

Cyclic AMP selectively enhances bradykinin receptor synthesis and expression in cultured arterial smooth muscle. Inhibition of angiotensin II and vasopressin response.

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Research Article

Bradykinin receptors on vascular smooth muscle may play an important role in regulating the endogenous effects of the vascular kallikrein-kinin system. The present study examined the effect of cyclic nucleotides on bradykinin-stimulated responses in cultured arterial smooth muscle cells. Short term stimulation (1 min) with cyclic AMP produced a variable inhibition of bradykinin-stimulated calcium mobilization which was lost in later passaged cells. However, long-term stimulation (24 h) produced a consistent increase in bradykinin-stimulated calcium mobilization in both early and late passaged cells. Further analysis demonstrated that chronic exposure to cAMP produced a twofold increase in both the number of cell surface bradykinin receptors and in bradykinin-stimulated phosphoinositide hydrolysis. The increase in bradykinin receptors was time dependent (> 7 h) and blocked by protein synthesis inhibitors, suggesting that cAMP enhanced the synthesis of new bradykinin receptors. The increase in bradykinin receptor binding and calcium mobilization was also stimulated by cholera toxin, forskolin, and isobutylmethylxanthine, but not isoproterenol or prostaglandin E₂. Of considerable interest, prolonged exposure to cAMP inhibited both angiotensin II and arginine vasopressin-stimulated phosphoinositide hydrolysis and intracellular calcium mobilization. In summary, prolonged treatment with cAMP selectively stimulates the synthesis and expression of bradykinin receptors on arterial smooth muscle while decreasing the responsiveness to vasoconstrictor agonists such as angiotensin II and vasopressin.

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Cyclic AMP Selectively Enhances Bradykinin Receptor Synthesis and Expression in Cultured Arterial Smooth Muscle

Inhibition of Angiotensin II and Vasopressin Response

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Abstract

Bradykinin receptors on vascular smooth muscle may play an important role in regulating the endogenous effects of the vascular kallikrein-kinin system. The present study examined the effect of cyclic nucleotides on bradykinin-stimulated responses in cultured arterial smooth muscle cells. Short term stimulation (1 min) with cyclic AMP produced a variable inhibition of bradykinin-stimulated calcium mobilization which was lost in later passaged cells. However, long-term stimulation (24 h) produced a consistent increase in bradykinin-stimulated calcium mobilization in both early and late passaged cells. Further analysis demonstrated that chronic exposure to cAMP produced a twofold increase in both the number of cell surface bradykinin receptors and in bradykinin-stimulated phosphoinositide hydrolysis. The increase in bradykinin receptors was time dependent (> 7 h) and blocked by protein synthesis inhibitors, suggesting that cAMP enhanced the synthesis of new bradykinin receptors. The increase in bradykinin receptor binding and calcium mobilization was also stimulated by cholera toxin, forskolin, and isobutylmethylxanthine, but not isoproterenol or prostaglandin E₂. Of considerable interest, prolonged exposure to cAMP inhibited both angiotensin II and arginine vasopressin-stimulated phosphoinositide hydrolysis and intracellular calcium mobilization. In summary, prolonged treatment with cAMP selectively stimulates the synthesis and expression of bradykinin receptors on arterial smooth muscle while decreasing the responsiveness to vasoconstrictor agonists such as angiotensin II and vasopressin. (*J. Clin. Invest.* 1994. 93:2535–2544.) Key words: intracellular calcium • phosphoinositides • vascular kallikrein-kinin system • cyclic nucleotides • rat mesenteric artery

Introduction

Evolving evidence has suggested that an endogenous kallikrein-kinin system, analogous to the tissue renin-angiotensin system is present within the vascular wall (1–3). Kininogenase activity as well as messenger RNA for kallikrein has been found in freshly isolated vascular tissue and the entire system (kininogen, kininogenase, and kininase activity) is localized within smooth muscle cells cultured from the vascular wall (1–3). The kinins which are generated can bind to bradykinin recep-

tors which have been found on both endothelial cells as well as on cultured vascular smooth muscle cells (4–6). Kinin receptors in both cell types are coupled to activation of a phosphoinositide-specific phospholipase C which in endothelial cells leads to increases in intracellular calcium and the release of nitrovasodilators and prostaglandins (4, 6, 7). The tissue level of kinins are further controlled by proteases such as kininase II (angiotensin converting enzyme) and other kininases which are present on endothelial cells and vascular smooth muscle cells (8). Although knowledge of the vascular kallikrein-kinin is relatively new, studies with the recently developed specific bradykinin B₂ receptor antagonists have suggested that this system may be important in a number of pathophysiologic processes involving the vascular system (9, 10). For instance, studies using bradykinin antagonists have suggested that endogenous bradykinin may play a role in both the antihypertensive and the antiproliferative actions of angiotensin converting enzyme inhibitors (9, 10). However, considerable work remains to determine the exact role of the vascular kallikrein-kinin system.

Most of the recent interest in bradykinin has focused on its ability to stimulate the release of vasoactive substances from the endothelium (11). However, there is evidence that bradykinin can also exert endothelium-independent effects on many isolated arteries and veins (12, 13). Depending upon the species of animal and the site of origin of the vessel, bradykinin has been shown to produce both relaxation and contraction in endothelium-denuded vessels (13). This underscores the heterogeneity of possible responses to bradykinin and demonstrates that in addition to its potent endothelial-dependent effects, bradykinin also has direct effects upon the vascular smooth muscle. These vascular smooth muscle effects of bradykinin are likely to be very important to understanding the role of the kinins generated by the endogenous kallikrein-kinin system within the vessel wall.

To better understand the role of the endogenous vascular kallikrein-kinin system, we have analyzed the biochemical effects of bradykinin on arterial smooth muscle cells cultured from rat mesenteric arteries; a vessel which displays both vasodilatation and contraction to bradykinin (14). These studies have demonstrated that bradykinin binds to a B₂ receptor coupled to activation of a phosphoinositide-specific phospholipase C and activation of protein kinase C (5, 6). During the course of these studies it was noted that the response to bradykinin tended to decrease with serial passage and we looked for factors which might be responsible for modulating bradykinin responsiveness. Since the vascular responses to bradykinin are likely to be influenced by endothelial-derived substances which act by modulating cyclic nucleotide levels in vascular smooth muscle, we explored the effects of cyclic nucleotides on bradykinin-stimulated calcium release. Preliminary results demonstrated

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that while short term exposure to cAMP had variable results, long term exposure produced a significant increase in bradykinin-stimulated calcium release (preliminary data were presented at the American Federation for Clinical Research meetings in April 1989 and published in abstract form [1989. *Clin. Res.* 37[2]:259A]. Subsequent results were also presented at the American Society for Cell Biology in December 1990 and published in abstract form [1990. *J. Cell Biol.* 111(5):215a]. This study was designed to further analyze the mechanism whereby cyclic nucleotides regulate bradykinin-stimulated calcium release in cultured arterial smooth muscle cells. The results demonstrate that long term exposure to agents which increase intracellular cAMP stimulated the synthesis of new bradykinin receptors coupled to activation of a phosphoinositide-specific phospholipase C. Moreover, this effect was selective for bradykinin and long term exposure to cAMP produced inhibition of both angiotensin II and arginine vasopressin-stimulated phosphoinositide hydrolysis and calcium release. Overall, the results demonstrate that cAMP can regulate bradykinin receptor synthesis and expression in cultured arterial smooth muscle cells.

Methods

Chemicals. Chemicals were from the following sources: Fura II, Molecular Probes Inc. (Eugene, OR); ionomycin and H-8 (*N*-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide), Calbiochem Corp. (San Diego, CA); [³H]bradykinin (110–121 Ci/mmol), DuPont-NEN (Boston, MA); [³H]myo-inositol (15 Ci/mmol), American Radiolabeled Chemicals (St. Louis, MO) and Du Pont-NEN; Dowex AG 1-×8 (200 mesh) anion exchange resin, Bio Rad Laboratories, (Richmond, CA); bradykinin, tyr⁰bradykinin and arginine vasopressin, Bachem California (Torrance, CA); Cholera toxin, List Biol. Labs. Inc. (Campbell, CA). Other chemicals and cell culture additives including p-8-(4-chlorophenylthio) cyclic AMP and 8-bromo cGMP were the highest grades available from Sigma Chemical Co. (St. Louis, MO).

