Tyrosine Kinase and Mitogen-Activated Protein Kinase/Extracellularly Regulated Kinase Differentially Regulate Intracellular Calcium Concentration Responses to Angiotensin II/III and Bradykinin in Rat Cortical Thick Ascending Limb

Annette Hus-Cittharel, Xavier Iturrio, Pierre Corvol, Jeannine Marchetti, and Catherine Llorens-Cortes

Institut National de la Santé et de la Recherche Médicale Unité 691 (A.H.-C., X.I., C.L.-C.) and Unité 36 (P.C.), Collège de France, 75231 Paris, France; and Institut National de la Santé et de la Recherche Médicale Unité 367 (J.M.), 75005 Paris, France

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The cortical thick ascending limb (CTAL) contains only one morphological cell type but is the target site of multiple hormonal controls (1). This segment expresses two key G protein-coupled receptors (GPCR): angiotensin (Ang) II/Ang III receptor type 1A (AT1A-R) and bradykinin (BK) receptor type 2 (B2-R). In several cell types, these two receptors share the same signaling pathways, although their physiological functions are often opposite. In CTAL, little is known about the intracellular transduction events leading to the final physiological response induced by these two peptides. We investigated and compared in this segment the action of Ang II/III and BK on intracellular calcium concentration ([Ca2+]i) response and metabolic CO2 production, an index of Na+ transport, by using inhibitors of protein kinase C (bisindolylmaleimide), Src tyrosine kinase (herbimycin A and PP2), and MAPK/ERK (PD98059 and UO126). Ang II/III and BK (10^-7 mol/liter) released Ca2+ from the same intracellular pools but activated different Ca2+ entry pathways. Ang II/III- and BK-induced [Ca2+]i increases were similarly potentiated by bisindolylmaleimide. Herbimycin A and PP2 decreased similarly the [Ca2+]i responses induced by Ang II/III and BK. In contrast, PD98059 and UO126 affected the effects of BK to a larger extent than those of Ang II/III. Especially, the Ca2+ influx induced by BK was more strongly inhibited than that induced by Ang II/III in the presence of both compounds. The Na+ transport was inhibited by BK and stimulated by Ang II/III. The inhibitory action of BK on Na+ transport was blocked by UO126, whereas the stimulatory response of Ang II/III was potentiated by UO126 but blocked by bisindolylmaleimide. These data suggest that the inhibitory effect of BK on Na+ transport seems to be directly mediated by an increase in Ca2+ influx dependent on MAPK/ERK pathway activation. In contrast, the stimulatory effect of Ang II/III on Na+ transport is more complex and involves PKC and MAPK/ERK pathways.


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stimulate rapid protein tyrosine phosphorylation (9–11). Similarly, in vascular endothelial cells, the stimulation of inositol 1,4,5-triphosphate production and calcium signaling by BK is dependent on tyrosine phosphorylation (12).

It has been suggested that MAPK cascades have an important role in the regulation of renal function in all rat nephron segments (13). Naidu et al. (14) showed that in vascular smooth muscle cells, both Ang II and BK significantly increase MAPK phosphorylation. In vascular smooth muscle cells from spontaneously hypertensive rats, Ang II-stimulated [Ca\(^{2+}\)] responses are significantly reduced by the selective MAPK kinase (MEK) inhibitor PD98059 (15).

Although AT\(_{1A}\)-R and B\(_2\)-R are thought to share signaling pathways, the physiological roles of Ang II and BK are often reversed. Recent data show that balance between the kaliuretic-kinin and renin-angiotensin systems is essential for normal renal function. Indeed, in low-kaliuretic rats, activity of Ang II appears to be responsible for increased glomerular hydrostatic pressure and augmented tubular reabsorption (16). More accurately, in the medullary thick ascending limb of the loop of Henle, high Ang II concentrations stimulated the Na\(^{-}/K\(^{+}/2Cl\(^{-}\) cotransport activity (17) whereas in this same segment, the B\(_2\)-R mediated inhibition of NaCl reabsorption (18). The search for putative differences in intracellular signaling events mediated by AT\(_{1A}\)-R and B\(_2\)-R in the CTAL, which could be responsible for the opposite biological responses of Ang II and BK, was still not undertaken. In an attempt to answer to this question, we have evaluated the action of Ang II and BK on Na\(^{+}\) transport in freshly microdissected CTAL, as estimated by measuring metabolic CO\(_2\) production and explored the respective roles of PKC, TK, and MAPK/ERK in Ang II/III- and BK-induced [Ca\(^{2+}\)] responses.

**Materials and Methods**

**Animals**

All procedures involving animals were carried out in accordance with institutional guidelines for the care and use of laboratory animals. Male Sprague-Dawley rats weighing 130–180 g were used. They were fed a normal standard diet and offered water *ad libitum*.

**Microdissection of nephron segments**

Rats were anesthetized by ip injection of pentobarbital. The left kidney was prepared for nephron microdissection by infusion of basal medium supplemented with 0.3% collagenase (Serva, Heidelberg, Germany) was prepared for nephron microdissection by infusion of basal medium containing 2 mmol/liter CaCl\(_2\) (2 Ca\(^{2+}\)) at 30 C for 15 min. Single pieces of CTAL and proximal convoluted tubule were perfused with 0.1% collagenase in basal medium through which filtered air was bubbled, and explored the respective roles of PKC, TK, and MAPK/ERK in Ang II/III- and BK-induced [Ca\(^{2+}\)] responses.

