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Role of phospholipase A_2 and myoendothelial gap junctions in melittin-induced arterial relaxation

Iain R. Hutcheson [1], Tudor M. Griffith *

Department of Diagnostic Radiology, Wales Heart Research Institute, University of Wales College of Medicine, Health Park, Cardiff CF14 4XN, UK

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Abstract

We have used preconstricted rings of rabbit superior mesenteric artery to investigate the contribution of phospholipase A_2 and gap junctional communication to endothelium-derived hyperpolarizing factor (EDHF)-type relaxations evoked by melittin, a polypeptide toxin known to mobilize arachidonic acid from the cell membrane. Arachidonyl trifluoromethyl ketone (30 μM), an inhibitor of the Ca^{2+}-dependent phospholipase A_2, and Gap 27 (300 μM), a connexin-mimetic peptide which attenuates intercellular communication via gap junctions, both abolished the endothelium-dependent component of EDHF-type responses evoked by melittin in the presence of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) and the cyclooxygenase inhibitor indomethacin (10 μM). By contrast, the sulfhydryl agent thimerosal (300 nM), which amplifies EDHF activity, potentiated nitric oxide (NO)/prostanoid-independent relaxations induced by melittin. Neither arachidonyl trifluoromethyl ketone nor thimerosal modulated relaxations evoked by the peptide toxin in the absence of L-NAME and indomethacin. We conclude that melittin evokes EDHF-type relaxations through activation of the endothelial Ca^{2+}-dependent phospholipase A_2 followed by the transmission of a chemical and/or electrical signal via myoendothelial gap junctions. This mechanism of vasorelaxation may be negatively regulated by NO.

Keywords: Melittin; Phospholipase A_2; Gap junction; Connexin; Endothelium-derived hyperpolarizing factor (EDHF); Endothelium; Smooth muscle, vascular

1. Introduction

In many artery types endothelium-dependent relaxations evoked by agonists such as acetylcholine are observed in the simultaneous presence of inhibitors of nitric oxide (NO) and prostanoid synthesis. Such responses are thought to be mediated by an endothelium-derived hyperpolarizing factor (EDHF) whose chemical identity remains controversial (Hecker et al., 1994; Campbell et al., 1996; Randall and Kendall, 1998; Edwards et al., 1998; Griffith and Taylor, 1999). Under specific experimental conditions, mechanical relaxations that are attributable to EDHF can be demonstrated in cascade bioassay and in 'sandwich' preparations constructed from closely apposed arterial strips (Mombouli et al., 1996; Hutcheson et al., 1999). Nevertheless, in rabbit arteries and veins, and guinea-pig and pig arteries, there is accumulating evidence that an EDHF will transfer from endothelium to smooth muscle via gap junction channels, rather than the extracellular space, following stimulation by agonists (Chaytor et al., 1998, 1999; Taylor et al., 1998; Dora et al., 1999; Hutcheson et al., 1999; Griffith and Taylor, 1999; Yamamoto et al., 1999; Edwards et al., 2000).

Gap junctions consist of two interlocked connexons, one contributed by each coupled cell, which are constructed from six connexin subunits arranged around a central aqueous pore that permits the passage of molecules < 1 kDa in size and provides electrical continuity between adjacent cells (Yeager and Nicholson, 1996; Brink, 1998). Direct coupling between endothelium and vascular smooth muscle has been confirmed using dye transfer techniques and also by the characteristic pentalaminar appearance of myoendothelial gap junction plaques in rabbit conduit arteries (Spagnoli et al., 1982; Little et al., 1995). Previous studies have shown that connexin 43 (Cx43) is the only connexin protein expressed in endothelium-denuded rabbit

* Corresponding author. Tel.: +29-20743070; fax: +29-20744726.
E-mail address: griffith@cardiff.ac.uk (T.M. Griffith).
[1] Present address: Tenovus Cancer Research Centre, Welsh School of Pharmacy, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff, CF10 3XF, UK

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superior mesenteric artery, and in this species EDHF-type relaxations and hyperpolarizations induced by agonists can be inhibited by a synthetic peptide that possesses homology with the Gap 27 domain of the second extracellular loop of Cx43 (Gap 27; amino acid sequence SRPTEKTIFII) (Chaytor et al., 1997, 1998, 1999; Dora et al., 1999; Hutcheson et al., 1999). Gap 27 also inhibits intercellular Lucifer yellow dye transfer in confluent monolayers of COS-7 cells, which express Cx43 as their only functional protein (Dora et al., 1999; Chaytor et al., 1999). Inhibition of EDHF-mediated responses by Gap 27 in rabbit vessels is rapid and reversible, suggesting that it modulates already-established connexon interactions in addition to preventing connexon docking (Warner et al., 1995; Chaytor et al., 1998).