Cultured arterial smooth muscle cells. Arterial smooth muscle cells were isolated from the mesenteric artery of male Sprague Dawley rats (Sasco Co., Omaha, NE) and grown in culture as previously described (5, 6). Immunofluorescent staining has demonstrated that passages 1–4 (P₁–P₄), uniformly express the smooth muscle specific isoform of actin, α -actin (5). Cultures were grown in minimal essential medium (MEM, containing Earle's salts) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% bovine calf serum (BCS). Studies were performed as soon as the cultures were confluent (5–8 d). All cultures were passaged at least once prior to study. Since a decreased responsiveness to bradykinin was noted with serial passage, only passages 1–4 (P₁–P₄) were used.

Pretreatment of cultures to modulate intracellular cAMP and cGMP. Cyclic AMP, cyclic GMP, cholera toxin, isoproterenol, and nitroprusside were prepared in culture medium and added with the appropriate dilution (usually 1:1,000) directly to the cultures for the indicated time. Forskolin was prepared from 20 mM stock in DMSO and PGE₂ was prepared from a 1 mM stock in ethanol (final carrier concentration \leq 0.1%). The initial studies examining the effects of cAMP on bradykinin-stimulated calcium mobilization were performed using confluent cultures rendered quiescent by incubating for 48 h in medium containing 0.1% BCS (including the period of incubation with the cAMP or other agents). Subsequent studies confirmed that the same results were observed in cells grown in 2% serum. Hence, in all subsequent studies, the cultures were grown in 2% serum for 24–48 h before and during treatment with the experimental agents. For studies of [³H]inositol phosphate release, cultures were loaded with [³H]myo-inositol in 2% BCS for 48 h and cAMP or other experimental agents were added during the last 24 h of this treatment. Treatment with cAMP produced a 10–20% decrease in total cellular protein (pro-

tein per 4-cm² well: control = 129 \pm 14 μ g/well vs. cAMP \times 24 h = 106 \pm 11 μ g/well; *n* = average protein content per well from eight independent experiments) without any apparent change in cellular morphology by light microscopy (Nikon Inc., Melville, NY).

Intracellular calcium. Cultured arterial smooth muscle cells were grown to confluence on glass coverslips and intracellular calcium was measured using the calcium-sensitive fluorescent dye, Fura II as previously described (5). Briefly, confluent cultures were loaded with the permeant acetoxymethyl ester form of Fura II (4 μ M) for 60 min at 37°C, then incubated in physiologic saline solution (PSS)¹ (in mM: NaCl 145, KCl 5.0, MgCl 1.0, CaCl 1.0, glucose 10, Hepes 10, pH 7.5 with Tris base) for at least 20 min before securing along the diagonal of a disposable plastic cuvette containing 2.5 ml of physiological saline solution. Fluorescence emission (Turner spectrofluorimeter) at 496 nm was measured in response to excitation at 340 nm (F₃₄₀) and 380 nm (F₃₈₀) and the intracellular calcium concentration determined using the formula: $[Ca^{2+}] = [(R - R_{min}) / (R_{max} - R)] \times (Sf/Sb) \times 224$, where *R* is the ratio F₃₄₀/F₃₈₀, *R*_{min} and *R*_{max} are the ratios in the absence of calcium and the presence of an excess of calcium, respectively and *Sf/Sb* is determined by the ratio of F₃₈₀ in the absence of calcium to F₃₈₀ in the presence of excess calcium. These parameters were determined experimentally at the end of every experiment by adding ionomycin (10 μ M) to permeabilize the cells to calcium and then determining F₃₄₀ and F₃₈₀ in the presence of excess calcium (10 mM Ca²⁺; *R*_{max}, and *Sb*) and after depletion of intracellular calcium using a Ca²⁺ free bath containing 7.5 mM EGTA (*R*_{min}, and *Sf*). All fluorescent measurements were corrected for autofluorescence using manganese (4 mM Mn²⁺ in the presence of 1% sodium dodecyl sulfate) to quench the Fura II fluorescence.

[³H] Inositol phosphate release. Inositol phosphates were analyzed by the techniques of Berridge et al. (15). Cells grown to confluence in 4-cm² wells were loaded with [³H]myo-inositol (5 μ Ci/well) for 48 h in MEM containing 2% BCS. After loading, the cells were gently washed with PSS (2 \times) to remove unincorporated [³H]inositol and allowed to equilibrate at the assay temperature (26°C) for 15 min. Then the buffer was aspirated and the experiment initiated by adding 1 ml of fresh buffer containing the indicated concentration of agonist for the indicated time before terminating the reaction by scraping the cells into 1 ml of ice cold 20% TCA. The TCA-insoluble material is removed by centrifugation at 3,000 *g* for 10 min and the resulting supernatant is extracted with 10 vol of diethyl ether. The sample is alkalinized by the addition of 2 ml of 50 mM Tris base and then applied to a 0.8 \times 2 cm column of Dowex AG 1-×8 DEAE cellulose (200–400 mesh) and the ³H-labeled compounds sequentially eluted with 40 ml of dH₂O ([³H]-inositol), 40 ml of 5 mM NaBO₄/60 mM sodium formate ([³H]-glycerol phosphatylinositol), 10 ml of 0.1 M NH₄ formate/0.1 mM formic acid ([³H]inositol phosphate (IP)), 10 ml of 0.5 M NH₄ formate/0.1 mM formic acid ([³H]inositol bisphosphate, IP₂) and 10 ml of 1.0 M NH₄ formate/0.1 mM formic acid ([³H]inositol trisphosphate, IP₃ + [³H]inositol tetrakisphosphate, IP₄). IP, IP₂, and IP₃ are then quantitated by liquid scintillation counting (Beckman LS 3801; Beckman Instruments, Inc., Fullerton, CA).

Preparation of [¹²⁵I-Tyr⁰ bradykinin. Tyr⁰ bradykinin (BK) (Bachem) was radioiodinated on the tyrosine using the chloramine-T (iodogen) method under conditions to yield predominantly the monoiodinated derivative (16). The iodination was performed in an appropriate fume hood. Briefly, 3.75 μ g of iodogen dissolved in 75 μ l of CHCl₃ was placed into a 12 \times 75-mm tube and the CHCl₃ removed by drying under N₂. To this tube was added sequentially, 15 μ g of Tyr⁰ BK dissolved in 115 μ l of sodium phosphate buffer, pH 7.5, followed by Na¹²⁵I (1 mCi in 15 μ l of buffer) and the reaction allowed to proceed for 8 min at room temperature. The reaction was stopped by adding 1.5 ml of 4% acetic acid. The tube was washed two additional times with 1.5 ml of acetic acid and all washes were pooled. The solution was applied to a C₁₈ Sep-pak which had been prewashed with methanol and

1. **Abbreviations used in this paper:** AVP, arginine vasopressin; BK, bradykinin; IP, inositol phosphate; PSS, physiologic saline solution.

unreacted Na¹²⁵I removed with 20 ml of 4% acetic acid. The bound peptide was then eluted with 3–5 ml of methanol. The methanol was removed by drying under N₂ and the peptide dissolved in 20 μ l of 0.1% acetic acid. The radiolabeled peptide was purified by reverse phase HPLC (Beckman Instruments, Inc.) using a C₁₈ column (Beckman US ODS) with an acetonitrile gradient in water (containing 0.1% TFA) from 20–50% at a flow rate of 1 ml/min. All fractions were collected in a fraction collector (400 μ l/tube). The mono-¹²⁵I-labeled tracer eluted as a sharp peak (~35% acetonitrile) which was collected and used for the binding studies. The monoiodinated peak was clearly separated from both the unlabeled peptide and the small fraction of diiodinated peptide. The tube containing the peak activity (typically 1–3 \times 10⁸ cpm/400 μ l) was selected and diluted with an equal volume of methanol and stored at –20°C. The ¹²⁵I-Tyr⁰ BK was used as soon as possible after the preparation but never more than 1 mo after the radioiodination.