**Western blotting**

Microdissected PCT (20 mm) and CTAL (10- or 20-mm) segments were solubilized in sample buffer 2× [300 mm Tris-HCl (pH 6.8), 10% SDS, 13% glycerol, 20 mg/ml dithiothreitol, and bromophenol blue]. Proteins were resolved by 10% SDS-PAGE. ERK protein and phosphorylated ERK were detected, with an anti-p44/42 MAPK rabbit antibody (Cell Signaling, Beverly, MA). α-Tubulin was detected with an anti-α-tubulin mouse antibody (Sigma-Aldrich, St. Louis, MO). The immune complex was detected with an antiimmunoglobulin antibody coupled to horse-radish peroxidase by enhanced chemiluminescence (Amersham, Piscataway, NJ). Densitometric analysis of p42 ERK phosphorylation and tubulin mouse antibody (Sigma-Aldrich, St. Louis, MO). The immune complex was detected with an antiimmunoglobulin antibody coupled to horseradish peroxidase by enhanced chemiluminescence (Amersham, Piscataway, NJ). Densitometric analysis of p42 ERK phosphorylation and α-tubulin blots were performed with Image J (NIH software). The values were expressed in arbitrary units.

**Measurement of metabolic CO\(_2\) production by CTAL**

We measured the rate of metabolic CO\(_2\) production from a uniformly \(^{14}\)C-labeled substrate by CTAL as previously described (19). Briefly, CTAL was transferred with 0.5 μl incubation buffer onto a small disc of dry BSA coating the hollow of a glass slide. The samples were then sealed with a glass coverslip, photographed, and kept at 4 C. Incubation was initiated by adding another 0.5 μl incubation buffer containing [\(^{14}\)C]lactate and the MEK inhibitor U0126 or BIM when necessary. The samples were sealed with a glass coverslip containing a 2-μl droplet of KOH and incubated for 60 min at 37 C. Finally, the KOH droplet
containing the metabolic $^{14}\text{CO}_2$ trapped was transferred to a counting vial. The results are expressed as femtomoles CO$_2$ formed per millimeter of tubule per minute of incubation.

**Statistical analysis**

All the results are mean values of replicate samples ± SEM. Statistical differences were assessed using Student’s $t$ test for unpaired data.

**Results**

**Characterization of the $[\text{Ca}^{2+}]_i$ response to BK**

BK ($10^{-7}$ mol/liter) elicited a rapid increase in $[\text{Ca}^{2+}]_i$ equal to $398 \pm 26$ nmol/liter, this value being reached $23 \pm 1$ sec after agonist application. Then $[\text{Ca}^{2+}]_i$ decreased rapidly and returned to a new basal level that was slightly higher ($92 \pm 8$ nmol/liter) but not significantly different from the initial value ($72 \pm 6$ nmol/liter) (Fig. 1A). The effect of BK was totally abolished by HOE-140, a specific B$_2$-R antagonist (22) (Fig. 1B). This abolition of the calcium response was not a result of a loss of cell viability, because the subsequent application of Ang III induced normal $[\text{Ca}^{2+}]_i$ responses ($202 \pm 26$ nmol/liter). BK had a dose-dependent effect on calcium mobilization (Fig. 1C). The increase in calcium mobilization was maximal with $10^{-7}$ mol/liter BK, and the half-maximal response was obtained at $2.32 \times 10^{-8}$ mol/liter.

Previously reported $[\text{Ca}^{2+}]_i$ responses to Ang II (23) and to Ang III (2) are listed in Table 1. We found that the magnitudes of the peak responses induced by BK in the presence or absence of external calcium were greater than those elicited by Ang II and Ang III. However, the integrals of the Ca$^{2+}$ signals obtained with Ang II, Ang III, and BK did not differ significantly, because the duration of the response to BK was shorter than that to Ang II or Ang III (in minutes, $3.16 \pm 0.24$ vs. $4.13 \pm 0.31$, $P < 0.05$ for Ang II, and $4.33 \pm 0.34$, $P < 0.01$, for Ang III). Thus, in most experiments, the integrals of the Ca$^{2+}$ signals were used to compare Ang II/III and BK effects. Because Ang II and Ang III induced similar $[\text{Ca}^{2+}]_i$ responses, only one of these agonists was tested in some experimental sets.

