NO-Independent Vasodilation to Acetylcholine in the Rat Isolated Kidney Utilizes a Charybdotoxin-Sensitive, Intermediate-Conductance Ca\(^{++}\)-Activated K\(^{+}\) Channel

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ABSTRACT

The role of K\(^{+}\) channels in the nitric oxide-independent renal vasodilator effect of acetylcholine (Ach) was examined to address the hypothesis that the mechanism underlying this response was different from that of bradykinin, because an earlier study indicated the possibility of different mediators. We used the rat isolated, perfused kidney that was constricted with phenylephrine and treated with nitroarginine and indomethacin to inhibit nitric oxide synthase and cyclooxygenase, respectively. The nonspecific K\(^{+}\) channel inhibitors, procaine and tetraethylammonium (TEA), reduced vasodilator responses to Ach and cromakalim, but not those to nitroprusside. Glibenclamide, an inhibitor of ATP-sensitive K\(^{+}\) channels, reduced vasodilator responses to cromakalim but did not affect those to Ach or nitroprusside. Charybdotoxin, an inhibitor of Ca\(^{++}\)-activated K\(^{+}\) channels, reduced vasodilator responses to Ach without affecting those to cromakalim or nitroprusside. Iberiotoxin and apamin, inhibitors of large- and small-conductance Ca\(^{++}\)-activated K\(^{+}\) channels, respectively, did not reduce vasodilation induced by Ach, cromakalim or nitroprusside. The inhibitor of cytochrome P450, clotrimazole, reduced the renal vasodilator effects of Ach and bradykinin but not those of nitroprusside or SCA 40, an agonist for Ca\(^{++}\)-activated K\(^{+}\) channels. These results suggest that in the rat kidney, Ach, like bradykinin, utilizes a charybdotoxin-sensitive Ca\(^{++}\)-activated K\(^{+}\) channel of intermediate conductance to elicit vasodilation and that this effect may be dependent on cytochrome P450 activity.

Endothelium-dependent vasodilation using Ach as the gold standard has generally been attributed to the release of NO. However, depending on the agonist, species, tissue and experimental conditions, NO-independent vasodilation can be demonstrated (Cowan and Cohen, 1991; Baydoun and Woodward, 1991; Nagao et al., 1992; Pacicca et al., 1992), a phenomenon attributed to the release of an endothelium-derived hyperpolarizing factor (EDHF), a term coined by Taylor and Weston (1988). In large conduit vessels such as the aorta, endothelium-dependent relaxation appears to be mediated solely by NO; responses can be abolished by inhibition of NOS (Palmer et al., 1988; Rees et al., 1989). In smaller vessels, the contribution of EDHF becomes more apparent, and endothelium-dependent vasodilation persists in the face of inhibition of NOS (Shimokawa et al., 1996). Thus, NO-independent vasodilation has been reported for Ach and bradykinin, although the identity of the mediator(s) and the type of K\(^{+}\) channel that is involved remain to be elucidated. Our studies using the rat heart and kidney strongly suggest a P450-dependent eicosanoid as the mediator of the vasodilator effect of bradykinin that persists in the presence of inhibitors of NOS and cyclooxygenase (Fulton et al., 1992, 1995). This NO-independent effect is dependent on phospholipase C and A\(_{2}\) and is susceptible to inhibitors of P450 and K\(^{+}\) channels (Fulton et al., 1994, 1995, 1996), which suggests an endothelium-derived P450-dependent metabolite of arachidonic acid as a hyperpolarizing factor. This concept is supported by several recent reports (Hecker et al., 1994; Bauersachs et al., 1994), and Campbell et al. (1996) have proposed epoxides as hyperpolarizing factors in bovine coronary arteries, on the basis of a comprehensive series of studies. However, it is possible that there are several hyperpolarizing factors, depending on the agonist, species and vascular tissue.