Bradykinin receptor binding assay. Binding studies were performed using either ¹²⁵I-Tyr⁰ BK (~2,100 Ci/mmol) or commercially available [³H]BK (121 Ci/mmol, DuPont-NEN) with exactly the same results. Cell surface binding was measured in confluent cultures of intact arterial smooth muscle cells grown in either 24-well panels (for binding studies with ¹²⁵I-Tyr⁰ BK) or 12-well panels (for [³H]BK). After treatment with the experimental agents, the cells were washed and allowed to equilibrate in binding buffer containing: 25 mM HEPES, pH 7.0, 140 mM NaCl, 2 mM MgCl₂, 1 mM phenanthroline, 30 μ M captopril (gift from Squibb), 50 μ g/ml bacitracin and 1 mM L-tyrosine (for ¹²⁵I-Tyr⁰ BK, to block nonspecific uptake by amino acid transporters) at 4°C. Binding experiments were performed either by saturation analysis (using a constant ratio of hot:cold ligand of 0.1 for ¹²⁵I-Tyr⁰ BK or 100% hot ligand for [³H]BK) or by displacement (50,000 cpm tracer per well) in either 300 μ l (24-well panel) or 750 μ l (12-well panel) of binding buffer per well and a total ligand concentration initially varying over a wide range from ~10 pM to 15 nM. Nonspecific binding was determined in the presence of 2 μ M unlabeled peptide and was typically 40–50% of total binding, as reported by other investigators (17). Preliminary experiments demonstrated that equilibrium was reached within 8–10 h, so binding was allowed to continue overnight (16 h). Analysis of the supernatant solution by reverse phase HPLC demonstrated <5% degradation of the peptide after incubation with the cultured cells overnight at 4°C. The next morning the buffer was rapidly aspirated and the cells rapidly washed with either 1.25 cc (24-well panel) or 3 ml (12-well panel) of ice cold binding buffer to remove unbound counts. Radioactivity bound to the cell surface was then determined by solubilizing the cells into 1 ml of 0.5 N NaOH and quantitating by either gamma counting (¹²⁵I-Tyr⁰ BK, Micromedic 4/200plus) or scintillation counting ([³H]BK, Beckman LS 3801, Beckman Instr. Inc.). Typically, 50 μ l of this solution was taken before counting to determine protein content per well by the Lowry method. The results are corrected for protein content per well and the data analyzed by a nonlinear curve fitting program ("LIGAND" developed by Peter Munson at the National Institutes of Health) to determine the best model and model parameters (K_{1,i} and R_{1,i}) which fit the observed nontransformed data. In addition to the usual statistical analysis, because of the high degree of negative cooperativity between the receptor affinity (K, in nM⁻¹) and receptor concentration (R, in moles/liter), the statistical significance of any change observed in K and R after treatment with cAMP was further evaluated by looking for overlap between the 95% confidence ellipses generated for each set of data (18).

Statistics. A paired *t* test was used where appropriate. For multiple comparisons the Kruskal-Wallis test was performed, if a significant difference was found (*P* < 0.05), then the Mann-Whitney test was used to further analyze differences between two groups. *P* < 0.05 was considered statistically significant.

Results

Effects of cAMP on BK-stimulated calcium release. The effects of cAMP on bradykinin-stimulated calcium release depended

upon how many times the cultures had been passaged and the length of time of exposure to cAMP (Fig. 1). In early passaged cells (P₁ and occasionally P₂) where the control response to bradykinin was very strong, short term exposure to cAMP caused a marked inhibition of bradykinin-stimulated calcium release. However, this inhibitory effect of cAMP was somewhat variable and was not always present even in early passaged cells. Longer exposure to cAMP for 24 h reversed the inhibition and produced a small increase in bradykinin-stimulated calcium release over control (Fig. 1A). As the cells were passaged they lost bradykinin receptors (see below) and exhibited a decreased calcium response to stimulation by bradykinin (Fig. 1B). In these latter passaged cells, the inhibitory effects of short term exposure to cAMP were no longer observed and now long term exposure to cAMP clearly induced a pronounced increase in bradykinin-stimulated calcium release (Fig. 1B). The effect of cAMP appeared to increase the early peak of bradykinin-

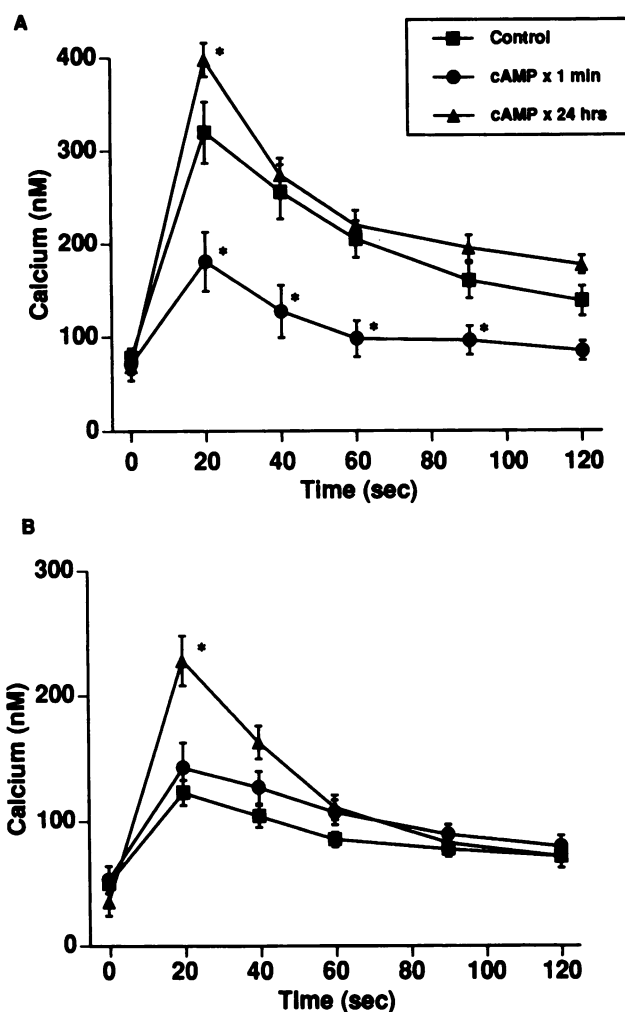


Figure 1. Effect of cyclic AMP on bradykinin-stimulated intracellular calcium mobilization. Confluent quiescent cultures of arterial smooth muscle cells were treated for either 1 min (●) or 24 h (▲) with 0.1 mM p-8-(4-chlorophenylthio) cyclic AMP or vehicle (■) before measuring intracellular calcium mobilization in response to 100 nM bradykinin. The results from early passaged cells (passages P₁ and occasionally P₂) are shown in A and results from later passaged cells are shown in B. The data represents the mean \pm SEM from eight separate coverslips for each treatment group. **P* < 0.05 compared with control.

stimulated calcium release whereas the later sustained plateau phase of intracellular calcium was no different between control and cAMP-treated cells. In some experiments, prolonged treatment with cAMP produced a significant bradykinin response in cells which had no baseline response to bradykinin (data not shown). There appeared to be a limit to this phenomena however because 24 h of exposure to cAMP did not restore responsiveness to bradykinin in cells which had lost all responsiveness to bradykinin for several passages. In the remainder of the study we have focused on the mechanism underlying the long term effects of cAMP on bradykinin-stimulated calcium release.

