (D-BK1) (Fig 4A). Costimulation with both agonists did not potentiate the effect. The B<sub>1</sub> receptor antagonist, Des-Arg<sup>9</sup> [Leu<sup>8</sup>]-bradykinin (D-BK3), present in 100-fold molar excess, had no direct effect on HK binding to unstimulated HUVEC, but partially inhibited the capacity of BK to stimulate HK binding (42% of the level seen with BK stimulation alone). A 100-fold molar excess of a B<sub>2</sub> receptor antagonist, D-Arg [Hyp<sup>3</sup>, Thi<sup>5</sup>, D-Tic, Oic<sup>8</sup>]-bradykinin (HOE140), which also had no effect alone on HK binding to HUVEC, did not significantly inhibit BK-induced binding of HK. The addition of both antagonists did not further augment the inhibition produced by the B<sub>1</sub> receptor antagonist alone (D-BK3) (41% of the level seen with BK stimulation alone) (Fig 4A).

Investigations were next performed to determine whether BK increased the number of HK binding sites by activating protein kinase C (Fig 4B). Preincubation of unstimulated HUVEC with the PKC inhibitors, H7 (10  $\mu$ mol/L) and sphingosine (1  $\mu$ mol/L), did not alter the binding of HK significantly (77.8%  $\pm$  8.1% and 93.5%  $\pm$  3.6% of control, respectively) (Fig 4B). However, preincubation of HUVEC with H7 and sphingosine completely blocked the induction of HK binding sites by BK (155.1%  $\pm$  2.0%  $\nu$  107.3%  $\pm$  10.4% and 91.2%  $\pm$  3.9%;  $n = 3$ ;  $P < .05$ , BK  $\nu$  BK + H7 and  $P < .005$ , BK  $\nu$  BK + sphingosine, respectively). In contrast, preincubation with HA1004, a weak inhibitor of PKC with a chemical configuration close to H7, had no inhibitory effect on BK-induced HK binding (Fig 4B). These findings suggest that the induction of HK binding sites on HUVEC by BK may be mediated, in part, by stimulation of intracellular PKC. To investigate this hypothesis further we studied the effect of the PKC activator, PMA, on the expression of kininogen binding sites.

