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Evidence against Anandamide as the Hyperpolarizing Factor Mediating the Nitric Oxide-Independent Coronary Vasodilator Effect of Bradykinin in the Rat[1]

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ABSTRACT

The mediator of nitric oxide-(NO) independent vasodilation attributed to endothelium-derived hyperpolarizing factor remains unidentified although there is evidence for a cytochrome P450-derived eicosanoid. Anandamide, the ethanolamide of arachidonic acid and an endogenous ligand for cannabinoid receptors, was proposed as an endothelium-derived hyperpolarizing factor-mediating mesenteric vasodilation to acetylcholine and the hypotensive effect of bradykinin. Using pharmacological interventions that attenuate responses to bradykinin, we examined the possibility of anandamide as a mediator of the NO-independent vasodilator effect of bradykinin in the rat perfused heart by determining responses to anandamide and arachidonic acid. Hearts were treated with indomethacin to exclude prostaglandins and nitroarginine to inhibit NO synthesis and elevate perfusion pressure. The cannabinoid receptor antagonist, SR 141716A (2 μM), reduced dose-dependent vasodilator responses to anandamide (1–10 μg) but was without effect on responses to AA (1–10 μg), bradykinin (10–1000 ng) or cromakalim (1–10 μg). Inhibition of voltage-dependent Ca^{++} channels with nifedipine (5 nM) attenuated vasodilation to anandamide and arachidonic acid whereas inhibition of Ca^{++}-activated K^+ channels with charybdotoxin (10 nM) reduced responses to arachidonic acid but had no effect on vasodilation induced by anandamide. Inhibition of cytochrome P450 with clotrimazole (1 μM) greatly reduced vasodilator responses to bradykinin with less effect on those to anandamide. Finally, the time course of the coronary vasodilator responses to anandamide and bradykinin were dissimilar. These results argue against a role of anandamide in the vasodilator effect of bradykinin in the rat heart.

The recognition by Furchgott and Zawadski (1980) of the requirement for an intact endothelium for responses to certain vasodilator agonists led to the identification of NO as EDRF. The introduction of inhibitors of NO synthesis underscored the importance of NO to the regulation of vascular tone. However, their use also resulted in the realization that NO could not fully account for endothelium-dependent responses to various agonists including bradykinin and acetylcholine, depending on the vascular bed and the species. Consequently, release of an unidentified hyperpolarizing factor, a term first coined by Taylor and Weston (1988), was invoked.

Currently, there is considerable support for a P450-derived metabolite of AA as an EDHF (Bauersachs *et al.*, 1994; Hecker *et al.*, 1994; Campbell *et al.*, 1996; Popp *et al.*, 1996) although problems with the specificity of inhibitors of P450 have culminated in several recent studies that question this hypothesis (Zygmunt *et al.*, 1996; Edwards *et al.*, 1996; Fukao *et al.*, 1997). Our studies with bradykinin in the rat heart and/or kidney demonstrate that the NO-independent vasodilator effect of this peptide is susceptible to inhibitors PLC and PLA_2, P450 and K^+ channels, supporting the concept of a P450-derived eicosanoid as a hyperpolarizing factor (Fulton *et al.*, 1992, 1994, 1995, 1996; Rapacon *et al.*, 1996). Studies using inhibitors of P450 that exhibit differential activity against epoxygenase *vs.* w-hydroxylase, *i.e.*, clotrimazole *vs.* 17-ODYA (Fulton *et al.*, 1995), suggest that of the AA metabolites, an EET is the most likely candidate. Moreover, GC-MS analysis of coronary perfusates revealed the release of EETs but not HETEs. EETs are vasodilator, synthesized by the endothelium and stimulate Ca^{++}-activated K^+ channels (Campbell *et al.*, 1996). Our pharmacological studies indicate that, of the four EET regioisomers, only 5,6 EET can fulfill the criteria for a putative mediator of the coronary vasodilator effect of bradykinin (Quilley *et al.*, 1997).

