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Role of cytosolic phospholipase A_2 in the enhancement of α_2-adrenoceptor-mediated vasoconstriction by the thromboxane-mimetic U46619 in the porcine isolated ear artery: Comparison with vasopressin-enhanced responses

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Abstract

Pre-contraction with the thromboxane-mimetic U46619 enhances the subsequent α_2-adrenoceptor-mediated vasoconstriction in the porcine ear artery through an enhanced activation of ERK-MAP kinase. In this study we determined the role of $cPLA_2$ in this enhanced response, and determined whether vasopressin is also able to enhance α_2-adrenoceptor-mediated vasoconstriction through the same pathway. The $cPLA_2$ inhibitors AACOCF3 (50 μM) and MAFP (50 μM) both inhibited the U46619-enhanced α_2-adrenoceptor response, but had no effect on the direct α_2-adrenoceptor response. AACOCF3 also inhibited the enhanced ERK activation associated with the enhanced α_2-adrenoceptor-mediated vasoconstriction. Pre-contraction with arachidonic acid mimicked the effect of U46619 by enhancing the contractile response to the α_2-adrenoceptor agonist UK14304 (1 μM) and enhancing the α_2-adrenoceptor-mediated ERK activation. Pre-contraction with vasopressin also enhanced the contractile response to UK14304, but neither PD98059 (50 μM) nor AACOCF3 (50 μM) had any effect this vasopressin-enhanced response, indicating that neither the ERK pathway, nor $cPLA_2$ are involved in vasopressin-enhanced responses. The α_2-adrenceptor-stimulated activation of ERK was also unaffected by pre-contraction with vasopressin. On the other hand, inhibition of PKCζ inhibited the enhanced α_2-adrenoceptor contraction after pre-contraction with both U46619 and vasopressin. This study demonstrates that α_2-adrenoceptor-mediated vasoconstriction can be enhanced through two different pathways—one dependent upon the enhanced activation of ERK-MAP kinase through activation of $cPLA_2$, and the other through a different, ERK/$cPLA_2$-independent pathway.

Keywords: Porcine; Adrenergic agonists; Contractile function; Second messengers

1. Introduction

α_2-Adrenoceptors cause vasoconstriction in porcine blood vessels through an ERK-MAP kinase pathway [1,2]. In the porcine ear artery, the α_2-adrenoceptor-mediated vasoconstriction is enhanced by pre-contraction with the thromboxane-mimetic U46619 [2] This enhancement occurs through an increased activation of ERK-MAP kinase, and is also dependent upon influx of extracellular calcium [2]. How ERK-MAP kinase activity is enhanced is not known.

Phospholipase A_2 (PLA_2) represents a family of structurally heterogeneous enzymes that catalyses hydrolysis of membrane phosphoplipids to liberate potent second messengers such as arachidonic acid, a precursor of eicosanoids including prostaglandins and leukotrienes [3]. Cytosolic PLA_2 ($cPLA_2$) is activated by calcium and is upstream of ERK [3,4]. It has been shown previously that arachidonic acid released by $cPLA_2$ is responsible for ERK activation in vascular smooth muscle cells. This can occur either through activation of protein kinase C (PKC), or

Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; ANOVA, analysis of the variance; E-64, trans-epoxysuccinyl-L-leucylamide-(4-guanidino) butane; ERK, extracellular signal-regulated kinase; HELSS, Haloenol lactone suicide substrate; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PKC, protein kinase C; PLA_2, phospholipase A_2; TBS-T, tris-buffered saline containing 0.1% tween-20

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through the products of lipoxygenase metabolism of arachidonic acid [4,5]. Arachidonic acid metabolites have also been implicated in the enhancement of vasoconstriction in rat and hamster blood vessels [6,7]. Therefore, $cPLA_2$ may form an important link between the influx of calcium, and activation of ERK leading to the enhanced α_2-adrenoceptor-induced vasoconstriction.

In order to try to understand the mechanisms further it is necessary to determine if the enhancement is specific to U46619 or can be mimicked by other vasoconstrictors as well. Vasopressin is a potent vasoconstrictor, and has been shown to enhance adrenergic vasoconstriction in a number of blood vessels, although the adrenoceptor subtype was not identified [8–10]. This enhancement was shown to be dependent upon influx of extracellular calcium, in a manner similar to the U46619-enhanced α_2-adrenoceptor-mediated vasoconstriction. Therefore, it is possible that vasopressin is also able to enhance α_2-adrenoceptor-mediated vasoconstriction in the porcine ear artery through a similar pathway.

The aim of this study was to determine the role of $cPLA_2$ and arachidonic acid in the U46619-enhancement of the α_2-adrenoceptor-mediated vasoconstriction. The vasopressin-enhanced α_2-adrenoceptor response was used as a comparison. In the rabbit femoral artery and portal vein, arachidonic acid released by $cPLA_2$ is thought to cause calcium sensitisation through activation of the atypical protein kinase C isozyme PKCζ [11]. Therefore, a further aim was to determine whether inhibition of PKCζ could also inhibit the enhanced α_2-adrenoceptor-mediated vasoconstriction.

2. Methods

2.1. Isometric tension recordings

Porcine ears were obtained from a local abattoir and transported to the laboratory on ice. Ear arteries were dissected out and placed in Krebs–Henseleit buffer containing 2% Ficoll which had been pre-gassed with 95% O_2/5% CO_2, and stored overnight at 4 °C. The following day ear arteries were dissected into 5 mm ring segments and suspended in an isolated organ bath containing Krebs–Henseleit buffer maintained at 37 °C and constantly gassed with 95% O_2/5% CO_2. The lower support was fixed and the upper support was connected to a force transducer (Lectromed, Letchworth, UK) linked to a PCLab data acquisition system (AD Instruments Ltd., Hastings, UK) via an amplifier. After a 20 min equilibration period, tension was applied to the tissue, which was allowed to relax to a final resting tension of between 0.5 and 1 g wt. Before each experiment the tissues were contracted at least three times with 60 mM KCl, until the final two responses to KCl differed by less than 10%. Contractile responses were expressed as a percentage of the final KCl response.

2.2. Effect of pre-contraction on α_2-adrenoceptor-mediated vasoconstriction

Tissues were pre-contracted with the thromboxane-mimetic U46619 (1–5 nM), vasopressin (1–40 nM) or arachidonic acid (400 μM) as appropriate. The degree of pre-contraction obtained under these conditions was between 10 and 20% of the response to 60 mM KCl. Tissues were then exposed to a single, near maximal concentration of the α_2-adrenoceptor agonist UK14304 (1 μM) [12]. UK14304 causes contractile responses in the porcine ear artery through activation of α_2-adrenoceptors [13]. We have shown previously that pre-contraction enhances the UK14304 response over the whole range of the concentration–response curve [2]. However, as the contractile responses to the lower concentrations of UK14304 were difficult to measure accurately, particularly in the absence of pre-constrictor agent, we decided to use a near maximum concentration of UK14304 (1 μM). Contractions to UK14304 were measured from the pre-UK14304 level of tone. In control tissues UK14304 was added in the absence of pre-contraction. In some experiments inhibitors (50 μM PD98059; 10 μM PKCζ pseudo-substrate inhibitor; 50 μM MAFP; 10 μM HELSS; 10 μM NDGA; 10 μM ETI; 10 μM indomethacin) were added 1 h prior to addition of UK14304 or pre-contraction/UK14304. In the experiments carried out in the presence of 50 or 100 μM AACOCF3, the inhibitor was added 3 h prior to addition of UK14304 or pre-contraction/UK14304. Control tissues received the appropriate vehicle (0.1% DMSO for PD98059, HELSS, and NDGA, 0.18% for 50 μM AACOCF3, 0.36% for 100 μM AACOCF3, 0.1% ethanol for ETI, indomethacin and 0.3% ethanol for MAFP).

