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Contribution of calcium-activated potassium channels to the vasodilator effect of bradykinin in the isolated, perfused kidney of the rat

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1 NO- and prostaglandin-independent, endothelium-dependent vasodilator responses to bradykinin are attributed to release of a hyperpolarizing factor. Therefore, the contribution of K^+ channels to the renal vasodilator effect of bradykinin was examined in rat perfused kidneys that were preconstricted with phenylephrine and treated with N^G-nitro-L-arginine (L-NOARG) and indomethacin to inhibit NO and prostaglandin synthesis.

2 The non-specific K^+ channel inhibitors, TEA and TBA reduced vasodilator responses to bradykinin and cromakalim but not those to nitroprusside.

3 Glibenclamide, an inhibitor of ATP-sensitive K^+ channels, blocked the vasodilator response to cromakalim without affecting responses to bradykinin.

4 Charybdotoxin, a selective inhibitor of Ca^{2+}-activated K^+ channels, greatly attenuated vasodilator responses to bradykinin without affecting those to cromakalim or nitroprusside.

5 Iberiotoxin and leiurotoxin, inhibitors of large and small conductance Ca^{2+}-activated K^+ channels, respectively, were without effect on vasodilator responses to bradykinin, cromakalim or nitroprusside.

6 These results implicate K^+ channels, specifically Ca^{2+}-activated K^+ channels of intermediate conductance, in the renal vasodilator effect of bradykinin and, thereby, support a role for a hyperpolarizing factor.

Keywords: Bradykinin; renal vasodilatation; K^+ channels; cytochrome P450; hyperpolarizing factor

Introduction

Endothelium-dependent vasodilatation is generally attributed to the release of endothelium-derived relaxing factor (EDRF) or NO (Vane *et al.*, 1990). However, as NO cannot fully account for endothelium-dependent vasodilatation, depending upon the agonist, tissue and species, release of an endothelium-derived hyperpolarizing factor (EDHF) has been invoked to explain the residual vasodilatation following inhibition of NO synthesis (Beny & Brunet, 1988; Feletou & Vanhoutte, 1988; Cowan & Cohen, 1991). In the rat kidney, for example, the vasodilator response to bradykinin consists of three components, two major ones susceptible to inhibitors of NO synthesis and cytochrome P450 and a minor component susceptible to inhibition of cyclo-oxygenase (Cachofeiro & Nasjletti, 1991; Fulton *et al.*, 1992). Thus, bradykinin is a useful probe for the investigation of vascular mechanisms as it is a well-established stimulus for the generation of lipid mediators as well as for NO. The present study was undertaken to address the contribution of hyperpolarizing factors, using K^+ channel inhibitors, to the component of the renal vasodilator effect of bradykinin remaining after inhibition of NO and prostaglandin synthesis. Similar experiments in the rat perfused heart in which the vasodilator effect of bradykinin is independent of NO (Baydoun & Woodward, 1991) but markedly reduced by inhibitors of phospholipases and cytochrome P450 (Fulton *et al.*, 1995a, b) revealed a role for Ca^{2+}-activated K^+ channels (Fulton *et al.*, 1994). These findings support the concept that a cytochrome P450-dependent hyperpolarizing factor is a product of arachidonic acid.

In the current study, the role of K^+ channels in the NO- and prostaglandin-independent vasodilator action of bradykinin in the rat kidney was initially determined by use of non-specific inhibitors, tetraethylammonium (TEA) and tetrabutylammonium, (TBA). Subsequently, the type of K^+ channel was characterized using selective inhibitors of specific types of K^+ channels. We found evidence for Ca^{2+}-activated K^+ channels in the vasodilator response to bradykinin.

