British Journal of Pharmacology (2003) 138, 107–116

www.nature.com/bjp

α_2 Adrenoceptor-mediated vasoconstriction in porcine palmar lateral vein: role of phosphatidylinositol 3-kinase and EGF receptor transactivation

*,[1]R.E. Roberts

[1]Institute of Cell Signalling, University of Nottingham, Medical School, Queen's Medical Centre, Nottingham, NG7 2UH

1 α_2 Adrenoceptors cause vasoconstriction in the porcine palmar lateral vein through a mechanism involving the ERK signal transduction cascade, calcium influx, and a Src tyrosine kinase. The aim of the present study was to determine if phosphatidylinositol 3-kinase (PI 3-kinase) and/or epidermal growth factor (EGF) receptor transactivation are also involved.

2 α_2 Adrenoceptor-mediated vasoconstriction and ERK2 activation in the porcine palmar lateral vein was inhibited in the presence of either the PI 3-kinase inhibitor LY294002, or the EGF receptor tyrosine kinase inhibitor AG1478 suggesting the involvement of both PI 3-kinase and EGF receptor transactivation.

3 Akt phosphorylation was increased in segments of porcine palmar lateral vein contracted with UK14304 indicating an increase in Akt activation. This is a further indication that PI 3-kinase is involved in α_2 adrenoceptor-mediated vasoconstriction. Akt activation was inhibited by the Src tyrosine kinase inhibitor PP2, and removal of extracellular calcium.

4 UK14304 (10 μM) stimulated an increase in intracellular calcium in segments of palmar lateral vein. This was inhibited by removal of extracellular calcium, but not by nifedipine suggesting the rise in calcium is due to influx of calcium through non-L type calcium channels. The increase in calcium was also inhibited by LY294002 indicating that PI 3-kinase is upstream of calcium influx.

5 These data indicate that α_2 adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein is dependent upon stimulation of PI 3-kinase, leading to an influx of calcium. This results in activation of the EGF receptor tyrosine kinase, and finally activation of ERK–MAP kinase.
British Journal of Pharmacology (2003) **138,** 107–116. doi:10.1038/sj.bjp.0705014

Keywords: ERK; α_2 adrenoceptors; vasoconstriction; calcium influx; phosphatidylinositol 3-kinase; EGF receptor

Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; ANOVA, analysis of the variance; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; E-64, trans-epoxysuccinyl-L-leucylamide-(4-guanidino) butane; EGF, epidermal growth factor; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PDK, phosphoinositide-dependent kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; TBS-T, tris-buffered saline containing 0.1% tween-20

Introduction

α_2 Adrenoceptors are Gi-protein coupled receptors and, as such, inhibit adenylyl cyclase resulting in a decrease in intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels (Bylund *et al*., 1994). As an increase in intracellular cyclic AMP causes relaxation of vascular smooth muscle (Yamagishi *et al*., 1994), a reduction of cyclic AMP levels by α_2 adrenoceptors would be expected to lead to an increase in blood vessel tone. However, direct α_2 adrenoceptor-mediated vasoconstriction does not seem to be due to inhibition of cyclic AMP production. For example, in the porcine palmar lateral vein, activation of α_2 adrenoceptors alone results in a large contraction, but does not decrease intracellular cyclic AMP levels (Wright *et al*., 1995). Recent studies have demonstrated that this α_2 adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein is dependent upon activation of the ERK–MAP kinase pathway (Roberts, 2001). The mechanism of ERK activation is unknown, but seems to involve activation of voltage-sensitive calcium channels and a Src tyrosine kinase (Roberts, 2001). However, the other intermediate steps in the pathway are unknown. One possible component of the signalling pathway is phosphatidylinositol 3-kinase (PI 3-kinase). PI 3-kinase is an early intermediate in G protein-coupled receptor stimulated ERK activation (Hawes *et al*., 1996). Furthermore, PI 3-kinase has been shown to regulate the opening of voltage-sensitive calcium channels by G protein-coupled receptors (Seki *et al*., 1999; Viard *et al*., 1999), and can be activated by Src tyrosine kinase (Ibitayo *et al*., 1998; Daulhac *et al*., 1999). As such, PI 3-kinase could link α_2-adrenoceptors to Src tyrosine kinase, calcium influx and ERK activation.

It is becoming increasingly apparent that G protein-coupled receptors can activate the ERK signal transduction pathway through the transactivation of tyrosine kinase-coupled growth factor receptors such as the epidermal growth factor (EGF) receptor (see Zwick *et al*., 1999).

*Author for correspondence;
E-mail: richard.roberts@nottingham.ac.uk.

Transactivation involves phosphorylation of the growth factor receptor and hence activation of the associated tyrosine kinase in the absence of the growth factor itself. Once activated, the EGF receptor tyrosine kinase activates the ERK–MAP kinase signal transduction cascade. One mechanism of EGF receptor transactivation is through Src tyrosine kinase (Luttrell *et al*., 1997; Gao *et al*., 2001). Transactivation, therefore, could link Src tyrosine kinase activity to activation of the ERK–MAP kinase pathway in α_2-adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein.