**Additivity of $[\text{Ca}^{2+}]_i$ responses to Ang II/III and BK**

We have previously shown that CTAL cells respond to vasoactive peptides such as Ang II with increases in intra-
cellular free calcium resulting from Ca\textsuperscript{2+} release from intracellular storage sites and entry across the cell plasma membrane (23). Here, we investigated whether [Ca\textsuperscript{2+}] responses to Ang II/III and BK involved different Ca\textsuperscript{2+} entry pathways and/or intracellular Ca\textsuperscript{2+} pools by simultaneously applying 10^{-7} mol/liter Ang II and BK (Fig. 2). In the presence of external calcium, the simultaneous application of maximal doses of Ang II and BK (Fig. 2A) gave a response stronger than either of the responses obtained with the agonists used alone but slightly lower (~20%; P \textless 0.05) than the sum of the [Ca\textsuperscript{2+}] responses caused by each of the agonists (theoretical additivity). In the absence of external calcium, the simultaneous application of Ang II and BK evoked [Ca\textsuperscript{2+}] increases that were not significantly different from those obtained with either agonist applied alone (Fig. 2B). We calculated Ca\textsuperscript{2+} influx and found that when Ang II and BK were applied simultaneously, the increase in [Ca\textsuperscript{2+}] caused by calcium influx (12,656 \pm 1,278 nmol/sec/liter) was equal to the theoretical sum of those caused by each agonist applied alone (11,418 \pm 1,149 nmol/sec/liter). Similar results were obtained with Ang III and BK (data not shown).

Lack of effect of voltage-operated channel blocker nifedipine

Ca\textsuperscript{2+} influx through the Ca\textsuperscript{2+} entry pathway mediated by either Ang III or BK was insensitive to L-type Ca\textsuperscript{2+} channel blockers, e.g. 1 \mu mol/liter nifedipine (15,414 \pm 1,249 nmol/sec/liter vs. controls, 16,052 \pm 1,485 nmol/sec/liter, and 14,503 \pm 948 nmol/sec/liter vs. controls, 13,660 \pm 1,236 nmol/sec/liter, respectively).

Effects of store-operated channel (SOC) blocker SKF96365

In the absence of involvement of L-type Ca\textsuperscript{2+} channel mediated by either Ang II/III or BK in CTAL, we have used

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<td></td>
<td>AUC (nmol/sec/liter)</td>
<td>Δ[Ca\textsuperscript{2+}] \textsubscript{i} (nmol/liter)</td>
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<tr>
<td>Ang II</td>
<td>18923 ± 1149</td>
<td>266 ± 14</td>
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<tr>
<td>Ang III</td>
<td>16635 ± 809</td>
<td>240 ± 23</td>
</tr>
<tr>
<td>BK</td>
<td>19206 ± 1066</td>
<td>398 ± 26</td>
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The results are expressed as the mean ± SEM. AUC is the integral of the Ca\textsuperscript{2+} signal, and Δ[Ca\textsuperscript{2+}]\textsubscript{i} is the variation in [Ca\textsuperscript{2+}]\textsubscript{i} expressed as the peak increase in [Ca\textsuperscript{2+}]\textsubscript{i} above basal values. n indicates the number of separate measurements.

* P < 0.01 when compared with values in the presence of external calcium.

** P < 0.01 when compared with values obtained with Ang II and Ang III.

A in the presence of external calcium  B in the absence of external calcium

Fig. 2. Effects of simultaneous applications of 10^{-7} mol/liter Ang II and BK on [Ca\textsuperscript{2+}] responses in the presence (A) or absence (B) of external calcium in CTAL. Each bar represents the AUC calculated for five to 14 determinations. Exp., Experimental additivity; Theo., theoretical additivity. ***, P < 0.0001; *, P < 0.05.
the inhibitor of SOC entry, SK96365. Data obtained were compared with [Ca\(^{2+}\)]\textsubscript{i}, responses induced by Ang II and BK in the absence of external calcium and are summarized on the Table 2. Results showed clearly that the presence of 30 μmol/liter SK96365 significantly inhibited integrals of the Ca\(^{2+}\) signal elicited either by Ang II or BK compared with that obtained in the presence of external calcium and reached a similar level to the calcium responses obtained in the absence of external calcium.

**Effects of selective inhibitors of intracellular signaling pathways on [Ca\(^{2+}\)]\textsubscript{i}, responses to Ang II/III and BK**

**Effect of the PKC inhibitor BIM.** As previously described for Ang II (3), 1 μmol/liter BIM, a specific PKC inhibitor (24), markedly potentiated the [Ca\(^{2+}\)]\textsubscript{i}, responses to 10^{-7} mol/liter Ang II (420 ± 50 nmol/liter vs. controls, 251 ± 30 nmol/liter; P < 0.01) and BK (620 ± 57 nmol/liter vs. controls, 395 ± 27 nmol/liter; P < 0.01).