2.3. Concentration–response curves to UK14304 after pre-contraction with vasopressin

Owing to the transient nature of the vasopressin responses it was necessary to perform non-cummulative response curves to UK14304. Tissues were pre-contracted with vasopressin to between 10 and 20% of the KCl response, and then a single concentration of UK14304 added (10 nM to 10 μM). After each response, tissues were washed out and allowed to recover for 30 min prior to the next addition.

2.4. Effect of PD98059 or MAFP on U46619 and vasopressin responses

Concentration–response curves to U46619 were carried out in the absence or presence of PD98059 (50 μM) or MAFP (50 μM) in order to determine the role of ERK or $cPLA_2$, respectively. The effects of PD98059 or MAFP on the response to vasopressin were also determined. However, owing to the fact that the vasopressin-induced contractions were transient, especially at higher

concentrations, the effects of PD98059 or MAFP on a single concentration of vasopressin (10 nM) were investigated.

2.5. Western blotting

Western blotting for phosphorylated or total ERK was carried out as described previously [1]. Briefly, segments of the porcine ear artery were set up in tissue baths as above. Tissues were exposed to UK14304 (1 μM), arachidonic acid (400 μM), or pre-constricted with arachidonic acid (400 μM, 10–20% of 60 mM KCl response) and treated with UK14304 (1 μM). In another set of experiment, tissues were exposed to UK14304 (1 μM), or pre-constricted with U46619 (1–4 nM, 10–20% of 60 mM KCl response) in the absence or presence of AACOCF3. In a further set of experiments, tissues were exposed to UK14304 (1 μM), vasopressin (10–20% of 60 mM KCl response), or pre-constricted with vasopressin (10–20% of 60 mM KCl response) and treated with UK14304 (1 μM). Control tissues were not exposed to any compound or vehicle where necessary (basal conditions). When the contractions reached a plateau, the segments were quickly removed from the tissue baths, and immediately frozen on dry ice. Frozen segments were then homogenised in ice cold buffer (80 mM sodium β-glycerophosphate, 20 mM imidazole [pH 7.0], 1 mM dithiothreitol, 1 mM sodium fluoride, 500 μM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 1 μM trans-epoxysuccinyl-L-leucylamide-(4-guanidino) butane (E-64), 10 μg ml^{-1} aprotonin, 1 μM leupeptin, 500 μM EDTA). Samples were diluted in 1:1 in Laemmli sample buffer, and heated at 95 °C for 5 min. Equal amounts of protein from each sample were separated on 10% SDS-PAGE gels, and then transferred onto nitrocellulose membranes by Western blotting. Membranes were probed for phosphorylated or total ERK using appropriate antibodies (both Cell Signaling Technology). Bands were detected by probing with a hydrogen peroxidase-conjugated secondary antibody, and visualised using the ECL system (Amersham Life Sciences). Both phosphorylated and total ERK bands were analysed by densitometry using the BioRad molecular analyst software package. Similar experiments were carried out to determine the effect of contraction of the ear artery with a high concentration of either U46619 (0.1 μM) or vasopressin (10 nM) on ERK activation.

2.6. Drugs

5-Bromo-6-[2-imidazolin-2-ylamine]-quinoxaline bitartrate (UK14304) (Tocris); (5Z, 9α, 11α, 13E, 15 (S))-15-hydroxy-9 (11) methanoepoxyprosta-5,13-dien-1 oic acid (U46619) (Cayman Chemicals); 2-amino-3-methoxyflavone (PD98059) (Calbiochem); methyl arachidonyl fluorophosphate (MAFP) (Tocris); arachidonyltrifluoromethyl ketone (AACOCF3) (Calbiochem); arachidonic acid (Alexis Biochemicals); PKCζ pseudosubstrate inhibitor (Biomol); E-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS) (Biomol); nordihydroguaiaretic acid (NDGA) (Calbiochem); 5,8,11-eicosatriynoic acid (ETI) (Calbiochem). All other compounds were obtained from Sigma, Poole, UK.

2.7. Statistics

Contractile responses were expressed as a percentage of the response to 60 mM KCl, and the results expressed as mean ± S.E.M. Bands obtained by immunoblotting were analysed by densitometry. Statistical evaluations were carried out using a two-tailed Student's unpaired or paired *t*-test for normally distributed data. Multiple comparisons between treatment groups were performed using analysis of the variance (ANOVA) followed by a Bonferroni test.

3. Results

3.1. Effect of $cPLA_2$ inhibitors on U46619-enhanced α_2-adrenoceptor-mediated vasoconstriction

The $cPLA_2$ inhibitor AACOCF3 (50 μM) had no effect on the contractile response to 1 μM UK14304 alone (Fig. 1a). On the other hand, AACOCF3 significantly reduced the enhanced response to UK14304 in the presence of U46619 (Fig. 1a). The degree of tone induced by U46619 was the same in both control (19 ± 1% of 60 mM KCl response, $n = 5$) and tissues exposed to AACOCF3 (19 ± 5%, $n = 5$) and the concentration range of U46619 required to induce this level of tone was the same in control and AACOCF3-treated tissues (3–5 nM). Increasing the concentration of AACOCF3 to 100 μM had no further effect on the size of the response to UK14304 after pre-contraction with U46619 (57 ± 6% ($n = 5$) inhibition in the presence of 50 μM AACOCF3 compared to 39 ± 8% ($n = 8$) inhibition in the presence of 100 μM AACOCF3). Similar effects were observed with MAFP, which also inhibits $cPLA_2$ (Fig. 1b). Pre-incubation with 50 μM MAFP had no effect on 1 μM UK14304 responses alone, but the U46619-enhanced responses were inhibited by MAFP. The degree of tone induced by U46619 was the same in both control (10 ± 1% of 60 mM KCl response, $n = 7$) and tissues exposed to MAFP (8 ± 1%, $n = 7$) and the concentration range of U46619 required to induce this level of tone was the same in control and MAFP-treated tissues (1–14 nM). Furthermore, in subsequent experiments, we found that 50 μM MAFP had no effect on the maximum response to U46619 in concentration–response curves (98 ± 8% in controls compared to 96 ± 9% in the presence of MAFP ($n = 4$). We were unable to use higher concentrations of MAFP as it came out of solution.