However, there are major reservations concerning the pro-

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ABBREVIATIONS: EDHF, endothelium-derived hyperpolarizing factor; AA, arachidonic acid; NO, nitric oxide; P450, cytochrome P450; EET, epoxide; HETE, hydroxyeicosatetraenoic acid; PLC; phospholipase C; PLA_2, phospholipase A_2; GC-MS, gas chromatography-mass spectrometry.

posal that a P450-AA metabolite may be a hyperpolarizing factor. These revolve primarily around the limited specificity of the inhibitors that have been used to implicate P450 (Edwards *et al.*, 1996; Fukao *et al.*, 1997; Ohlmann *et al.*, 1997). As a result, other potential mediators have been sought and Randall *et al.* (1996) proposed that anandamide, the ethanolamide of AA and the putative endogenous ligand for cannabinoid receptors (Devane *et al.*, 1992), may be an EDHF in the rat. Thus, a product with the chromatographic properties of authentic anandamide was released from the perfused mesenteric vascular bed labeled with ^{3}H-AA and challenged with carbachol and the mesenteric vasodilator effect of anandamide was greatly reduced in the presence of depolarizing concentrations of KCl, suggesting a role for activation of K^+ channels (Randall *et al.*, 1996). This group also reported that the NO-independent hypotensive effect of bradykinin in the anesthetized rat was attenuated by pretreatment with a cannabinoid receptor antagonist that also blocked the effect of anandamide in the mesenteric vasculature (Randall *et al.*, 1996). In contrast to these studies in the rat, Pratt *et al.* (1998) reported that the vasorelaxant effect of anandamide in the bovine coronary artery was independent of cannabinoid receptors but involved the release of AA and its subsequent conversion to vasodilatory eicosanoids.

Consequently, we used pharmacological criteria, based on our studies with bradykinin, to examine whether anandamide could fulfill the requirements for a putative mediator for bradykinin-induced vasodilation in the isolated heart of the rat. Thus, we determined coronary vasodilator responses to anandamide in the presence and absence of nifedipine to prevent vasodilation resulting from closure of voltage-dependent Ca^{++} channels, charydotoxin to inhibit Ca^{++}-activated K^+ channels and SR 141716A to antagonize cannabinoid receptors. The effects of SR 141517A on responses to bradykinin were also determined. As anandamide is readily cleaved by an amidase to yield AA, the effects of these interventions on responses to AA were also examined. We also compared the effects of a P450 inhibitor, clotrimazole, on responses to bradykinin and anandamide as this compound is a substrate for P450 (Bornheim *et al.*, 1993). The results indicate that anandamide is unlikely to be the mediator of bradykinin-induced, NO-independent vasodilation in the rat heart.

Methods

Male Wistar rats, weight 360 to 460 g, were anaesthetized with pentobarbital, 65 mg/kg i.p., and heparin, 1000 U/kg, was administered i.v. After thoracotomy, the heart with attached aorta was excised and flushed free of blood with ice-cold Krebs' buffer. The heart was then cannulated via the aorta and perfused retrogradely with oxygenated Krebs' buffer at 37°C at a constant flow rate (8–10 ml/min) to obtain an initial basal perfusion pressure of 30 to 40 mmHg. The perfusate contained indomethacin (2.8 μM) to inhibit cyclooxygenase and nitroarginine (50 μM) was added to inhibit NO synthase and elevate perfusion pressure to 130 to 140 mmHg and also to reproduce the experimental conditions that were used to address the mechanism of bradykinin-induced vasodilation (Fulton *et al.*, 1994, 1995, 1996).

Once a stable elevated perfusion pressure was obtained, vasodilator responses to increasing doses of anandamide (1, 3 and 10 μg) were determined followed by responses to increasing doses of AA (1, 3 and 10 μg) in the absence ($n = 8$) and presence of nifedipine (5 nM; $n = 4$), charydotoxin (10 nM; $n = 5$) and SR 141716A (2 μM; $n = 6$). Thus, we have previously reported that the coronary vasodilator activity of bradykinin is reduced by nifedipine and charybdotoxin (Fulton *et al.*, 1994) whereas the vasodilator effect of anandamide in the rat mesenteric vascular bed is inhibited by SR 141716A (Randall *et al.*, 1996). The antagonists were added to the perfusate at least 10 min before obtaining responses to anandamide and AA. The concentration of SR 141716A was twice that used by Randall *et al.* (1996) whereas the concentration of nifedipine and charybdotoxin were those we had previously shown to inhibit coronary vasodilator responses to bradykinin (Fulton *et al.*, 1994). Three to four preparations per day were completed and at least one served as a control; the others were assigned randomly to each of the treatment groups. In the experiments with SR 141716A, responses to nitroprusside (1 μg) were used an index of effects apparently unrelated to antagonism of cannabinoid receptors. In the experiments with nifedipine and SR 141716A which both reduced coronary vascular tone, U46619 was added to the perfusate (10 ng/ml for nifedipine and 0.5–1.0 ng/ml for SR 141716A) to restore perfusion pressure to its previous level.