Methods

Male Wistar rats (weight 350-450 g) were used in these experiments. Animals were anaesthetized with pentobarbitone, 65 mg kg^{-1}, i.p. Following midline laparotomy, the right renal artery was cannulated via the mesenteric artery to avoid interruption of blood flow. The vena cava was ligated above and below the right renal vein and cut for exit of perfusate. The right ureter was transected and the rat killed by an intracardiac injection of pentobarbitone. The kidney was then perfused *in situ* with oxygenated Krebs buffer (37°C) containing indomethacin (2.8 μM) at constant flow (8-10 ml min^{-1}) to obtain a basal perfusion pressure of 75-90 mmHg. N^G-nitro-L-arginine (L-NOARG) (50 μM) was added to the perfusate to inhibit NO synthesis. This concentration of L-NOARG has been shown to prevent bradykinin-stimulated increases in guanosine 3':5'-cyclic monophosphate (cyclic GMP) release from the isolated perfused kidney of the rat (Cachofeiro & Nasjletti, 1991). Subsequently, one of the inhibitors of K^+ channels or vehicle was added to the perfusate and perfusion pressure was then elevated to approximately 200 mmHg with phenylephrine ($1-4 \times 10^{-7}$ M) to amplify vasodilator responses. This sequence was adopted to obtain equivalent elevated perfusion pressures in the various groups and, thereby, avoid further increases in pressure resulting from inhibition of some K^+ channels. Once a stable elevated perfusion pressure was obtained, dose-response curves to bradykinin (10-100 ng) were determined in the presence of vehicle or one of several inhibitors of K^+ channels. Tetraethylammonium

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(TEA; 10 mM) and tetrabutylammonium (TBA; 30 μM) were used as non-selective inhibitors of K^+ channels to determine that the P450-dependent component of the vasodilator response to bradykinin was mediated via activation of K^+ channels. Glibenclamide (10 μM) was used to inhibit ATP-sensitive K^+ channels and charybdotoxin (CTX; 10 nM) to inhibit Ca^{2+}-activated K^+ channels. In addition, iberiotoxin (IBX; 20 nM) and leiurotoxin (LTX; 20 nM) were used to inhibit large (>200 pS) and small (10–20 pS) conductance Ca^{2+}-activated K^+ channels, respectively. In all preparations vasodilator responses to nitroprusside (NP; 1 μg), an NO donor, and cromakalim (3 μg), an ATP-sensitive K^+ channel opener, were determined to ascertain any vascular effects of the K^+ channel inhibitors unrelated to inhibition of K^+ channels and to confirm that the concentrations chosen were effective in blocking K^+ channels. Three to four experiments per day were performed where one kidney preparation served as a vehicle control and the others were assigned to the K^+ channel inhibitors. Consequently, many of the controls are common for several experimental groups, e.g., TEA, TBA, glibenclamide and CTX.

Data analysis

Vasodilator responses in the control and experimental groups were compared by ANOVA and individual points by Neuman-Keuls test. A P value $<0.05\%$ was considered statistically significant.

Materials

Tetraethylammonium, tetrabutylammonium, glibenclamide, N^G-nitro-L-arginine, nitroprusside and indomethacin were obtained from Sigma Chemical Co. St. Louis, U.S.A. Charybdotoxin, iberiotoxin and leiurotoxin were obtained from Peptide International, Louisville, Kentucky, U.S.A. The composition of the Krebs buffer (mM) was as follows: NaCl 118, KCl 4.75, $CaCl_2$ 1.9, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.19, $NaHCO_3$ 25.6 and glucose 5.6.

Results

Basal perfusion pressures in the various groups were not different. TEA, TBA and charybdotoxin all produced transient elevations (20–100 mmHg) in basal perfusion pressure that returned to baseline values within 5 min. Mean elevated perfusion pressures in the vehicle and experimental groups ranged between 205±5 mmHg and 248±18 mmHg.

Non-specific inhibitors of K^+ channels

TEA (10 mM) and TBA (30 μM) were effective inhibitors of K^+ channels as vasodilator responses to cromakalim were markedly reduced. Both agents reduced the vasodilator effect of bradykinin by approximately 50% (Figures 1, 2). Neither K^+ channel inhibitor affected vasodilator responses to nitroprusside.

ATP-sensitive K^+ channels

As these results indicated a role for K^+ channels in the renal vasodilator action of bradykinin, the contribution of ATP-sensitive K^+ channels was investigated by the use of glibenclamide (10 μM) which almost abolished the vasodilator effect of cromakalim, the ATP-sensitive K^+ channel opener. Glibenclamide also reduced the renal vasodilator effect of nitroprusside, the decrease in perfusion pressure being 69±5 mmHg versus 92±6 mmHg for the control ($P<0.05$). However, glibenclamide was without effect on responses to bradykinin (Figure 3), excluding a role of ATP-sensitive K^+ channels in the renal vasodilator action of bradykinin.

