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SPECIAL REPORT

Evidence that anandamide and EDHF act via different mechanisms in rat isolated mesenteric arteries

[1]F. Plane, *M. Holland, G.J. Waldron, C.J. Garland & *J.P. Boyle

Department of Pharmacology, University of Bristol, University Walk, Bristol, BS8 1TD and *Department of Cell Physiology & Pharmacology, University of Leicester, PO Box 138, Leicester LEI 9HN

The endogenous cannabinoid, anandamide, has been suggested as an endothelium-derived hyperpolarizing factor (EDHF). We found that anandamide-evoked relaxation in isolated segments of rat mesenteric artery was associated with smooth muscle hyperpolarization. However, although anandamide-evoked relaxation was inhibited by either charybdotoxin (ChTX) or iberiotoxin, inhibition of the relaxation to EDHF required a combination of ChTX and apamin. The relaxations induced by either anandamide or EDHF were not inhibited by the cannabinoid receptor (CB_1) antagonist SRI41716A, or mimicked by selective CB_1 agonists. Thus, anandamide appears to cause smooth muscle relaxation via a CB_1 receptor-independent mechanism and cannabinoid receptor activation apparently does not contribute to EDHF-mediated relaxation in this resistance artery.

Keywords: Anandamide; EDHF; smooth muscle relaxation; hyperpolarization

Introduction Recently, it was shown that endothelium-derived hyperpolarizing factor (EDHF)-induced relaxation of the rat isolated mesenteric bed could be inhibited by the CB_1 cannabinoid receptor antagonist SR141716A, and that anandamide, an endogenous cannabinoid, may be an EDHF in mesenteric arteries (Randall *et al*., 1996). In the present study, the mechanism underlying anandamide-evoked relaxation in rat single, isolated mesenteric arteries was examined and compared to EDHF-mediated relaxation.

Methods Male Wistar or Sprague-Dawley rats (250–350 g,) were stunned and killed by cervical dislocation. Segments (2 mm in length) of third order branches of the superior mesenteric artery (D_{100} 315±12 μm; $n=24$) were removed and mounted in a Mulvany-Halpern myograph, under normalized tension, for simultaneous recording of smooth muscle membrane potential and tone as previously described (Waldron & Garland, 1994). The tissues were maintained in a static bath and concentration-response curves were constructed by cumulative addition of anandamide to arterial segments preconstricted with phenylephrine (1–3 μM). All experiments were carried out in endothelium-intact arteries plus indomethacin (2.8 μM) and the nitric oxide (NO) synthase inhibitors N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester (both 100 μM). In the presence of the potassium channel inhibitors, the concentration of phenylephrine was adjusted to ensure a similar level of tone to control conditions. Data are expressed as mean±s.e.mean and the significance of differences between means calculated by Student's *t* test. All drugs were supplied by Sigma except for anandamide (CalBiochem), 3-(1,1-dimethylheptyl)-0-11-hydroxy-Δ^8-tetrahydrocannabinol (HU210; Tocris), **R**(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo [1,2,3-de] -1,4-benz-oxazon-yl] - (1-napthalenyl) methanone mesylate (WIN 55,212-2; RBI) and N-piperidino-5-(4-chlorophenyl) -1-(2,-dichloophenyl)-4-methyl-3-pyrazole-carb-oxamide (SR141716A; gift from Sanofi). Stock solutions of anandamide, HU210, WIN55,2212-2 and SR141716A were prepared in ethanol.

Results The resting membrane potential of smooth muscle cells in the mesenteric artery was −59.3±3.7 mV ($n=14$ cells from 5 tissues). Anandamide (1–300 nM) caused endothelium-independent repolarization and relaxation of arterial segments precontracted with phenylephrine (mean contraction and depolarization 15.4±3.4 mN ($n=16$) and 28.0±4.3 mV ($n=4$); Figure 1a). The maximal repolarization and relaxation to anandamide were 89.0±6.1% ($n=4$) and 93.0±5.6% ($n=26$), respectively, with pD_2 values of 7.99±0.09 ($n=4$) and 8.0±0.04 ($n=26$), respectively. Reproducible repolarization and relaxation required frequent washing for at least 60 min between dose-response curves. Increasing the extracellular potassium concentration to 60 mM abolished relaxation to both anandamide and EDHF ($n=6$).