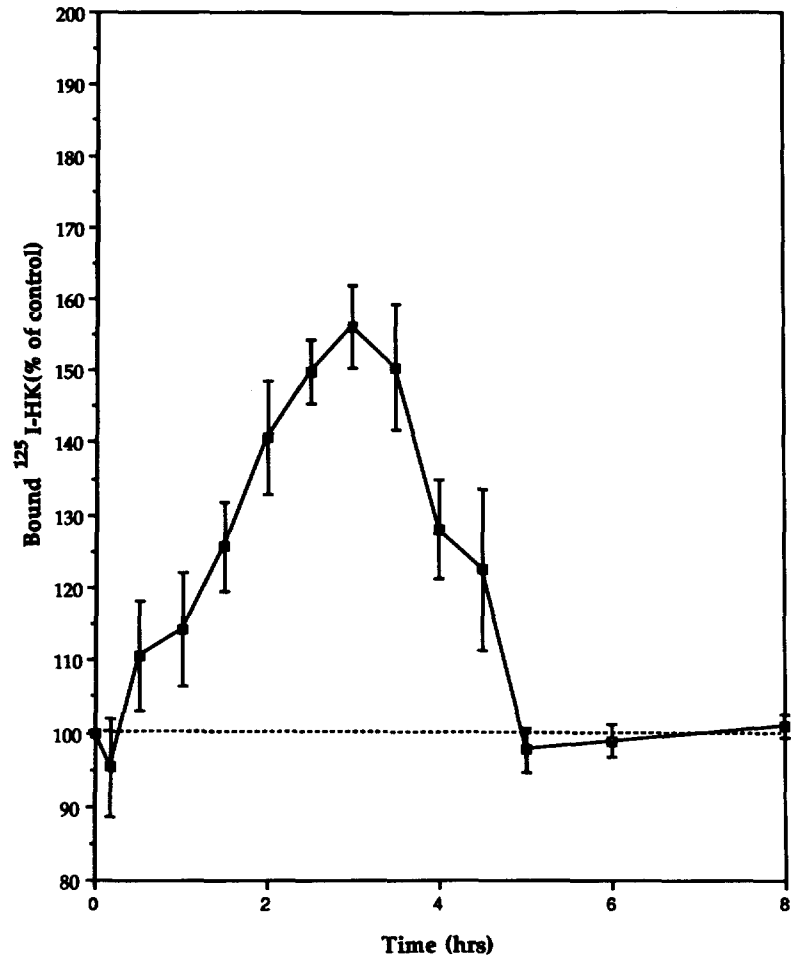


Fig 5. Time course of PMA induction of HK-binding sites. HUVEC were incubated with control media or media containing PMA (10 nmol/L) for various periods of time and the specific binding of <sup>125</sup>I-HK (8.3 nmol/L) was determined. Each point shown represents the mean  $\pm$  SEM of six experiments.

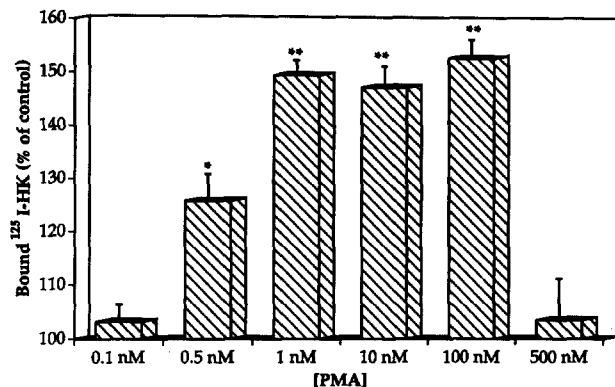
#### Binding of HK and LK to HUVEC Stimulated With PMA

**Binding of HK.** PMA (10 nmol/L) stimulated an increase in HK binding sites on HUVEC, first detected 30 minutes after addition of the agonist (Fig 5). Binding reached maximum 3 hours after PMA was added (156.1%  $\pm$  14.1% binding to PMA-stimulated *v* control cells;  $P < .001$ ;  $n = 6$ ), after which binding returned to basal levels by 5 hours and remained stable over the next 19 hours (data not shown). The induction of HK binding sites by PMA was concentration-dependent. The increase in binding sites became detectable when PMA was added at a concentration of 0.5 nmol/L (Fig 6) and reached a plateau at 1 to 100 nmol/L PMA. Higher concentrations of PMA were associated with cell loss. Scatchard analysis of HK binding at equilibrium to HUVEC preincubated with PMA for 3 hours indicated a 45.5%  $\pm$  5.6% ( $P < .005$ ;  $n = 3$ ) increase in the number of binding sites (14.9  $\pm$  0.1  $\times 10^5$  sites/cell) with an affinity similar to control cells (kd 53.3  $\pm$  3 nmol/L).

The induction of HK receptors by PMA was mediated, at least in part, by activating PKC. Mezerein, phorbol 12,13 didecanoate (P1), and PMA itself, each of which activates PKC, stimulated HK binding to a similar extent (Fig 7A). Structurally related phorbols, phorbol 12-myristate 13 acetate 4-0 methyl ether (P2) and 4 $\alpha$  phorbol 12,13 didecanoate (P3),

which do not activate PKC, were inactive (Fig 7A). Preincubation of HUVEC with H7 or sphingosine inhibited the induction of HK binding sites by 10 nmol/L PMA (149.3%  $\pm$  2.2% binding with PMA alone *v* 115.1%  $\pm$  2.6% binding with PMA + H7, and 112%  $\pm$  4.0% binding with PMA + sphingosine), while HA1004 had no inhibitory effect (Fig 7B). Costimulation of HUVEC with PMA (10 nmol/L) and BK (100 nmol/L) were not additive (160.1%  $\pm$  13.5% after PMA + BK *v* 149.3%  $\pm$  2.2% after PMA alone) (Fig 7B). When HUVEC were preincubated in media containing PMA for 24 hours, a situation in which PKC is downregulated, neither BK, D-BK1, nor PMA alone were able to induce HK binding sites above control levels (102%  $\pm$  5%, 107%  $\pm$  3%, 98.3%  $\pm$  10%, respectively, *v* control;  $n = 3$ ) (Fig 7B).