**Effects of tyrosine kinase inhibitors herbimycin A and PP2 and of EGF receptor kinase inhibitor AG1478.** Herbimycin A, a specific TK inhibitor (25), decreased peak [Ca\(^{2+}\)]\textsubscript{i}, responses to Ang II and BK by about 60% (Fig. 3, A and B). Herbimycin A also inhibited [Ca\(^{2+}\)]\textsubscript{i}, responses to Ang III (76 ± 9%). This inhibitor caused a small but significant increase in basal calcium levels. We carried out experiments in the absence of external calcium to determine whether TK influenced intracellular Ca\(^{2+}\) release rather than Ca\(^{2+}\) influx. The inhibition of intracellular Ca\(^{2+}\) release (Fig. 4A) by herbimycin A was more important for Ang II and Ang III (86 ± 4 and 75 ± 6%, P < 0.01, respectively) than that induced for BK (38 ± 13%). In contrast, the inhibition of Ca\(^{2+}\) influx by herbimycin A (Fig. 4B) was more marked for BK (87 ± 5%) than for Ang II (52 ± 6%, P < 0.01) and Ang III (64 ± 7%, P < 0.05). To specify the nature of the tyrosine kinase(s) involved, we first tested a more selective inhibitor of the Src-family tyrosine kinases, PP2. A significant inhibition of Ang II- and BK-induced [Ca\(^{2+}\)]\textsubscript{i}, responses was evidenced (Fig. 5), suggesting that Src signaling is involved in Ang II/III and BK action in CTAL. The degree of inhibition induced by PP2 was not significantly different from that obtained with herbimycin A. Then we examined the involvement of the epidermal growth factor receptor (EGFR) in Ang II- and BK-induced [Ca\(^{2+}\)]\textsubscript{i}, responses by using the selective EGFR kinase inhibitor AG1478. Results obtained indicate that AG1478 was without effect on the [Ca\(^{2+}\)]\textsubscript{i}, responses elicited by Ang II and BK (174 ± 16 nmol/liter vs. controls, 185 ± 21 nmol/liter, and 366 ± 51 nmol/liter vs. controls, 373 ± 33 nmol/liter, respectively).

**Effects of MEK inhibitors PD98059 and UO126.** We investigated the effects of PD98059, a specific MEK inhibitor (26), on CTAL. PD98059 did not affect basal calcium levels (not shown) but significantly decreased [Ca\(^{2+}\)]\textsubscript{i}, responses to Ang II (146 ± 41 nmol/liter vs. controls, 250 ± 24 nmol/liter; P < 0.05). Ang III (142 ± 12 nmol/liter vs. controls, 208 ± 14 nmol/liter; P < 0.01) and BK (192 ± 40 nmol/liter vs. controls, 537 ± 62 nmol/liter; P < 0.01). The highest level of inhibition was observed for BK. This observation was confirmed by calculations based on the AUC, with inhibition also more marked for BK (73 ± 5%) than for Ang II (45 ± 12%) and Ang III (38 ± 7%). We investigated whether PD98059 inhibited intracellular Ca\(^{2+}\) mobilization and external Ca\(^{2+}\) influx differently by performing additional experiments in the absence of extracellular calcium (Fig. 6). PD98059 inhibited intracellular Ca\(^{2+}\) release (Fig. 6A) to a similar extent for all the peptides considered (Ang II, 56 ± 6%; Ang III, 48 ± 9%; and BK, 44 ± 5%), whereas it clearly affected the Ca\(^{2+}\) influx (Fig. 6B) induced by BK (85 ± 2%, P < 0.01) to a greater extent than that induced by Ang II (30 ± 8%, not significant) and Ang III (27 ± 9%, not significant). To ensure the differences observed between Ca\(^{2+}\) responses induced by Ang II/III and BK after MAPK inhibition, the effects of another specific MEK inhibitor, UO126 (27), were evaluated at various concentrations. Whatever the concentrations of UO126 used (10–50 μmol/liter), this inhibitor did not affect basal calcium levels (not shown). As described for PD98059, inhibitions of integrals of the Ca\(^{2+}\) signals in the presence of external calcium by 20 μmol/liter UO126 were more marked for BK, 68 ± 7% (Fig. 7B), than for Ang II, 39 ± 6% (Fig. 7A), whereas 10 μmol/liter UO126 did not significantly affect the calcium responses induced by BK and Ang II. Moreover, inhibition of the Ca\(^{2+}\) influx induced by BK was significantly more pronounced than that induced by Ang II for 20 and 50 μmol/liter UO126 (85 ± 7% vs. 52 ± 7%, P < 0.01, and 87 ± 7 vs. 62 ± 8%, P < 0.05, respectively). No major difference was observed between BK and Ang II concerning intracellular Ca\(^{2+}\) release (Fig. 7, C and D). To check whether the decreases in [Ca\(^{2+}\)]\textsubscript{i}, responses induced by these two inhibitors similarly occurred after a shorter pretreatment time, CTAL were preincubated for 15 min in the presence of either inhibitor or vehicle and immediately stimulated by either Ang II or BK. Results obtained clearly showed that the inhibition of [Ca\(^{2+}\)]\textsubscript{i}, responses induced by either PD98059 (Ang II, 61 ± 6%; BK, 59 ± 6%) or UO126 (Ang II, 36 ± 6%; BK, 59 ± 6%) was not significantly different from those obtained with a longer incubation time. Determinations of times necessary to reach the maximal [Ca\(^{2+}\)]\textsubscript{i}, responses were performed after stimulation by both agonists in the presence or in the absence of MEK inhibitors were similar (~27 sec), indicating that the magnitude of [Ca\(^{2+}\)]\textsubscript{i}, increase was weaker in the presence than in the absence of inhibitor. In addition, a more accurate analysis of recordings of [Ca\(^{2+}\)]\textsubscript{i}, responses shows that inhibition induced by either PD98059 or UO126 was already detectable less than 15 sec after the agonist application (data not shown).