In the perfused rat heart and mesentery and in isolated rabbit superior mesenteric arteries, mobilization of arachidonic acid by a Ca^{2+}-dependent phospholipase A_2 also appears to play a central role in the mediation of EDHF-type responses (Fulton et al., 1996; Adeagbo and Henzel, 1998; Hutcheson et al., 1999). In these studies, however, pharmacological inhibitors of phospholipase A_2 were administered to intact preparations, thereby precluding a clear distinction between their effects on endothelial and smooth muscle cells. The aim of the present study was to clarify the site of phospholipase A_2 activation involved in the EDHF phenomenon by studying relaxations evoked by melittin, a polypeptide toxin found in bee venom. This agent is a potent activator of phospholipase A_2 and mobilizes arachidonic acid in a variety of cell types, including the endothelium (Hassid and Levine, 1977; Shier, 1979; Rosenthal and Jones, 1988; Choi et al., 1992). Although melittin is known to evoke endothelium-dependent relaxations of rat and rabbit aorta by stimulating endothelial NO production (Thomas et al., 1986; Forstermann and Neufang, 1985; Loeb et al., 1988; Rapoport et al., 1989), we reasoned that it should also evoke a gap-junction dependent EDHF-type relaxation by stimulating phospholipase A_2 activity. Arachidonyl trifluoromethyl ketone, a specific inhibitor of the Ca^{2+}-dependent phospholipase A_2 (Street et al., 1993), and Gap 27 were used to test this hypothesis, with the site of action of melittin being defined in experiments with rings of endothelium-intact and -denuded rabbit superior mesenteric artery.

2. Materials

2.1. Isolated ring preparations

Experiments were performed with superior mesenteric arteries from male New Zealand white rabbits (2.5 kg) which had been killed by injection of sodium pentobarbitone (120 mg/kg; i.v.). The tissues were transferred to cold Holman's solution of the following composition (mM): 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, and 10 sucrose. Rings (2–3 mm wide) were cut and suspended by thread from Dynamometer UFI force transducers (Lectromed, UK) connected to a Maclab 4e system (ADInstruments, UK) in 3 ml organ baths containing gassed (95% O_2, 5% CO_2, pH 7.4) Holman's solution at 37°C to measure isometric force development. Tension was set at 0.5 g and during an equilibrium period of 1 h, the tissues were washed with fresh Holman's solution every 10–15 min with tension being restored to its initial level as necessary following stress relaxation. Endothelium-denuded rings were prepared by gentle abrasion of the luminal surface of the vessel. All tissues were initially tested for the presence or absence of an endothelium by constriction with 10 μM phenylephrine and addition of 1 μM acetylcholine followed by washout.

2.2. Experimental protocols

Endothelium-intact and -denuded rings were preconstricted with 10 μM phenylephrine and cumulative concentration–response curves to melittin (10 ng/ml–10 μg/ml) constructed before and after a 45 min incubation with the specific inhibitors of NO synthase, N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) and cyclooxygenase, indomethacin (10 μM). The involvement of phospholipase A_2 activation in the response was determined using the Ca^{2+}-dependent phospholipase A_2 inhibitor, arachidonyl trifluoromethyl ketone (30 μM). This agent was added to the organ bath 45 min prior to construction of concentration–response curves to melittin both in the absence and presence of L-NAME and indomethacin. To investigate the role of gap junctional communication in the response to melittin, endothelium-intact rings were preincubated for 20 min with Gap 27 (300 μM) again in the absence and presence of L-NAME and indomethacin. Using an identical protocol, the effects of a 20 min preincubation with thimerosal (300 nM) were also assessed.