**Binding of LK.** Studies were next performed to determine if the upregulation of LK binding sites by BK was also mediated through activation of PKC. Unexpectedly, the binding of <sup>125</sup>I-LK to HUVEC was not stimulated by PMA in three separate experiments, even in the presence of optimal concentrations of Zn<sup>2+</sup> (data not shown). Therefore, the binding of <sup>125</sup>I-LK, <sup>125</sup>I-HK, and <sup>125</sup>I-LC was compared in the same experiment (Fig 8). PMA (10 nmol/L) caused a transient increase in the binding of both HK and LC to HUVEC, with peak binding occurring between 2.5 and 4 hours, whereas



**Fig 6.** Concentration dependence of PMA induction of HK-binding sites. HUVEC were incubated with various concentrations of PMA for 3 hours at 37°C and the specific binding of <sup>125</sup>I-HK (8.3 nmol/L) was determined. The results shown are the mean ± SEM of three experiments. (\*) and (\*\*) denote results statistically different ( $P < .05$  or  $P < .005$ , respectively) from control.

the binding of LK was not increased at any of the times studied (Fig 8).

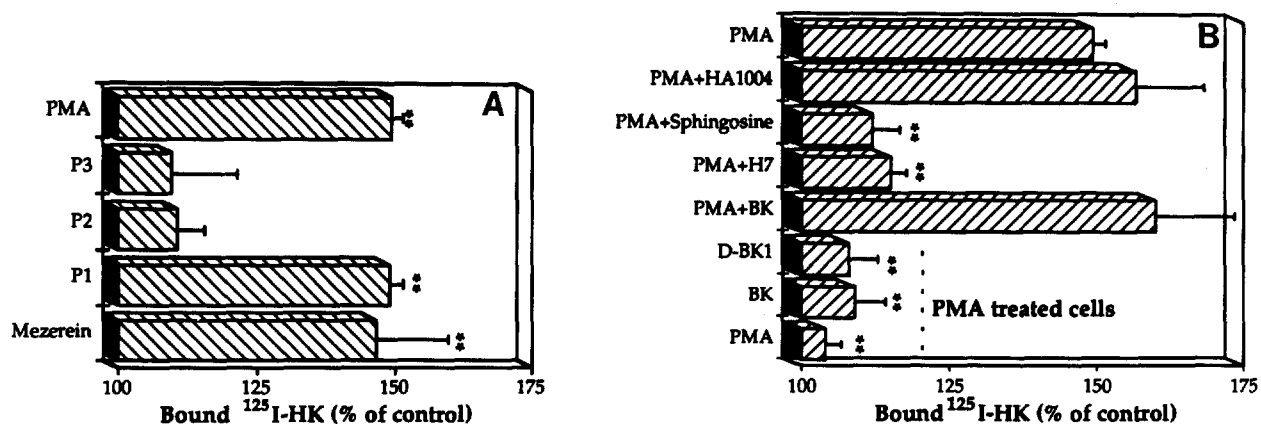
To investigate the basis of this difference between the binding of HK and LK to PMA-stimulated HUVEC, studies were performed to determine if divalent cations other than zinc might affect the binding of LK to control or PMA-stimulated HUVEC. The effect of varying concentrations of Ca<sup>2+</sup> on HK and LK binding to unstimulated and PMA-stimulated HUVEC was investigated. Addition of calcium ion over a range from 0 to 2.0 mmol/L had no influence on HK or LK binding to unstimulated HUVEC; calcium values greater than 2.0 mmol/L inhibited binding of both ligands. <sup>125</sup>I-LK binding to HUVEC preincubated with BK was independent of added calcium (166.6% ± 3% binding in the presence of calcium v

163.1% ± 5.6% binding in its absence) (Fig 9A). In contrast, PMA stimulated <sup>125</sup>I-LK binding in the presence, but not in the absence, of exogenous Ca<sup>2+</sup> (179.8% ± 3% v 97.2% ± 5% binding in the presence and absence of calcium, respectively;  $P < .005$ ;  $n = 3$ ) (Fig 9A). A small additive effect was also noted when HUVEC were costimulated with PMA and BK in the presence of calcium (205.2% ± 7% binding for BK + PMA v 166.6% ± 3% binding for BK alone and 179.8% ± 3% binding for PMA alone). Additional studies were performed to determine if PKC was involved in the calcium-dependent induction of <sup>125</sup>I-LK binding sites by PMA (Fig 9B). Sphingosine and H7 alone induced a slight increase in LK-binding sites in the presence of calcium. However, the induction of the calcium-dependent binding sites for <sup>125</sup>I-LK by PMA (10 nmol/L) was only partially downregulated by the PKC inhibitors H7 and sphingosine (179.8% ± 3% over control PMA alone v 139.4% ± 5% and 146.3% ± 5% for PM + H7 and PMA + sphingosine, respectively;  $P < .05$ ,  $n = 3$ ). These studies suggest that induction of LK binding sites by PMA in the presence of calcium may be mediated by additional intracellular pathways as well as by PKC.