Ca^{2+}-activated K^+ channels

The contribution of Ca^{2+}-activated K^+ channels to the vasodilator action of bradykinin was addressed. Charybdotoxin (10 nM), a specific inhibitor of Ca^{2+}-activated K^+ channels, markedly reduced the renal vasodilator effects of bradykinin (Figure 4) without affecting responses to nitroprusside or cromakalim, the ATP-sensitive K^+ channel opener, providing evidence for its specificity. Blockade of large conductance Ca^{2+}-activated K^+ channels (>200 pS) with the selective inhibitor, iberiotoxin (20 nM), did not affect vasodilator responses to bradykinin. Thus, vasodilator responses to 10, 30 and 100 ng bradykinin were 25±1, 41±11 and 54±11 mmHg, respectively for the control group ($n=4$) versus 18±2, 45±3 and 55±9 mmHg, respectively, for the iberiotoxin-treated group ($n=3$). Therefore, the selective inhibitor of small conductance Ca^{2+}-activated K^+ channels (10–20 pS), leiurotoxin (20 nM), was used. Like iberiotoxin, leiurotoxin was without effect on renal vasodilator responses to bradykinin. Falls in perfusion pressure in response to 10, 30 and 100 ng bradykinin were 13±4, 31±5 and 45±3 mmHg, respectively, in the control group ($n=4$) compared to 11±4, 26±5 and 37±5 mmHg, respectively, in the leiurotoxin group ($n=3$).

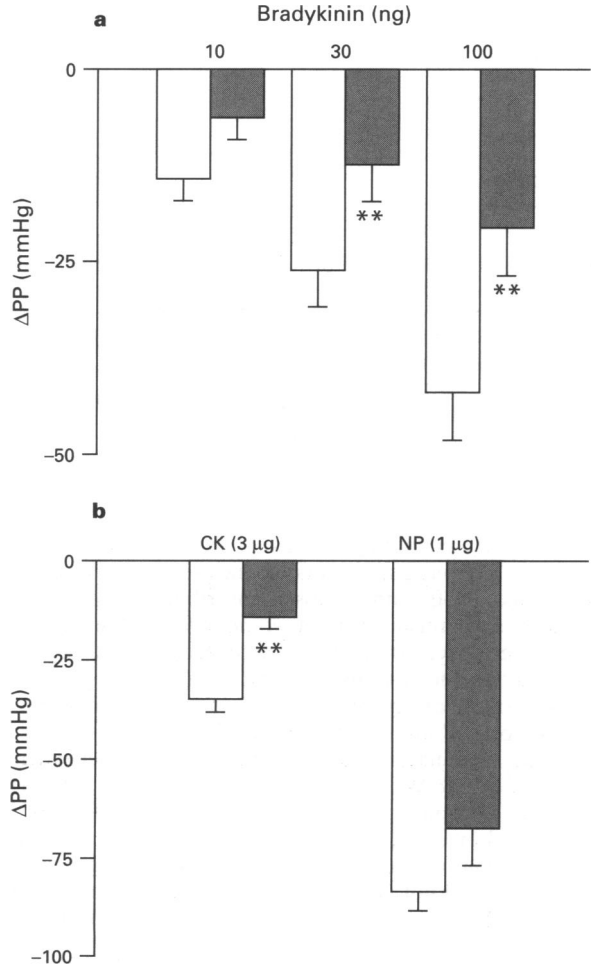

Figure 1 Effects of inhibition of K^+ channels with tetraethylammonium (10 mM) on vasodilator responses, expressed as decreases in perfusion pressure (PP), to (a) bradykinin, and (b) cromakalim (CK) and nitroprusside (NP) in isolated perfused kidneys treated with N^G-nitro-L-arginine (50 μM) and indomethacin (2.8 μM) and preconstricted with phenylephrine to elevate perfusion pressure to approximately 200 mmHg. Control: open columns, $n=19$ and tetraethylammonium: stippled columns, $n=6$: $^*P<0.05$, $^{**}P<0.01$.