The main aims of this present study were 2 fold. Firstly to determine whether PI 3-kinase is involved in α_2-adrenoceptor-mediated vasoconstriction and ERK activation in the porcine palmar lateral vein, and secondly to determine whether EGF receptor transactivation is involved. Further aims were to determine the relationship of these signalling components to the other known signalling components.

Methods

Isometric tension recordings

Porcine trotters were obtained from a local abattoir and transported to the laboratory on ice. Palmar lateral veins were dissected out and placed in Krebs–Henseleit buffer containing 2% ficoll, which has been pre-gassed with 95% O_2/5% CO_2, and stored overnight at 4°C (see Wright *et al*., 1995). The following day veins were carefully cleaned of fat and connective tissue, dissected into 5 mm ring segments, and suspended in a 5 ml isolated tissue 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 (World Precision Instruments, Sarasota, FL, U.S.A.) linked to a MacLab data acquisition system (AD Instruments Ltd., Hastings, U.K.) *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 0.5–1.0 g wt. Before each experiment the tissues were contracted with 60 mM KCl, until the final two responses differed by less than 10%.

Effect of inhibitors on UK14304 responses

Tissues were incubated for 1 h with one of the following inhibitors: the PI 3-kinase inhibitor LY294002 (1–50 μM); the EGF receptor tyrosine kinase inhibitor AG1478 (0.1 and 1 μM). Control tissues received just vehicle (0.1% DMSO). Cumulative concentration response curves to UK14304 (1 nM to 10 μM) were then performed.

Immunoblotting for ERK, Akt or Src

Segments of porcine palmar lateral vein were set up in tissue baths as above. Tissues were contracted with 10 μM UK14304 in the absence or presence of one of the following inhibitors: the MEK inhibitor PD98059 (50 μM); the L-type calcium channel blocker nifedipine (50 μM); the selective Src tyrosine kinase inhibitor PP2 (10 μM), LY294002 (50 μM); AG1478 (1 μM). Control tissues were not exposed to any compound (basal conditions). In experiments in which UK14304 was added in the absence of extracellular calcium, the Krebs–Henseleit buffer was replaced with calcium-free Krebs–Henseleit in which the calcium was replaced with 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 5 min before UK14304 was added. When the contractions to UK14304 reached a plateau (3–4 min after addition of the agonist), the segments were quickly removed from the tissue baths, and immediately frozen on dry ice. Frozen segments were then homogenized 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} aprotinin, 1 μM leupeptin, 500 μM EDTA). After removal of a sample for a protein assay, the homogenate was diluted 1 : 1 in 2 × 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. After incubating in blocking solution (5% powdered milk in tris-buffered saline containing 0.1% tween-20 (TBS–T)), nitrocellulose blots were incubated overnight at 4°C with antibodies recognizing one of the following: the double phosphorylated (activated) forms of both isoforms of ERK (ERK1 and 2), Akt phosphorylated at Ser 473, Src kinase phosphorylated at Tyr416, total ERK, total Akt, or total Src (all from New England Biolabs). After washing in TBS–T, the blots were incubated with the appropriate, hydrogen peroxidase-conjugated secondary antibody. Proteins were visualized using the ECl system (Amersham Life Sciences). Bands were analysed by densitometry.

Immunoprecipitation

Segments of porcine palmar lateral vein were set up in tissue baths as above. Tissues were contracted with 10 μM UK14304. When the contractions to UK14304 reached a plateau, the segments were quickly removed from the tissue baths, and immediately frozen on dry ice. Frozen segments were then homogenized in ice-cold immunoprecipitation buffer (20 mM Tris [pH 7.5], 150 mM sodium chloride, 1 mM EGTA, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM sodium β-glycerophosphate, 1 mM sodium vanadate, 500 μM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 1 μM trans-epoxysuccinyl-L-leucylamide-(4-guanidino) butane (E-64), 10 μg ml^{-1} aprotinin, 1 μM leupeptin). After homogenization, 1% sodium dodecyl sulphate was added, and the samples heated at 95°C for 10 min with occasional vortexing. Samples were then centrifuged at 10,000 × g for 2 min, and supernatants removed. An aliquot of the supernatant was removed and assayed for protein content using the Bradford method. Equal amounts of protein were then diluted 1 : 10 in immunoprecipitation buffer (final volume 300 μl). An antibody against the EGF receptor (New England Biolabs) was then added (1 : 50), and samples incubated overnight at 4°C with shaking. The following day 20 μl of a 50 : 50 mixture of protein A/G sepharose beads (Santa Cruz) was added to each sample, and mixed at 4°C for 2 h. Samples were then centrifuged at 10,000 × g for 2 min, and supernatants removed and discarded. The sepharose beads were then washed four times with ice-cold immunoprecipitation buffer,

before being resuspended in 20 μl of Laemmli sample buffer. Samples were heated at 95°C for 5 min prior to running on a 7% SDS–PAGE gel. After transferring the proteins onto nitrocellulose by Western blotting, the samples were probed for phosphotyrosine using 4G10 antibody (Upstate Biotech), or with an antibody against total EGFR (New England Biolabs). Proteins were visualized using the ECl system (Amersham Life Sciences). Bands were analysed by densitometry.