Because the results of the studies cited above (Fig 1 and Table 1) suggest that LK and HK as well as both of the isolated chains of HK appear to bind to a common site on HUVEC, we then determined whether calcium was also an essential cofactor for the induction of binding sites for <sup>125</sup>I-HC by PMA, as observed for <sup>125</sup>I-LK. BK stimulated the binding of <sup>125</sup>I-HC in the absence of added calcium (Fig 10). In contrast, binding of additional <sup>125</sup>I-HC, like LK, to PMA-stimulated HUVEC, required exogenous calcium (Fig 10).

## DISCUSSION

These studies indicate first that HUVEC express binding sites for LK as well as for HK. Several findings suggest that a single receptor may bind both molecules to the surface of



**Fig 7.** Effect of inhibitors of PKC, phorbol esters, and Mezerein on HK binding. (A) HUVEC were incubated for 3 hours with four different phorbol esters: PMA, P1 or phorbol 12,13 didecanoate, P2 or phorbol 12-myristate 13-acetate 4-0-methyl ether, P3 or 4 $\alpha$  phorbol 12,13-didecanoate, and Mezerein (all added at a concentration of 100 nmol/L). (B) HUVEC were incubated for 3 hours with PMA (10 nmol/L) without or with H7 (10  $\mu$ mol/L), HA1004 (40  $\mu$ mol/L), sphingosine (1  $\mu$ mol/L), or BK (100 nmol/L) for 3 hours. In other experiments, the cells were incubated with PMA (10 nmol/L) for 24 hours before addition of D-BK1 (100 nmol/L), BK (100 nmol/L), or PMA (100 nmol/L). Specific binding of <sup>125</sup>I-HK (8.3 nmol/L) was determined. The results shown are the mean ± SEM of three experiments. (\*\*) Denotes values that are statistically higher or lower than control values.