### TABLE 2. Effects of SKF96365 on 10^{-7} mol/liter Ang II- and BK-stimulated [Ca\(^{2+}\)]\textsubscript{i}, responses in CTAL

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<th>Ang II</th>
<th>BK</th>
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<tr>
<td>In the presence of external calcium</td>
<td>21,899 ± 3,219 (9)</td>
<td>18,762 ± 2,593 (8)</td>
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<tr>
<td>In the absence of external calcium</td>
<td>6,858 ± 1,060 (6)</td>
<td>4,908 ± 1,193 (6)</td>
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<tr>
<td>In the presence of 30 μmol/liter SKF96365 and external calcium</td>
<td>9,254 ± 569 (6)*</td>
<td>8,987 ± 1,075 (6)*</td>
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The results are expressed as the mean ± SEM. AUC is the integral of the Ca\(^{2+}\) signal measured either in the presence or the absence of external calcium or in the presence of 30 μmol/liter SKF96365 and external calcium. Numbers in parentheses indicate the number of individual determinations.

* P < 0.05 when compared with values in the presence of external calcium.
Then, we investigated whether ERK1 and ERK2 were present in the CTAL by Western blotting with an anti-p44/42 ERK antibody and an anti-phospho-p44/42 ERK antibody. PCT were used as controls because ERK1 and ERK2 have been shown to be present in this segment (28). In both CTAL and PCT (20 mm of tubular length), we obtained two specific bands at 44 and 42 kDa, corresponding to ERK1 and ERK2, respectively, with the strongest expression in the PCT compared with the CTAL (Fig. 8A). Interestingly, the basal levels of phosphorylated p44/42 ERK were higher in CTAL than in PCT (Fig. 8A). To investigate the effects of UO126, Ang II, and BK on the phosphorylation status of ERK, 10 mm of CTAL were microdissected and treated with 20 μmol/liter UO126 and 10^{-7} mol/liter Ang II and BK, and phosphorylated ERK were analyzed by Western blot. Basal phosphorylation of p44/42 ERK is completely blocked in the presence of UO126 (Fig. 8B). Because of the presence of a high phosphorylated p44/42 ERK steady-state level in CTAL, only a moderate additional increase (as corrected by α-tubulin) in the phosphorylation of p42 ERK (Fig. 8C) was observed after treatment with 10^{-7} mol/liter Ang II and BK in our experimental conditions (results expressed in arbitrary units: control, 0.70; Ang II, 1.05; and BK, 1.13).

Effects of combined herbimycin A and PD98059 treatment. The inhibitory effects of herbimycin A and PD98059 on [Ca^{2+}+]_{i} responses to Ang III and BK were not additive (Table 3). The application of these two inhibitors together had an effect of a similar magnitude to that caused by herbimycin A alone.

Furthermore, we investigated the sequence of signaling events involving TK, MAPK/ERK, and PKC by studying the effects of herbimycin A or PD98059 in the presence of BIM. Figure 9 shows that the inhibition of TK elicited by herbimycin A prevented BIM-induced potentiation of the [Ca^{2+}+]_{i}
responses elicited by Ang III or BK. In contrast, the inhibition of MAPK/ERK by PD98059 did not prevent BIM-induced potentiation of the [Ca\(^{2+}\)]\(i\) responses to Ang III or BK.

**Effect of Ang II and BK on metabolic CO\(_2\) production**

We previously showed that oxidative metabolism in the CTAL was largely coupled to active Na\(^+\) transport because it is inhibited by over 50% with either the Na\(^+-\)K\(^+\)-ATPase inhibitor ouabain or the Na\(^+-\)K\(^+\)-2Cl\(^-\) cotransport inhibitor furosemide (29). We investigated the effects of Ang II and BK on Na\(^+\) transport by measuring metabolic CO\(_2\) production from [U\(^{14}\)C]lactate in the presence of these two agonists and also in the presence of the MEK inhibitor UO126. A concentration of 10\(^{-7}\) mol/liter Ang II and BK clearly had opposite effects on Na\(^+\) transport, with Ang II significantly increasing metabolic CO\(_2\) production and BK inhibiting it (Fig. 10A). A similar increase of the metabolic CO\(_2\) production was found with 10\(^{-7}\) mol/liter Ang III (data not shown). To examine whether differences observed in intracellular signaling events and opposite physiological effects induced by Ang II and BK were linked, we tested the effects of the MEK inhibitor UO126 on metabolic CO\(_2\) production. Figure 10A showed that 20 \(\mu\)mol/liter UO126 used alone did not change the basal metabolic CO\(_2\) production. However, UO126 potentiated metabolic CO\(_2\) production elicited by Ang II, whereas it abolished the inhibitory effect of BK on CO\(_2\) production. We then evaluated the effect of 1 \(\mu\)mol/liter BIM on the metabolic CO\(_2\) production induced by Ang II. Figure 10B shows that CO\(_2\) production was unaffected by BIM alone, whereas the increase in CO\(_2\) production induced by Ang II was suppressed in the presence of BIM.

**Discussion**

As previously described for the medullary thick ascending limb of rat (17, 18) and by us in this work, Ang II increases Na\(^+\) transport, whereas BK inhibits it. This major difference in the physiological actions of the two peptides in the CTAL led us to look for similarities and differences in their signal transduction pathways.