2.3. Drugs

Acetylcholine, phenylephrine, melittin, indomethacin, L-NAME and thimerosal were obtained from Sigma, Poole, UK. Arachidonyl trifluoromethyl ketone was obtained from Affiniti Research Products Gap 27 (SRPTEKTIFII) was synthesised by Sigma Genosys, Cambridge, UK. Purity was $< 95\%$. All drugs were dissolved in Holman's buffer with the exception of arachidonyl trifluoromethyl ketone (dimethylsulphoxide), melittin (distilled water), and indomethacin (5% w/v $NaHCO_3$ in distilled water).

2.4. Statistical analysis

Data are given as mean ± S.E.M., where *n* denotes the number of animals studied for each data point. Concentration–response curves were assessed by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple

comparisons test. EC_{50} and maximal responses were compared by the Student's *t*-test for paired and unpaired data as appropriate. $P < 0.05$ was considered as significant.

3. Results

3.1. Effects of phospholipase A_2 inhibition on melittin-induced relaxations in endothelium-intact rings

Melittin evoked relaxations of endothelium-intact mesenteric rings, preconstricted to 3.0 ± 0.2 g with 10 μM phenylephrine, with an EC_{50} of 0.49 ± 0.05 μg/ml and a maximal response of $64 \pm 4\%$ at a concentration of 3 μg/ml ($n = 17$, Fig. 1a). Inhibition of the Ca^{2+}-dependent phospholipase A_2 using arachidonyl trifluoromethyl ketone (30 μM) had no effect on melittin-induced relaxations either in terms of the EC_{50} (0.52 ± 0.09 μg/ml, $n = 6$) or the maximal response ($67 \pm 6\%$, $n = 6$) (Fig. 1a). Preincubation with L-NAME (300 μM) and indomethacin (10 μM) had no significant effect on steady-state phenylephrine-induced tone (3.2 ± 0.2 g, $n = 20$), but significantly attenuated melittin-induced relaxations causing a shift in the EC_{50} to 1.06 ± 0.13 μg/ml ($P < 0.001$, $n = 20$) and a reduction in the maximal response to $28 \pm 3\%$ ($P < 0.001$, $n = 20$) (Fig. 1a). Preincubation with arachidonyl trifluoromethyl ketone in the presence of L-NAME and indomethacin, further increased the EC_{50} to 1.40 ± 0.23 μg/ml ($P < 0.001$, $n = 7$) and reduced the maximal response to $12 \pm 2\%$ ($P < 0.01$, $n = 7$) (Fig. 1a). There was again no significant effect of these treatments on phenylephrine-induced tone (3.3 ± 0.2 g, $n = 7$).

3.2. Effects of phospholipase A_2 inhibition on melittin-induced relaxations in endothelium-denuded rings

In endothelium-denuded rings, precontracted with phenylephrine, melittin evoked relaxations with an EC_{50} of 0.98 ± 0.24 μg/ml and a maximal response of $16 \pm 3\%$ ($n = 7$) at a concentration of 3 μg/ml (Fig. 1b). Arachidonyl trifluoromethyl ketone had no effect on these relaxations either in terms of the EC_{50} (0.67 ± 0.12 μg/ml, $n = 5$) or maximal response ($17 \pm 4\%$, $n = 5$) (Fig. 1b). In the presence of L-NAME and indomethacin, there was no significant change in the EC_{50} value for melittin-induced relaxation (0.84 ± 0.15 μg/ml, $n = 11$) or the maximal response to this agent ($10 \pm 2\%$, $n = 11$) (Fig. 1b). Addition of arachidonyl trifluoromethyl ketone to the L-NAME- and indomethacin-treated rings had no effect on melittin-induced relaxations either in terms of the EC_{50} (0.61 ± 0.13 μM, $n = 5$) or maximal response ($12 \pm 2\%$, $n = 5$) (Fig. 1b). There was no significant effect of any of these treatments on phenylephrine-induced tone (control: 3.5 ± 0.4 g, $n = 7$; arachidonyl trifluoromethyl ketone: 3.3 ± 0.4

