Arginine-vasopressin (AVP), angiotensin II (AII), and norepinephrine (NE) are known to stimulate prostaglandin (PG) synthesis in the intact rat kidney perfused with Tyrode's solution by a mechanism that requires intracellular Ca\(^{2+}\), while PG synthesis elicited by bradykinin (BK) is independent of Ca\(^{2+}\). To elucidate further the differences in the mechanism of action of BK and other vasoactive agents, in this preparation we have investigated the effect of 1 mM caffeine, an agent known to interfere with the uptake and storage of Ca\(^{2+}\) in intracellular sites, on renal output of PGE\(_2\) and 6-keto-PGF\(_{1\alpha}\), elicited by AVP, AII, NE, and BK; 2) various combinations of the maximal doses of BK, AII, AVP, and NE on renal PG synthesis; and 3) RHC 80267, an inhibitor of diglyceride and monoglyceride lipase, on the output of PGs produced by these vasoactive agents. Infusion of 1 mM caffeine inhibited PG output elicited by AVP, AII, and NE but not that caused by BK in the absence of extracellular Ca\(^{2+}\). Combined administration of maximal doses of BK (2.8 nmol) with that of AII (0.28 nmol), AVP (0.27 nmol), or NE (3.2 nmol) but not AVP and AII, NE and AVP, or NE and AII produced an additive effect on renal PG output in the presence or absence of Ca\(^{2+}\). The renal vasoconstrictor effect of AVP, AII, and NE produced in the presence of Ca\(^{2+}\) was not additive and remained unaltered when given together with BK. Administration of 12 \(\mu\)M RHC 80267 attenuated BK-induced renal PG output in concentrations that did not alter the effect of either AVP, AII, or NE to increase PG output or renal vasoconstriction in the presence of Ca\(^{2+}\). These data suggest that BK stimulates renal PG synthesis by a mechanism that is different from that of other vasoactive agents and probably involves activation of a distinct lipase that releases arachidonic acid from diglycerides or monoglycerides derived from phospholipids or triglycerides. (Circulation Research 1987;60:914–922)
additive. To determine if BK and other vasoactive agents stimulate release of arachidonic acid for renal PG synthesis by causing activation of a distinct or common lipase, the effect of a reported inhibitor of diglyceride and monoglyceride lipase (1,6-di(0-carbamoyl)cyclohexane oxime)hexane on renal PG output elicited by this peptide and other vasoactive agents was examined.

Materials and Methods
Male Sprague-Dawley rats, weighing 325—400 g, were anesthetized with ether, the abdomen was opened by a midline incision, and the left kidney was exposed. The aorta was ligated proximal and distal to the left renal artery, and a small catheter was inserted into the artery. The kidney was flushed with 100 U/ml heparinized saline, removed from the animal, and immediately transferred to a perfusion chamber. Kidneys were perfused at a constant flow rate of 5 ml/min with Tyrode’s solution that was maintained at 37° C and was perfused at a constant flow rate of 5 ml/min as previously described. The renal perfusate was allowed to flow out of the cut ends of the renal vein and ureter, and the perfusion fluid was not recirculated. The composition of Tyrode’s solution in mmol/l was NaCl 137, KCl 2.7, CaCl2 1.8, MgCl2 1.1, NaHCO3 12, NaH2PO4 0.42, and D(+)-glucose 5.6. Perfusion pressure was measured with a Statham P23 ID pressure transducer (Statham Co., Huto Rey, Puerto Rico) and recorded on an Esterline Angus physiograph (Indianapolis, Ind.). Since the flow rate remained constant, changes in perfusion pressure reflect alterations in renal vascular resistance.