In the rat kidney, we found that the vasodilator effect of Ach, as well as that of bradykinin, exhibited a substantial component that was unaffected by inhibition of NOS (Fulton et al., 1992). In this study, the renal vasodilator response to Ach, unlike that to bradykinin, was unaffected by inhibitors of P450, clotrimazole and 7-ethoxyresorufin, which suggests

ABBREVIATIONS: Ach, acetylcholine; NO, nitric oxide; NOS, nitric oxide synthase; EDHF, endothelium-derived hyperpolarizing factor; P450, cytochrome P450; TEA, tetraethylammonium.
the possibility that a P450 product was not involved in this action of Ach (Fulton et al., 1992). These observations suggested, therefore, that the mediator of the NO-independent response to Ach was not the same as that mediating the response to bradykinin. Because the NO- and prostaglandin-independent renal and coronary vasodilator effects of bradykinin were dependent on activation of K+ channels, specifically charybdoxin-sensitive K+ channels, the primary aim of the present study was to determine whether Ach-induced vasodilation used a similar mechanism, the premise being that a different mediator may use a different mechanism. Consequently, we first determined the role of K+ channels in the renal vasodilator action of Ach and then characterized the type of K+ channel. The initial experiments utilized nonspecific antagonists of K+ channels, and subsequent experiments investigated the contribution of ATP-sensitive K+ channels and of small-, intermediate- and large-conductance Ca++-activated K+ channels. Thus, studies addressing the type of K+ channel involved in NO-independent vascular responses that are attributed to EDHF have yielded variable results. Both small- and large-conductance Ca++-activated K+ channels (Cowan et al., 1993; Adeagbo and Triggle, 1993; Hecker et al., 1994) and, in a few instances, ATP-sensitive K+ channels (Standen et al., 1989) have been implicated, depending on the tissue. Recently, Zygmunet et al. (1997) proposed that a subtype of a small-conductance Ca++-activated K+ channel figures in the response to Ach in rat hepatic artery.

The results of the present study show that the renal vasodilator effect of Ach in the presence of inhibitors of NOS and cyclooxygenase is dependent on activation of K+ channels, specifically charybdoxin-sensitive Ca++-activated K+ channels. Because the renal vasodilator effects of Ach and bradykinin exhibited similar characteristics in terms of the contribution of K+ channels, thereby suggesting a common mediator, we subsequently conducted additional studies with clotrimazole to investigate a potential role of P450 in the vasodilator action of Ach when NOS and cyclooxygenase were inhibited. Thus, earlier experiments suggested the possibility that the NO-independent renal vasodilator responses to Ach and bradykinin utilized different mediators. The present results show that the renal vasodilator effect of Ach is attenuated by clotrimazole.

Materials and Methods

The isolated perfused kidney of the rat has been described previously (Fulton et al., 1992; Rapacon et al., 1996). Briefly, after pentobarbitone anesthesia (65 mg/kg i.p.), the right kidney was exposed via a midline laparotomy. The right renal artery was cannulated via the mesenteric artery to avoid interruption of blood flow to the kidney, which was perfused at constant flow with oxygenated Krebs’ buffer (37°C) containing indomethacin (2.8 μM) using a pulsatile pump (Watson-Marlow, 502S). The vena cava was ligated above and below the right renal vein and cut for exit of the perfusate, and the right ureter was transected. Perfusate flow rate (8–10 ml/min), which was maintained constant throughout the experiment, was adjusted to obtain a basal perfusion pressure of approximately 65 to 85 mmHg, and nitroarginine (50 μM) was added to the perfusate to inhibit NO synthesis and isolate the NO-independent component of the vasodilator effect of Ach (Fulton et al., 1992). This concentration of nitroarginine was shown by Cacheiro and NasiLetti (1991) to prevent increases in cGMP release from the kidney in response to bradykinin, and we found that doubling the concentration to 100 μM produced no further inhibition of renal vasodilator responses to Ach or bradykinin (Fulton et al., 1992). Inhibitors of K+ channels were added to the perfusate at least 10 min before vascular tone was elevated with phenylephrine (0.2–0.4 μM) that was titrated to raise perfusion pressure to about 180 to 200 mmHg to amplify vasodilator responses. Successive doses of Ach were added at intervals of at least 5 min and not before perfusion pressure had returned to the control value after the preceding dose. Once a stable elevated perfusion pressure was obtained, vasodilator responses to Ach (10–100 ng) were determined as maximal reductions in perfusion pressure. In the first series of experiments, responses to Ach were obtained under control conditions (n = 8) and in the presence of procaine (1 mM; n = 3) and TEA (10 mM; n = 3) to inhibit all types of K+ channels and of glibenclamide (10 μM; n = 4) to inhibit ATP-sensitive K+ channels. Responses to cromakalim (3 μg), an activator of ATP-dependent K+ channels, and to nitroprusside (1 μg) were used as indices of the effectiveness of K+ channel blockade and vascular effects unrelated to inhibition of K+ channels, respectively. The control group was the same for each of these interventions; 3 to 4 preparations a day were completed, where at least one was a control.