Pre-incubation with the Ca^{2+}-independent PLA_2 selective inhibitor HELSS reduced the contraction to 1 μM

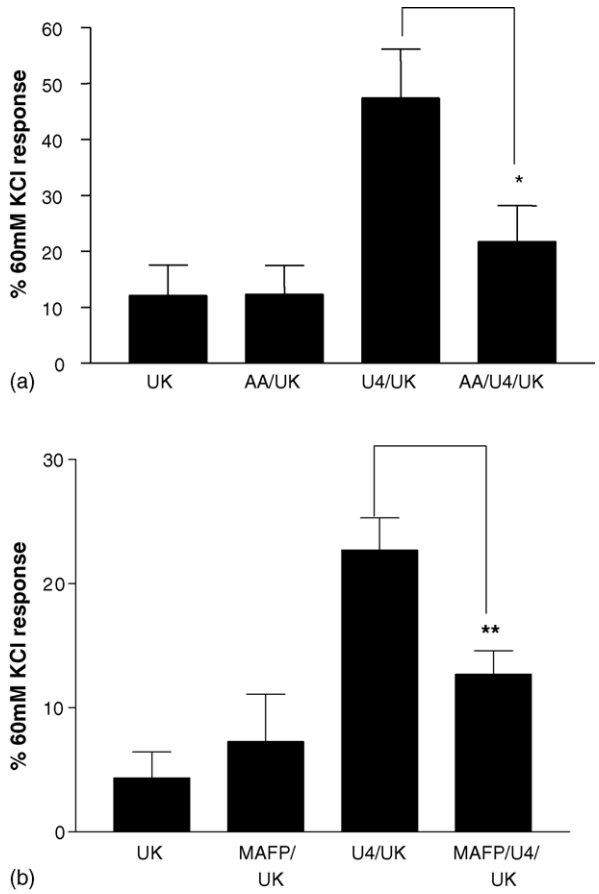

Fig. 1. (a) Contractile responses to 1 μM UK14304 alone (UK), or after pre-contraction with U46619 (U4/UK) in the absence or presence of 50 μM AACOCF3 (AA/UK or AA/U4/UK) in the porcine ear artery. Results are expressed as a percentage of the response to 60 mM KCl (mean ± S.E.M., $n = 5$). $^{*}p < 0.05$, Student's two-tailed, unpaired t-test vs. U4/UK. (b) Contractile responses to 1 μM UK14304 alone (UK), or after pre-contraction with U46619 (U4/UK) in the absence or presence of 50 μM MAFP (MAFP/UK or MAFP/U4/UK) in the porcine ear artery. Results are expressed as a percentage of the response to 60 mM KCl (mean ± S.E.M., $n = 7$). $^{**}p < 0.01$, Student's two-tailed, unpaired t-test vs. U4/UK.

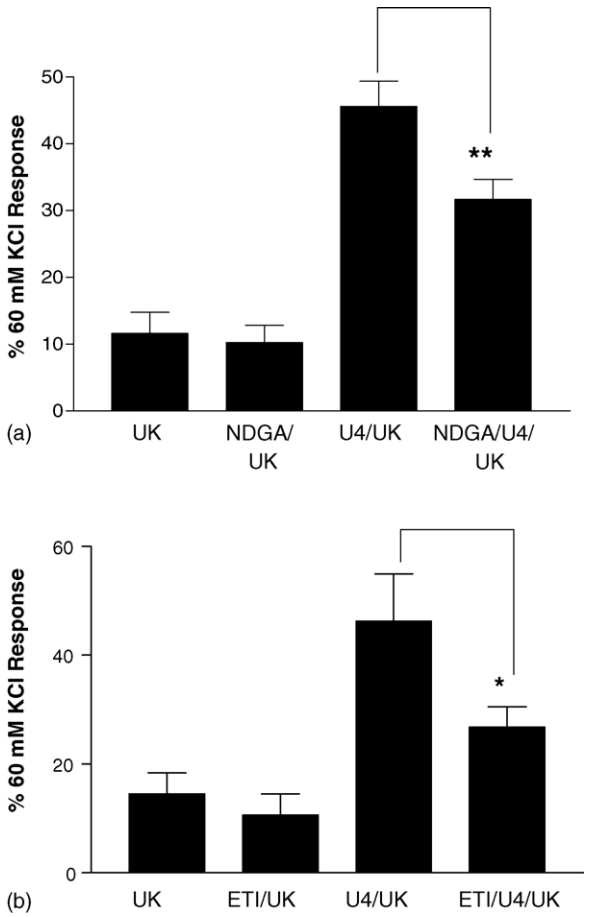

Fig. 2. (a) Contractile responses to 1 μM UK14304 alone (UK), or after pre-contraction with U46619 (U4/UK) in the absence or presence of 10 μM NDGA (NDGA/UK or NDGA/U4/UK) in the porcine ear artery. Results are expressed as a percentage of the response to 60 mM KCl (mean ± S.E.M., $n = 5$). $^{**}p < 0.02$, Student's two-tailed, unpaired t-test vs. U4/UK. (b) Contractile responses to 1 μM UK14304 alone (UK), or after pre-contraction with U46619 (U4/UK) in the absence or presence of 10 μM ETI (ETI/UK or ETI/U4/UK) in the porcine ear artery. Results are expressed as a percentage of the response to 60 mM KCl (mean ± S.E.M., $n = 7$). $^{*}p < 0.05$, Student's two-tailed, unpaired t-test vs. U4/UK.

UK14304 from 3 ± 1% of 60 mM KCl response to 0.3 ± 0.3% ($p < 0.02$, two-tailed, Student's unpaired t-test, $n = 8$). Pre-incubation with HELSS also reduced the U46619-enhanced response to UK14304 from 25 ± 2 to 12 ± 4% ($p < 0.02$, two-tailed, Student's unpaired t-test, $n = 8$).

Pre-incubation with the selective lipoxygenase inhibitor NDGA (10 μM) had no effect on the response to 1 μM UK14304, but caused a significant reduction in the U46619-enhanced response to UK14304 (Fig. 2a). The degree of tone induced by U46619 was the same in both control (12 ± 3% of 60 mM KCl response) and tissues exposed to NDGA (12 ± 3%) and the concentration range of U46619 required to induce this level of tone was the same in control and NDGA-treated tissues (1–3 nM). Likewise ETI (10 μM), another lipoxygenase inhibitor also had no effect on the direct response to 1 μM UK14304, but reduced the U46619-enhanced response (Fig. 2b). The degree of tone induced by U46619 was the same in both control (13 ± 1% of 60 mM KCl response) and tissues exposed to ETI (10 ± 2%). However, a higher concentration of U46619 was generally required to induce this level of tone in ETI-treated tissues (1–40 nM) compared to controls (2–4 nM). In subsequent experiments, we found that 10 μM ETI caused a significant reduction in the maximum response in U46619 concentration–response curves (93 ± 4% in controls compared to 64 ± 7% in the presence of ETI ($n = 4$). In contrast, 10 μM NDGA had no effect on the maximum response to U46619 in concentration–response curves (88 ± 6% in controls compared to 81 ± 6% in the presence of 10 μM NDGA, $n = 7$).