In a second series of experiments, we compared the effects of SR 141716A (2 μM; $n = 4$) or vehicle ($n = 4$) on coronary vasodilator responses to bradykinin (10–1000 ng) as the hypotensive response to bradykinin in anesthetized rats has been reported to be attenuated by pretreatment with SR 141517A (Randall *et al.*, 1996). Responses to cromakalim (1, 3 and 10 μg) were used to assess any direct effects of SR 141716A on K^+ channels and unrelated to cannabinoid receptor antagonism.

In a third series of experiments, vasodilator responses to anandamide (3 and 10 μg) and bradykinin (30 and 100 ng) were compared in the absence ($n = 6$) and presence ($n = 5$) of the P450 inhibitor, clotrimazole (1 μM), as coronary vasodilator responses to bradykinin have been shown to be attenuated by clotrimazole (Fulton *et al.*, 1995) and anandamide has been reported to be a substrate for P450 (Bornheim *et al.*, 1993). However, if anandamide is the mediator of bradykinin-induced vasodilation, then clotrimazole should be without effect on responses to anandamide although attenuating those to bradykinin. We chose clotrimazole, despite reports of effects on K^+ channels, because it is considered to be more specific for epoxygenase than ω-hydroxylase. Moreover, at the concentration chosen (1 μM), we have no evidence for effects on K^+ channels as clotrimazole did not affect vasodilator response to cromakalim or SCA 40 (Fulton *et al.*, 1994) which has been reported to stimulate Ca^{++}-activated K^+ channels (Laurent *et al.*, 1993).

Statistics. Vasodilator responses in control and treatment groups were compared by analysis of variance and individual points were compared by Neuman-Keuls test. Differences were considered statistically significant when $P < .05$.

Materials. Anandamide was obtained from Biomol (Plymouth Meeting, PA) and was dissolved in ethanol. Indomethacin, nitroarginine, bradykinin, nifedipine, cromakalim, clotrimazole and nitroprusside were purchased from Sigma Chemical Co. (St. Louis, MO). Indomethacin was dissolved in 4.2% $NaHCO_3$, clotrimazole in ethanol and cromakalim in ethanol before dilution with saline. The other agents were dissolved in distilled water. Charybdotoxin was purchased from Peptides International (Louisville, KY) and was dissolved in distilled water. SR141716A was a gift from RBI (Natick, MA) supported by NIMH Chemical Synthesis Program and was dissolved in ethanol. U46619 was obtained from UpJohn (Kalmazoo, MI) and was dissolved in ethanol and diluted with distilled water. Arachidonic acid (NuChek, Elysian, MN) was dissolved in distilled water.

Results

Initial basal perfusion pressures were not different in the various groups: vehicle, 37 ± 2 mmHg; SR 141716A, 38 ± 2 mmHg; charybdotoxin, 39 ± 2 mmHg and nifedipine, 42 ± 3

mmHg. Elevated perfusion pressures were comparable in all the groups except the charybdotoxin group where pressure was further increased by inhibition of Ca^{++}-activated K^+ channels to 155 ± 4 mmHg compared to 134 ± 2 mmHg for vehicle, 138 ± 3 mmHg for SR 141716A and 131 ± 6 mmHg for nifedipine.

In the vehicle control group, 1, 3 and 10 μg anandamide elicited dose-dependent falls in perfusion pressure of 11 ± 2, 24 ± 3 and 40 ± 3 mmHg, respectively (fig. 1). The cannabinoid receptor antagonist, SR 141716A, reduced the coronary vasodilator response to the two lower doses of anandamide, 6 ± 1 and 15 ± 2 mmHg ($P < .05$), but was without effect on the highest dose, 36 ± 3 mmHg. In contrast, the dose-dependent coronary vasodilator response to AA was unaffected by SR 141716A (fig. 1). SR 141716A did not affect vasodilator responses to nitroprusside, 37 ± 4 *vs.* 44 ± 7 mmHg for the control.