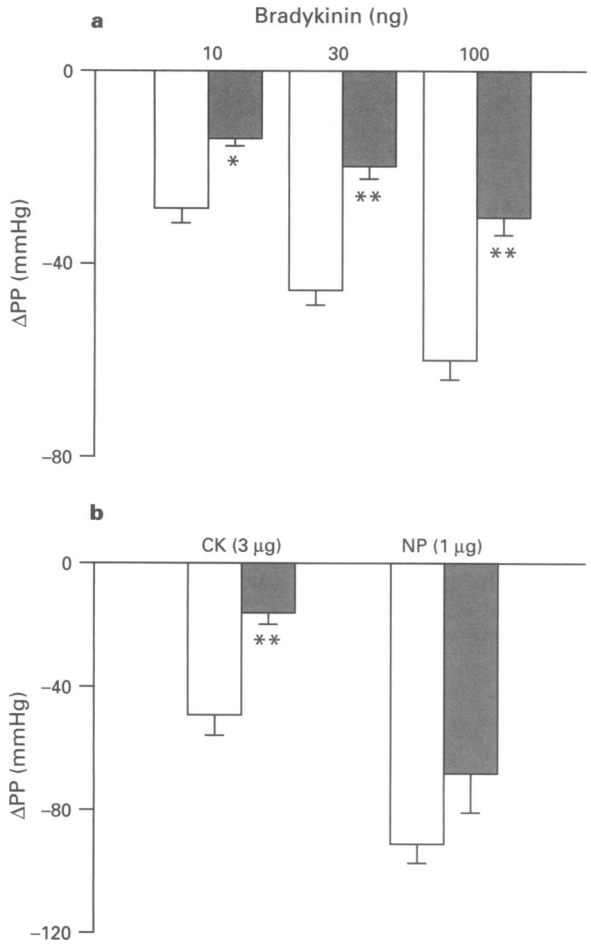

Figure 2 Effect of inhibition of K^+ channels with tetrabutylammonium (30 μM) on perfusion pressure (PP) responses to (a) bradykinin, and (b) cromakalim (CK) and nitroprusside (NP) in N^G-nitro-L-arginine (50 μM)- and indomethacin (2.8 μM)- treated perfused kidneys constricted with phenylephrine to elevate perfusion pressure to approximately 200 mmHg. Control: open columns, $n=25$ and tetrabutylammonium: stippled columns; $n=6$; $^*P<0.05$; $^{**}P<0.01$.

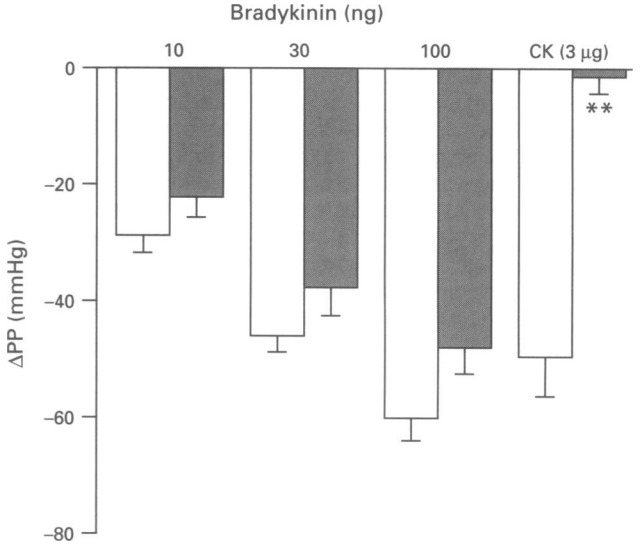

Figure 3 Effects of inhibition of ATP-sensitive K^+ channels with glibenclamide (10 μM; $n=12$; stippled columns) versus vehicle ($n=25$; open columns) on vasodilator responses, expressed as decreases in perfusion pressure (PP), to bradykinin, and cromakalim (CK) in perfused kidneys treated with N^G-nitro-L-arginine (50 μM) and indomethacin (2.8 μM) and constricted with phenylephrine to elevate perfusion pressure to approximately 200 mmHg. $^*P<0.05$; $^{**}P<0.01$.