Pre-incubation with the selective cyclooxygenase inhibitor indomethacin (10 μM) had no effect on the response to 1 μM UK14304 alone (1 ± 0.5% compared to 1 ± 0.3% in the presence of indomethacin ($n = 4$). Furthermore,

indomethacin had no significant effect on the response to 1 μM UK14304 after pre-contraction with U46619 (37 ± 3% compared to 30 ± 5% in the presence of indomethacin, $n = 7$). The degree of tone induced by U46619 was the same in both control (19 ± 3% of 60 mM KCl response, $n = 7$) and tissues exposed to indomethacin (15 ± 3%, $n = 7$) and the concentration range of U46619 required to induce this level of tone was the same in control and indomethacin-treated tissues (1–4 nM).

3.2. Effect of pre-incubation with AACOCF3 on ERK activation

Levels of phosphorylated and total ERK were measured by Western blotting. In tissues contracted with 1 μM UK14304 on its own there was a significant increase in levels of phosphorylated ERK2 over control levels (Fig. 3). Levels of total ERK2 were unaffected, indicating an increase in ERK2 activity (Fig. 3b). In tissues pre-contracted with U46619 and then exposed to 1 μM UK14304, levels of both phosphorylated ERK1 and ERK2 were significantly enhanced. This was inhibited by pre-incubation with AACOCF3 (Fig. 3).

Neither NDGA (10 μM) nor ETI (10 μM) had any effect on ERK2 phosphorylation after contraction with 1 μM UK14304 in the presence of U46619. The level of ERK2 phosphorylation in the presence of NDGA was 108 ± 33% of the level of ERK2 phosphorylation in the absence of NDGA. Likewise, the level of ERK2 phosphorylation in the presence of ETI was 81 ± 12% of the level of ERK2 phosphorylation in the absence of ETI.

3.3. Effect of arachidonic acid on α_2-adrenoceptor-mediated responses

Activation of $cPLA_2$ generates arachidonic acid. Therefore, as inhibition of $cPLA_2$ inhibits the U46619-enhanced α_2-adrenoceptor-mediated vasoconstriction, pre-incubation with arachidonic acid itself should mimic the U46619 response. In tissues pre-contracted with arachidonic acid to between 10 and 20% of the 60 mM KCl response, the response to 1 μM UK14304 was enhanced from 11 ± 2 to 34 ± 4% of the 60 mM KCl response (mean ± S.E.M., $n = 8$, $p < 0.05$ Student's two-tailed, unpaired t-test). Arachidonic acid up to1 mM did not cause a contraction in the presence of 50 μM PD98059; therefore, we were unable to determine the effect of PD98059 on the arachidonic acid-enhanced response.

3.4. Effect of pre-contraction with arachidonic acid on ERK activation

Levels of phosphorylated and total ERK were measured by Western blotting. In this set of experiments, in tissues contracted with 1 μM UK14304 on its own there was a significant increase in levels of phosphorylated ERK1 and ERK2 over control levels (Fig. 4). Levels of total ERK were unaffected, indicating an increase in ERK activity (Fig. 4b). ERK1 and ERK2 phosphorylation was also increased in the presence of arachidonic acid alone, and this response was not significantly different from the UK14304 response (Fig. 4). After pre-contraction with arachidonic acid and addition of 1 μM UK14304 the level of phosphorylated ERK1 and ERK2 were greatly enhanced above the response to UK14304 alone (Fig. 4).

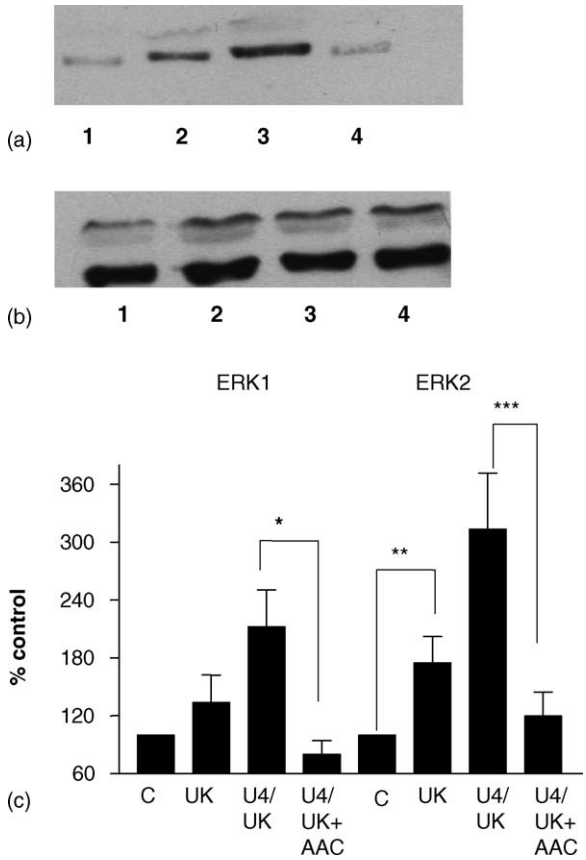

Fig. 3. (a and b) Representative immunoblot of porcine ear artery proteins separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with a primary antibody against phosphorylated ERK1/2 (a) or total ERK1/2 (b). Segments of porcine ear artery were set up in a tissue bath and exposed to the following conditions: no UK14304 (basal conditions) (1), 1 μM UK14304 (2), or pre-contracted with U46619, and exposed to 1 μM UK14304 (3), or incubated with 50 μM AACOCF3 for 3 h and pre-contracted with U46619 and exposed to 1 μM UK14304 (4). (c) Bar charts showing changes in phosphorylated ERK1 and 2 in segments of porcine ear artery contracted with: control tissues were not exposed to any agent (basal condition) (C), 1 μM UK14304 (UK), or pre-contracted with U46619 and exposed to 1 μM UK14304 (U4/UK), or incubated with 50 nM AACOCF3 for 3 h and pre-contracted with U46619 and exposed to 1 μM UK14304 (U4/UK + AAC). Responses are expressed as a percentage of control values, and are means ± S.E.M. of seven experiments. *Significant difference from UK14304 in the presence of U46619 and AACOCF3, $p < 0.05$, ANOVA followed by a Bonferroni test. **Significant difference from control, $p < 0.05$, Student's two-tailed paired t-test (direct comparison of densitometric values). ***Significant difference from UK14304 in the presence of U46619 and UK14304 in the presence of U46619 and AACOCF3, $p < 0.05$ ANOVA followed by a Bonferroni test.