Calcium imaging

Segments of porcine palmar lateral vein were incubated with 5 μM fura 2/AM in Krebs–Henseleit buffer in the presence of 0.02% Pluronic F-127, 0.1% Cremophor FL, and 1% dimethylsulphoxide for 1 h at room temperature with shaking. Segments were then cut open, and placed lumen-side down on a heated coverslip and held in position with two metal supports. The heated coverslip was placed on an inverted microscope (Leica) equipped for dual excitation wavelength fluorescent measurements. The objective was a Nikon CF Fluor (10×0.5). The light source was a 75 W Xenon lamp. The tissue was bathed in 200 μl Krebs–Henseleit buffer maintained at 37°C and constantly gassed with 95% O_2/CO_2. There was a 30 min equilibration period after the tissue had been set up prior to any calcium measurements. Tissues were challenged with 60 mM KCl twice before UK14304 (10 μM) was added.

Changes in intracellular calcium levels were assessed by alternatively exciting the preparation with 340 nm and 380 nm wavelength light with a 3 s delay between exposures. Emitted light (measured at 510 nm) was collected by a photomultiplier (Photonic Science, Tunbridge Wells, U.K.). The system was controlled by an Apple Macintosh Power PC using IonVision software (Improvision, Coventry). In its free form fura-2 produces a high fluorescence at 380 nm, and a low fluorescence at 340 nm. When fura-2 binds to calcium the opposite its true such that the amount of fluorescence at 380 nm decreases, and the amount of fluorescence at 340 nm increases. The ratio of fluorescence at these wavelengths (340/380 nm) is an index of calcium concentration. Therefore, changes in this ratio are recorded as a change in intracellular calcium. The Grynkiewicz equation (Grynkiewicz *et al*., 1985) can be used to calculate the absolute calcium concentration from the ratio values. However, the constants required for the equation (the dissociation constant for fura-2, R_{max} and R_{min}) are difficult to determine accurately, and are likely to vary between tissues. Therefore, as this study is concerned purely with the changes in intracellular calcium levels, we have quoted the 340 nm/380 nm fluorescence ratios, which are directly proportional to the absolute values.

Drugs

5-bromo-6-[2-imidazolin-2-ylamine]-quinoxaline bitartrate (UK14304), (Pfizer); 2-amino-3-methoxyflavone (PD98059), (Calbiochem); nifedipine (Alexis Biochemicals); LY294002 (Tocris); AG1478 (Alexis Biochemicals); Fura-2AM (Calbiochem); 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) (Calbiochem). All other compounds were obtained from Sigma, Poole, U.K.

Data analysis

Contractile responses and calcium responses were expressed as a percentage of the response to 60 mM KCl, and results expressed as mean$\pm$s.e.mean. Multiple comparisons between treatment groups were performed using analysis of the variance (ANOVA) followed by a Bonferroni test or Dunnett's multiple comparison test. Bands obtained by immunoblotting were analysed by densitometry. Responses obtained from segments from the same animals were compared using a two-tailed, Student's paired *t*-test for normally-distributed data, or a two-tailed, Wilcoxon Signed Rank test for non-parametric data.

Results

Role of PI 3-kinase in α_2 adrenoceptor-mediated contractions

The α_2 adrenoceptor agonist UK14304 caused a large, concentration-dependent increase in contraction of porcine palmar lateral vein segments (see Figure 1). This response was significantly inhibited by the PI 3-kinase inhibitor LY294002 (Figure 1). The effect of LY294002 was concentration-dependent with 1 μM having no effect, but 10 μM caused a significant shift in the response-curves to the right with no significant reduction in maximum response (pD2 7.1 ± 0.16 (control) compared to 6.5 ± 0.17 (LY294002), $P<0.05$, ANOVA followed by Bonferroni test, $n=9$). Increasing the concentration of LY294002 to 50 μM caused a significant reduction in the maximum response to UK14304 (Figure 1).

Role of PI 3-kinase in α_2 adrenoceptor-mediated activation of ERK

Segments of porcine palmar lateral vein were contracted with a single concentration of UK14304 (10 μM), and then rapidly

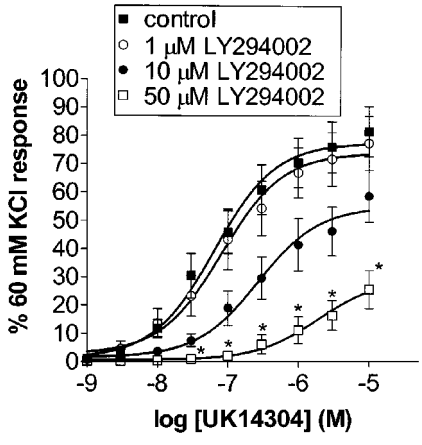

Figure 1 Log concentration-response curves to UK14304 in segments of porcine palmar lateral vein, in the absence (control) or presence of 1, 10, or 50 μM LY294002. Responses are expressed as per cent 60 mM KCl response, and shown as means$\pm$s.e.mean of 10 experiments. *Indicates significant difference from control responses, $P<0.01$, ANOVA followed by Bonferroni test.

frozen, homogenized, and the proteins separated by SDS–PAGE. The proteins were transferred on to nitrocellulose membranes, and the nitrocellulose blots probed with an antibody against the double phosphorylated (activated) form of ERK. Development of the immunoblots revealed just two bands of around 40–45 kDa in size (Figure 2). Densitometric analysis of the bands revealed that there was a significant increase in the amount of phosphorylated ERK1 and ERK2 in UK14304-contracted palmar lateral vein segments compared to control tissues (tissue segments not stimulated with UK14304; see Figure 2). Table 1 shows the densities of the ERK2 bands expressed as a percentage of control values. There was no apparent difference in the levels of total ERK