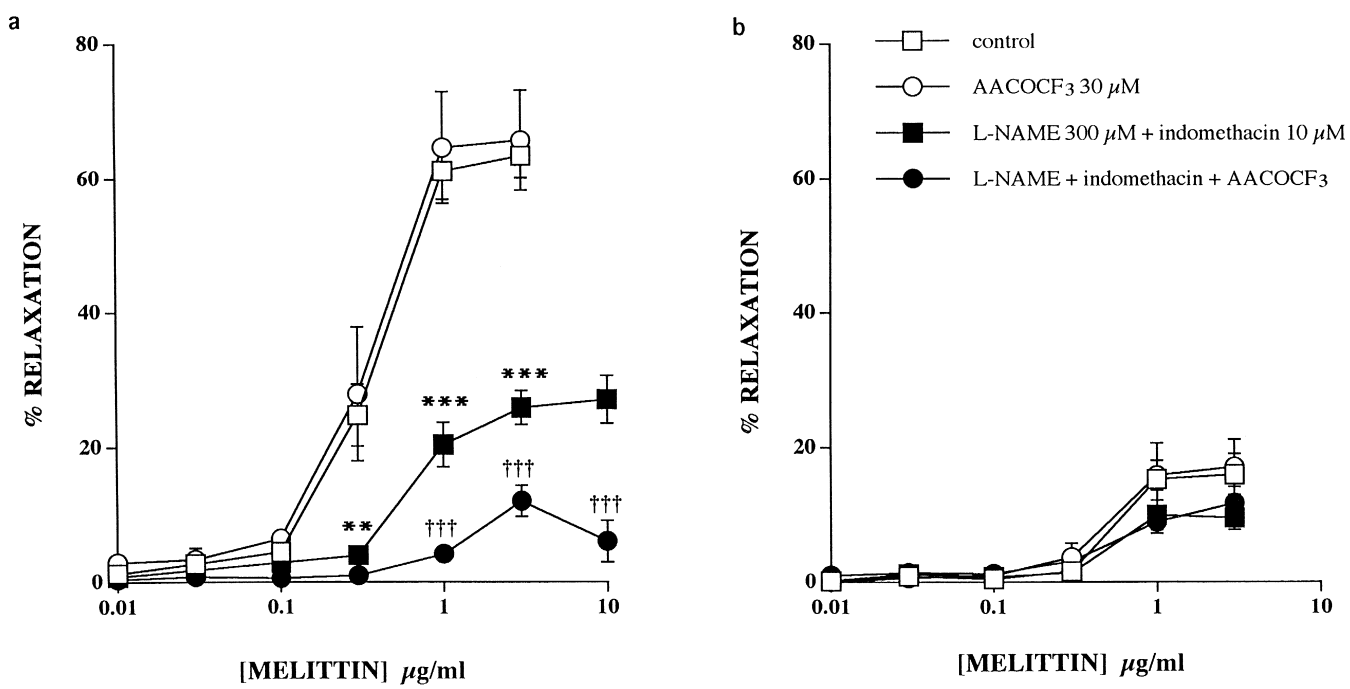

Fig. 1. Concentration–response curves showing the effects of phospholipase A_2 inhibition on melittin-induced relaxations in endothelium-intact and -denuded rings of rabbit superior mesenteric artery. (a) In endothelium-intact tissues a combination of L-NAME (300 μM) and indomethacin (10 μM) attenuated responses to melittin by ~60% whereas arachidonyl trifluoromethyl ketone ($AACOCF_3$, 30 μM) alone was without effect. In the presence of L-NAME and indomethacin, however, arachidonyl trifluoromethyl ketone significantly attenuated melittin-induced relaxations. (b) In endothelium-denuded vessels melittin evoked only a small relaxant response which was not significantly affected by pretreatment with L-NAME and indomethacin, arachidonyl trifluoromethyl ketone alone, or the combination of these inhibitors. $^{**}P < 0.01$ cf. control, $^{***}P < 0.001$ cf. control, $^{†††}P < 0.001$ cf. L-NAME + indomethacin.

g, $n = 5$; L-NAME + indomethacin: 3.5 ± 0.2 g, $n = 11$; L-NAME + indomethacin + arachidonyl trifluoromethyl ketone: 3.3 ± 0.2 g, $n = 5$).