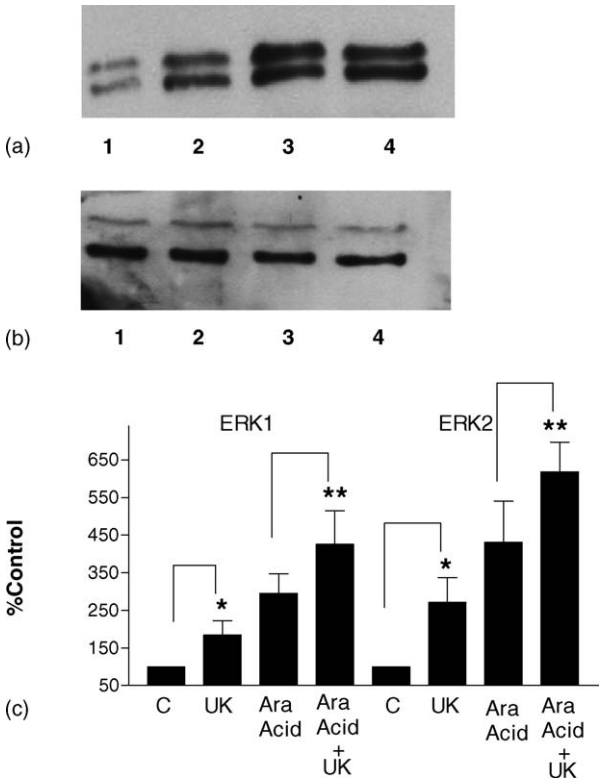

Fig. 4. (a and b) Representative immunoblots of porcine ear artery proteins separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with a primary antibody against phosphorylated ERK1/2 (a) or total ERK1/2 (b). Segments of porcine ear artery were set up in a tissue bath and exposed to the following conditions: no UK14304 (basal conditions) (1), or 1 μM UK14304 (2), or pre-contracted with arachidonic acid (3), or pre-contracted with arachidonic acid and exposed to 1 μM UK14304 (4). (c) Bar charts showing changes in phosphorylated ERK1 and 2 in segments of porcine ear artery contracted with: control tissues (C) were not exposed to any agent (basal condition), 1 μM UK14304 (UK), pre-contracted with arachidonic acid (Ara Acid), or pre-contracted with arachidonic acid and exposed to 1 μM UK14304 (Ara Acid + UK). Responses are expressed as a percentage of control values, and are means ± S.E.M. of seven experiments. *Significant difference from control, $p < 0.05$, Student's two-tailed paired *t*-test (direct comparison of densitometric values). **Significant difference from arachidonic acid (Ara Acid), $p < 0.05$, ANOVA followed by a Bonferroni test.

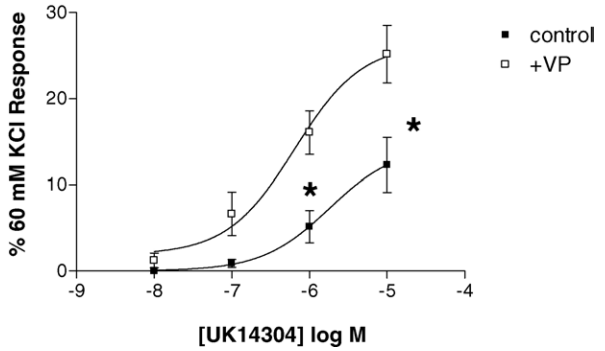

Fig. 5. Concentration–response curves to UK14304 in the absence (control) or presence of pre-contraction with vasopressin (+VP). Results are expressed as a percentage of the response to 60 mM KCl and are means ± S.E.M. of 10 experiments. *$p < 0.01$, Student's two-tailed, unpaired *t*-test vs. control.

3.5. Vasopressin enhances α_2-adrenoceptor-mediated vasoconstriction: role of ERK

Pre-contraction with vasopressin enhanced the responses to UK14304 (Fig. 5). A single concentration of UK14304 (1 μM) produced a small response in the porcine ear artery when added on its own (Fig. 6a). Pre-contraction of the tissues with vasopressin to 10–20% of the 60 mM KCl response enhanced the subsequent response to 1 μM UK14304 (Fig. 6a). In the presence of the MEK inhibitor PD98059 (50 μM), the responses to UK14304 alone were inhibited. However, PD98059 had no effect on the vasopressin-enhanced responses (Fig. 6a). The degree of tone induced by vasopressin was the same in both control (14 ± 1% of 60 mM KCl response) and tissues exposed to PD98059 (13 ± 1%) and the concentration range of vasopressin required to induce this level of tone was the same in control and PD98059-treated tissues (0.3–1 nM), suggesting that PD98059 had no effect on the vasopressin-induced vasoconstriction. These data were backed up by subsequent experiments that showed that 50 μM PD98059 had no effect on the response to a single, maximum concentration of 10 nM vasopressin (28 ± 10% of the 60 mM KCl response compared to 28 ± 10% in the presence of PD98059, $n = 9$). Furthermore, Western blotting experiments demonstrated that this maximum concentration of vasopressin (10 nM) had no effect on ERK2 phosphorylation (117 ± 13% of control, $n = 5$). In contrast, PD98059 (50 μM) caused a slight, but significant reduction in the U46619 response at high concentrations of U46619 measured in concentration–response curves (78 ± 5% in controls compared to 58 ± 5% in the presence of PD98059, $n = 8$, $p < 0.01$, Student's two-tailed, unpaired *t*-test). U46619 (0.1 μM) also caused an increase in ERK2 phosphorylation (158 ± 17% of control, $p < 0.05$, Student's two-tailed paired *t*-test, $n = 4$). There was no effect on total levels of ERK2 (112 ± 27% of control, $n = 4$).

In tissues contracted with 1 μM UK14304 on its own there was a significant increase in levels of phosphorylated ERK2 over control levels. Levels of total ERK2 were unaffected, indicating an increase in ERK2 activity (Fig. 6b and c). In tissues pre-contracted with vasopressin, and exposed to 1 μM UK14304 there was no measurable effect on ERK2 phosphorylation (Fig. 6b and c). Contracting the tissues with vasopressin alone also had no effect on ERK2 phosphorylation (Fig. 6b and c).

3.6. Effect of Inhibition of $cPLA_2$ on vasopressin-enhanced α_2-adrenoceptor-mediated vasoconstriction

Contrary to the effects in the presence of U46619, the $cPLA_2$ inhibitor AACOCF3 had no effect on the enhanced response to UK14304 in the presence of vasopressin