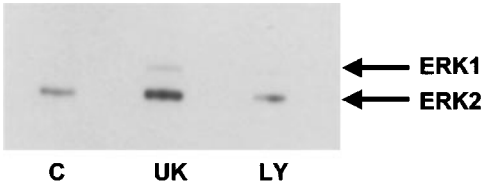

Figure 2 Representative immunoblot of porcine palmar lateral vein proteins separated by SDS–PAGE, transferred onto nitrocellulose membranes, and incubated with a primary antibody against phosphorylated ERK1/2. Segments of porcine palmar lateral vein were set up in a tissue bath and contracted with 10 μM UK14304 in the absence (UK) or presence of 50 μM LY294002 (LY). Non-stimulated segments kept under basal conditions were also obtained (C). Tissues were rapidly frozen, homogenized, and then subjected to SDS–PAGE.

Table 1 Changes in the levels of phosphorylated and total ERK2

a	*Phospho ERK2* (% control)	*Total ERK2* (% control)
Cont	100	100
UK14304 (10 μM)	184.7±27.5* ($n=6$)	106.1±13.1 ($n=6$)
+LY294002 (50 μM)	97.8±18.3† ($n=6$)	104.9±18.8 ($n=6$)
b		
Cont	100	100
UK14304 (10 μM)	178.4±18.5* ($n=6$)	117.3±9.1 ($n=6$)
+AG1478 (1 μM)	117.0±7.3‡ ($n=6$)	117.2±5.5 ($n=6$)

Phosphorylated (Phospho ERK2 and total ERK2 levels were determined by Western blotting as outlined in the Methods section. Densitometric analysis of the bands on the immunoblots was carried out, and the results expressed as a per cent of control (unstimulated) levels. (**a**) Shown are the levels of phosphorylated and total ERK2 in the presence of 10 μM UK14304 in the absence, or presence of 50 μM LY294002. *Indicates significant difference ($P<0.05$) from control values (direct comparison of densitometric values), Student's two-tailed, paired t-test. †Indicates significant difference ($P<0.05$) from UK14304 alone values, Student's two-tailed, paired t-test. (**b**) Shown are the levels of phosphorylated and total ERK2 in the presence of 10 μM UK14304 in the absence, or presence of 1 μM AG1478. *Indicates significant difference ($P<0.05$) from control values (direct comparison of densitometric values), Wilcoxon signed rank test. ‡Indicates significant difference ($P<0.05$) from UK14304 alone values, Wilcoxon signed rank test.

between control and UK14304-stimulated palmar lateral vein segments (Table 1). The PI 3-kinase inhibitor LY294002 (50 μM) caused significant inhibition of ERK2 phosphorylation. There was no effect on total ERK2 levels (Table 1a) indicating that LY294002 was preventing activation of ERK2. Although there was a significant increase in the phosphorylation of ERK1 after contraction with UK14304 (153.7±16.4% of control, $n=8$; $P<0.05$ two-tailed, paired t-test), levels of ERK1 were not always detectable by Western blotting. This made it difficult to determine the effect of inhibitors on ERK1 phosphorylation. In the presence of LY294002 ERK1 phosphorylation was only 104.7±2.9% of control levels ($n=5$), but this reduction was not statistically significant ($P=0.06$, Wilcoxon Signed Rank test).

Activation of Akt by UK14304

Segments of palmar lateral vein were contracted with 10 μM UK14304, frozen, and prepared for SDS–PAGE as above. Western blots were probed for the phosphorylated form (Ser473) of Akt, or total Akt. Both the phosphorylated and total Akt antibodies revealed a single band on the immunoblot of around 60 kDa. Figure 3 shows typical immunoblots. In tissues contracted with 10 μM UK14304 there was a significant increase in the phosphorylation of Akt (Figure 3a). Phosphorylation in the presence of 10 μM UK14304 was 168.7±11.1% of control values (mean±s.e.mean, $n=25$; $P<0.0001$, Wilcoxon Signed Rank test (direct comparison of densitometric values)). There was no significant difference in levels of total Akt (Figure 3b). The level of total Akt in the presence of 10 μM UK14304 was 116.3±4.6% of control values ($n=25$). The phosphorylation of Akt was inhibited by the PI 3-kinase inhibitor LY294002 (50 μM; Figure 3 and Table 2). Akt phosphorylation was also inhibited by the Src tyrosine kinase inhibitor PP2, and by removal of calcium from the Krebs-Henseleit buffer (calcium-free Krebs; see Table 2). The MEK inhibitor PD98059 (50 μM), the L-type calcium channel blocker nifedipine

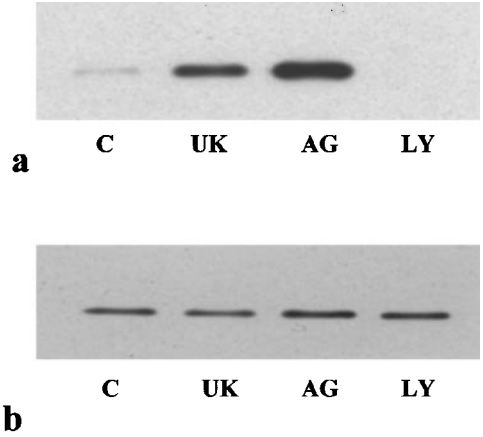

Figure 3 Representative immunoblots of porcine palmar lateral vein proteins separated by SDS–PAGE, transferred onto nitrocellulose membranes, and incubated with a primary antibody against phosphorylated Akt (Ser473, a), or total Akt (b). Segments of porcine palmar lateral vein were set up in a tissue bath and contracted with 10 μM UK14304 in the absence (UK) or presence of AG1478 (AG) or LY294002 (LY). Non-stimulated segments kept under basal conditions were also obtained (C).