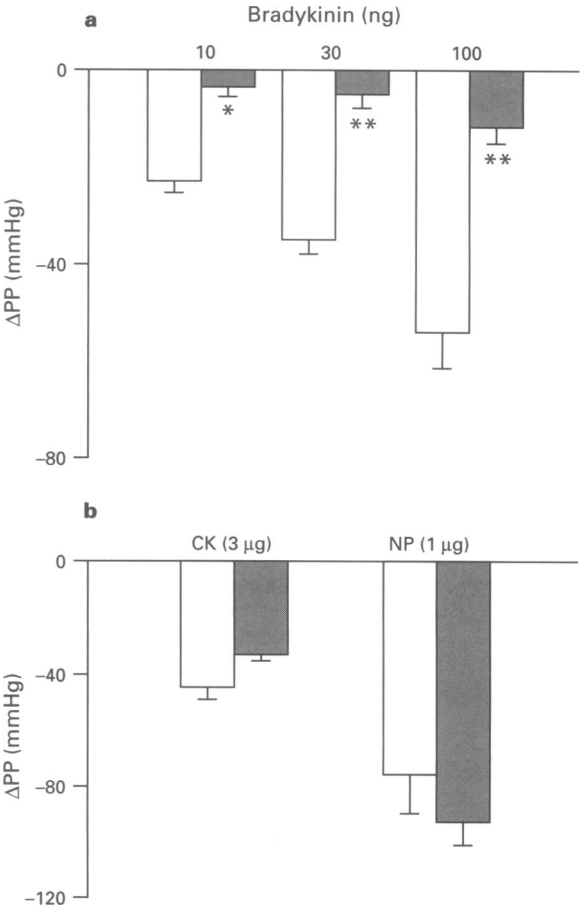

Figure 4 Effect of inhibition of Ca^{2+}-activated K^+ channels with charybdotoxin (10 nM) on vasodilator responses (changes in perfusion pressure; ΔPP) to (a) bradykinin, and (b) cromakalim (CK) and nitroprusside (NP) in kidneys with N^G-nitro-L-arginine (50 μM) and indomethacin (2.8 μM) and constricted with phenylephrine to elevate perfusion pressure to approximately 200 mmHg. Control; open columns, $n=19$ and charybdotoxin: stippled columns, $n=6$. $^*P<0.05$, $^{**}P<0.01$.

Discussion

In the rat perfused kidney, the vasodilator action of bradykinin has three components, two major ones subserved by NO and cytochrome P450 with a lesser contribution from a prostaglandin mechanism (Fulton *et al.*, 1992). We have considerable evidence for a major cytochrome P450-dependent component to the response (Fulton *et al.*, 1992), presumably via metabolism of arachidonic acid as bradykinin stimulates phospholipase C and A_2. Indeed, in the rat perfused heart in which the vasodilator effect of bradykinin is independent of NO (Baydoun & Woodward, 1991; Fulton *et al.*, 1995a) inhibitors of phospholipase C and A_2 inhibit the effects of bradykinin (Fulton *et al.*, 1995b) supporting a role for arachidonic acid metabolism. As inhibitors of cyclo-oxygenase and lipoxygenase did not affect the coronary vasodilator actions of bradykinin (Baydoun & Woodward, 1991), a role for cytochrome P450 was investigated (Fulton *et al.*, 1995a). Thus, several inhibitors of cytochrome P450, including 17-ODYA, a mechanism based inhibitor of long chain fatty acid metabolism by cytochrome P450, all attenuated coronary vasodilatation to bradykinin (Fulton *et al.*, 1995a). As NO-independent, endothelium-dependent vasodilatation has been attributed to release of hyperpolarizing factor, we also studied the role of K^+ channels in the coronary vasodilator effect of bradykinin which is mediated via stimulation of Ca^{2+}-activated K^+ channels (Fulton *et al.*, 1994). The results of these studies led to

the hypothesis that bradykinin stimulates the release of a hyperpolarizing factor that is formed as the result of cytochrome P450-dependent metabolism of arachidonic acid. Of the P450 arachidonate metabolites, an epoxide is considered the most likely candidate for the putative hyperpolarizing factor as epoxides, but not HETEs, can be demonstrated in the coronary perfusate in response to bradykinin (Quilley *et al.*, 1993; Fulton *et al.*, 1994). Moreover, epoxides are vasodilator and elicit hyperpolarization of vascular smooth muscle by stimulating large conductance Ca^{2+}-activated K^+ channels which are selectively inhibited by low concentrations of tetraethylammonium (Pratt *et al.*, 1995).