In the second series of experiments, the role of Ca++-activated K+ channels was investigated by comparing vasodilator responses to Ach (30 and 100 ng) in the presence (n = 5) and absence (n = 4) of charybdoxin at a concentration (10 nM) that markedly reduced renal vasodilator responses to bradykinin without affecting those to cromakalim or nitroprusside.

In the third series of experiments, we investigated the contribution of large-conductance Ca++-activated K+ channels to the renal vasodilator effect of Ach, on the basis of the results with charybdoxin, which inhibits both intermediate- and large-conductance Ca++-activated K+ channels. Thus, responses to Ach (30 and 300 ng) were determined in the presence (n = 6) and absence (n = 5) of iberiotoxin (10–50 nM). The doses of Ach represent an intermediate dose and a maximal dose that were used in anticipation of inhibition of the vasodilator effect by iberiotoxin.

In a fourth series of experiments, the role of small-conductance Ca++-activated K+ channels was addressed by comparing responses to Ach (10–100 ng) in the presence (n = 4) and absence (n = 4) of apamin (250 nM).

In the final series of experiments, vasodilator responses to Ach (30 and 100 ng) were determined in phenylephrine-constricted kidneys in the absence and presence of clotrimazole (1 μM) to inhibit P450. Clotrimazole was included in the perfusate from the start of the experiment. Responses to a single dose of bradykinin were also assessed; we reported previously that clotrimazole attenuated the renal vasodilator effect of this peptide. Responses to SCA 40, an agent reported to stimulate Ca++-activated K+ channels (Laurent et al., 1993), and nitroprusside were used to assess any effects of clotrimazole on vasodilator responses mediated via activation of K+ channels and guanylate cyclase, respectively. SCA 40 was used in these experiments, because clotrimazole has been reported to affect the function of Ca++-activated K+ channels (Alvarez et al., 1992).

Statistical analysis. Data from the various groups were compared by ANOVA and individual points by Newman-Keuls test. P < .05 was considered statistically significant.

Materials. Indomethacin, nitroarginine, procaine, TEA, glibenclamide, Ach, cromakalim, apamin and sodium nitroprusside were obtained from Sigma Chemical Co. (St. Louis, MO), and charybdoxin and iberiotoxin were obtained from Peptides International (Louisville, KY). Indomethacin was dissolved in 4.2% NaHCO3, glibenclamide and cromakalim were dissolved in ethanol and the other compounds were dissolved in saline or distilled water.
Results

Basal perfusion pressures (65–85 mmHg) were not different between the various groups treated with K⁺ channel inhibitors.

**Nonselective inhibition of K⁺ channels.** Elevated perfusion pressure in the control group was 189 ± 4 mmHg compared with 165 ± 10 mmHg for the procaine group and 190 ± 5 mmHg for the TEA-treated group. Ach elicited dose-dependent vasodilation in phenylephrine-constricted kidneys in which NO and prostaglandin synthesis was inhibited (figs. 1 and 2). Thus, 10, 30, and 100 ng of Ach reduced perfusion pressure by 6 ± 3, 25 ± 5 and 46 ± 8 mmHg, respectively. Procaine and TEA greatly reduced the vasodilator effect of cromakalin (fig. 2), a result that shows their effectiveness against K⁺ channels. These inhibitors abolished the NO-independent vasodilator action of Ach, which indicates that K⁺ channels play a role in the response. TEA also reduced the vasodilator effect of nitroprusside (P < .05), which suggests effects other than inhibition of K⁺ channels.