Table 2 Changes in the levels of phosphorylated Akt

Inhibitor	*UK14304* (10 μM) (% control)	*+inhibitor* (% control)
LY294002 (50 μM)	176.5±19.9 (n=4)	93.8±9.6† (n=4)
PD98059 (50 μM)	186.0±8.5† (n=4)	148.8±19.5 (n=4)
PP2 (10 μM)	167.4±19.9 (n=8)	121.9±18.8† (n=8)
AG1478 (1 μM)	141.9±13.9 (n=5)	163.9±42.3 (n=5)
Nifedipine (50 μM)	176.2±21.3 (n=5)	136.3±10.9 (n=5)
Ca-free Krebs	176.5±19.9 (n=4)	93.8±9.6† (n=4)

Phosphorylated Akt (Phospho Akt) levels were determined by Western blotting as outlined in the Methods section. Densitometric analysis of the bands on the immunoblots was carried out, and the results expressed as a per cent of control (unstimulated) levels. Shown are the levels of phosphorylated Akt stimulated by 10 μM UK14304 in the presence of 50 μM PD98059, 50 μM LY294002, 50 μM nifedipine, calcium-free Krebs–Henseleit buffer (Ca-free Krebs), 10 μM PP2, or 1 μM AG1478. Each inhibitor is shown with its paired UK14304-stimulated value. †Indicates significant difference ($P<0.05$) from paired UK14304 alone values, Student's two-tailed paired t-test.

(50 μM), and the EGF receptor tyrosine kinase inhibitor AG1478 (1 μM) did not inhibit the phosphorylation of Akt, although they did inhibit UK14304-mediated contractions (Table 2). None of the inhibitors altered the levels of total Akt (data not shown).

Role of the EGF tyrosine kinase in α_2 adrenoceptor-mediated contractions and ERK activation

The EGF receptor tyrosine kinase inhibitor AG1478 caused a concentration-dependent inhibition of the α_2 adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein (Figure 4). Both 0.1 μM and 1 μM AG1478 caused significant reduction in the maximum response to UK14304. AG1478 (1 μM) also caused significant inhibition of ERK2 phosphorylation (see Figure 5 and Table 1b). There was no effect on total ERK2 levels (Table 1b) indicating that AG1478 was preventing activation of ERK2. As with the experiments in the presence of LY294002, the levels of ERK1 phosphorylation were at or below the level of detection. Therefore, it was difficult to determine the effect of AG1478 on ERK1 phosphorylation.

Tyrosine phosphorylation of EGF receptor

EGF receptor was immunoprecipitated from segments of porcine palmar lateral vein that had been contracted with UK14304. Immunoprecipitated proteins were then run on a 7% SDS–PAGE gel and transferred on to nitrocellulose. Immunoblots were then probed with an anti-phosphotyrosine antibody or total EGF receptor antibody. Figure 6 shows typical immunoblots probed with either the anti-phosphotyrosine antibody or anti-EGF receptor antibody. The immunoblots probed with the anti-phosphotyrosine antibody revealed a major band at around 170 kDa, and corresponded to a

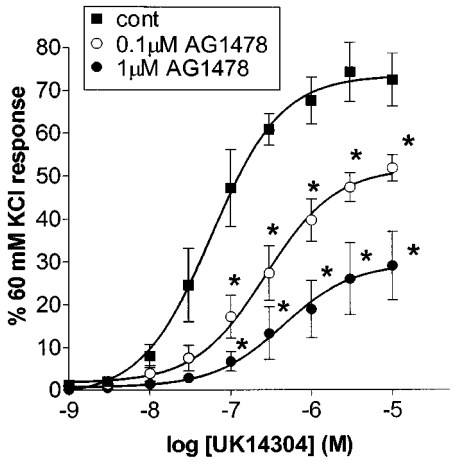

Figure 4 Log concentration-response curves to UK14304 in segments of porcine palmar lateral vein in the absence (Cont) or presence of 0.1 or 1 μM AG1478. Responses are expressed as per cent 60 mM KCl response, and shown as means±s.e.mean of six experiments. *Indicates significant difference from control responses,

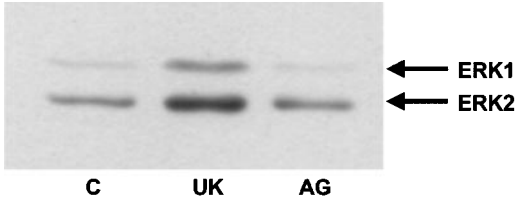

Figure 5 Representative immunoblot of porcine palmar lateral vein proteins separated by SDS–PAGE, transferred onto nitrocellulose membranes, and incubated with a primary antibody against phosphorylated ERK1/2. Segments of porcine palmar lateral vein were set up in a tissue bath and contracted with 10 μM UK14304 in the absence (UK) or presence of 1 μM AG1478 (AG). Non-stimulated segments kept under basal conditions were also obtained (C). Tissues were rapidly frozen, homogenized, and then subjected to SDS–PAGE.