Inhibition of voltage-dependent Ca^{++} channels with nifedipine diminished the vasodilator effects of 3 and 10 μg anandamide ($P < .05$) and AA ($P < .05$) to a similar degree without affecting the responses to the lowest doses of these agents (fig. 2).

Inhibition of Ca^{++}-activated K^+ channels with charydotoxin did not reduce the coronary vasodilator response to anandamide (fig. 3), rather, the response to the lowest dose of anandamide was slightly increased from 11 ± 2 to 16 ± 2 mmHg ($P < .05$). In contrast, the coronary vasodilator effect of AA was significantly reduced in the presence of charydotoxin (fig. 3).

In the second series of experiments to determine the effects of SR 141716A on vasodilator responses to bradykinin and cromakalim, basal and elevated perfusion pressures in the control and treatment groups were 34 ± 2 and 134 ± 6

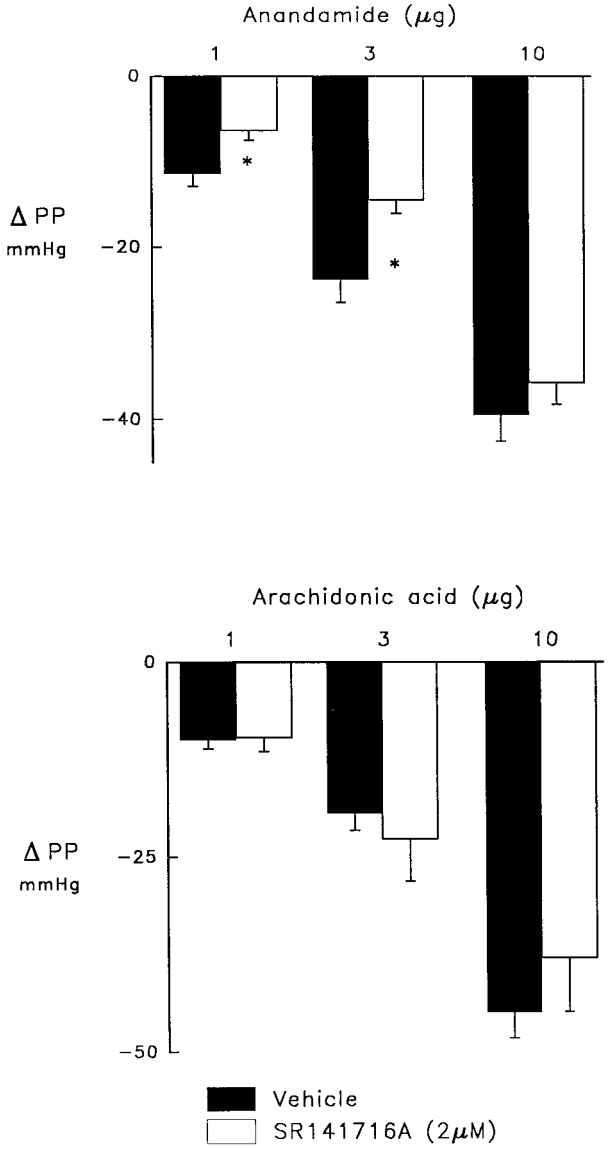

Fig. 1. Effect of the cannabinoid receptor antagonist, SR 141716A, (2 μM; open bars) or vehicle (solid bars) on vasodilator responses to anandamide (upper panel) and arachidonic acid (lower panel) in the rat isolated perfused heart treated with indomethacin (2.8 μM) and nitroarginine (50 μM) which elevated perfusion pressure from 30–40 to 130–140 mmHg.