The CB_1 antagonist SR141716A (5 μM) did not modify anandamide-induced relaxation; (maximum relaxation, 94.1±3.2%; pD_2 value, 8.04±0.19 ($n=4$)). Similarly, SR141716A did not alter EDHF-mediated relaxation to either acetylcholine (maximum, control 97.4±1.9%; test 94.5±3.8%; $n=5$) or A23187 (maximum, control 95.1±1.79%; test 92.2±1.5%; $n=5$). In addition, HU210 (0.01–3 μM) and WIN 55,212-2 (0.01–3 μM), CB_1 agonists structurally unrelated to anandamide, caused maximum relaxant responses of only 24.9±6.8% ($n=5$) and 0% ($n=15$), respectively.

Anandamide-induced relaxations were not altered by apamin (50 nM; $n=6$), but were significantly inhibited by either charybdotoxin (ChTX; 100 nM; $n=4$; $P<0.01$) or iberiotoxin (100 and 300 nM; Figure 1b). In contrast, EDHF-mediated relaxation was significantly attenuated by apamin (maximum relaxation reduced to 33±4.7% ($n=3$; $P<0.05$)) and, although ChTX alone was without effect ($n=4$), exposure of arterial segments to a combination of apamin and ChTX abolished EDHF-mediated relaxation and repolarization ($n=4$; $P<0.01$; Figure 1c).

Discussion The clear correlation between relaxation and repolarization to anandamide, and the sensitivity of these responses to increases in the extracellular potassium concentration, clearly indicate a link between the two parameters. This is very similar to the link between EDHF and smooth muscle relaxation in this vascular bed (Garland & McPherson, 1992). However, relaxation to anandamide appears to be mediated by potassium channels which are different from those opened by EDHF and does not appear to be mediated through activation of CB_1 cannabinoid receptors.

Anandamide evoked a concentration-dependent relaxation in depolarized and precontracted arterial segments which was

[1] Author for correspondence.