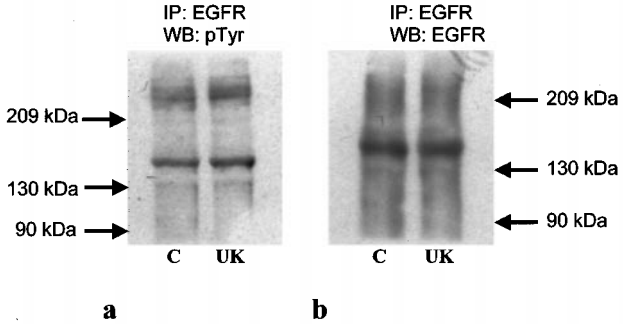

Figure 6 Representative immunoblots of porcine palmar lateral vein proteins immunoprecipitated with total EGFR antibody, separated by SDS–PAGE, transferred onto nitrocellulose membranes, and incubated with a primary antibody against phosphorylated tyrosine (pTyr, a) or total EGFR (b). Segments of porcine palmar lateral vein were set up in a tissue bath and contracted with 10 μM UK14304 (UK). Non-stimulated segments kept under basal conditions were also obtained (C).

band of the same molecular weight in the blots probed with total EGF receptor. There was a slight, but significant

increase in the level of phosphotyrosine labelling in UK14304-stimulated samples compared to control. UK14304-stimulated bands were 110.4±3.8% of control levels ($P<0.05$, two-tailed Student's paired t-test, $n=4$).

Effect of UK14304 on Src phosphorylation

Samples of porcine palmar lateral vein contracted with 10 μM UK14304 were analysed for activation of Src kinase by looking at the phosphorylation of Src at the autophosphorylation site Tyr 416. The immunoblotting with the anti-phospho Src antibody detected three bands at around 60 kDa (Figure 7a). However, there was no difference in the density of the bands from samples from tissues stimulated with UK14304 compared to controls (bands in the presence of UK14304 were 106.3±7.9% of control, $n=4$). There was also no significant difference in levels of total Src (bands in the presence of UK14304 were 95.9±9.0% of control, $n=4$; Figure 7b).

Effect of UK14304 on intracellular calcium responses

Segments of porcine palmar lateral vein were loaded with fura-2AM, and then changes in intracellular calcium on the lumen side of the segments were determined. KCl (60 mM) caused a rapid, transient increase in intracellular calcium. There was no significant difference in the size of the responses to KCl obtained at the second challenge compared to the responses obtained at the first challenge. UK14304 (10 μM) also caused a rapid rise in intracellular calcium, but this was well maintained compared to the KCl response (Figures 8 and 9d). The maximal response to UK14304 was obtained at around 1–2 min after addition of the agonist. The calcium response then dropped to around 50% of the maximal response after 5 min, and then remained at this level (Figure 9d).

Calcium responses to UK14304 were inhibited in the presence of 50 μM LY294002 (Table 3), and were virtually abolished in the absence of extracellular calcium (Table 3). On the other hand, 50 μM nifedipine appeared to have no significant effect on the UK14304-induced calcium response (Table 3).

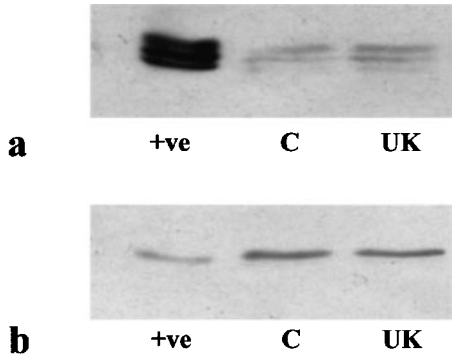

Figure 7 Representative immunoblots of porcine palmar lateral vein proteins separated by SDS–PAGE, transferred onto nitrocellulose membranes, and incubated with a primary antibody against phosphorylated Src (Tyr416, a) or total Src (b). Segments of porcine palmar lateral vein were set up in a tissue bath and contracted with 10 μM UK14304 (UK). Non-stimulated segments kept under basal conditions were also obtained (C). The positive control (+ve) is lysate of A-431 cells stimulated with EGF.

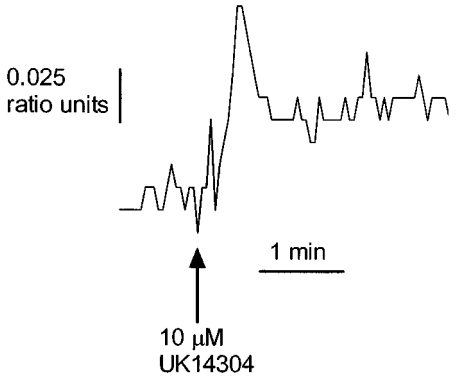

Figure 8 A typical trace showing the effect of 10 μM UK14304 on intracellular calcium levels in a segment of porcine palmar lateral vein measured by a change in the 340 : 380 nm ratio. Changes in the calcium levels were measured on the lumen side of the vessel segment as outlined in the Methods section.