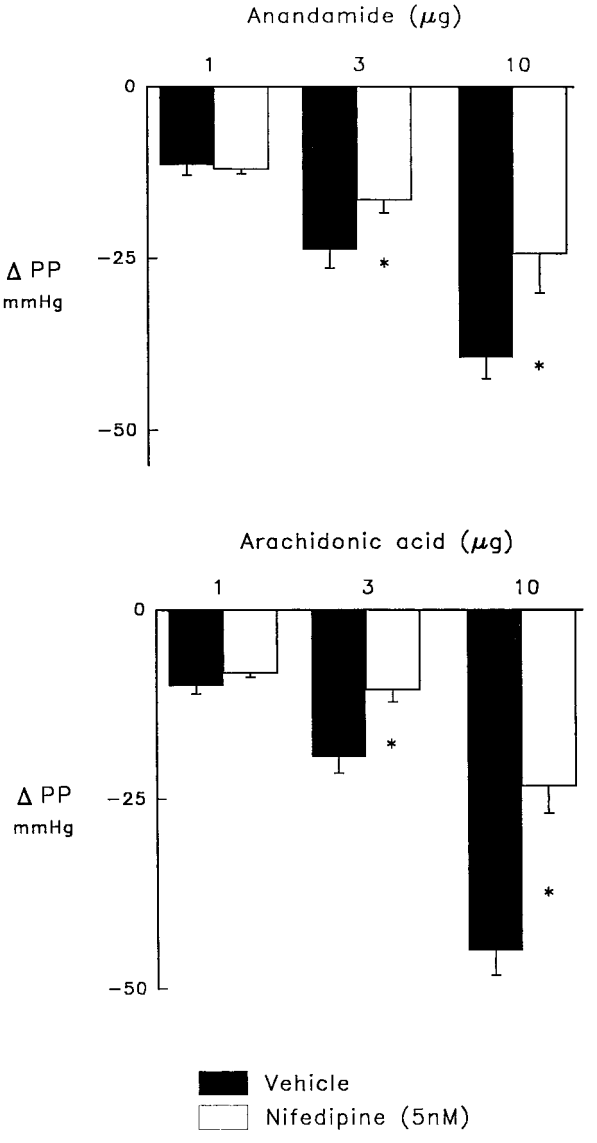

Fig. 2. Vasodilator responses to anandamide (upper panel) and arachidonic acid (lower panel) in control hearts (solid bars) and in the presence of nifedipine (5 nM; open bars). Heart were treated with indomethacin (2.8 μM) and nitroarginine (50 μM) to inhibit cyclooxygenase and NO synthase and elevate perfusion pressure from 30–40 to 130–140 mmHg. Nifedipine reduced elevated perfusion pressure that was restored with U46619 (10 ng/ml).