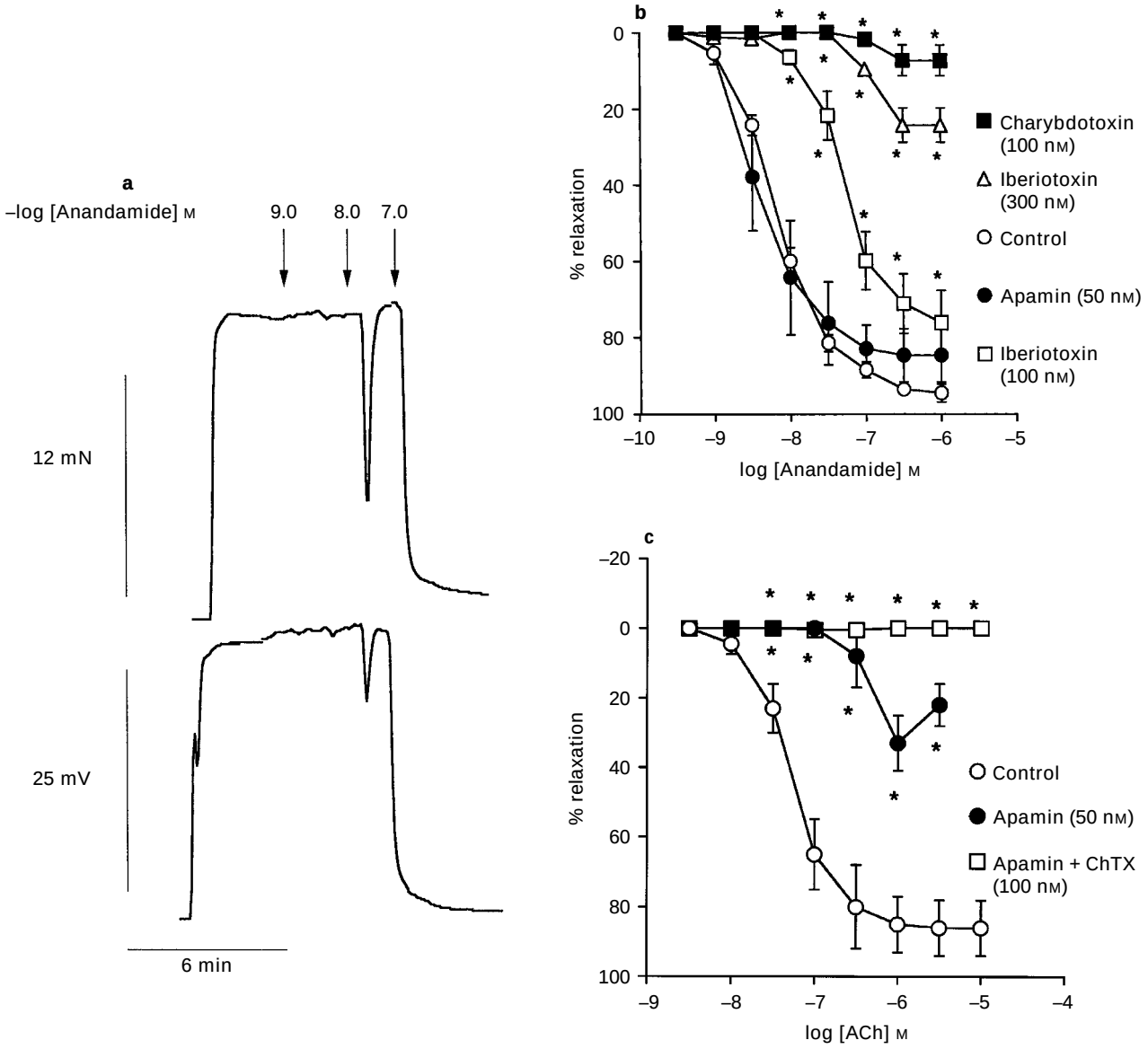

Figure 1 (a) Representative traces showing simultaneous recording of anandamide (1–100 nM)-evoked changes in smooth muscle membrane potential and tension in tissues stimulated with phenylephrine. (b and c) Mean concentration-response curves for (b) anandamide- and (c) acetylcholine-evoked relaxation of arterial segments in the presence and absence of potassium channel inhibitors. All values are means of 3 to 6 experiments with s.e.means shown by vertical lines $*P<0.01$ compared to control values.

mirrored by repolarization of the smooth muscle cell membrane potential. These responses were abolished by 60 mM extracellular potassium, a possible indication that potassium channel opening is required for relaxation to anandamide. However, 25 mM potassium, which has been shown to abolish EDHF-mediated relaxation in the same vessels (Adeagbo & Triggle, 1993; Waldron & Garland, 1994), only slightly inhibited relaxation to anandamide (data not shown).

Anandamide has been shown to cause relaxation via activation of cannabinoid CB_1 receptors (Randall *et al*., 1996). However, in the present study, the selective CB_1 antagonist SR141716A did not alter anandamide-evoked relaxation of segments of mesenteric artery. Also, HU210 and WIN 55,212-2, selective CB_1 agonists which are structurally unrelated to anandamide, were either much less effective or ineffective as relaxants. These observations indicate that anandamide evokes arterial repolarization and relaxation independently of CB_1 receptors. Anandamide is structurally similar to arachidonic acid, and might therefore interact directly with potassium channels or an associated protein (Clark *et al*., 1997).

SR141716A had no effect on EDHF-induced relaxations to either acetylcholine or A23187, providing further evidence that cannabinoid receptors are not involved in the actions of EDHF. These observations clearly contrast with SR141716A-induced block of dilatation to both carbachol and A23187 in the rat isolated, perfused mesenteric bed found previously (Randall *et al*., 1996). However, in that study, around 40–50% and 60–70%, respectively of the carbachol and A23187-induced dilatation persisted in the presence of the antagonist. Thus, as in the present investigation, a large component of the EDHF response appeared to be independent of CB_1 receptor activation.

Relaxation to anandamide was unaffected by apamin, an inhibitor of small-conductance calcium-activated potassium channels, but almost completely inhibited by either ChTX or iberiotoxin. This finding suggests that activation of large-conductance calcium-activated potassium channels may underlie relaxation to anandamide. In contrast, ChTX did not affect EDHF-mediated relaxation, whereas apamin caused a significant reduction in the response and a combination of apamin and ChTX together abolished EDHF-evoked relaxa-

tion (as previously described in other arteries: Corriu *et al*., 1996; Zygmunt & Högestatt, 1996).

In conclusion, although anandamide-evoked relaxation in rat isolated mesenteric arteries appears to be associated with repolarization of the smooth muscle membrane potential, the characteristics of this response differ markedly from EDHF-mediated relaxation in these arteries. This suggests that anandamide is not an EDHF in this tissue.

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