Time-dependency of vasoconstriction, Akt activation and ERK2 activation

Tissues were set up in a tissue bath and exposed to 10 μM UK14304. After 5, 10, or 20 min tissues were removed, frozen on dry ice, and analysed for Akt or ERK2 activation. The comparisons between vasoconstriction, Akt activation and ERK2 activation can be seen in Figure 9. Palmar lateral veins produced a maximum contraction at around 3–4 min after addition of UK14304. The tension then dropped gradually over time, being only 50% of the maximum response after 20 min (Figure 9a). ERK2 activity was raised at the 5 min time point, and remained fairly constant over the 20 min time period (Figure 9b). Akt activity, on the other hand, was raised after 5 min, and continued to rise over the next 15 min (Figure 9c). In a separate set of experiments the effect of the MEK inhibitor PD98059 (50 μM) on 10 μM UK14304-induced contractions was studied over 20 min. In this set of experiments 10 μM UK14304 produced a maximum contraction of 110.0±6.8% of the 60 mM KCl response. PD98059 inhibited this initial contraction reducing the maximum response to 48.6±9.8% ($P<0.001$, Student's two-tailed, unpaired t-test, $n=5$). The UK14304 response dropped over the next 20 min in both sets of tissue to 50% of the maximum response. The response in the presence of PD98059 after 20 min was still significantly different from the response to UK14304 alone ($P<0.05$, Student's two-tailed, unpaired t-test, $n=5$).

Discussion

The aim of this present study was to further understand the signal transduction pathways that mediate the α_2 adrenoceptor-mediated, adenylyl cyclase-independent mechanism of vasoconstriction. Previous studies have demonstrated that α_2 adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein is dependent upon activation of the ERK–MAP kinase signal transduction cascade, as well as influx of

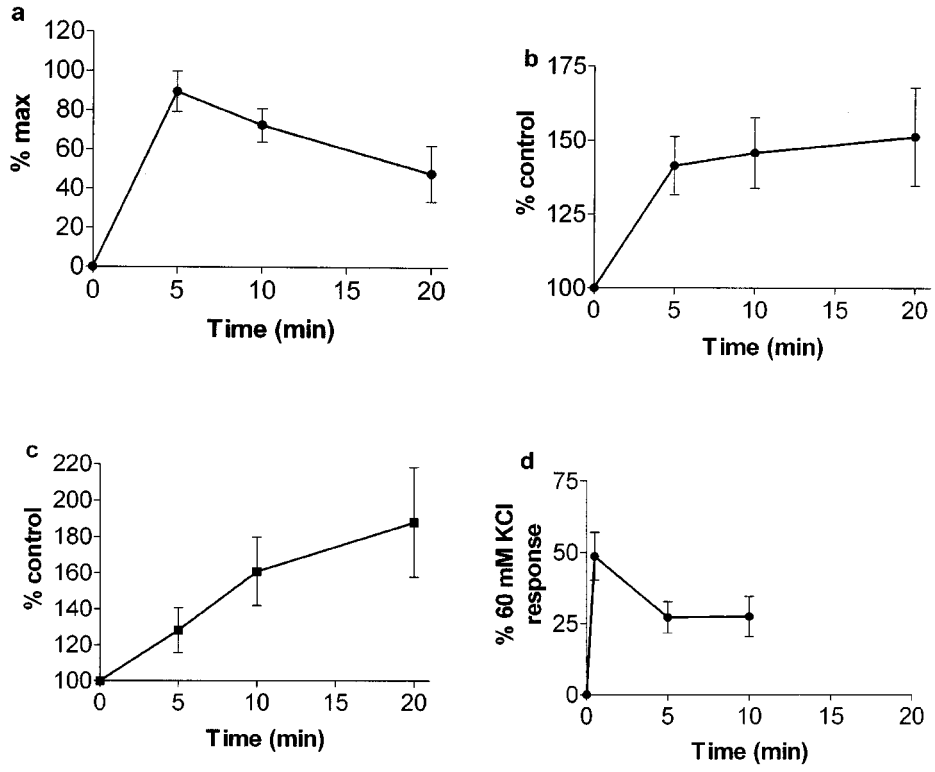

Figure 9 Effect of 10 μM UK14304 on vasoconstriction, activation of ERK2, activation of Akt and intracellular calcium in segments of porcine palmar lateral vein over time. (a) Contractions to UK14304 in segments of porcine palmar lateral vein expressed as per cent maximum response, and shown as means ± s.e.mean of five experiments. (b) Changes in phosphorylation of ERK2 in segments of porcine palmar lateral vein in response to UK14304 over time. Results are expressed as a per cent of control (unstimulated segments), and are means ± s.e.mean of five experiments. (c) Changes in phosphorylation of Akt in segments of porcine palmar lateral vein in response to UK14304 over time. Results are expressed as a per cent of control (unstimulated segments), and are means ± s.e.mean of five experiments. (d) Changes in intracellular calcium in segments of porcine palmar lateral vein in response to UK14304 over time. Results are expressed as per cent 60 mM KCl response, and shown as means ± s.e.mean of eight experiments.