3.3. Effects of Gap 27 on melittin-induced relaxations in endothelium-intact rings

Preincubation of endothelium-intact rings with Gap 27 (300 μM) caused a small but not significant shift in the EC_{50} value for melittin from 0.49 ± 0.05 μg/ml ($n = 20$) to 0.71 ± 0.07 μg/ml ($n = 3$) but had little effect on maximal relaxation (control: $64 \pm 4\%$, $n = 20$; Gap 27: $62 \pm 6\%$, $n = 3$) (Fig. 2). However, in the presence of L-NAME and indomethacin preincubation with Gap 27 nearly abolished melittin-induced relaxations ($n = 4$; Fig. 2). Gap 27 had no effect on phenylephrine-induced tone either in the absence (2.8 ± 0.2 g, $n = 3$) or presence (3.2 ± 0.2 g, $n = 4$) of L-NAME and indomethacin.

3.4. Effects of thimerosal on melittin-induced relaxations in endothelium-intact rings

Preincubation with thimerosal (300 nM) had no effect on either phenylephrine-induced tone (2.9 ± 0.2 g, $n = 4$) or melittin-induced relaxations in the absence of L-NAME (EC_{50}: 0.45 ± 0.11 μg/ml, $n = 4$; maximal response: $61 \pm 8\%$, $n = 4$; Fig. 3). In L-NAME- and indomethacin-treated rings thimerosal again had no effect on phenylephrine-induced tone (3.2 ± 0.2 g, $n = 4$) but enhanced relaxations to melittin with a significant reduction in the EC_{50} from 1.06 ± 0.13 μg/ml ($n = 17$) to 0.52 ± 0.09 μg/ml ($n = 4$) ($P < 0.001$) and a significant increase in the maximal response from $28 \pm 3\%$ ($n = 17$) to $43 \pm 6\%$ ($n = 4$) ($P < 0.05$) (Fig. 3).

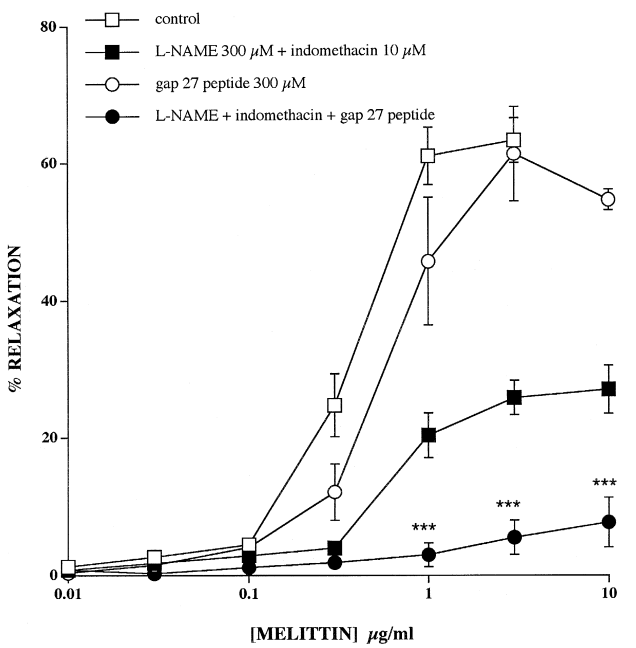

Fig. 2. Concentration–response curves showing the effects of Gap 27 peptide (300 μM) on melittin-induced relaxations in endothelium-intact rings of rabbit superior mesenteric artery. Maximal melittin-induced responses were not significantly affected by Gap 27 in the absence of L-NAME, but in its presence relaxations were almost abolished. $^{***}P < 0.001$ cf. L-NAME + indomethacin.