Effect of chronic cAMP on BK-stimulated phosphoinositide hydrolysis. As we have previously reported, exposure to BK stimulates phosphoinositide hydrolysis in cultured vascular smooth muscle cells leading to the rapid release of IP₃ followed by the more gradual and sustained formation of IP₂ and IP (6). The effect of cAMP on BK-stimulated inositol phosphate formation is shown in Fig. 2. Treatment with 0.1 mM cAMP for 24 h produced a further 2–3-fold increase in BK-stimulated IP₂ release which began at 10 s and continued for up to 60 s. Despite the marked stimulation of IP₂ release, BK-stimulated IP₃ release was variable and not consistently increased by chronic exposure to cAMP (Fig. 2, *top inset*). In addition, BK-stimulated IP release was not significantly affected by chronic exposure to cAMP (Fig. 2, *bottom inset*). The dose response curve for BK-stimulated IP₂ formation was also compared before and after 24 h of treatment with cAMP. As shown in Fig. 3, cAMP

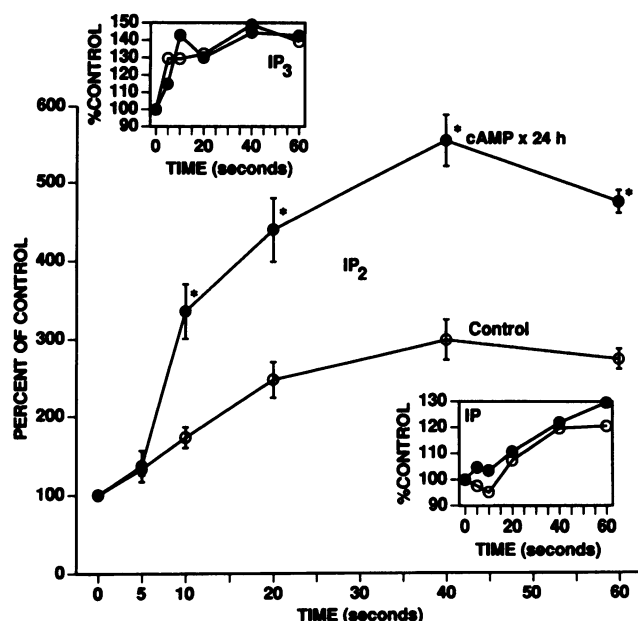


Figure 2. Effect of cyclic AMP on bradykinin-stimulated inositol phosphate formation. Confluent cultures arterial smooth muscle cells were loaded with [³H]myoinositol in 2% serum for 48 h. During the last 24 h the cells were treated with either 0.1 mM *p*-8-(4-chlorophenylthio) cyclic AMP (●) or vehicle (○). The cells were washed and exposed to 100 nM bradykinin for the stated times before measuring [³H]inositol phosphate as described in Methods. Inositol bisphosphate (IP₂) is shown in the main graph, inositol trisphosphate (IP₃) is shown in the top inset and inositol phosphate (IP) is shown in the bottom inset. The results represent the mean±SEM from four independent experiments. **P* < 0.05 compared with control.

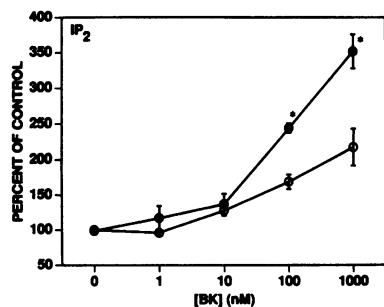


Figure 3. Effect of cyclic AMP on the dose response curve for bradykinin-stimulated IP₂ formation. Confluent cultures of arterial smooth muscle cells were loaded with [³H]myoinositol and treated with either 0.1 mM *p*-8-(4-chlorophenylthio) cyclic AMP (●) or vehicle (○) for 24 h. The cells were washed and exposed to 100 nM bradykinin for 20 s before measuring [³H]inositol bisphosphate as described in Methods. The results represent the mean±SEM of 4–7 measurements in two independent experiments. Similar results were found in four additional experiments done at different incubation times. **P* < 0.05 compared with control.

produced a twofold increase in the maximal effect of BK with no clear difference in the dose response to BK.

Effect of chronic cAMP on BK receptor binding. The effect of cAMP to increase maximal phosphoinositide hydrolysis suggested that cAMP was acting to increase the number of BK receptors coupled to phosphoinositide-specific phospholipase C. Studies were therefore performed to measure cell surface BK receptors in cultured vascular smooth muscle cells before and after treatment with cAMP. Experiments in control cells revealed a single population of cell surface receptors which was best fit by a one site model (Fig. 4, *open symbols*). As shown in Fig. 4 (*closed symbols*), treatment with cAMP for 24 h produced a twofold increase in cell surface BK receptors. The results from eight separate paired experiments, done in cells at different levels of passage are shown in Fig. 5. In control cells the *K*_d was 0.88±0.16 nM, however the *B*_{max} decreased with increased passage from a maximum of 102 fmol/mg in a P₁ passage to a minimum of 3 fmol/mg in a P₄ passage (average *B*_{max} = 38±11 fmol/mg). Chronic treatment with cAMP produced a consistent and statistically significant 116% increase in the number of cell surface BK receptors, regardless of the preexisting number of receptors in the control cells (average *B*_{max} after 24 h treatment with cAMP = 83±15 fmol/mg). The receptor affinity was also slightly decreased (41%, *K*_d = 1.28±0.18 nM) after treatment with cAMP. Since negative cooperativity exists between the determinations of *B*_{max} (i.e., the receptor concentration, *R* in mole/liter) and receptor affinity *K* (in nM⁻¹; a decrease in *K* will produce a dependent increase in *R*), we compared the 95% confidence ellipses generated for *R* and *K* from control and cAMP-treated cells, as shown in Fig. 6 (18). The results confirm that the observed increase in *B*_{max} is statistically significant and independent of the decrease in receptor affinity.

Mechanism of cAMP effect to increase BK receptor binding. To better understand the mechanism by which cAMP stimulated an increase in BK receptor binding we examined the time course and dose-response curve for cAMP-mediated effects. As shown in Fig. 7, treatment with cAMP for up to 7 h had no effect on BK receptor binding. Longer treatment for 24 h stimulated a marked increase in BK receptor binding which remained significantly increased over control values for up to 56 h. The decrease in receptor binding at longer time points was in part a consequence of a generalized decrease in protein content per well, however the cells remained attached and appeared