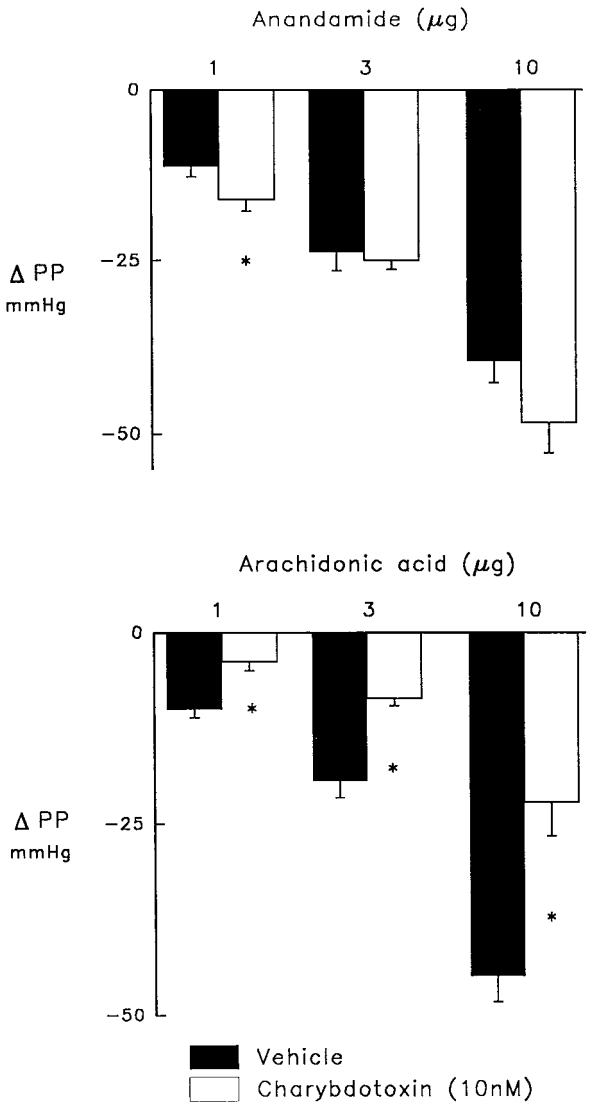

Fig. 3. Effects of charybdotoxin (10 nM; open bars) compared to vehicle (solid bars) on vasodilator responses to anandamide (upper panel) or arachidonic acid (lower panel) in the isolated perfused heart treated with indomethacin (2.8 μM) and nitroarginine (50 μM) to inhibit cyclooxygenase and NO synthase and elevate perfusion pressure from 30–40 to 130–140 mmHg. Charybdotoxin caused a further elevation of perfusion pressure to 155 mmHg.

mmHg, respectively, and 36 ± 2 and 138 ± 5 mmHg, respectively. SR 141716A did not affect responses to bradykinin (fig. 4) but tended to reduce those to cromakalim although the differences were not significant. In control hearts, 1, 3 and 10 μg cromakalim decreased perfusion pressure by 6 ± 1, 22 ± 3 and 58 ± 7 mmHg, respectively, compared to 5 ± 3, 13 ± 4 and 43 ± 4 mmHg, respectively, for hearts treated with SR 141716A.

In the presence of clotrimazole to inhibit P450, vasodilator responses to bradykinin were almost abolished, confirming our previous results (Fulton *et al.*, 1995). Thus, reductions in perfusion pressure to 30 and 100 ng bradykinin were 2 ± 1 and 6 ± 2 mmHg, respectively, compared to control values of 20 ± 3 and 39 ± 4 mmHg, respectively. Clotrimazole also reduced coronary vasodilator responses to 3 and 10 μg anandamide from 21 ± 2 and 33 ± 2 mmHg, respectively, to 12 ± 1 and 23 ± 1 mmHg, respectively. Elevated perfusion pressure in the control group was 131 ± 1 mmHg compared to 128 ± 2 mmHg in the clotrimazole group.

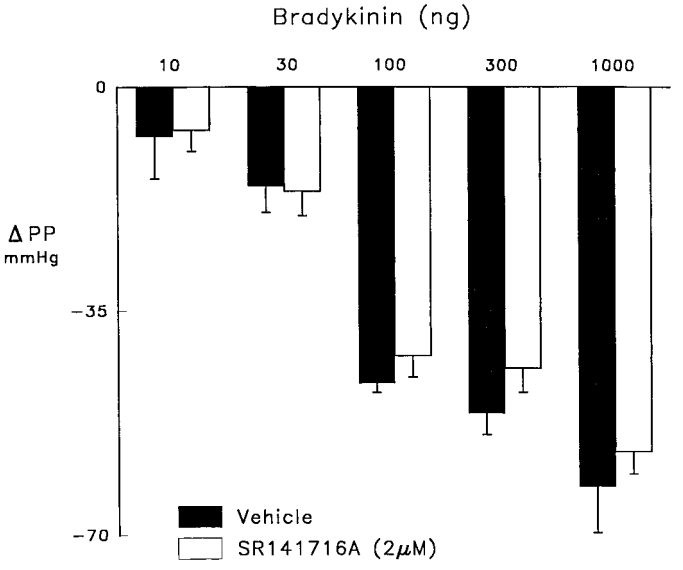

Fig. 4. Vasodilator responses to bradykinin in control hearts (solid bars) and those treated with SR 141716A (2 μM; open bars). The coronary perfusate contained indomethacin (2.8 μM) and nitroarginine (50 μM) to inhibit cyclooxygenase and NO synthase and elevate perfusion pressure from 30–40 to 130–140 mmHg. SR 141716A reduced elevated perfusion pressure which was restored with U46619 (0.5–1.0 ng/ml).

Figure 5 shows a recording of perfusion pressure from a vehicle-treated heart and the vasodilator responses to bradykinin and anandamide. The response to bradykinin was rapid in onset and of short duration whereas the response to anandamide developed more slowly and was of longer duration.