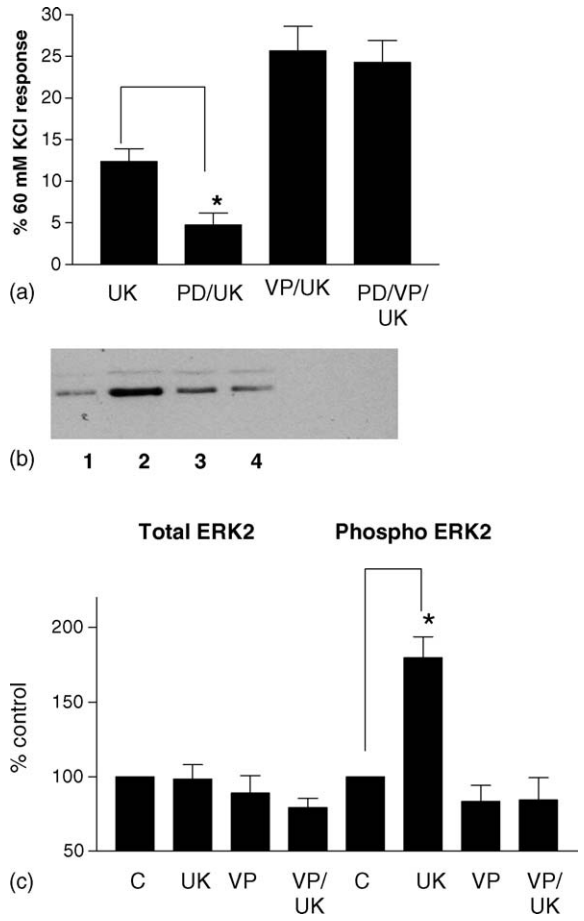

Fig. 6. (a) Contractile responses to 1 μM UK14304 alone (UK), or after pre-contraction with vasopressin (VP/UK) in the absence or presence of 50 μM PD98059 (PD/UK or PD/VP/UK) in the porcine ear artery. Results are expressed as a percentage of the response to 60 mM KCl (mean ± S.E.M., $n = 6$). $^{*}p < 0.05$, Student's two-tailed, unpaired t-test vs. UK. (b) Representative immunoblot of porcine ear artery proteins separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with a primary antibody against phosphorylated ERK1/2. Segments of porcine ear artery were set up in a tissue bath and exposed to the following conditions: no UK14304 (basal conditions) (1), 1 μM UK14304 (2), contracted to 20% of the 60 mM KCl response with vasopressin (3), or pre-contracted with vasopressin, and exposed to 1 μM UK14304 (4). (c) Bar charts showing changes in phosphorylated (phospho) or total ERK2 in segments of porcine ear artery contracted with 1 μM UK14304 (UK), vasopressin (contracted to <20% of the 60 mM KCl response, VP), or pre-contracted with vasopressin and exposed to 1 μM UK14304 (VP/UK). Control tissues (C) were not exposed to any agent (basal condition). Responses are expressed as a percentage of control values, and are means ± S.E.M. of seven experiments. *Significant difference from control, $p < 0.05$, Student's two-tailed paired t-test (direct comparison of densitometric values).

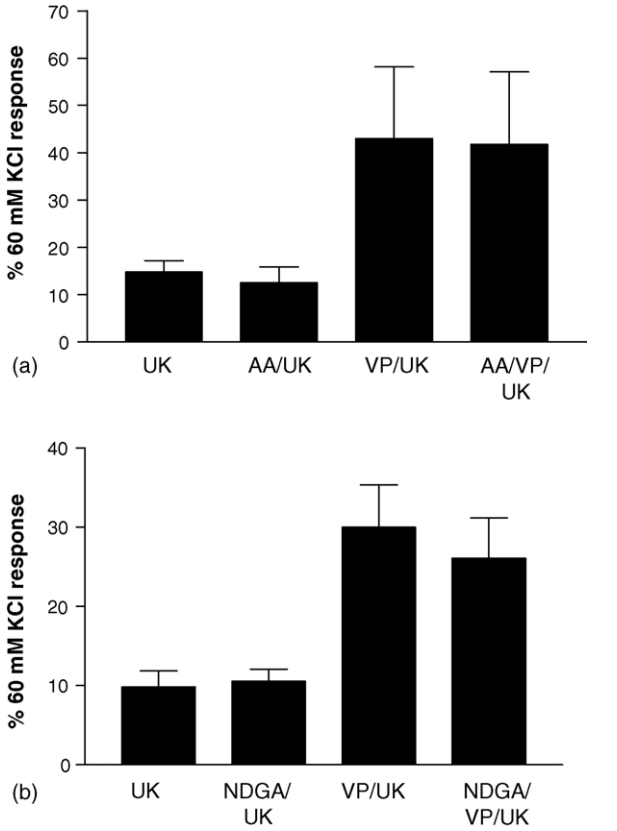

Fig. 7. (a) Contractile responses to 1 μM UK14304 alone (UK), or after pre-contraction with vasopressin (VP/UK) in the absence or presence of 50 μM AACOCF3 (AA/UK or AA/VP/UK) in the porcine ear artery. Results are expressed as a percentage of the response to 60 mM KCl (mean ± S.E.M., $n = 6$). (b) Contractile responses to 1 μM UK14304 alone (UK), or after pre-contraction with vasopressin (VP/UK) in the absence or presence of 10 μM NDGA (NDGA/UK or NDGA/VP/UK) in the porcine ear artery. Results are expressed as a percentage of the response to 60 mM KCl (mean ± S.E.M., $n = 5$).

(Fig. 7a). The degree of tone induced by vasopressin was the same in both control (20 ± 2% of 60 mM KCl response, $n = 6$) and tissues exposed to AACOCF3 (20 ± 6%, $n = 6$). The concentration range of vasopressin required to induce this degree of tone was the same in control and AACOCF3-treated tissues (5–40 nM) suggesting that inhibition of $cPLA_2$ has no effect on the vasopressin-induced contraction. These data were backed up by the observation that 50 μM of the $cPLA_2$ inhibitor MAFP had no effect on the response to a single, maximum concentration (10 nM) of vasopressin (45 ± 14% in controls compared to 39 ± 12% in the presence of MAFP, $n = 7$).

Pre-incubation with the selective lipoxygenase inhibitor NDGA (10 μM) also had no effect on the vasopressin-enhanced UK14304 response (Fig. 7b). The degree of tone induced by vasopressin was the same in both control (12 ± 2% of 60 mM KCl response) and tissues exposed to NDGA (12 ± 2%) and the concentration range of vasopressin required to induce this level of tone was the same in control and NDGA-treated tissues (1–2 nM).

3.7. Effect of PKCζ pseudosubstrate inhibitor on α_2-adrenoceptor-mediatedresponses

In order to try to identify signalling components downstream of arachidonic acid involved in the enhancement of the α_2-adrenoceptor contractile responses by U46619,

tissues were exposed to the PKCζ pseudosubstrate inhibitor (10 μM) for 1 h. The responses to UK14304 alone were unaffected by pre-incubation with the PKCζ pseudosubstrate inhibitor (Fig. 8a and b). On the other hand, the enhanced response in the presence of U46619 was significantly reduced by the PKCζ pseudosubstrate inhibitor (see Fig. 8a). The degree of tone induced by U46619 was the same in both control ($16 \pm 1\%$, $n = 5$) and tissues exposed to the PKCζ pseudosubstrate inhibitor ($16 \pm 3\%$, $n = 5$) and the concentration range of U46619 required to induce this tone was the same in both control and the PKCζ pseudosubstrate inhibitor-treated tissues (2 nM–4 nM). PKCζ inhibition also caused a significant inhibition of the enhanced response to UK14304 in the presence of vasopressin (Fig. 8b). The degree of tone induced by vasopressin was the same in both control ($11 \pm 5\%$, $n = 5$) and tissues exposed to the inhibitor ($11 \pm 7\%$, $n = 5$) and the concentration range of vasopressin required to induce this tone was the same in both control and inhibitor-treated tissues (1–2 nM). On the other hand, the PKCζ pseudosubstrate inhibitor had no effect on UK14304 responses in the presence of arachidonic acid ($14 \pm 7\%$ compared to $17 \pm 5\%$ in the presence of the pseudosubstrate inhibitor ($n = 4$)).