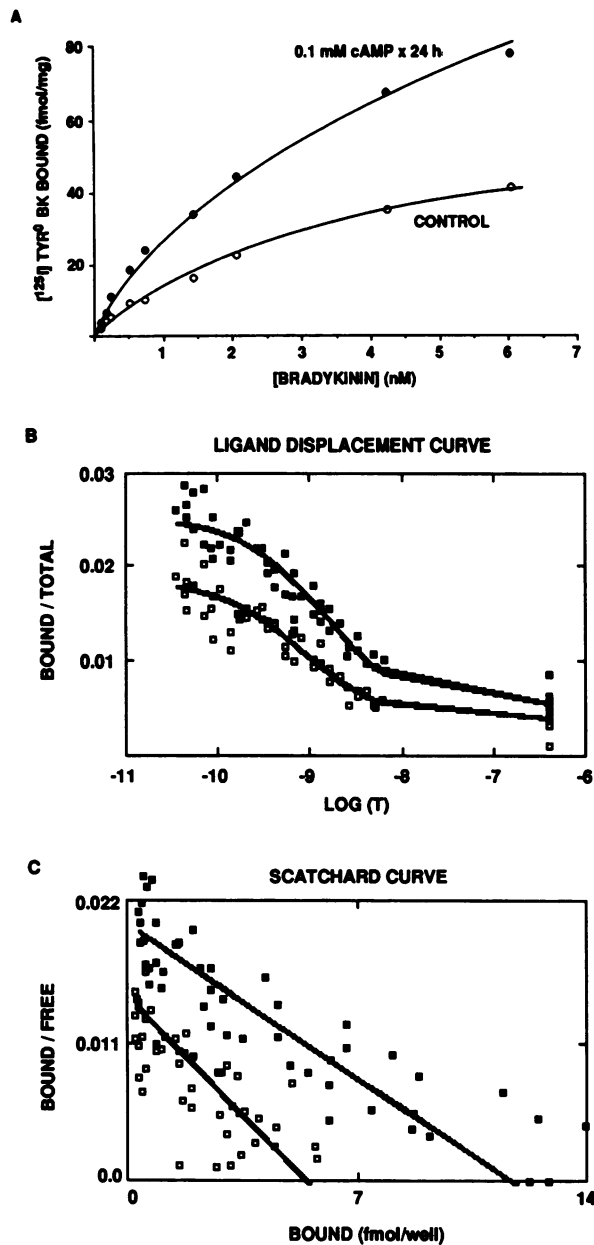


Figure 4. Effect of cyclic AMP on bradykinin receptor binding. Confluent cultures of arterial smooth muscle cells were treated with either 0.1 mM *p*-8-(4-chlorophenylthio) cyclic AMP (closed symbols) or vehicle (open symbols) for 24 h and then cell surface bradykinin receptors were measured as described in Methods. A typical association binding isotherm using ^{125}I -Tyr⁰ bradykinin is shown in *A*. The untransformed data was then analyzed by LIGAND 4.1 and the results from three separate paired experiments are combined and presented as a displacement curve (*B*) and Scatchard curve (*C*). The data was best fit by a model with one independent binding site ($K_d = 0.77$ nM for control and 1.16 nM for cAMP treated) as shown by the solid curve in *B* and *C*. The data in *C* is expressed as fmol bound per well (there was ~ 100 μg protein/well).

viable as assessed morphologically by light microscopy. In separate experiments, an increase in bradykinin receptor binding was seen within 16 h after treatment with cAMP. The dose response curve, shown in Fig. 8 demonstrates that the threshold for stimulation of BK receptor binding with cAMP was seen at 10 μM and maximal at 100 μM . Further increases in

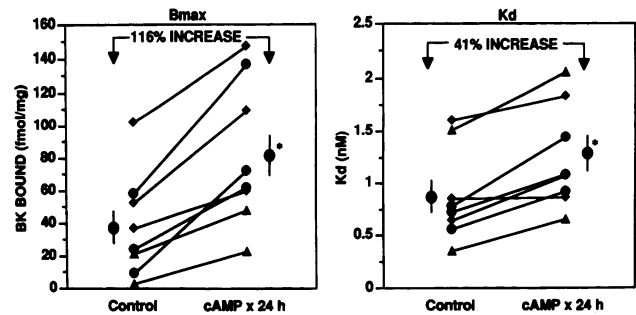


Figure 5. Cyclic AMP enhances bradykinin receptor number in cultured arterial smooth muscle cells. The results for B_{max} and K_d from eight separate paired binding studies which compared treatment with either 0.1 mM cAMP or vehicle (control) for 24 h on cell surface bradykinin binding as described in Fig. 4 are shown. A single line connects the results from one paired experiment. The large closed circles represent the mean \pm SEM of all eight data points for either control or cAMP treated cells as indicated ($*P < 0.05$ compared with control). The results from P_1 cultures are represented as (\diamond), P_2 cultures are represented with (\bullet), and P_3 and P_4 cultures are represented with (\blacktriangle). In addition to the obvious effect of cAMP to increase the number of bradykinin receptors, the figure also shows that there is a decrease in the number of cell surface bradykinin receptors in latter passaged cells.

cAMP may have been toxic and produced a decrease in maximal bradykinin receptor binding.

The time course experiments suggested that cAMP was acting to induce the synthesis of new BK receptors rather than insertion of preformed receptors. Therefore, the effect of the protein synthesis inhibitor, cycloheximide and the transcriptional inhibitor, actinomycin D were examined for their effect on cAMP-stimulated BK receptor binding. As shown in Fig. 9, 24 h of exposure to cAMP produced the expected twofold increase in BK receptor binding. Coincubation with either cycloheximide or actinomycin D caused a small increase in BK receptor binding in control cells and completely inhibited any further increase produced by cAMP. Hence, chronic exposure to cAMP appears to act by increasing the synthesis of new BK receptors in cultured arterial smooth muscle cells.

Effects of various agents which increase cAMP on BK-stimulated calcium release and receptor binding. To further confirm that increases in intracellular cAMP were responsible for stimulating the increase in BK receptor synthesis and calcium release, we examined the effect of various agents which increase

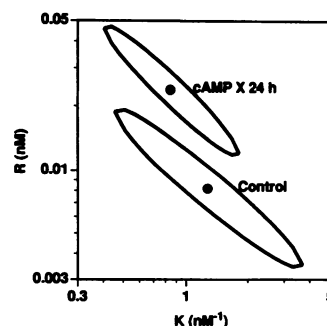


Figure 6. 95% confidence ellipses for bradykinin receptor binding in control and cAMP treated cells. The data from the three separate experiments shown in Fig. 4 were combined and the 95% confidence ellipses calculated and plotted as a function of the receptor number (R , in nM) and affinity (K , in nM^{-1}) as described by Munson and Rodbard (18). The data demonstrates that

cAMP produces a statistically significant ($P < 0.05$) increase in both the B_{max} as well as K_d for the bradykinin receptor on cultured arterial smooth muscle cells.

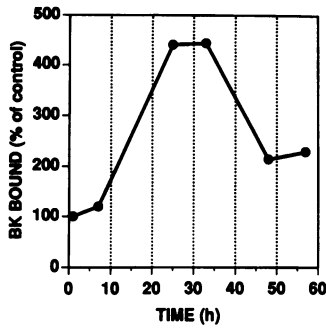


Figure 7. Time course for cAMP stimulation of bradykinin receptor binding. Confluent cultures of arterial smooth muscle cells were treated with 0.1 mM *p*-8-(4-chlorophenylthio) cyclic AMP for the stated times before washing and measuring bradykinin receptor binding as described in Methods. The results represent the data from a typical experiment. In separate experiments it was determined that a significant increase in bradykinin receptor binding could be observed within 16 h of treatment with cAMP.

either cAMP or cGMP in cultured arterial smooth muscle cells. Fig. 10 demonstrates the effect of chronic treatment with these agents on BK-stimulated calcium release. With the exception of the receptor agonist, isoproterenol, which we have previously shown increases intracellular cAMP levels (5), all post-receptor agents which increase cAMP (cAMP, forskolin, and cholera toxin) produced a similar 2–2.5-fold increase in BK-stimulated calcium release. Alternatively, agonists which increase intracellular cGMP (e.g., 8-bromo-cGMP and nitroprusside) had no significant effect on BK-stimulated calcium release. The results were further examined in Table I where we compared the effect of several of these agents to stimulate cAMP levels and enhance BK-receptor binding. Again, treatment with another receptor agonist, PGE₂, produced a small increase in cAMP levels but did not increase BK receptor binding in most experiments. By contrast, chronic treatment with several post-receptor agonists which acted either by stimulating the production of cAMP (forskolin and cholera toxin) or preventing its breakdown (IBMX) produced larger increases in cAMP levels and significant 60–150% increases in BK receptor binding. Overall, the results demonstrate bradykinin receptor expression is increased by multiple agonists which increase cAMP. However, prolonged receptor activation of adenylate cyclase alone may not be sufficient to stimulate significant increases in BK receptor synthesis.

Effect of inhibition of cAMP kinase. To further determine whether the effects of cAMP were mediated by activation of cAMP-dependent kinase (protein kinase A) we studied the effects of the kinase inhibitor, H-8. H-8 is reported to have selectivity for inhibiting cyclic nucleotide-dependent kinases with a *K_i* for cAMP- and cGMP-dependent protein kinase of 0.48 and 1.2 μM, respectively (19). As shown in Table II, both cAMP and cholera toxin increased bradykinin receptor binding, however, this increase was not inhibited by coinubation with either 5 or 20 μM of H-8. In fact, incubation with H-8 alone, produced a numerical increase in bradykinin receptor binding which was not statistically significant. The results suggest that the effects of cAMP and cholera toxin are not mediated via activation of the catalytic subunit of cAMP-dependent kinase. However, the effectiveness of H-8 as an inhibitor of cyclic nucleotide-dependent kinase in rat vascular tissue is unclear, since it has been reported that similar concentrations of H-8 also failed to prevent (and in fact enhanced) cAMP- and cGMP-mediated vasodilatation in the rat aorta (20). Higher doses of H-8 were not employed because specificity for cAMP-dependent kinase could not be assured.