Experiment Protocol
PROTOCOL 1. This series of experiments was performed to determine the effect of BK, AVP, All, NE, and arachidonic acid (AA) before, during, and after the infusion of caffeine in a final concentration of 1 mM in kidneys perfused with Ca2+-free Tyrode’s solution containing ethylene glycerol bis(β-aminoethyl ether)N,N,N'-tetraacetic acid (EGTA) (23 μM). In preliminary experiments, this concentration of caffeine did not affect the renal vasoconstrictor response to NE, All, and AVP or PG output elicited by exogenous AA in kidneys perfused with Tyrode’s solution containing Ca2+ (1.8 mM). Although 10 mM caffeine has been used in vascular smooth muscle to promote Ca2+ efflux,46 in our study no difference was found between 1 mM and 10 mM caffeine on PG synthesis. The 1 mM concentration was chosen to avoid nonspecific actions on PG synthesis due to reduction in the vasoconstrictor response to vasoactive agents by higher concentrations of caffeine. Following an 80-minute stabilization period, 2.8 nmol BK, 0.27 nmol AVP, 0.28 nmol All, 3.2 nmol NE, 33 nmol AA, or their vehicle were injected into the renal arterial circuit as a bolus in a volume of 0.1 ml at 15-minute intervals in random order. Samples of the renal perfusate were collected for 2-minute periods immediately prior to and following the administration of vasoactive agents and were frozen for later PG analysis.

PROTOCOL 2. The purpose of this series of experiments was to determine the effects of the following combinations of vasoactive substances on renal PG output and vascular tone in kidneys perfused with Ca2+-containing and Ca2+-free Tyrode’s solution with and without EGTA (23 M): 2.8 nmol BK plus 0.27 nmol AVP, 2.8 nmol BK plus 0.28 nmol All, 2.8 nmol BK plus 3.2 nmol NE, All plus AVP, All plus NE, and AVP plus NE. Following an appropriate stabilization period (35 minutes in the presence of Ca2+ and 90 minutes in the absence of Ca2+), 2.8 nmol BK, 0.27 nmol AVP, 0.28 nmol All, 3.2 nmol NE, or their various combinations or their vehicles were administered as a bolus in a volume of 0.1 ml at 15-minute intervals in random order. Samples of renal perfusate were collected for PG analysis as described in Protocol 1.

PROTOCOL 3. The purpose of this series of experiments was to examine the effect of a diglyceride and monoglyceride lipase inhibitor RHC 80267 (12 μM) on PG output and changes in perfusion pressure with or without Ca2+-containing Tyrode’s solution and 0.27 nmol AVP, 0.28 nmol All, 3.2 nmol NE, 2.8 nmol BK, or 33 nmol AA administered as a bolus at 15-minute intervals in random order. Renal perfusate samples for PG analysis were collected as described above. The concentration of RHC 80267 (12 μM) that was selected from the preliminary experiments did not alter the vasoconstrictor response to NE, All, and AVP or AA-induced PG output in kidneys perfused with Ca2+ (1.8 mM).

Drugs. Drugs that were purchased were norepinephrine bitartrate (Levophed, Winthrop Laboratories, Inc., New York), angiotensin II (Beckman Instruments, Inc.), bradykinin diacetate (Protein Research Foundation, Minoh-shi, Japan), Argen 1-vasopressin (Bachem Inc., Torrance, Calif.), arachidonic acid (Nu Chek Prep. Inc., Elyssian, Minn.), caffeine, and EGTA (Sigma Chemical Co., St. Louis Mo.). Drugs given as gifts were unlabelled prostaglandins (Upjohn Co., Kalamazoo, Mich.), PGE2 and 6-keto-PGF1α antiserum (Dr. Charles Leftler, University of Tennessee, Department of Physiology), and RHC 80267 (Revlon Co., New York).

NE, All, BK, and AVP were dissolved in 1 mg/ml saline, stored in small aliquots at −20° C, and diluted with saline just before use. Arachidonic acid was dissolved in (20 mg/ml) absolute ethanol divided into 5-μl aliquots, and stored at −20° C in small vials protected from light with aluminum foil. The contents of the vials were diluted with 0.1 ml Na2CO3 (1 mg/ml) and 0.9 ml of 0.15 NaCl just prior to use. RHC 80267 was dissolved in dimethyl sulfoxide (2 mg/ml) and diluted with Tyrode’s solution for infusion into the renal arterial circuit. Caffeine was dissolved in a small volume of Tyrode’s solution and added to the perfusion fluid to obtain the final concentration. NE, All, BK, AVP, and various combinations and AA were injected in a volume of 0.1 ml into the renal arterial
The concentration of caffeine (1 mM) and RHC 80267 (12 μM) used in our studies represents the final concentration in the perfusion medium.