Discussion

Several studies have provided evidence to support a P450-derived metabolite of AA as an EDHF mediating the NO-independent vasodilator/vasorelaxant response to bradykinin and/or acetylcholine (Hecker *et al.*, 1994; Bauersachs *et al.*, 1995; Campbell *et al.*, 1996; Popp *et al.*, 1996). Our studies are consistent with this concept as the coronary and/or renal vasodilator action of bradykinin is susceptible to inhibitors of PLC and PLA_2, P450 and Ca^{++}-activated K^+ channels (Fulton *et al.*, 1992, 1994, 1995, 1996). Of the P450-AA metabolites, an EET is considered the most likely as an EDHF as EETs are produced by the endothelium and are vasodilator, presumably by their ability to activate K^+

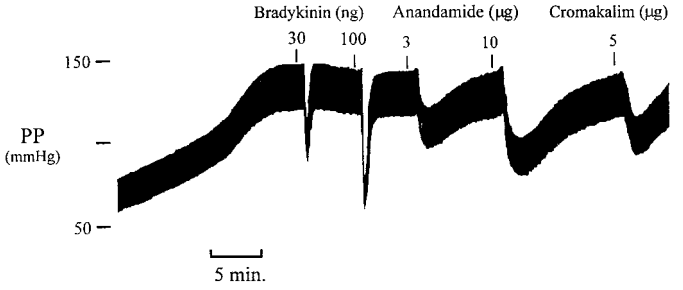

Fig. 5. Recording of perfusion pressure in response to bradykinin, anandamide and cromakalim in the isolated heart treated with indomethacin (2.8 μM) and nitroarginine (50 μM) to inhibit prostaglandin and NO synthesis and elevate perfusion pressure to approximately 130 mmHg.

channels (Hu and Kim, 1993; Campbell *et al.*, 1996). However, a role of P450 has been questioned as inhibitors of this pathway exhibit a variety of actions apparently unrelated to inhibition of P450 and including effects on K^+ channels (Oyekan *et al.*, 1994; Edwards *et al.*, 1996). Moreover, the administration of EETs has been reported to be without effect in some vascular preparations (Zygmunt *et al.*, 1996). Consequently, alternative mediators have been sought and Randall *et al.* (1996) proposed the ethanolamide of AA (anandamide) which is the putative endogenous ligand for cannabinoid receptors. We considered anandamide an attractive possibility for mediating vasodilator responses to bradykinin because our previous results would not be inconsistent with this concept; as an analogue of AA, anandamide would presumably be stored in phospholipids and released by the actions of phospholipases whereupon it could also serve as a substrate for P450 (Bornheim *et al.*, 1993) to produce a vasodilator that activates K^+ channels.

To address this possibility, we determined the vasodilator activity of anandamide in the presence of pharmacological interventions that inhibit NO-independent coronary vasodilator responses to bradykinin. Under identical experimental conditions of inhibition of prostaglandin and NO synthesis, coronary vasodilator responses to anandamide were tested after treatment of hearts with nifedipine, charybdotoxin, clotrimazole and SR141716A and compared to those obtained with AA or bradykinin. The results obtained argue against anandamide as the mediator of bradykinin-induced vasodilation. First, inhibition of Ca^{++}-activated K^+ channels with charybdotoxin at a concentration that almost abolished coronary vasodilator responses to bradykinin (Fulton *et al.*, 1994) was without effect on vasodilator responses to anandamide. The only explanation for these observations that permits consideration of anandamide as the vasodilator mediator for bradykinin is that bradykinin stimulates a charydotoxin-sensitive K^+ channel in the endothelium to result in the release of the mediator, in this case anandamide. In this scenario, administration of the mediator, anandamide, would by-pass the processes involved in its synthesis and/or release. Consequently, any intervention that modifies the response to anandamide should also modify that to the initiating stimulus, *i.e.*, bradykinin. However, the failure of the cannabinoid receptor antagonist, SR 141716A, to inhibit the vasodilator effect of bradykinin although reducing that to anandamide argues against this possibility regardless of whether the effect of SR 141716A is via inhibition of cannabinoid receptors or an alternative mechanism. Thus, the inhibitory effect of SR 141716A on responses to anandamide was not pronounced and may reflect functional antagonism (White and Hiley, 1997). Nonetheless, if anandamide is the mediator of bradykinin-induced vasodilation, then SR 141716A should also attenuate the response to bradykinin which was not the case.