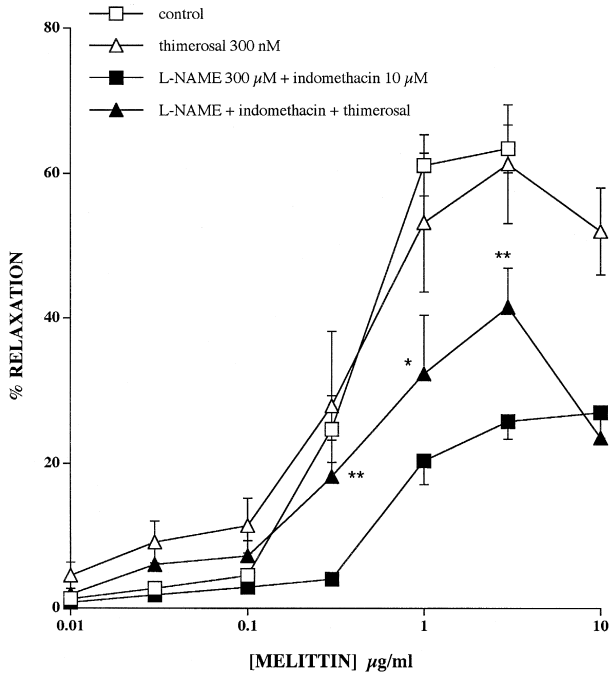

Fig. 3. Concentration–response curves showing the effects of thimerosal (300 nM) on melittin-induced relaxations in endothelium-intact rings of rabbit superior mesenteric artery. Thimerosal significantly enhanced melittin-evoked relaxations observed in the presence, but not the absence, of L-NAME. $^{*}P < 0.05$ cf. L-NAME + indomethacin, $^{**}P < 0.01$ cf. L-NAME + indomethacin.

4. Discussion

In the present study, we have investigated the pathways through which melittin induces endothelium-dependent relaxations of the rabbit superior mesenteric artery and have confirmed previous reports that this peptide stimulates NO synthesis. The major new finding is that melittin-induced activation of a Ca^{2+}-dependent phospholipase A_2 located within the endothelial cell additionally promotes an EDHF-type relaxation, which is dependent on intercellular communication via gap junctions.

Approximately 60% of the initial relaxation induced by melittin in endothelium-intact rings was susceptible to combined inhibition of NO and prostanoid synthesis. The remaining endothelium-dependent component of this response was almost abolished by preincubation with Gap 27, which attenuates EDHF-type relaxations and hyperpolarizations evoked by agonists such as acetylcholine in rabbit arteries and veins (Chaytor et al., 1998; Taylor et al., 1998; Dora et al., 1999; Griffith and Taylor, 1999).

L-NAME and indomethacin-insensitive relaxations to melittin were also markedly attenuated by arachidonyl trifluoromethyl ketone, consistent with previous observations that activation of a Ca^{2+}-dependent phospholipase A_2 plays a crucial role in EDHF-type relaxations in the rabbit superior mesenteric artery (Hutcheson et al., 1999). Relaxations induced by melittin in endothelium-denuded preparations were much smaller (~10% of initial tone) than in those possessing an intact endothelium, and were not significantly affected by L-NAME plus indomethacin, arachidonyl trifluoromethyl ketone, or these inhibitors in combination. These observations are in general agreement with a previous report that melittin has little or no direct relaxant activity in vascular smooth muscle (Rapoport et al., 1989), and indicate that the activation of phospholipase A_2 associated with EDHF-type relaxations occurs within the endothelium rather than smooth muscle. The mechanisms that underlie the small residual direct relaxant effect of melittin in endothelium-denuded rings of rabbit mesenteric artery require further evaluation.

Melittin has previously been shown to increase free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in bovine aortic endothelial cells, and this action of the polypeptide is likely to underlie the NO-mediated component of its relaxant response since the constitutive endothelial NO synthase is Ca^{2+}/calmodulin-dependent (Loeb et al., 1988; Whatley et al., 1989; Moncada et al., 1991). Melittin-induced elevations in endothelial $[Ca^{2+}]_i$ could also contribute to activation of the cytosolic arachidonyl trifluoromethyl ketone-sensitive form of phospholipase A_2, which is a Ca^{2+}-dependent enzyme, thereby explaining the known Ca^{2+}-dependency of EDHF formation, at least in part (Street et al., 1993; Fukao et al., 1997). The rise in $[Ca^{2+}]_i$ induced by melittin may be multifactorial in origin and involve: (i) the formation of pores in the plasma membrane that permit influx of extracellular Ca^{2+} (Alder et al., 1991; Choi et al., 1992), (ii) Ca^{2+} release from stores following increased synthesis of inositol (1,4,5)-trisphosphate ($InsP_3$) (Loeb et al., 1988), and (iii) inhibition of the endoplasmic reticulum Ca^{2+}-ATPase which prevents sequestration of Ca^{2+} within stores (Baker et al., 1995; Voss et al., 1995).