Effect of cAMP on AII and AVP-stimulated calcium release and phosphoinositide hydrolysis. To determine whether the effects of cAMP were specific for the BK signal transduction pathway or represented a more generalized response to cAMP,

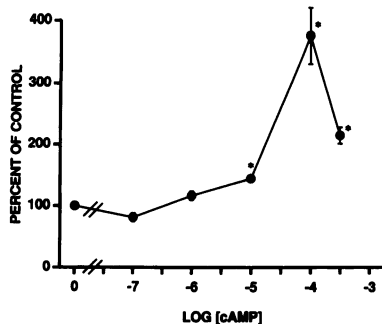


Figure 8. Dose response for cAMP-stimulation of bradykinin receptor binding. Confluent cultures of arterial smooth muscle cells were treated with the indicated concentrations of *p*-8-(4-chlorophenylthio) cyclic AMP for 24 h before measuring bradykinin receptor binding as described in

Methods. The results represent the mean±SEM of four cultures in a single experiment, similar results were found in three separate experiments, but they were not combined because the maximal stimulation varied between 2–4-fold. **P* < 0.05 compared with control.

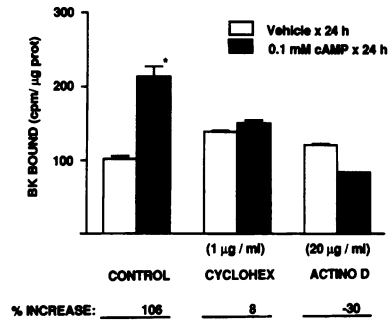


Figure 9. Effect of inhibiting protein synthesis on cAMP-stimulated increase in bradykinin receptor binding. Confluent cultures of arterial smooth muscle cells were treated with either vehicle (open bars) or 0.1 mM *p*-8-(4-chlorophenylthio) cyclic AMP (solid bars) for 24 h.

As indicated, cycloheximide (1 μg/ml) or actinomycin D (20 μg/ml) were added simultaneously with the addition of either vehicle or cAMP. After 24 h the cells were washed and cell surface bradykinin receptors were measured as described in Methods. The results represent the mean±SEM from eight separate cultures. The results were confirmed in separate experiment. **P* < 0.05 compared with vehicle. Cycloheximide also produced a small but significant increase in bradykinin receptor binding in control cells.

Effect of various agonists on bradykinin-stimulated intracellular calcium mobilization. Confluent quiescent cultures of arterial smooth muscle cells were treated with either vehicle, 0.1 mM *p*-8-(4-chlorophenylthio) cyclic AMP (cAMP), 10 μM forskolin (FSK), 1 μg/ml cholera toxin (CT), 0.1 mM isoproterenol (ISP), 0.1 mM 8-bromo cGMP (cGMP), or 1 μM nitroprusside (NP) for 24 h before measuring bradykinin-stimulated intracellular calcium mobilization as described in Methods. The results represent the mean±SEM of the percent increase in peak intracellular calcium compared with vehicle; *n* = 11–33 separate determinations in six different experiments. **P* < 0.05 compared with vehicle alone.

Effect of various agonists on bradykinin-stimulated intracellular calcium mobilization. Confluent quiescent cultures of arterial smooth muscle cells were treated with either vehicle, 0.1 mM *p*-8-(4-chlorophenylthio) cyclic AMP (cAMP), 10 μM forskolin (FSK), 1 μg/ml cholera toxin (CT), 0.1 mM isoproterenol (ISP), 0.1 mM 8-bromo cGMP (cGMP), or 1 μM nitroprusside (NP) for 24 h before measuring bradykinin-stimulated intracellular calcium mobilization as described in Methods. The results represent the mean±SEM of the percent increase in peak intracellular calcium compared with vehicle; *n* = 11–33 separate determinations in six different experiments. **P* < 0.05 compared with vehicle alone.

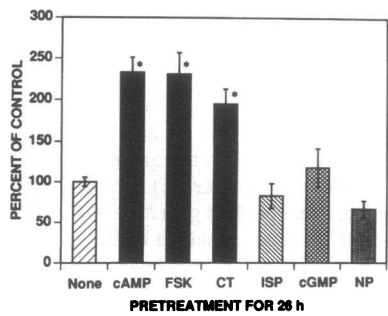


Figure 10. Effect of various agonists on bradykinin-stimulated intracellular calcium mobilization. Confluent quiescent cultures of arterial smooth muscle cells were treated with either vehicle, 0.1 mM *p*-8-(4-chlorophenylthio) cyclic AMP (cAMP), 10 μM forskolin (FSK), 1 μg/ml cholera toxin (CT), 0.1 mM isoproterenol (ISP), 0.1 mM 8-bromo cGMP (cGMP), or 1 μM nitroprusside (NP) for 24 h before measuring bradykinin-stimulated intracellular calcium mobilization as described in Methods. The results represent the mean±SEM of the percent increase in peak intracellular calcium compared with vehicle; *n* = 11–33 separate determinations in six different experiments. **P* < 0.05 compared with vehicle alone.

Table I. Effect of Agonists which Increase Cyclic AMP on Bradykinin Receptor Binding

Agent	Concentration	cAMP <i>fmol/well</i>	Bradykinin binding	
			Specific binding <i>cpm</i>	% of Control
Control		49.7±4.5 (12)	36.7±2.5 (42)	
cAMP	0.1 mM	ND	58.6±4.1 (8)*	160
PGE ₂	100 nM	144±11.6 (12)*	35.4±3.7 (8)	97
Cholera Toxin	1 µg/ml	5120±347 (6)*	91.8±8.1 (8)*	250
Forskolin	20 µM	337±60.5 (6)*	58.8±6.2 (12)*	160
IBMX	0.5 mM	474±120 (12)*	68.0±8.1 (8)*	185

Confluent cultures of vascular smooth muscle cells were incubated with the indicated concentration of agonist for 24 h at 37°C. The medium was removed to measure cAMP production as described in Methods. The cells were washed and bradykinin receptor binding measured at 4°C as described in Methods. Total binding (TB) was measured in the presence of 0.625 nM [³H]BK and nonspecific binding (NSB) in the presence of 2 µM unlabeled BK. Specific binding is calculated from the difference between TB and NSB measured in cpm. The results are expressed as mean±SEM. The sample number, *n*, given in parentheses, refers to the number of independent wells assayed in one experiment. The results for bradykinin binding were confirmed in four separate experiments. Protein content was ~ 100 µg/well. * *P* < 0.05 compared with control.

we examined the effect of chronic exposure to cAMP on AII and AVP-stimulated calcium and phosphoinositide release. As shown in Fig. 11, treatment of the cultured cells for 24 h with cAMP produced an increase in BK-stimulated calcium release but an opposite, inhibitory effect on both AII- and AVP-stimulated calcium release. The mechanism of the inhibitory effect of cAMP was further analyzed by measuring angiotensin II- and vasopressin-stimulated phosphoinositide hydrolysis. As demonstrated in Fig. 12, prolonged exposure to cAMP significantly enhanced BK-stimulated IP₂ release but inhibited both AII and AVP-stimulated IP₂ release. Similar results were seen for measurement of IP₃, however the results did not reach statistical significance (data not shown). The results demonstrate that prolonged exposure to cAMP selectively enhanced BK receptor synthesis and expression in arterial smooth muscle cells while inhibiting the effects of other vasoactive hormone receptors coupled to phosphoinositide-specific phospholipase C.