In CTAL, BK was found to elicit [Ca\(^{2+}\)]\(i\) increases with pharmacological characteristics similar to those for Ang II and Ang III. The maximal calcium response was obtained at 10\(^{-7}\) mol/liter BK, and the half-maximal response was observed at a concentration of 10\(^{-8}\) mol/liter (2, 23). BK acts via the B\(_2\)-R and Ang II/III via the AT\(_{1A}\)-R (2). However, one interesting finding is that the peak responses induced by BK were greater than those observed with Ang II or Ang III but that the amplitude of Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) entry were of the same order of magnitude for the three peptides. In addition, additivity experiments performed in the presence or absence of external calcium clearly showed that Ang II/III and BK released Ca\(^{2+}\) from the same intracellular pools but activated different Ca\(^{2+}\) entry pathways. Taken together, such differences in intracellular calcium kinetics and in activation of Ca\(^{2+}\) entry could contribute to different physiological effects. To determine the nature of the channels involved or at least to identify some different properties of these channels and to relate them to their different physiological effects, we have used the L-type Ca\(^{2+}\) channel (voltage-operated channel) blocker nifedipine and the most commonly used inhibitor of SOC, SKF96365, also reported as an inhibitor of receptor-operated channel (ROC) (30). The best-characterized Ca\(^{2+}\) entry pathway uses voltage-operated calcium channels, particularly the L-type Ca\(^{2+}\) channels. In CTAL, we have shown that Ca\(^{2+}\) influx through Ca\(^{2+}\) entry pathways mediated by either Ang or BK was insensitive to L-type Ca\(^{2+}\) channel blocker nifedipine but completely blocked by the SOC inhibitor SKF96365. However, in different cell types are present several types of calcium-permeable channels that are not voltage dependent, including ROC, activated by agonists acting on a range of GPCR, and SOC, activated after depletion of intracellular Ca\(^{2+}\) stores. Recently, McFadzean and Gibson (31) in smooth muscle cells presented evidence that ROC and SOC may in fact be members of the same ion channel family, differing only in their composition of transient receptor potential channel protein subunits. In our study, data obtained with SKF96365 did not allow us to precisely determine the ROC- or SOC-type channels involved in the Ca\(^{2+}\) influx induced by Ang II/III and BK in CTAL.

We then compared the intracellular signaling events stimulated by Ang II/III and BK in the CTAL by investigating whether PKC, TK, and MAPK/ERK were differentially activated by these hormones.
Potentiation of the \([\text{Ca}^{2+}]_i\) responses to Ang II/III and BK by the PKC inhibitor suggest that both peptides activated PKC and that this activation was probably a result of the inhibition of short-term PKC-mediated desensitization (3). These observations are consistent with data from previous studies showing the presence of phosphorylation sites for PKC on AT1-R and B2-R sequences (32–34). No difference in the PKC activation on \([\text{Ca}^{2+}]_i\) responses induced by Ang II/III or BK was observed. Dixon et al. (35) indicated that in arterial smooth muscle cells, both Ang II and BK activated PKC, as shown by phosphorylation of the endogenous PKC substrate. However, despite similar patterns of phosphorylation, only Ang II induced a significant increase in membrane-bound PKC activity, suggesting that Ang II and BK activated different isoforms of PKC. This hypothesis was supported by data showing that both PKC\(_{\alpha}\) and PKC\(_{\beta}\) were present in the luminal membrane of the cortical and medullary thick ascending limb (36).

Recent studies have implicated GPCR in coupling to PLC\(\gamma\) (37). Indeed, it has been shown that PLC\(\gamma\) is involved in \([\text{Ca}^{2+}]_i\) signaling in response to the stimulation of receptors classically defined as receptors activating PLC\(\beta\). Such coupling may be mediated by nonreceptor TK activation (11, 38). We therefore investigated whether TK in the CTAL were involved in the \([\text{Ca}^{2+}]_i\) responses induced by activation of AT1A-R and B2-R, both of which lack intrinsic TK activity, by using the protein TK inhibitor herbimycin A. The \([\text{Ca}^{2+}]_i\) increases induced by Ang II/III and BK were partially inhibited by herbimycin A and PP2, a more selective Src TK inhibitor, indicating that both PLC\(\beta\) and PLC\(\gamma\) were involved in these \([\text{Ca}^{2+}]_i\) responses. After herbimycin A treatment in the presence or absence of external calcium, we observed differences between the actions of Ang II/III and BK. TK displayed similar levels of involvement in the mobilization of intracellular calcium and \([\text{Ca}^{2+}]_i\) influx induced by Ang II/III. In contrast, they were primarily involved in the \([\text{Ca}^{2+}]_i\) influx induced by BK. These results are consistent with those of Lee et al. (39), who demonstrated in human foreskin fibroblast cells that TK are involved in the regulation of a BK-induced \([\text{Ca}^{2+}]_i\) entry pathway. Similarly, in 3T3-like em-
bryonic lines derived from wild-type and Src−/− transgenic mice, the level of Ca²⁺ influx after store depletion by BK has been reported to be much lower in Src−/− than in wild-type fibroblasts (40).