The possibility that anandamide yields AA that then undergoes transformation by P450 to generate a vasodilator product was also addressed in this study. Thus, the relatively slow onset of vasodilation to anandamide compared with bradykinin is consistent with conversion to an active product. The observation that nifedipine reduced the coronary vasodilator effects of both anandamide and AA is consistent with a common vasodilator mechanism that involves closure of voltage-dependent Ca^{++} channels in response to hyperpolarization, for example. However, the K^+ channels responsible for the effects of anandamide and AA must be different, based on the results with charybdotoxin that markedly reduced the coronary vasodilator effect of AA but not that of anandamide. These observations are good evidence against anandamide as a source of AA which then exerts a direct effect or serves as a precursor for the formation of a product that elicits vasodilation via a charybdotoxin-sensitive mechanism. The alternative explanation, that AA stimulates an endothelial K^+ channel to initiate the release of a vasodilator is untenable as inhibition of cannabinoid receptors with SR141716A reduced responses to anandamide but failed to influence responses to AA. The effect of SR141716A to reduce responses to anandamide is unlikely to be due to an effect on K^+ channels as SR141716A did not affect responses to cromakalim and did not alter responses to bradykinin or AA which are dependent on activation of K^+ channels.

Finally, we addressed the effect of an inhibitor of P450, clotrimazole, on the coronary vasodilator action of anandamide as we have previously shown this agent reduces the coronary and renal vasodilator actions of bradykinin. If anandamide itself is the mediator of the bradykinin effect, then inhibition of P450 with clotrimazole should be without effect. Alternatively, if anandamide, after its release in response to bradykinin, requires conversion by P450 for activity, then clotrimazole should inhibit the vasodilator effect of both anandamide and bradykinin to the same degree. Clotrimazole virtually abolished vasodilator responses to bradykinin in this series of experiments, consistent with our previous observations (Fulton *et al.*, 1995). In contrast, inhibition of vasodilation induced by anandamide was much less pronounced, a result that provides further evidence against anandamide as the mediator for bradykinin. However, the observation that clotrimazole reduced the vasodilator activity of anandamide indicates that an intact P450 system may be required. Thus, anandamide can be a substrate for P450 (Bornheim *et al.*, 1993) although the activity of any products to elicit vasodilation remains to be determined. It is unlikely that anandamide first releases AA which is then converted by P450 to vasodilatory eicosanoids as suggested by Pratt *et al.* (1998) because charybdotoxin failed to affect dilator responses to anandamide but inhibited those to AA. An alternative explanation for the inhibitory effects of clotrimazole on vasodilation induced by anandamide is that clotrimazole exerts effects on K^+ channels or even the cannabinoid receptor in addition to inhibiting P450.

The results from this study, therefore, do not support the hypothesis proposed by Randall *et al.* (1996) that anandamide is an EDHF in the rat. However, they used the perfused mesentery and studied vasodilation to acetylcholine which was inhibited by SR141716A as was the endothelium-independent vasodilator effect of anandamide, suggesting a role for CB_1 receptors. This is in contrast to our studies where the effects of bradykinin in the heart were addressed and which provides a possible explanation for the different results. Thus, depending on the tissue and the agonist, different hyperpolarizing factors may be involved. However, the observation of Randall *et al.* (1996) that the NO-independent hypotensive effect of bradykinin in the rat is also attenuated by SR141716A, suggesting a mechanism operating through cannabinoid receptors, is not supported by our study.

In summary, our observations, when viewed collectively,

strongly suggest that anandamide is unlikely to be the EDHF mediating the NO-independent vasodilator effect of bradykinin in the rat heart and support the conclusions reached by Plane *et al.* (1997) and Pratt *et al.* (1998). Although nifedipine reduced the response to anandamide as was reported for bradykinin, charybdotoxin was without effect and, more conclusively, the cannabinoid receptor antagonist reduced the vasodilation to anandamide but was without effect on that to bradykinin. Further, the time course of the vasodilator response to bradykinin and anandamide was dissimilar; that to anandamide was slow in onset and of prolonged duration.

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