Discussion

The role of the recently discovered vascular kallikrein-kinin system is uncertain. However, studies using selective bradykinin antagonists have suggested that the vascular kallikrein-kinin system may play a role in both the antihypertensive and antiproliferative actions of angiotensin converting enzyme inhibitors (9, 10). These antihypertensive and antiproliferative effects of bradykinin on vascular smooth muscle are presumed to be due to the indirect action of bradykinin to release vasoactive substances from the endothelium (10, 11). However, in addition to the indirect endothelial effects of bradykinin, there are also direct effects of bradykinin upon vascular smooth muscle cells (5, 6, 12, 13). Since bradykinin is likely produced and

Table II. Effect of the cAMP Kinase Inhibitor, H-8 on Cholera Toxin- and cAMP-stimulated Increase in Bradykinin Receptor Binding

	5 µM		20 µM	
	Vehicle	H-8	Vehicle	H-8
	<i>cpm</i>			
Cholera toxin				
Control	108±19 (8)	183±21 (16)	307±14 (8)	331±24 (12)
Cholera toxin	222±15* (8)	297±14* (16)	892±21* (8)	946±40* (12)
Absolute Δ (cpm)	114±17	121±25	584±20	615±31
Percent Δ (%)	106	66	190	186
Cyclic AMP				
Control	ND	ND	175±35 (7)	203±27 (16)
PCT cAMP	ND	ND	283±35* (8)	314±29* (16)
Absolute Δ (cpm)	—	—	87±21	110±38
Percent Δ (%)	—	—	50	54

Confluent cultures grown in 12-well panels were placed on media containing 2% bovine calf serum. Each 12-well panel received either vehicle (water) or the indicated concentration of H-8 (5 or 20 µM). After 5 min, half of the wells on each plate (six wells) were additionally treated with either vehicle (control) or an agent to increase intracellular cAMP levels. The table shows the effects of cholera toxin (1 µg/ml) and the effects of p-8-(4-chlorophenylthio) cyclic AMP (0.1 mM). Incubation was continued for 24 h and cell surface bradykinin receptor binding was measured as described in Methods. For these studies total binding was measured in the presence of 0.41 nM [³H]bradykinin (4 of the 6 wells) and nonspecific binding by the addition of 2 µM unlabeled bradykinin (2 of the 6 wells). The results are reported as specific binding in cpm and the data expressed as mean±SEM. The total number of individual wells assayed is given in parentheses. ND, not done. * *P* < 0.01 for the effect of cholera toxin or cAMP compared with control by *t* test.

released by vascular smooth muscle cells within the vessel wall, then the ultimate effects of the vascular kallikrein-kinin system will involve an interplay between the indirect (paracrine) effects of bradykinin to release vasoactive mediators from endothelial cells and the direct (autocrine) effects on vascular smooth muscle. The direct effects of bradykinin on vascular

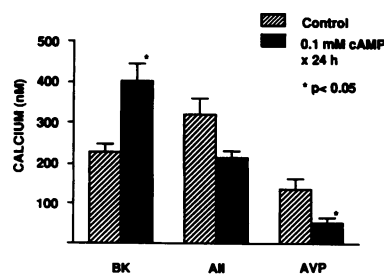


Figure 11. Differential effects of long-term exposure to cAMP on hormone-stimulated intracellular calcium mobilization. Confluent cultures of arterial smooth muscle cells were treated with either vehicle (□) or 0.1 mM p-8-(4-chlorophenylthio) cyclic AMP (■) for 24 h before measuring the peak intracellular calcium response to either 100 nM bradykinin (BK), 100 nM angiotensin II (AII), or 100 nM arginine vasopressin (AVP). The results represent the mean±SEM of eight separate determinations for each hormone. * *P* < 0.05 compared with control.

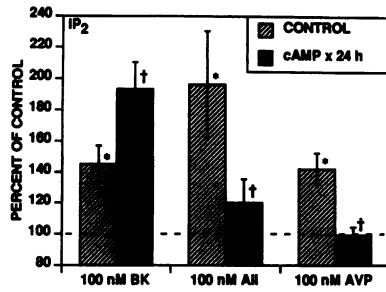


Figure 12. Differential effects of long-term exposure to cAMP on hormone-stimulated inositol phosphate formation. Confluent cultures of arterial smooth muscle cells were loaded with [³H]myo-inositol in 2% serum for 48 h. During the last 24 h the cells were treated with

either 0.1 mM *p*-8-(4-chlorophenylthio) cyclic AMP (■) or vehicle (control, ▨). The cells were then washed and exposed to either 100 nM bradykinin (BK), 100 nM angiotensin II (AII), or 100 nM arginine vasopressin (AVP) for 20 s and [³H]inositol phosphates measured as described in Methods. The data for inositol bisphosphate (IP₂) is shown as mean±SEM for 8–12 independent measurements in four separate experiments. **P* < 0.05 comparing hormone-stimulated vehicle-treated control cells with unstimulated control cells (represented by the dotted line, control data had SEM of ±4%), †*P* < 0.05 comparing cAMP-treated cells to vehicle-treated cells for each hormone.

smooth muscle cells may be synergistic, antagonistic, or completely independent of the effects mediated by endothelial cells. Therefore to better understand the role of the vascular kallikrein-kinin system, it is important to understand what regulates the expression of bradykinin receptors on vascular smooth muscle cells and what transmembrane signaling pathways are activated by these receptors.

The present study examined the effects of cyclic nucleotides on bradykinin-stimulated calcium release in cultured arterial smooth muscle cells. While short term treatment with cyclic nucleotides had a variable inhibitory effect on bradykinin-stimulated calcium release that disappeared completely as the cells were passaged, long-term exposure to cAMP caused a reproducible increase in bradykinin-stimulated calcium release. Further examination of the mechanism of this effect revealed that cAMP stimulated the synthesis and expression of new cell surface bradykinin receptors coupled to the activation of a phosphoinositide-specific phospholipase C and release of intracellular calcium. The stimulatory effect of cAMP was selective for the bradykinin-stimulated transmembrane signaling pathway and cAMP actually inhibited the effects of the polypeptide vasoconstrictor hormones, angiotensin II and AVP. The physiological effects of these observations are not yet clear. We have recently found that both bradykinin and angiotensin II receptors are coupled to a similar transmembrane signaling pathway leading to activation of phosphoinositide-specific phospholipase C (6). Although differences were noted in the ability of bradykinin and angiotensin II to translocate protein kinase C to the membrane, both hormones were able to activate protein kinase C, as measured by a similar increase in the phosphorylation of MARCKS, a major intracellular substrate for protein kinase C (6). This suggests that the direct effects of bradykinin on vascular smooth muscle may be similar to those produced by angiotensin II. Recent studies in the balloon-denuded carotid artery model of vascular damage seem to confirm this hypothesis (10). While increases in bradykinin were shown to be beneficial to the antiproliferative effects of angiotensin converting enzyme inhibitors, probably by increasing the endothelial release of nitric oxide (10). The direct effects of bradykinin

on vascular smooth muscle appeared to be similar to those of angiotensin II, since both the angiotensin II receptor antagonist, losartan as well as the bradykinin receptor antagonist, HOE 140 decreased neointimal proliferation (10). The overall effect of cAMP can not be predicted however, since the effects of cAMP on bradykinin receptor expression in endothelial cells is not yet known. Taken together, these observations suggest that prolonged increases in cyclic AMP could modulate the responsiveness of vascular smooth muscle cells by decreasing the responsiveness to known vasoconstrictor hormones, angiotensin II, and vasopressin while increasing the responsiveness to bradykinin. However, the ultimate effect of this shift in hormone responsiveness on the overall physiologic responses of the blood vessel remains to be explored.