The results of the present study demonstrate that the NO- and prostaglandin-independent component of the renal vasodilator response to bradykinin, which was isolated by inhibition of NO synthesis and prostanoid synthesis and amplified by increasing vascular tone with phenylephrine, utilizes a similar mechanism to that observed in the heart, i.e., Ca^{2+}-activated K^+ channels. Thus, tetraethylammonium and tetrabutylammonium, at concentrations that partially inhibited ATP-sensitive K^+ channels, reduced the dose-dependent renal vasodilator effect of bradykinin to a similar degree to that of cromakalim. The lack of effect of glibenclamide, at a concentration that almost abolished responses to cromakalim, on vasodilator responses to bradykinin excluded a role for ATP-sensitive K^+ channels. The tendency for glibenclamide to reduce the vasodilator response to bradykinin was attributed to actions independent of inhibition of K^+ channels as responses to nitroprusside were also reduced. In contrast, the potent inhibitory effect of charybdotoxin is presumptive evidence that bradykinin-induced renal vasodilatation is dependent on Ca^{2+}-activated K^+ channels. The specificity of the effect of charybdotoxin is supported by the lack of effect on vasodilator responses to either nitroprusside or cromakalim. Charybdotoxin is considered to be more selective for large conductance Ca^{2+}-activated K^+ channels although, depending on the source and type of tissue, the actions of charybdotoxin may not only be limited to this type of channel (Kuriyama *et al.*, 1995). In vascular smooth muscle various subtypes of Ca^{2+}-activated K^+ channels have been identified: a large conductance or maxi K^+ channel with conductance of 200–300 pS; an intermediate conductance channel, 90–150 pS; and a small conductance channel (Kuriyama *et al.*, 1995). Consequently, we used two additional toxins, iberiotoxin and leiurotoxin, to inhibit large and small conductance channels, respectively. The absence of an effect of leiurotoxin excludes a role of small conductance K^+ channels which are also resistant to the inhibitory effects of charybdotoxin. Similarly, the lack of effect of iberiotoxin tends to exclude a role of large conductance K^+ channels that are generally considered to be susceptible to charybdotoxin (Kuriyama *et al.*, 1995). However, it seems likely that subtypes of large conductance Ca^{2+}-activated K^+ channels exist that exhibit differential sensitivity to charybdotoxin and iberiotoxin. Alternatively, insufficient concentrations of the K^+ channel inhibitors were used in the present study. This is unlikely, however, as we used 20 nM concentrations of iberiotoxin and leiurotoxin which is far in excess of that shown to inhibit activity of large and small Ca^{2+}-activated K^+ channels, respectively (Chicchi *et al.*, 1988; Galvez *et al.*, 1990). Therefore, these results suggest, by exclusion, that bradykinin utilizes a Ca^{2+}-activated K^+ channel of intermediate conductance (20–200 pS) to elicit NO-independent renal vasodilatation. These results also raise questions regarding the identity of the putative hyperpolarizing factor. Thus, epoxides have been proposed as likely candidates as they are vasodilator and activate K^+ channels. However, epoxides are reported to increase activity of large conductance (250 pS) Ca^{2+}-activated K^+ channels which should, therefore, be inhibited by iberiotoxin (Pratt *et al.*, 1995). Our present results in the kidney as well as the heart (unpublished observations) with iberiotoxin do not support this contention. Moreover, the results of this study invite a further consideration, namely, that hyperpolarization of endothelial cells by bradykinin receptor stimulation which, in turn, elevates intracellular Ca^{2+}, may result in the release of vasorelaxant mediators (Johns *et al.*, 1987). Although we can exclude NO and prostaglandins under our experimental conditions, we cannot exclude the release of an unknown vasorelaxant mediator resulting from stimulation of endothelial Ca^{2+}-activated K^+ channels in response to bradykinin. Thus, endothelial cells possess Ca^{2+}-activated K^+ channels that are susceptible to inhibition by tetraethylammonium, tetrabutylammonium and charybdotoxin (Rusko *et al.*, 1992), consistent with the results of this study. A limitation of the use of an isolated organ to address hyperpolarization as a mechanism for bradykinin-induced vasodilatation is the inability to measure membrane potential of vascular endothelial and smooth muscle cells of resistance vessels. Consequently, studies of this nature rely upon the use of pharmacological agents which potentially can interfere at several steps in signal transduction pathways. For example, several cytochrome P450 inhibitors can affect vasorelaxant responses to a variety of agents that utilize different mechanisms (Oyekan *et al.*, 1994). Consequently, the use of such agents is generally a compromise between a maximum effect at a specific target and the appearance of unrelated effects. However, this does not appear to be the case for the specific inhibitors of Ca^{2+}-activated K^+ channels that were used in the present study as they were without effect on vasodilator responses to either cromakalim or nitroprusside. Moreover, they are relatively large peptides that bind to extracellular sites of the K^+ channel and do not readily gain access to intracellular sites.

In summary, we have shown that the NO-independent renal vasodilator response to bradykinin that is dependent on cytochrome P450 is mediated via Ca^{2+}-activated K^+ channels: we assume, by exclusion, that this is a Ca^{2+}-activated K^+ channel of intermediate conductance.

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