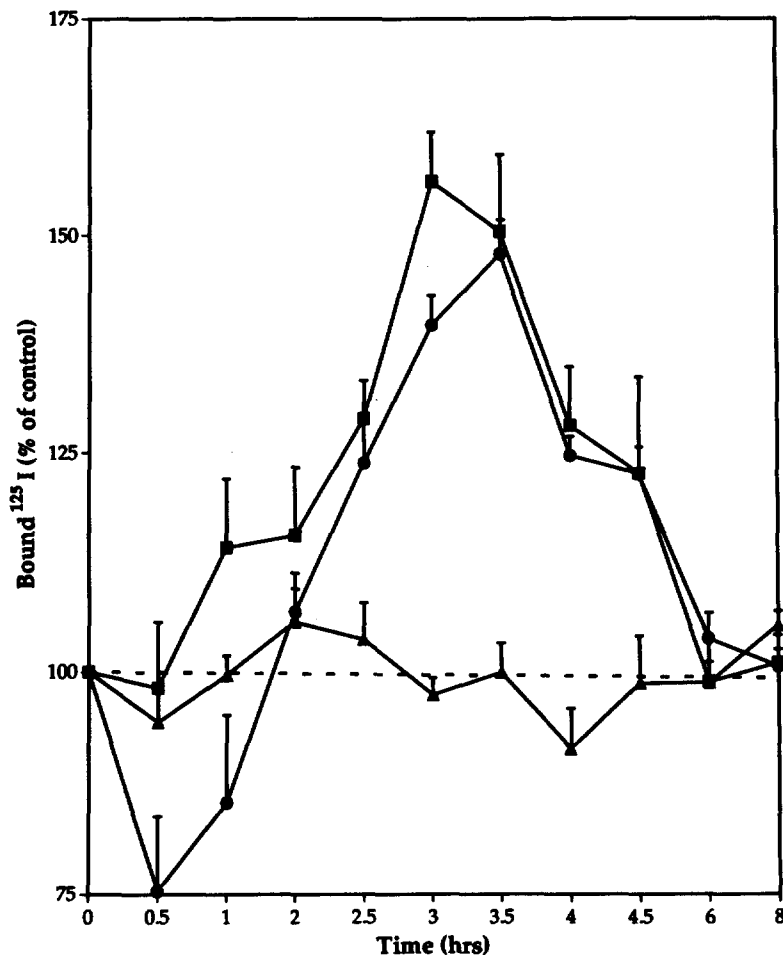


Fig 8. Time course of PMA stimulation of HK, LK, and LC binding. HUVEC were treated with PMA (10 nmol/L) for varying periods of time and the specific binding of  $^{125}\text{I}$ -HK (8.3 nmol/L,  $\blacksquare$ ),  $^{125}\text{I}$ -LK (10 nmol/L,  $\blacktriangle$ ), and  $^{125}\text{I}$ -LC (5 nmol/L,  $\bullet$ ) were determined. The data shown are the mean  $\pm$  SEM of three experiments.

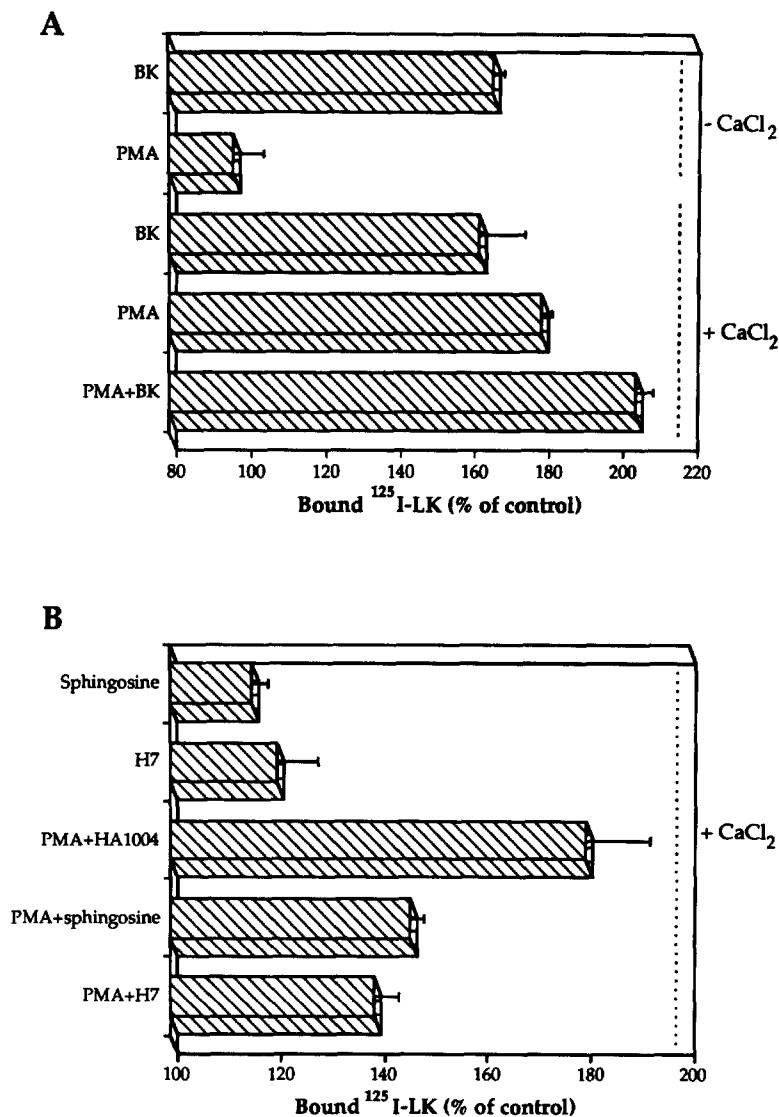
unstimulated HUVEC, as has been previously suggested for human platelets.<sup>10</sup> Both ligands bind with similar affinity to the same number of sites (Fig 1), both require zinc for specific binding (Table 1), and each native kininogen inhibits the binding of the other with a similar affinity. The latter result is consistent with the proposed involvement of the cell binding region on domain 3 of their common HC.<sup>6</sup>