Cyclic nucleotides are known to play a central role in modulating the responsiveness of vascular smooth muscle to vasoconstrictor agonists (21). Although cAMP may exert a direct inhibitory effect on the contractile proteins (e.g., possibly by phosphorylation of MLCK) most evidence suggests that cyclic nucleotides act primarily by inhibiting vasoconstrictor-induced increases in intracellular calcium (22, 23). Cyclic nucleotides can decrease hormone-stimulated increases in intracellular calcium via the following mechanisms: (a) by activating calcium pumps located on the sarcolemma and sarcoplasmic reticulum leading to a decrease in both basal and hormone-stimulated intracellular calcium (24, 25); (b) by inhibiting receptor-mediated activation of phospholipase C, possibly via phosphorylation of a small molecular weight G protein which couples the receptor to phospholipase C (26, 27); and (c) possibly by inhibiting the IP₃-mediated release of calcium from intracellular stores (28). Our results in early passaged cells are also compatible with an inhibitory effect of cAMP on bradykinin-stimulated calcium release. However, this effect was variable and rapidly lost with further cell passage. Of interest, recent studies by Lincoln et al. (23) have suggested that the inhibitory effects of cAMP on agonist-induced increases in intracellular calcium may be mediated via activation of cGMP-dependent kinase, which was lost during the passage of vascular smooth muscle cells in culture. Loss of cGMP-dependent kinase or other factors might explain the variability we observed in the acute inhibitory effects of cAMP in our early passaged cells. In addition, since there was no inhibitory effect of cAMP (or cGMP, data not shown) in later passaged cells, the loss of cGMP kinase or other factors may be more rapid and complete in arterial smooth muscle cells cultured from the mesenteric artery than those traditionally obtained from rat aorta. Finally, it is of interest to note that the effects of cAMP are not always inhibitory on hormone-stimulated calcium mobilization. In particular, short term increases in cAMP have been shown to enhance bradykinin-stimulated calcium mobilization and nitric oxide release in cultured porcine endothelial cells (29). These observations suggest that the acute effects of cAMP may differentially enhance the hormone-stimulated release of vasodilatory substances from the endothelium while inhibiting the effects of calcium-mobilizing vasoconstrictor agonists on the vascular smooth muscle.

The results of this study suggest that after prolonged exposure to cAMP, there is a dissociation between the increase in bradykinin receptor binding and bradykinin-stimulated calcium release in early passage cells. Several factors may account for this observation. First, as discussed above, cAMP may be exerting an inhibitory effect on bradykinin-stimulated calcium

mobilization that may be present only in early passaged cells. In addition to the inhibitory mechanisms discussed above, we also noted that prolonged treatment with cAMP increased bradykinin-stimulated IP₂ formation without measurable increases in IP₃ formation. This might suggest that chronic cAMP has additional effects to increase the dephosphorylation of IP₃ to IP₂ or alter the metabolism of phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol phosphate (PIP) by phospholipase C. Although cAMP can affect some of the enzymes involved in inositol phosphate metabolism, these particular effects of cAMP on inositol phosphate metabolism have not yet been reported (30). An alternative possibility is that early passage cells already have maximal bradykinin-stimulated calcium release and further increases in receptor number or phosphoinositide hydrolysis will not elicit any further release of intracellular calcium. Using image analysis to study the intracellular calcium response in individual cells, we have observed that in early passaged cells virtually all cells respond with large increases in intracellular calcium suggesting that the presence of additional receptors coupled to phosphoinositide turnover may not serve to significantly further enhance the release of calcium from intracellular stores (6). However, in later passaged cells there are fewer cells responding to bradykinin and the response in each individual cell is lower. In these cells prolonged treatment with cAMP appears to cause an increase in both the number of cells responding to bradykinin and in the maximal response within each cell (B. S. Dixon and R. V. Sharma, unpublished observations). Further studies will be necessary to determine if it is the increase in cAMP or saturation of calcium release mechanisms (or both) which produces the dissociation between increased bradykinin receptor expression and bradykinin-stimulated calcium release in early passages of cultured arterial smooth muscle cells.

Much less is known about the chronic effects of cyclic nucleotides on vascular responsiveness. In particular, there have been very few studies of the long term effects of cyclic nucleotides on hormone receptor expression in vascular smooth muscle cells. Colluci reported that 24 h of incubation with cAMP inhibited the synthesis of α_1 -adrenergic receptors in rabbit aortic smooth muscle cells (31). Although norepinephrine-stimulated calcium efflux was not inhibited, the effect of prolonged exposure to cyclic nucleotides on norepinephrine-stimulated phosphoinositide hydrolysis and intracellular calcium release were not determined (31). Studies in other cell types have demonstrated that prolonged exposure to cAMP can inhibit the synthesis of polypeptide hormones linked to activation of phosphoinositide hydrolysis (32, 33). In particular, Guillon et al. (33) have shown that chronic treatment with agents that elevate intracellular cAMP produced a 50–60% decrease in both vasopressin and angiotensin II receptors in rat granulosa cells. The decrease in receptors was paralleled by a similar decrease in both vasopressin and angiotensin II-stimulated phosphoinositide hydrolysis (33). These results are compatible with our observations and suggest that cyclic nucleotides may inhibit the synthesis of angiotensin II and vasopressin receptors in vascular smooth muscle as well. Overall, these results suggest that cAMP may inhibit the synthesis of receptors for several of the known vasoconstrictor hormones while stimulating the synthesis of bradykinin receptors on vascular smooth muscle cells.

The mechanisms whereby cAMP regulates gene transcription are under active investigation (34). One well described mechanism involves the phosphorylation and activation of a

transcription factor (CREB) which binds to the cAMP response element (CRE, 5'-TGACGTCA). This *cis*-acting element has been shown to be present in a number of genes which are regulated by cAMP (34). In addition, for some genes, cAMP also appears to act via a different *cis*-acting element known as the AP-2 (activator protein-2) binding site (34). Neither of these cAMP responsive elements have been identified in the recently reported sequence for the rat bradykinin receptor (35). However, further cloning and analysis of the regulatory regions of the bradykinin receptor will clearly be necessary before a firm conclusion regarding the presence or absence of the CRE or AP-2 can be reached. In addition to the rapid regulation of gene transcription via modification of preformed DNA binding proteins, cAMP may also regulate gene expression via a slower mechanism which requires preceding protein synthesis and can be blocked by protein synthesis inhibitors (34). Further studies of the effects of cycloheximide on mRNA for the bradykinin receptor will have to be performed to differentiate between these two possibilities. Although some authors have suggested that the regulatory subunit of cyclic AMP-dependent kinase might be involved in the regulation of gene transcription, most of the available evidence suggests that cAMP regulates gene transcription via activation of phosphorylation by the catalytic subunit of cyclic AMP-dependent kinase (34). However, in the present study, simultaneous treatment with the cyclic nucleotide-dependent protein kinase inhibitor, H-8 failed to prevent the increase in bradykinin receptor expression produced by either cAMP or cholera toxin. While these results are compatible with a putative role for the regulatory subunit of cAMP-dependent kinase, a more likely explanation is that H-8 failed to inhibit cAMP-dependent kinase in our vascular tissue. A similar phenomena in which H-8 was unable to inhibit cyclic nucleotide-mediated vasodilatation has also been reported for rat aorta (20). Finally, it is noted in passing, that bradykinin receptor synthesis has also been shown to be stimulated several fold in *ras*-transformed fibroblasts (36). However, since *ras*-proteins do not appear to activate adenylate cyclase or produce significant increases in cAMP they presumably stimulate bradykinin receptor synthesis by a mechanism different than that observed in the present study (37). Clearly additional studies will be necessary to determine the mechanism whereby cAMP stimulates bradykinin receptor synthesis and to evaluate the significance of these observations to interactions between the endothelium and arterial smooth muscle *in vivo*.

In summary, prolonged increases in cAMP stimulate the synthesis and expression of new cell surface bradykinin receptors coupled to activation of phosphoinositide hydrolysis and mobilization of intracellular calcium in cultured arterial smooth muscle cells. In distinct contrast, prolonged increases in cAMP significantly inhibit both angiotensin II- and vasopressin-stimulated phosphoinositide hydrolysis and calcium mobilization. These studies, suggest that prolonged increases in cAMP may shift the biologic responsiveness of vascular smooth muscle away from known vasoconstrictor hormones towards a cellular program regulated by bradykinin. However, the physiologic effects of these observations is not yet clear. Although there may be subtle differences in the transmembrane signaling pathways activated by bradykinin and angiotensin II, the overall effects of these hormones to increase intracellular calcium and activate protein kinase C appear to be the same. These studies may have important implications to mech-

anisms whereby cyclic nucleotides regulate vascular contractility and proliferation. Moreover, they begin to provide a clearer understanding of the factors regulating the expression and function of bradykinin receptors on vascular smooth muscle cells.

Acknowledgments

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