**Analysis of Data.** The basal and the increase in prostaglandin output elicited by various agents is expressed as ng/min of immunoreactive PGE₂ and 6-keto-PGF₁α. The basal output is the amount of PG in the samples collected for 2-minute periods prior to drug administration. The increase in the output of PGs caused by a drug was calculated by subtracting the basal efflux of PGs from that obtained during 2-minute periods following injection of a drug. Results are expressed as mean ± SEM. Data were analyzed by one-way analysis of variance and paired Student's *t* test to determine the difference between means. The difference between means were considered significant if the probability (*p*) of the null hypothesis being true was less than 0.05.

**Results**

**Effect of Caffeine on Actions of AVP, All, NE, BK, and AA on Output of PGs in Kidneys Perfused With Ca²⁺-Free Tyrode's Solution**

The basal perfusion pressure remained steady (63 ± 4 mm Hg, *n* = 8) for 3–4 hours in kidneys perfused with Ca²⁺-containing and Ca²⁺-free Tyrode's solution (see Figure 1). During perfusion, the wet weight of the kidney, which averaged 1.6 g, was unaltered. In kidneys perfused with Ca²⁺-free Tyrode's solution, infusion of caffeine (1 mM) inhibited the effect of AVP, All, and NE, but not that of BK, to enhance the output of PGE₂ and 6-keto-PGF₁α (Figure 1). Perfusion of kidneys with caffeine-free Tyrode's solution restored PG output elicited by AVP, All, and NE. Administration of AA into the kidney increased the output of PGE₂ from 1.31 ± 0.3 ng/min to 7.36 ± 0.4 ng/min and that of 6-keto-PGF₁α from 0.98 ± 0.2 ng/min to 6.92 ± 0.3 ng/min; this increase in PG output was not altered during caffeine infusion. Caffeine did not alter the basal PG output or basal perfusion pressure (62 ± 6 mm Hg, *n* = 9). Infusion of caffeine vehicle did not alter PG output elicited by the vasoactive substances.

**Effect of BK, AVP, All, and NE Alone and in Various Combinations on Renal PG Output and Vascular Tone in Presence and Absence of Extracellular Ca²⁺**

In kidneys perfused with Ca²⁺-free Tyrode's solution, the basal output of PGs was not altered during the time course of the experiment (see Figures 2–6). Administration of BK, AVP, All, or NE in the isolated rat kidney enhanced the output of both PGE₂ and 6-keto-PGF₁α in a dose-related manner; AVP, All, and NE, but not BK, increased perfusion pressure in a dose-dependent fashion.1,25 The time course of PG output and the changes in perfusion pressure produced by these vasoactive agents have been reported in the latter publications. The effect of maximal doses of BK and other vasoactive substances alone and in various combinations on renal PG output and vascular tone in the presence and absence of extracellular Ca²⁺ in the iso-
Effect of BK and AVP. Renal arterial administration of AVP or BK enhanced the output of PGE$_2$ and 6-keto-PGF$_{1α}$ in both the presence and absence of Ca$^{2+}$ (see Figure 2). AVP, but not BK, increased the renal perfusion pressure in the presence of Ca$^{2+}$. Combined administration of BK and AVP in maximal doses enhanced renal PG output that was approximately equal to the sum of that produced by either peptide alone. The rise in perfusion pressure produced by this combination was not significantly different from that elicited by AVP alone. Combined administration of another vasoconstrictor peptide, AII, and BK also produced an additive effect on the renal output of PGE$_2$ and 6-keto-PGF$_{1α}$.

Effect of NE and AVP. Combined administration of NE and AVP did not produce additive effects on either PG synthesis or perfusion pressure in the presence or absence of Ca$^{2+}$ (see Figure 5).

Effect of NE and AII. Renal arterial administration of the combination of NE and AII did not produce additive effects on PG output or perfusion pressure in the presence or absence of Ca$^{2+}$ (see Figure 6).