**ATP-sensitive K⁺ channels.** Elevated perfusion pressure in the glibenclamide-treated group was 184 ± 9 mmHg compared with 189 ± 4 mmHg in the control group, a value the same as that in the procaine and TEA experiments. The vasodilator effect of cromakalin was abolished by glibenclamide (fig. 3), which shows that this agent blocked ATP-sensitive K⁺ channels. However, vasodilator responses to Ach were unaffected by glibenclamide, a result that excludes a role for ATP-sensitive K⁺ channels. Responses to nitroprusside were also unaffected by glibenclamide, which provides evidence for its specificity.

**Ca²⁺-activated K⁺ channels.** Elevated perfusion pressure in the charybdotoxin-treated group was 189 ± 4 compared with 177 ± 9 in the respective control group. In the control group, 30 and 100 ng of Ach lowered perfusion pressure by 28 ± 5 and 39 ± 7 mmHg, respectively (fig. 4), compared with 3 ± 2 and 12 ± 3 mmHg, respectively, in the presence of charybdotoxin; this indicates a role for Ca²⁺-activated K⁺ channels. In contrast, charybdotoxin had no effect on vasodilator responses to cromakalin or nitroprusside, a reflection of its specificity.

Whether large-conductance Ca²⁺-activated K⁺ channels play a role was addressed using the specific inhibitor, iberiotoxin. Elevated perfusion pressure in the iberiotoxin-treated group was 203 ± 7 vs. 191 ± 4 in the respective control group. In the control group, 30 and 300 ng of Ach reduced perfusion pressure by 27 ± 2 and 55 ± 5 mmHg, respectively, responses that were unaffected by iberiotoxin at concentrations ranging from 10 to 50 nM. Consequently, the data from experiments using different concentrations of iberiotoxin were pooled. Responses to 30 and 300 ng of Ach in the combined group were 30 ± 2 and 67 ± 5 mmHg, respectively. Because iberiotoxin had no effect on responses to Ach, nonspecific effects were not addressed.

We examined whether small-conductance Ca²⁺-activated K⁺ channels play a role by using apamin. Elevated perfusion pressure in the apamin-treated group was 200 ± 5 mmHg vs. 197 ± 3 mmHg in the control group, in which 10, 30, and 100 ng of Ach reduced perfusion pressure by 10 ± 1, 23 ± 3 and 41 ± 2 mmHg, respectively, with 25 ± 4, 26 ± 1 and 60 ± 3 mmHg, respectively, in the apamin-treatment group. Responses to nitroprusside were not different in the control and apamin groups (63 ± 11 vs. 70 ± 9 mmHg), whereas responses to cromakalin (5 μg) were slightly greater with apamin treatment (72 ± 1 vs. 53 ± 7 mmHg).

**Inhibition of P450.** The results with the K⁺ channel inhibitors suggested that Ach utilizes a type of channel similar to that used by bradykinin (Fulton et al., 1994). Consequently, we questioned whether these endothelium-dependent vasodilators use different mediators, a proposal based on an earlier study in which the vasodilator effect of bradykinin was reduced by inhibitors of P450, whereas that of Ach was unaffected (Fulton et al., 1992). In those experiments, NO and prostaglandin synthesis were intact. Therefore, we conducted experiments in nitroarginine- and indomethacin-treated kidneys to determine the effects of the P450 inhibitor clotrimazole on responses to Ach.

Phenylephrine elevated perfusion pressure from 74 ± 3 mmHg to 223 ± 5 mmHg in the clotrimazole-treated group (n = 4) and from 76 ± 6 to 214 ± 4 mmHg in the control group (n = 5). In clotrimazole-treated kidneys, responses to Ach were approximately 50% of those obtained in the control.
group (fig. 5), which suggests a role for P450. As previously reported, the vasodilator effect of bradykinin was also reduced by clotrimazole, whereas responses to nitroprusside and SCA 40 were unaffected (fig. 5).