These findings indicate that nonreceptor TK, by activating PLCγ isoforms, have a role in both the intracellular Ca²⁺ release and Ca²⁺ influx induced by Ang II/III and BK. As recently demonstrated (41), the action of PLCγ in the agonist-induced activation of Ca²⁺ entry appears to be independent of the lipase activity of these enzymes and is consistent with a conformational role of PLCγ regulating Ca²⁺ entry.

In the literature, there is abundant evidence for the involvement of EGFR transactivation in GPCR, not only in Ang II- but also in BK-mediated ERK activation (42–44). Because EGFR expression was detected along the thick ascending limb (45), we evaluated the effects of the selective EGFR kinase inhibitor AG1478 on the [Ca²⁺]i responses induced by Ang II and BK. In CTAL, we found that [Ca²⁺]i responses induced by Ang II and BK were not altered by AG1478, showing that in our experimental conditions, EGFR transactivation was not required.

The GPCR regulation of MAPK activity depends on the nature of the G protein, the receptor, and the cell type considered (46). Our experiments with MEK inhibitors PD98059 and UO126 indicate that MAPK/ERK activation is involved in regulation of the intracellular Ca²⁺ mobilization and external Ca²⁺ influx induced by Ang II/III and BK in CTAL. As for TK activity, MAPK/ERK effects differed between Ang II/III and BK regarding intracellular calcium mobilization. The activation of MAPK/ERK seemed to have a major role in Ca²⁺ mobilization and influx, while TK activity played a minor role, as indicated by the similar responses to Ang II/III and BK.
in BK-induced [Ca\textsuperscript{2+}], responses, especially in the regulation of external Ca\textsuperscript{2+} influx, as previously shown in endothelial cells (47). Because MAPK/ERK is less involved in the [Ca\textsuperscript{2+}], increases induced by Ang II/III than in those induced by BK, the induction of Ca\textsuperscript{2+} entry by these agonists may involve direct activation by PLC\textgamma, in line with the findings of Patterson et al. (41). This is also consistent with differential regulation of Ca\textsuperscript{2+} entry depending on whether it is induced by BK or Ang II/III.

In agreement with the involvement of the MAPK/ERK pathway in the Ang II/III- and BK-induced [Ca\textsuperscript{2+}], responses in CTAL, we found a high basal phosphorylation of p44/42 ERK in rat CTAL by comparison with PCT as recently shown in cortical collecting duct of the mouse kidney (48). We then studied the PKC dependence of these signaling events, especially in the regulation of Ca\textsuperscript{2+} entry. We found that whereas UO126 applied alone did not change Ca\textsuperscript{2+} entry by these agonists may involve direct activation by PLC\textgamma, in line with the findings of Patterson et al. (41). This is also consistent with differential regulation of Ca\textsuperscript{2+} entry depending on whether it is induced by BK or Ang II/III.

To investigate whether differences observed in intracellular signaling events and opposite physiological effects induced by Ang II and BK were linked, we first tested the effect of UO126 on the metabolic CO\textsubscript{2} production elicited by BK. We found that whereas UO126 applied alone did not change basal metabolic CO\textsubscript{2} production, it completely blocked the inhibitory action of BK on CO\textsubscript{2} production, similar to its inhibitory action on BK-induced Ca\textsuperscript{2+} influx. This suggests that the inhibition of Na\textsuperscript{+} transport by BK could be a consequence of the increase in Ca\textsuperscript{2+} influx induced by BK via a stimulation of the MAPK/ERK pathway. Then, we evaluated the effects of UO126 and BIM on Ang II-induced metabolic CO\textsubscript{2} production. We found that in contrast to BK, the increase in Ca\textsuperscript{2+} influx induced by Ang II was not affected by UO126 or BIM.

Inhibition of AUC (percent) induced by 33 \textmu mol/liter herbinycin A and 50 \textmu mol/liter PD98059 used alone or together (experimental additivity) were calculated as compared with controls in the presence of 2 \textmu mol/liter external calcium. The results are expressed as the mean ± SEM. Numbers in parentheses indicate the number of individual determinations.

Statistically different from Ang III + herbimycin A and from BK + PD98059.

\textsuperscript{a}Statistically different from Ang III + herbimycin A and from BK + PD98059.

\textsuperscript{b}Statistically different from combined (sum of inhibitions separately induced by herbimycin A and PD98059 alone). P < 0.01 (one-way ANOVA on weighted means followed by Fisher's test).

### TABLE 3. Effects of herbimycin A and PD98059 used alone or together on 10\textsuperscript{-7} mol/liter Ang III- and BK-stimulated [Ca\textsuperscript{2+}], responses in CTAL

<table>
<thead>
<tr>
<th></th>
<th>Herbimycin A</th>
<th>PD98059</th>
<th>Herbimycin A + PD98059</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>% Inhibition as compared with controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang III</td>
<td>72 ± 9 (6)</td>
<td>38 ± 7 (11)</td>
<td>72 ± 4 (6)</td>
</tr>
<tr>
<td>BK</td>
<td>74 ± 6 (7)</td>
<td>73 ± 5 (7)</td>
<td>88 ± 3 (6)</td>
</tr>
<tr>
<td></td>
<td>110 ± 7 (11)</td>
<td>147 ± 5 (7)</td>
<td></td>
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</tbody>
</table>