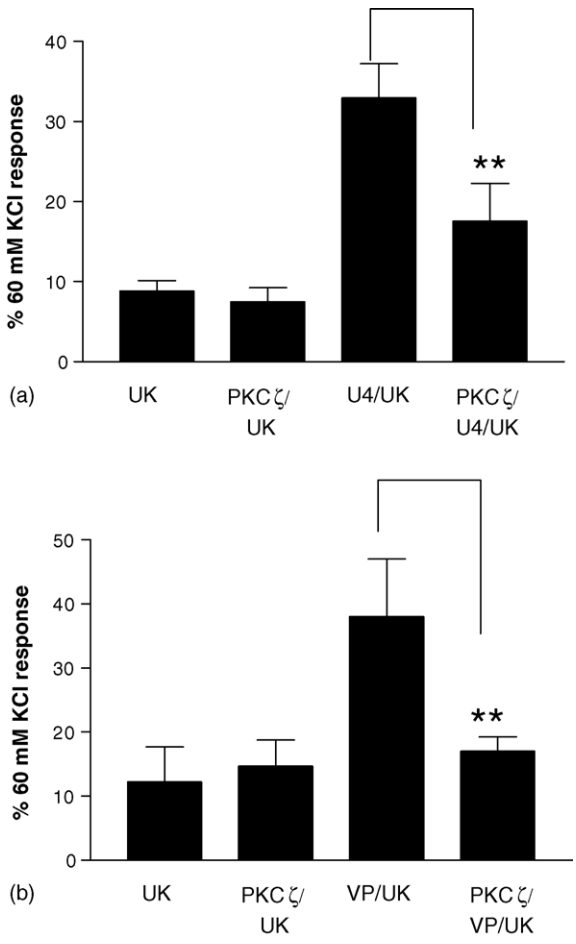

Fig. 8. (a) Contractile responses to 1 μM UK14304 alone (UK), or after pre-contraction with U46619 (U4/UK) in the absence or presence of 10 μM PKCζ pseudosubstrate inhibitor (PKCζ/UK or PKCζ/U4/UK) in the porcine ear artery. Results are expressed as a percentage of the response to 60 mM KCl (mean ± S.E.M., $n = 5$). $^{**}p < 0.05$ Student's two-tailed, unpaired *t*-test vs. U4/UK. b. Contractile responses to 1 μM UK14304 alone (UK), or after pre-contraction with vasopressin (VP/UK) in the absence or presence 10 μM PKCζ pseudosubstrate inhibitor (PKCζ/UK or PKCζ/VP/UK) in the porcine ear artery. Results are expressed as a percentage of the response to 60 mM KCl (mean ± S.E.M., $n = 5$). $^{**}p < 0.05$ Student's two-tailed, unpaired *t*-test vs. VP/UK.

4. Discussion

The current study suggests the role of cytosolic phospholipase A_2 ($cPLA_2$) and arachidonic acid as important signalling components in U46619-mediated enhancement of the α_2-adrenoceptor vasoconstriction. $cPLA_2$ is a ubiquitous enzyme which catalyses the release of arachidonic acid which is metabolized by different enzyme systems to produce prostaglandins, thromboxanes, leukotrienes, etc. The $cPLA_2$ inhibitors AACOCF3 [14] and MAFP [15] had no effect on the response to UK14304 alone indicating that $cPLA_2$ plays no role in the direct α_2-adrenoceptor-mediated vasoconstriction. On the other hand, AACOCF3 and MAFP caused large inhibitions of the U46619-enhanced response. Similarly, AACOCF3 had no effect on activation of ERK by UK14304 alone, but significantly reduced the U46619-enhanced ERK activation. Both AACOCF3 and MAFP can also inhibit Ca^{2+}-independent PLA_2 at the concentrations used in this study [16,17]. However, HELSS, which possesses a 1000-fold selectivity for the Ca^{2+}-independent PLA_2 over $cPLA_2$ [18], inhibited both the direct UK14304 response and the U46619-enhanced UK14304 response. Taken together, these data would seem to indicate that inhibition of $cPLA_2$ causes selective inhibition of the U46619-enhanced UK14304 response. The effect of HELSS on both the direct and the enhanced α_2-adrenoceptor response could indicate a role for Ca^{2+}-independent PLA_2 in α_2-adrenoceptor-mediated responses. Inhibition of $cPLA_2$ with MAFP also had no effect on the U46619-induced contractions demonstrating that although $cPLA_2$ is not involved in the direct contractions to either UK14304 or U46619, it is involved in the U46619-enhancement of the UK14304 response.

$cPLA_2$ catalyses the formation of arachidonic acid from phospholipids. Arachidonic acid, or its lipoxygenase (12-HETE and 15-HETE) or cytochrome P450 metabolites (20-HETE) are able to activate ERK-MAP kinase in vascular smooth muscle cells [5,11]. As the U46619-induced α_2-adrenoceptor-mediated vasoconstriction is dependent upon an increased activation of ERK [2], it is possible that release of arachidonic acid by $cPLA_2$ leads to the increase in ERK activation, resulting in the enhanced vasoconstriction. If so, arachidonic acid itself should be able to mimic the effects of U46619. Indeed we found that pre-contraction of the porcine ear artery with arachidonic acid did result in an enhanced α_2-adrenoceptor-mediated

vasoconstriction. This enhanced response should be sensitive to inhibition by PD98059 in the same way as the U46619-enhanced response [2]. However, we found that in the presence of PD98059 arachidonic acid failed to produce a contraction up to 1 mM, suggesting that arachidonic acid-mediated vasoconstriction is dependent upon activation of ERK-MAP kinase. Indeed, we found that arachidonic acid causes an increase in ERK phosphorylation, indicative of an increased activation. The enhanced α_2-adrenoceptor-mediated contractile response in the presence of arachidonic acid was associated with an enhanced ERK activation. This is as expected as the U46619-enhanced contractile response is associated with an enhanced ERK activation, and this is inhibited by the $cPLA_2$ inhibitor AACOCF3. The increased ERK activation in the presence of arachidonic acid and UK14304 appears to be additive, whereas the contractile response appears to be enhanced. This suggests that the arachidonic acid induced activation of ERK does not lead to a large vasoconstriction on its own, but when combined with α_2-adrenoceptor activation, can lead to an enhancement of the α_2-adrenoceptor-mediated vasoconstriction. Arachidonic acid activation of ERK can lead to further activation of $cPLA_2$ resulting in more arachidonic acid production through a positive feedback mechanism [4]. It may be this positive feedback that is important in the enhancement of the α_2-adrenoceptor-mediated vasoconstriction.