However, our data also suggest that there appear to be at least two domains on HK molecule and one on LK that are involved in the binding to HUVEC, similar to observations made previously on platelets.<sup>10</sup> This interpretation is supported by the finding that the isolated light and heavy chains of HK bind specifically to HUVEC. The observation that the isolated light chain of HK does not completely inhibit the binding of native HK or LK (Table 1) may reflect a loss of a preferred three-dimensional structure required for optimal binding which is retained in the native molecules. An alternative explanation is that the putative kininogen receptor recognizes two independent sites on HK, and that LK and to a lesser extent the isolated chains of HK sterically block the recognition of the remaining portions of the molecule. The data also do not allow us to distinguish between two potential models for the kininogen receptor itself; ie, that the receptor consists of two independent domains, each of which interact with a complementary independent region on HK,

or that the receptor consists of a single domain that recognizes a three-dimensional structure formed through an interaction between at least two portions of the HK molecule. In accord with either model, the important structural features that permit kininogens to bind to its receptor appear to be preserved in LK but are only partially retained by the isolated light and heavy chains of the HK molecule.

The second conclusion from these studies is that BK stimulates the expression of the putative kininogen receptor for both of its parent proteins. BK induced a comparable increase in the binding of HK and LK compatible with induction of a single common receptor (Fig 2). The increase in binding of each radioligand was observed only after a delay (2 to 3 hours) that could be consistent with a requirement for either protein synthesis or redistribution of an internal pool of binding sites to the cell surface. Further study will be required to distinguish between these possibilities. A second, unexpected feature of BK's induction of kininogens' putative receptor was the apparent transient nature of the increase. After a 70% to 80% increase in the number of binding sites at 2 to 3 hours, binding returned to basal levels by 6 hours and remained stable over the next 19 hours. The mechanism underlying the transient expression of the putative receptor is unknown, but could be explained by internalization or shedding of kininogen receptors, or



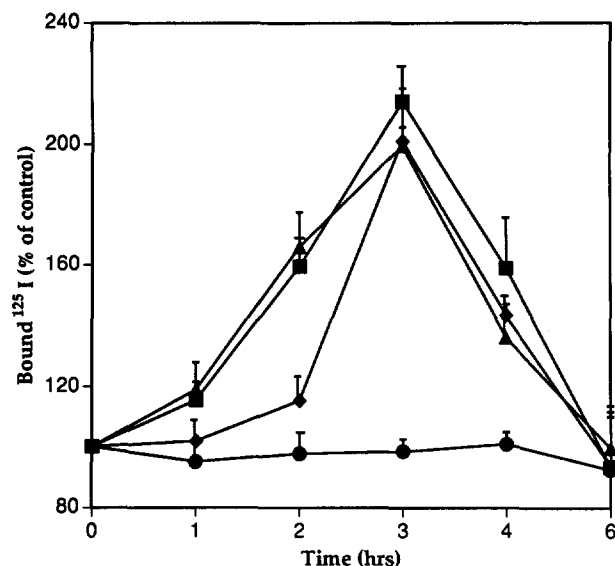


**Fig 9.** Effect of calcium on the binding of <sup>125</sup>I-LK to PMA- or BK-stimulated cells. (A) HUVEC were incubated for 3 hours with PMA (10 nmol/L), BK (100 nmol/L), or both agonists in the absence or presence of 1 mmol/L calcium. Specific binding of <sup>125</sup>I-LK (10 nmol/L) was determined in Tyrode's HEPES buffer containing 50  $\mu$ mol/L ZnCl<sub>2</sub>. The values shown are the mean  $\pm$  SEM of three experiments. (B) HUVEC were incubated for 3 hours in the presence of 1 mmol/L calcium and the inhibitors of PKC, H7 (10  $\mu$ mol/L) or sphingosine (1  $\mu$ mol/L), or with HA1004 (40  $\mu$ mol/L), in the absence or presence of PMA (10 nmol/L). The specific binding of <sup>125</sup>I-LK (10 nmol/L) was determined. The results shown are the mean  $\pm$  SEM of three experiments.

by the delayed induction of a counter-regulatory intracellular pathway.