The effect of BK and other vasoactive substances alone or in various combinations on renal output of PGE$_2$ and 6-keto-PGF$_{1α}$ in the absence of Ca$^{2+}$ was not altered by the addition of EGTA (23 μM) into the perfusion fluid; the basal output of PGs was also not altered by EGTA. The small increase in perfusion pressure response to AVP and NE obtained in the absence of Ca$^{2+}$ was abolished by the addition of EGTA to the perfusion fluid (data not shown).

Effect of RHC 80267 on Actions of AVP, AII, NE, BK, and AA on Output of PGs in Kidneys Perfused With and Without Ca$^{2+}$-Containing Solution

Infusion of RHC 80267 (12 μM) inhibited BK-induced output of both PGE$_2$ and 6-keto-PGF$_{1α}$ but failed to alter the output of PGs or the renal vasoconstrictor effect of AVP, AII, or NE in kidneys perfused with Ca$^{2+}$-containing Tyrode's solution (see Figure 7). In kidneys perfused with Ca$^{2+}$-free Tyrode's solution, RHC 80267 also inhibited BK-induced PG output without altering the output of PGs elicited by other vasoactive agents. The basal as well as arachidonic acid-induced output of PGs was not altered by RHC.
Discussion

BK stimulates PG synthesis in renal medullary slices and in aortic and pulmonary endothelial cells in culture by a mechanism dependent on extracellular Ca\(^{2+}\). However, in the isolated rabbit perfused ear artery, the ability of BK to increase PGE\(_2\) output was not altered by omission of Ca\(^{2+}\) from the medium and was reduced only by addition of large amounts of EGTA in the absence of extracellular Ca\(^{2+}\). Our recent finding that in the absence of extracellular Ca\(^{2+}\), calmodulin inhibitors, and intracellular Ca\(^{2+}\) antagonists attenuated AVP, All, and NE, but not BK-induced renal PG output, in the isolated rat kidney suggests that BK stimulates renal PG synthesis by a mechanism independent of Ca\(^{2+}\) and calmodulin. The present study, which was performed to elucidate further the mechanism of action of BK on PG output in the isolated rat kidney perfused with Tyrode's solution, provides additional evidence that the action of this peptide on renal PG synthesis is different from that of other vasoactive agents and probably is due to activation of a distinct lipase. This conclusion is based on our demonstration that in the absence of extracellular Ca\(^{2+}\), caffeine, which is reported to enhance Ca\(^{2+}\) efflux from the vascular smooth muscle and increase release of Ca\(^{2+}\) from and inhibit its reuptake into sarcoplasmic reticulum, the major organelle regulating cytosolic Ca\(^{2+}\), attenuated renal PG output elicited by AVP, All, and NE, but not that caused by BK.

That proposal that bradykinin stimulates renal PG synthesis in the intact perfused kidney by a mechanism that is different from other vasoactive agents is also supported by our observations that the combined administration of maximal doses of BK with that of All, AVP, or NE increased renal output of PGs that were approximately equal to the sum of that produced by separate administration of these agents. The increase in PG output elicited by combined administration of BK with All, AVP, or NE does not appear to be due to alterations in the action of AVP, All, or NE by the kinin because it did not effect the renal vasoconstrictor action of these agents. Moreover, combined administration of AVP and All, NE and AVP, or NE and All failed to produce an additive effect on renal PG output or vascular tone. The additive effect of BK with other vasoactive agents together with the lack of the inhibitory effect of caffeine on BK-induced PG synthesis and the reported lack of requirement for Ca\(^{2+}\) and calmodulin by BK but not by other vasoactive agents to stimulate PG synthesis in the intact kidney could be due to differences in the response of various renal cellular structures to BK and other vasoactive hormones. Although this possibility cannot be excluded totally, it appears to be less likely for the following reasons. First, BK stimulates PG synthesis in all renal
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FIGURE 4. Effect of 0.27 nmol AVP, 0.28 nmol AII, and the combination of AVP plus AII on the output of PGE$_2$ (upper panel) and 6-K-PGF$_{1alpha}$ (middle panel) and on changes in perfusion pressure (bottom panel) in kidneys perfused with Ca$^{2+}$-containing (left side) and Ca$^{2+}$-free (right side) Tyrode's solution. *, values significantly greater than basal (p<0.05).