**Discussion**

In the rat perfused kidney, the vasodilator effect of bradykinin exhibits three components subserved by NO and P450 with a lesser contribution of prostaglandins, a conclusion based on the use of inhibitors of these pathways (Fulton et al., 1992). Similarly, in the rat heart, the endothelium-dependent vasodilator effect of bradykinin, which was independent of NO, was susceptible to inhibitors of P450, phospholipases and K⁺ channels (Fulton et al., 1994, 1995, 1996); this suggests that a P450-dependent metabolite of arachidonic acid exhibited the essential properties of EDHF. As with the heart, the renal vasodilator action of bradykinin involved a
charybdotoxin-sensitive K\(^+\) channel (Rapacon et al., 1996). In the studies of bradykinin in the kidney, we also noted that a vasodilator effect of Ach could be demonstrated in the presence of an inhibitor of NO synthesis (Fulton et al., 1992), a finding supported by Vargas et al. (1994). However, in the absence of inhibitors of NOS and cyclooxygenase, the vasodilator response to Ach, unlike that to bradykinin, was not affected by the inhibitors of P450, clotrimazole and 7-ethoxyresorufin (Fulton et al., 1992). Because NO-independent responses to Ach have been attributed to the release of EDHF, these results suggest the possibility that bradykinin and acetylcholine utilize different hyperpolarizing factors. Consequently, the primary aim of the present study was to examine whether Ach and bradykinin use different mechanisms to elicit NO-independent renal vasodilation. The experimental approach was threefold: 1) to determine whether K\(^+\) channels contribute to the action of acetylcholine, 2) to examine whether Ach and bradykinin utilize a similar type of K\(^+\) channel and 3) to assess whether the vasodilator activity of Ach could be distinguished from that of bradykinin in terms of a P450 component using clotrimazole. We used clotrimazole in this series of experiments because it was one of the agents used in previous studies with bradykinin (Fulton et al., 1992). Moreover, clotrimazole, like other imidazoles, may have a more selective action on the formation of epoxides (Zou et al., 1994) that exhibit some of the properties required of an EDHF. Although the imidazole derivatives have been reported to elicite effects that may be unrelated to inhibition of P450, our experiments show that clotrimazole had no effect on vasodilator responses to nitroprusside and SCA 40, which indicates that, under these experimental conditions, it did not affect vasodilation mediated by NO or activation of K\(^+\) channels.

The results of this study demonstrate that the NO- and prostaglandin-independent component of the renal vasodilator effect of Ach, which was isolated by inhibition of NO and prostaglandin synthesis and amplified by elevation of vascular tone, utilizes a mechanism similar to that of bradykinin in the rat heart and kidney—i.e., stimulation of Ca\(^++\)-activated K\(^+\) channels. Thus, TEA and procaine, at concentrations that greatly reduced the vasodilator effect of cromakalim by inhibiting ATP-sensitive K\(^+\) channels, abolished the renal vasodilator action of Ach. The lack of effect of glibenclamide, at a concentration that abolished responses to cromakalim, on vasodilator responses to Ach excluded a role for ATP-sensitive K\(^+\) channels; this was also true of bradykinin (Fulton et al., 1994). In contrast, the potent inhibitory effect of charybdotoxin is presumptive evidence of a role for Ca\(^++\)-activated K\(^+\) channels in the vasodilator effect of Ach. The specificity of the action of charybdotoxin is supported by the lack of effect on vasodilator responses to cromakalim and nitroprusside. Charybdotoxin is considered to be more selective for large-conductance Ca\(^++\)-activated K\(^+\) channels, although, depending on the source and type of tissue, the actions of charybdotoxin may not be limited to this type of channel (Kuriyama et al., 1995) but may also affect intermediate-conductance Ca\(^++\)-activated K\(^+\) channels and delayed rectifier-type K\(^+\) channels (Kaczorowski et al., 1996).