In marked contrast to the attenuation of relaxations to mellitin observed following inhibition of NO and prostanoid synthesis, responses to this peptide were unaffected by arachidonyl trifluoromethyl ketone in the absence of L-NAME and indomethacin. This is analogous to findings with acetylcholine in the rabbit superior mesenteric artery in which arachidonyl trifluoromethyl ketone is also ineffective as an inhibitor of endothelium-dependent relaxation under control conditions (Hutcheson et al., 1999). A possible explanation for these findings is that NO activity in some way suppresses EDHF-type relaxations (Olmos et al., 1995; Kessler et al., 1999). Under conditions where NO dominates melittin- and acetylcholine-induced relaxations, the phospholipase A_2-dependent component of the response would then be masked, and arachidonyl trifluoromethyl ketone without significant effect, whereas EDHF-type responses may be enhanced in the presence of L-NAME, and relaxation then more susceptible to inhibition of phospholipase A_2. Consistent with this interpretation of the present experimental findings, there is a negative correlation between the magnitude of NO- and EDHF-dependent relaxations in superior mesenteric artery rings from individual rabbits (Hutcheson et al., 1999). The biochemical basis for this apparently reciprocal interaction between NO and EDHF remains to be elucidated in detail, although there is some evidence for the participation of a cGMP-dependent mechanism (Olmos et al., 1995).

Further evidence that the pathways underlying EDHF-type responses evoked by melittin are similar to those activated by other pharmacological stimuli was provided by experiments with thimerosal, an organic sulfhydryl reagent which stimulates endothelial NO synthesis and EDHF-type hyperpolarizations and relaxations when administered at concentrations in the high micromolar range (Forstermann et al., 1986; Bény, 1990; Mombouli et al., 1996). In the rabbit superior mesenteric artery, subthreshold nanomolar concentrations of thimerosal have also been shown to amplify EDHF-mediated relaxations evoked by acetylcholine and the Ca^{2+} ionophore A23187, which acts through a receptor-independent mechanism (Hutcheson et al., 1999). A similar phenomenon was evident in the present study with melittin in which maximal relaxations observed in the presence of L-NAME and indomethacin were enhanced ~1.5-fold in the presence of 300 nM thimerosal. This potentiation of EDHF-type responses may reflect the ability of thimerosal to inhibit the endothelial acyl-coenzyme A/lysolecithin acyltransferase and thereby elevate free arachidonic acid levels within the cell (Irvine, 1982; Forstermann et al., 1986). Indeed, we have previously shown that 5,6-epoxyeicosatrienoic acid (5,6-EET) and the cannabinoid *N*-arachidonylethanolamide (anandamide), which are both endothelial products of arachidonic acid, induce EDHF-type relaxations of the rabbit superior mesenteric artery that are susceptible to inhibition of gap junctional communication by Gap 27 (Hutcheson et al., 1999; Chaytor et al., 1999). Measurements of cell membrane potential also suggest that the 11,12-epoxyeicosatrienoic acid regioisomer (11,12-EET) promotes an EDHF-type hyperpolarization in the porcine coronary artery (Edwards et al., 2000). Further research is necessary to establish the extent to which such arachidonic acid derivatives act via the endothelium in other artery types, as EETs and anandamide can activate hyperpolarizing K^+ channels in vascular smooth muscle, leading to proposals that they may also act as freely diffusible EDHFs (Hecker et al., 1994; Rosolowsky and Campbell, 1996; Campbell et al., 1996; Lin et al., 1996; Li and Campbell, 1997; Li et al., 1997; Randall and Kendall, 1998; Fisslthaler et al., 1999). Whether gap junctional communication contributes to the EDHF phenomenon by allowing the transmission of a hyperpolarizing current

from the endothelium to smooth muscle and subsequently between smooth muscle cells in successive layers of the vessel wall likewise remains uncertain. However, in porcine ciliary arteries electrotonic conduction of endothelial hyperpolarization extends only as far as immediately subjacent smooth muscle, suggesting that the endothelium may be unable to serve as a major source of electrical current in thick-walled conduit arteries (Bény, 1999).

We conclude that, melittin, like acetylcholine, activates a Ca^{2+}-dependent phospholipase A_2 in endothelial cells that leads to the formation of a signal which is transmitted from endothelium to smooth muscle via gap junctions and results in an EDHF-type relaxation. This mechanism of vasorelaxation may normally be attenuated by NO under physiological conditions.

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