Inhibition of AUC (percent) induced by 33 \textmu mol/liter herbinycin A and 50 \textmu mol/liter PD98059 used alone or together (experimental additivity) were calculated as compared with controls in the presence of 2 \textmu mol/liter external calcium. The results are expressed as the mean ± SEM. Numbers in parentheses indicate the number of individual determinations.

	extsuperscript{a}Statistically different from Ang III + herbimycin A and from BK + PD98059.

	extsuperscript{b}Statistically different from combined (sum of inhibitions separately induced by herbimycin A and PD98059 alone). P < 0.01 (one-way ANOVA on weighted means followed by Fisher's test).
FIG. 9. Effects of BIM treatment on the TK and MAPK/ERK activation induced by 10^{-7} mol/liter Ang III and BK in CTAL. Each bar represents the mean of the integral of the Ca^{2+} signal calculated from six to 25 determinations. a, Significantly different from Ang III alone (P < 0.01); b, significantly different from Ang III + BIM (P < 0.01); c, significantly different from Ang III + PD98059 (P < 0.01); d, significantly different from BK alone (P < 0.05); e, significantly different from BK + BIM (P < 0.01); f, significantly different from BK + PD98059 (P < 0.01) (one-way ANOVA on weighted means followed by Fisher’s test). Concentrations used for BIM, herbimycin A (Herb A), and PD98059 were 1, 33, and 50 μmol/liter, respectively.

FIG. 10. Effects of 10^{-7} mol/liter Ang II and 10^{-7} mol/liter BK on metabolic CO2 production by CTAL. Each bar is the mean of four to 21 determinations. a, Significantly different from control (P < 0.01); b, significantly different from Ang II (P < 0.01); c, significantly different from control (P < 0.05); d, significantly different from BK (P < 0.05); e, significantly different from control (P < 0.01); f, significantly different from Ang II (P < 0.05). Concentrations used for UO126 and BIM were 20 and 1 μmol/liter, respectively.
of the metabolic CO₂ production induced by Ang II was potentiated by UO126 and, in addition, inhibited by BIM. These data suggest that Ang II stimulated Na⁺ transport in large part via an increase in Ca²⁺ release mediated by PKC as found by Amlal et al. (17) in the rat medullary thick ascending limb (MTAL). But on the other hand, direct stimulation of MAPK/ERK pathway by Ang II in CTAL could also inhibit Na⁺ transport as shown by Watts and Good (54), who identified an inhibitory action of ERK on Na⁺/H⁺ exchange activity in MTAL. However, the net effect of Ang II in CTAL, by activating PKC- and MAPK/ERK-dependent pathways, would be to stimulate Na⁺ transport. Thus, the PKC-dependent pathway would exert a dominant effect on the final biological action of Ang II.

Additional events may influence the biological effects of these two peptides, and two hypotheses can be considered. First, because the CTAL contains both AT1A-R and B2-R, heterodimerization of these two receptors may occur, as demonstrated by AbdAlla et al. (55) in HEK cells. This would affect specific [Ca²⁺]i, responses induced by Ang II/III or BK, modifying Na⁺ transport in this segment. Second, another relationship between intracellular signaling events and the opposite physiological effects induced by these agonists can be envisaged if we assume a compartmentalized distribution of intracellular calcium, facilitating the selective control of distinct cellular responses, as suggested by Ferairel and Doucet (56) and Yu et al. (57). As underlined by Petersen (58), different patterns of Ca²⁺ signals can be created, in space and time, which allow specific cellular responses to be elicited. One of the perhaps most remarkable features of Ca²⁺ signaling is the ability, in the same cell, to regulate entirely different processes. For instance, in pancreatic acinar cells, Ca²⁺ signals not only control the normal secretion of digestive enzymes but can also activate autodigestion and programmed cell death (58). Not all stimuli that increase Ca²⁺ elicit the same physiological response, implying differences in their [Ca²⁺]i handling (59). In our study, because Ang II/III and BK activate different Ca²⁺ entry pathways that are differentially regulated, we could speculate that calcium does not diffuse homogeneously in the cytosol, and according to the localization of activated channels, calcium could be confined in well determined cell areas under apical or basolateral membranes (60, 61). Such different spatial distribution of calcium could induce different biological responses in CTAL cells.

In conclusion, in CTAL, our data show that Ang II/III and BK stimulate Ca²⁺ release from the same intracellular pools and by activating different calcium entry pathways. The increases in [Ca²⁺]i depended partly on TK and MAPK/ERK pathways. However, the MAPK/ERK pathway strongly influences Ca²⁺ influx induced by BK and, to a lesser extent, Ca²⁺ influx induced by Ang II/III. Ang II/III and BK have opposite actions on Na⁺ transport. The inhibitory effect of BK on Na⁺ transport seems to be directly mediated by an increase in Ca²⁺ influx dependent on MAPK/ERK pathway activation. In contrast, the stimulatory effect of Ang II/III on Na⁺ transport is more complex and involves both PKC and MAPK/ERK pathways. Taken together, our study brings new insights for the understanding of the opposite biological effects mediated by AT1A-R and B₂-R in rat CTAL.
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