FIGURE 5. Effect of 3.2 nmol NE, 0.27 nmol AVP, and the combination of NE and AVP on the renal output of PGE$_2$ (upper panel) and 6-K-PGF$_{1alpha}$ (middle panel) and on changes in perfusion pressure (bottom panel) in kidneys perfused with Ca$^{2+}$-containing (left side) and Ca$^{2+}$-free (right side) Tyrode's solution. *, values significantly greater than basal (p<0.05).

cellular structures that have been reported to produce PGs in response to other vasoactive agents.\textsuperscript{5-11,36,37} Second, in the present study, the profile of PGs released from the kidney in response to BK was similar to that produced by other vasoactive agents. Regardless of the site and the type of cells involved in PG synthesis in response to BK, the experimental evidence obtained in this study suggests that the mechanism by which BK releases AA from tissue lipids for PG synthesis is different and might involve activation of a distinct lipase. Arachidonic acid may be released from tissue lipids by activation of phospholipase A$_2$ \textsuperscript{38} or phospholipase C through the formation of diglyceride and subsequent deacylation by diglyceride or monoglyceride lipase\textsuperscript{39,40}.
Figure 6. Effect of 3.2 nmol NE, 0.28 nmol All, and the combination of NE and All on the output of PGE2 (upper panel) and 6-K-PGF1α (middle panel) and on changes in perfusion pressure (bottom panel) in kidneys perfused with Ca2⁺-containing and Ca2⁺-free Tyrode's solution. * values significantly greater than basal (p<0.05).

or through phosphorylation to phosphatidic acid, which in turn is deacylated by a phosphatidic acid-specific phospholipase A2. Since renal medulla triglycerides are rich in AA, they may also serve as a source of AA. Although the mechanism by which vasoactive agents release AA from tissue lipids in the kidney has not yet been established, several studies indicate that BK, All, AVP, or NE stimulate phospholipase A2 activity, as indicated by reduced PG synthesis or AA release by inhibitors of phospholipase A2. The demonstration that these vasoactive agents stimulate breakdown of polyphosphoinositides and generation of diglycerides, which is associated with release of AA or PG synthesis in several cell types, including renal cellular structures, indicates that phospholipase C may also be involved in the action of these agents on renal PG synthesis. That AVP, All, NE, and BK stimulate release of AA by causing activation of phospholipase A2 and/or phospholipase C in the intact rat kidney was suggested by our demonstration that mepacrine, an inhibitor of phospholipase A2 and phospholipase C, attenuated PG output elicited by these agents. However, the above findings together with our demonstration that RHC 80267, a reported inhibitor of diglyceride and monoglyceride lipase, attenuated BK-induced PG output in concentrations that did not alter either the renal vasoconstrictor response or PG output elicited by AVP, All, and NE supports our contention that BK stimulates release of AA by causing activation of a distinct lipase. Whether diglycerides or monoglycerides that might serve as the source of AA in response to BK originate from phospholipids or triglycerides remains to be determined. Although RHC 80267 has been reported to exhibit nonspecific effects, including inhibition of cyclooxygenase activity in other tissues, in the present study, this agent did not alter the basal or the AA-induced output of PGE2 and 6-keto-PGF1α in concentrations that inhibited BK-induced PG output. The concentration of RHC 80267 used by these investigators was much higher (25 μM or higher) than that employed in the present study (12 μM). Since BK in our study did not alter the vascular tone, it is not known whether the inhibitory effect of RHC 80267 on BK-induced renal PG output was due to alterations in the action of the kinin at its receptor sites and/or at some postreceptor event leading to activation of the lipase involved in release of arachidonic acid from tissue lipids. Our preliminary experiments on the isolated rat uterus indicate that concentrations of RHC 80267 that inhibited renal PG synthesis elicited by BK did not alter the effect of this peptide to produce contraction of the uterus (C.L. Cooper and K.U. Malik, unpublished work).

In conclusion, this study demonstrates that BK stimulates renal PG synthesis in the intact rat kidney by a mechanism that is different from that of AVP, All, and NE.
NE, and the stimulation probably is due to activation of a distinct lipase involved in release of AA from tissue lipids for renal PG synthesis.

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