In vascular smooth muscle, various subtypes of Ca\(^++\)-activated K\(^+\) channels have been identified and include large-, intermediate- and small-conductance channels (Kuriyama et al., 1995). Consequently, we also used iberiotoxin to inhibit large-conductance Ca\(^++\)-activated K\(^+\) channels. The lack of effect of iberiotoxin on vasodilator responses to Ach tends to exclude large-conductance channels that are generally considered to be sensitive to charybdotoxin (Kuriyama et al., 1995). However, it is possible that subtypes of large-conductance Ca\(^++\)-activated K\(^+\) channels exist that exhibit differential sensitivity to charybdotoxin and iberiotoxin. Alternatively, an insufficient concentration of iberiotoxin may have been used, although this is unlikely because the concentrations up to 50 nM that we employed are far in excess of the concentration shown to inhibit large-conductance Ca\(^++\)-activated K\(^+\) channels (Galvez et al., 1990). The results with iberiotoxin coupled with those with charybdotoxin, which does not inhibit small-conductance channels, suggest by exclusion that Ach, like bradykinin, utilizes an intermediate-conductance Ca\(^++\)-activated K\(^+\) channel to elicit NO-independent renal vasodilation (Rapacon et al., 1996). Moreover, a role for small-conductance Ca\(^++\)-activated K\(^+\) channels was excluded because apamin did not reduce the renal vasodilator response to Ach; rather, it tended to increase it. These results contrast with those of Zygmun et al. (1997), who reported that neither apamin alone nor charybdotoxin alone affected responses to Ach in rings of hepatic artery. Thus, a combination of apamin and charybdotoxin was necessary to inhibit the effect of Ach, and Zygmun et al. (1997) proposed a subtype of a small-conductance channel as the target for EDHF. Our results indicate the involvement of an intermediate-conductance Ca\(^++\)-activated K\(^+\) channel; neither iberiotoxin nor apamin reduced responses to Ach, whereas charybdotoxin alone markedly reduced the vasodilator effect of Ach. However, we cannot exclude a role for voltage-dependent K\(^+\) channels, because charybdotoxin also inhibits some subtypes of these channels (Kaczorowski et al., 1996).

Unlike the results of an earlier study showing that the vasodilator response to Ach was unaffected by inhibitors of P450 (Fulton et al., 1992), the results of the present study show that clotrimazole attenuated the renal vasodilator effect of Ach to a similar extent to that seen with bradykinin. The different results observed in these two studies are not readily explained, although the experimental conditions were not identical. In the current investigation, the effect of clotrimazole was assessed in the presence of nitroarginine and indomethacin, whereas all vasodilator mechanisms were intact in the earlier study. Thus, it may be that when one system is compromised, another mechanism can elicit a full vasodilator response. Second, in the earlier study, only one dose of Ach was tested. Nonetheless, it is difficult to reconcile the previous observations of a differential effect of clotrimazole on the responses to bradykinin and Ach with the current results: a similar inhibitory action on responses to bradykinin and Ach unless the responses are unequally dependent on two or more mediators. The effects of clotrimazole could not be attributed to actions on vasodilator mechanisms in general or to actions on K\(^+\) channels, because dilator responses to nitroprusside and SCA 40, respectively, were unaffected, a result consistent with our previous observations. Thus, these findings indicate that the vasodilator effect of Ach, like that of bradykinin, exhibits a substantial NO-independent component that depends on P450 and on activation of Ca\(^++\)-activated K\(^+\) channels.

Another interpretation of the results is that charybdotoxin
blocks a Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel in the endothelium that is stimulated by both Ach and bradykinin and results in the release of vasodilator mediators. Thus, endothelial cells possess channels that can be inhibited by TEA and charybdotoxin (Rusko et al., 1992). The use of an isolated organ to address hyperpolarization as a mechanism of Ach-induced vasodilation is limited because it is not possible to measure the membrane potential of endothelial and smooth muscle cells in resistance vessels. Thus, studies of this nature rely on the use of pharmacological agents that can potentially interfere at more than one step in the signal transduction pathway—in this case, at the level of the endothelium rather than the smooth muscle.

In summary, we have shown that the NO-independent renal vasodilator effect of Ach, like that of bradykinin, is mediated via Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, presumably of intermediate conductance, and may involve the participation of a P450-related mechanism.

References


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