Stimulation of kininogen receptors by Des-Arg<sup>9</sup>-bradykinin, a B<sub>1</sub> agonist, followed the same time course seen after addition of BK (Fig 4A). Moreover, BK and Des-Arg<sup>9</sup>-bradykinin stimulated expression of HK binding sites to the same extent. Because our experiments were performed in the presence of fetal calf serum, it is possible that this B<sub>1</sub> agonist was generated by proteolysis of BK in serum and accounts for all the stimulatory activity.<sup>27</sup> However, the B<sub>1</sub> antagonist Des-Arg<sup>9</sup>[Leu<sup>8</sup>]-bradykinin only partially blocked the induction of HK binding sites by BK (Fig 4A). A B<sub>2</sub> antagonist, Des-Arg<sup>9</sup>[Hyp<sup>3</sup>, Thi<sup>5</sup>, D-Tic, Oic<sup>8</sup>]-bradykinin, caused minimal inhibition and added little to the inhibition caused by Des-Arg<sup>9</sup>[Leu<sup>8</sup>]-BK (Fig 4A). Because the B<sub>1</sub> receptor antagonist did not cause complete inhibition, it is possible that additional heterogeneity exists among BK receptors including a receptor subtype not fully influenced by these BK receptor antagonists.<sup>27</sup>

The induction of kininogen binding sites on HUVEC by BK appears to be mediated, at least in part, by activation of PKC. PMA also stimulated HK and LC binding to HUVEC to the same extent and with the same kinetics as receptor induction by BK. Moreover, phorbol 12,13 didecanoate, which activates PKC, stimulated HK binding to a similar extent as PMA, while the structurally related phorbols, phorbol 12-myristate 13 acetate 4- $\alpha$  methyl ether and 4 $\alpha$  phorbol 12,13 didecanoate, which do not activate PKC, did not stimulate binding (Fig 7A). Furthermore, receptor induction by BK was almost completely inhibited when HUVEC were preincubated with either H7 or sphingosine, but not HA1004 (Fig 4B). Taken together, it is unlikely that BK upregulated kininogen binding primarily through cyclic adenosine monophosphate and cyclic guanosine monophosphate dependent kinases.<sup>28</sup> However, the possibility that sphingosine may inhibit by blocking diacylglycerol and Ca<sup>2+</sup>/calmodulin-dependent kinases has not been totally excluded.<sup>29</sup>



**Fig 10.** Effect of calcium on the binding of  $^{125}\text{I}$ -HC of HK to PMA- and BK-stimulated cells. HUVEC were incubated for 1 to 6 hours with PMA (10 nmol/L) and specific binding of 10 nmol/L  $^{125}\text{I}$ -LK (—◆—) in the presence of 1 mmol/L calcium or 5 nmol/L  $^{125}\text{I}$ -HC in the absence (—●—) or presence (—■—) of 1 mmol/L calcium were determined. HUVEC were also incubated with BK (100 nmol/L) for the times incubated and specific binding of 5 nmol/L  $^{125}\text{I}$ -HC was determined (—▲—). The data presented are the mean  $\pm$  SEM of three experiments.

The third conclusion from these studies is that the kininogen binding site induced by PMA differs from that found on unstimulated or BK-stimulated HUVEC. Specifically, binding of LK and HK to HUVEC stimulated by BK does not require added calcium (Fig 2), and PMA stimulated the binding of HK (and its purified LC) in the absence of calcium (Fig 8). PMA stimulated the binding of LK and the HC of HK only in the presence of added calcium (Figs 9 and 10). There are two possible explanations for these results. One possibility is that PMA stimulates intracellular pathways in addition to PKC that modify the kininogen receptor. This may generate or expose a calcium-modulated domain on the receptor itself, or a novel protein that recognizes a region common to LK and HC and a calcium-independent domain to which the LC of HK binds. It is also possible that calcium may alter the conformation of LK or isolated HC through its interaction with domain 1, which facilitates their interaction with the modified binding site induced by PMA.<sup>30</sup> Clarification of the two hypotheses must await the isolation of the receptor(s) for the kininogens.

Assuming that BK induces expression of HK/LK receptors *in vivo*, it is likely that these receptors would be saturated with ligand at plasma concentrations of reactants. Induction of HK/LK receptors on the cell surface could initiate a positive feedback system that would lead to the production of additional BK by the kallikreins at sites of inflammation. However, because binding of kininogens to HUVEC has the same effect as binding to platelets, induction of additional receptors may serve to retard the liberation of additional

BK<sup>10,11</sup> and thus limit its effects on blood pressure, inflammation, permeability, and pain. Additional experiments are required to determine how the additional kininogen receptors modulate the production of BK as well as other known effects of kininogens.<sup>5,6,31-33</sup>

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