Reports indicate that lipoxygenase metabolites of arachidonic acid are able to activate ERK in vascular smooth muscle cells [5]. Therefore, we determined the effect of lipoxygenase inhibition on the α_2-adrenoceptor-mediated contractile responses. The lipoxygenase inhibitors NDGA [19] and ETI [20] both caused a selective inhibition of the U46619-enhanced α_2-adrenoceptor-mediated vasoconstriction, with no effect on the direct α_2-adrenoceptor-mediated response. This inhibition corresponds with the selective effect of the $cPLA_2$ inhibitors on the U46619-enhanced contractile responses. NDGA and ETI may also inhibit cyclooxygenase. However, the selective cyclooxygenase inhibitor indomethacin had no effect on the enhanced response to UK14304 after pre-contraction with U46619. These data, therefore, indicate that lipoxygenase products may be responsible for the enhanced contractile responses. On the other hand, neither NDGA nor ETI had any effect on the UK14304 activation of ERK in the presence of U46619. This indicates that either lipoxygenase is downstream of ERK activation, or lipoxygenase products are not involved in the enhancement of ERK activation.

ETI but not NDGA also inhibited the U46619-induced contraction, suggesting that some of the effects of ETI may not be specific to inhibition of lipoxygenase. ETI has been shown to inhibit calcium influx in vascular smooth muscle cells [21], although whether this was due to inhibition of lipoxygenase or a non-specific effect is not clear. As the U46619-induced contraction in the porcine ear artery is dependent upon influx of extracellular calcium (unpublished observations), this may explain the effect of ETI on the U46619-induced contraction.

4.1. Comparison of U46619 and vasopressin-enhanced responses

In order to determine whether this mechanism of enhancement of α_2-adrenoceptor-mediated vasoconstriction is specific to U46619, we decided to investigate whether other vasoconstrictors were able to mimic the effects of U46619. Vasopressin has been shown to enhance α-adrenoceptor-mediated vasoconstriction in the human renal artery, although the α-adrenoceptor subtype involved was not investigated [10]. Vasopressin has also been shown to enhance sympathetic nerve-stimulated vasoconstriction, but this was thought to be due to a direct effect on the nerves [22]. In the porcine ear artery, pre-contraction with vasopressin to the same degree as with U46619 (10–20% of the 60 mM KCl response) caused a similar enhancement of the response to UK14304. However, unlike the U46619-induced enhancement [2], this response was not inhibited by PD98059, an inhibitor of the ERK-MAP kinase pathway [23], and vasopressin did not enhance the UK14304-stimulated ERK activation. This suggests that U46619 and vasopressin enhance α_2-adrenoceptor-mediated vasoconstriction through different mechanisms. Indeed, we found that inhibition of $cPLA_2$ also had no effect on vasopressin-enhanced responses, providing further evidence that the vasopressin-enhanced response is mediated through a different pathway. Similarly, the lipoxygenase inhibitor NDGA had no effect on the vasopressin-enhanced α_2-adrenoceptor-mediated vasoconstriction. The U46619-enhanced α_2-adrenoceptor-mediated vasoconstriction is dependent upon influx of extracellular calcium [2]. However, we were unable to compare the effects of removal of extracellular calcium on the vasopressin-enhanced responses as vasopressin did not stimulate a contraction in the absence of calcium in the porcine ear artery (unpublished observations). These data also demonstrate that the enhancement of the α_2-adrenoceptor-mediated vasoconstriction by U46619, which appears to be mediated through a pathway dependent upon $cPLA_2$ and enhancement of ERK activation, is not a non-specific effect caused by increased resting load.

Inhibition of ERK activation with the MEK inhibitor PD98059 did cause a slight inhibition of the contractile response to U46619, demonstrating that U46619-induced contractions have at least a partial dependence on ERK activation. The vasopressin contraction, on the other hand, was unaffected by the MEK inhibitor PD98059 suggesting that ERK activation is not involved in vasopressin-induced vasoconstriction. A high concentration of U46619 also caused an increase in ERK activation. Previously we have been unable to detect an increase in ERK phosphorylation at the low concentrations of U46619 required to produce a

pre-contraction of 10–20% of the KCl response [2]. This may be because at these lower concentrations U46619 does not stimulate ERK activation, or the change in phosphorylation of ERK is too small to detect by Western blotting. Therefore, it is possible that pre-contraction with U46619 might cause a small activation of ERK, thus priming the ERK signalling pathway, resulting in an enhanced α_2-adrenoceptor-mediated ERK activation and vasoconstriction. As ERK is not involved in the vasopressin-induced contraction, the ERK pathway is not primed and so there is no enhanced α_2-adrenoceptor-mediated ERK activation. However, there are a few unresolved questions. The U46619-enhanced α_2-adrenoceptor contraction and ERK activation are dependent upon $cPLA_2$. However, the U46619-induced contraction is not dependent upon $cPLA_2$ itself. How priming of the ERK signalling pathway leads to an enhancement of the α_2-adrenoceptor-stimulated ERK activation is unknown.

4.2. Role of protein kinase Cζ

Protein kinase Cζ is a member of the atypical PKC subfamily and is a calcium and diacylglycerol (DAG) independent isoform. PKCζ is activated by phosphatidylinositols, arachidonic acid and other lipids (see [24]). It has also been reported that atypical protein kinase C activated by arachidonic acid is involved in calcium sensitization of rabbit smooth muscle [11]. Since the α_2-adrenoceptor-mediated enhanced vasoconstriction by U46619 is dependent on cytosolic phospholipase A_2 and arachidonic acid enhances the α_2-adrenoceptor-mediated response, we decided to observe the effects of PKCζ inhibition. PKCζ inhibition significantly reduced the enhanced α_2-adrenoceptor-mediated vasoconstriction by U46619 while no reduction was observed in the direct α_2-adrenoceptor response. However, PKCζ inhibition also significantly inhibited the vasopressin-mediated enhancement of the α_2-adrenoceptor vasoconstriction. On the other hand, the PKCζ inhibitor had no effect on the arachidonic acid-enhanced α_2-adrenoceptor responses. Taken together, these data suggest that while PKCζ may be involved in the enhanced α_2-adrenoceptor-mediated vasoconstriction in the presence of U46619, or vasopressin, but not the direct α_2-adrenoceptor-mediated response, it is not downstream of arachidonic acid production.

We have previously demonstrated that the U46619-enhanced α_2-adrenoceptor-mediated vasoconstriction is dependent upon influx of extracellular calcium [2]. This appears to be in conflict with the finding that the calcium-independent PKC isozyme PKCζ is involved in the U46619-enhanced contraction. However, as PKCζ is involved in the vasopressin and the U46619-enhanced α_2-adrenoceptor-mediated responses, the possibility exists that PKCζ may play a role in a separate pathway, common to both vasopressin and U46619, which is independent of calcium.

The data presented here and in our previous paper [2] demonstrate that α_2-adrenoceptor-mediated vasoconstriction in the porcine ear artery can be enhanced through two different pathways. In the first, the thromboxane-mimetic U46619 enhances the α_2-adrenoceptor-mediated response through an ERK-dependent pathway that involves activation of $cPLA_2$ leading to an increased activation of α_2-adrenoceptor-stimulated ERK-MAP kinase activation. On the other hand, vasopressin enhances the α_2-adrenoceptor-mediated response through an ERK- and $cPLA_2$-independent pathway. The mechanism of the enhancement of the α_2-adrenoceptor-mediated vasoconstriction by vasopressin is unknown, and further work is required to identify this pathway.

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