Table 3 Changes in the levels of intracellular calcium

	Calcium (% 60 mM KCl)
UK14304 (10 μM)	62.1 ± 11.1 (n = 10)
+ Nifedipine (50 μM)	47.5 ± 14.1 (n = 4)
+ LY294002 (50 μM)	16.6 ± 7.4† (n = 4)
+ Ca-free Krebs	2.5 ± 2.5† (n = 3)

Effect of UK14304 (10 μM) on intracellular calcium levels in segments of porcine palmar lateral vein in the absence or presence of 50 μM nifedipine, 50 μM LY294002, or calcium-free Krebs–Henseleit buffer (Ca-free Krebs). Results are expressed as a percentage of the response to 60 m KCl, and are means ± s.e.mean. †Indicates significant difference (P < 0.05) from UK14304 alone values, Dunnett's multiple comparison test.

extracellular calcium and activation of a Src-family tyrosine kinase (Roberts, 2001). Both the calcium influx and Src tyrosine kinase are upstream of the ERK signal transduction pathway. However, it is not clear how these different pathways relate to one another.

One possible intermediate is PI 3-kinase which has been shown to activate the ERK signal transduction pathway, and open voltage-sensitive calcium channels (Hawes *et al*., 1996; Seki *et al*., 1999; Viard *et al*., 1999). PI 3-kinase can also be activated by Src tyrosine kinase (Ibitayo *et al*., 1998; Daulhac *et al*., 1999). This present study demonstrates that PI 3-kinase is involved in α_2 adrenoceptor-mediated vasoconstriction in porcine palmar lateral vein, and that it is part of the signalling pathway upstream of ERK activation. The evidence for this is as follows. Firstly, the selective PI 3-kinase inhibitor LY294002 (Vlahos *et al*., 1994) inhibited α_2 adrenoceptor-mediated vasoconstriction in segments of porcine palmar lateral vein suggesting that PI 3-kinase is involved in the contractile process. As well as inhibiting α_2 adrenoceptor-mediated vasoconstriction, LY294002 also inhibited the activation of ERK2 associated with the vasoconstriction. This indicates that PI 3-kinase is upstream of ERK2 activation. In order to be certain that PI 3-kinase is activated it was necessary to look for evidence of PI 3-kinase activity. The enzyme Akt or protein kinase B (PKB) is a direct target of PI 3-kinase and is often used as a tool for studying PI 3-kinase activation (Datta *et al*., 1996). Akt is phosphorylated at Thr 308 and Ser 473 by phosphoinositide-dependent kinase 1 (PDK 1). Phosphorylation at both of these sites is required for full activation of Akt (Vanhaesebroeck & Alessi, 2000). PDK 1 is activated by phosphatidyl 3,4,5 trisphosphate (PIP_3), the product of PI 3-kinase activation (Vanhaesebroeck & Alessi, 2000). Therefore, phosphorylation of Akt can be used as an indicator of PI 3-kinase activation. The level of phosphorylated Akt was increased in segments of porcine palmar lateral vein

contracted with 10 μM UK14304, but there was no increase in total Akt indicating an increase in Akt activation. This activation was inhibited in the presence of LY294002 demonstrating that it is dependent upon PI 3-kinase activity. Akt phosphorylation was not inhibited by the MEK inhibitor PD98059. This is in keeping with the data that showed that LY294002 inhibited ERK2 activation. Taken together, these data demonstrate that α_2 adrenoceptor-mediated vasoconstriction is dependent on PI 3-kinase activation which is upstream of ERK2.

Previously it has been demonstrated that the Src tyrosine kinase inhibitor PP2 inhibited α_2 adrenoceptor-mediated vasoconstriction, and ERK activation in porcine palmar lateral vein (Roberts, 2001). In this present study, PP2 was shown to inhibit Akt activation suggesting that a Src-like tyrosine kinase is upsteam of PI 3-kinase. This is in line with studies in cultured cells demonstrating that a Src-like tyrosine kinase is upstream of PI 3-kinase activation (Ibitayo *et al*., 1998; Daulhac *et al*., 1999). The identity of the Src tyrosine kinase involved in α_2 adrenoceptor-mediated vasoconstriction is still unclear. In order to determine the relationship between Src tyrosine kinase and the other signalling components, Src kinase activity was determined by measuring the change in phosphorylation of Src at the autophosphorylation site Tyr416. However, segments of palmar lateral vein contracted with UK14304 did not show any significant increase in Src phosphorylation at this site. Total Src levels were unaffected. This would suggest that Src tyrosine kinase is not activated by α_2 adrenoceptors, which would seem to be at odds with the previous contractile data (Roberts, 2001). One possible explanation is that in the porcine palmar lateral vein, Src tyrosine kinase is not phosphorylated at Tyr416 during activation. Activation of Src tyrosine kinases is not properly understood, and seems to involve different mechanisms depending upon the stimulus (see Thomas & Brugge, 1997). Src activation may not require phosphorylation, but could involve dephosphorylation (Rodriguez-Fernandez & Rosengurt, 1996), particularly if it involves removal of the Csk-induced Tyr 527 phosphorylation which inhibits Src activity (see Thomas & Brugge, 1997). Src tyrosine kinase could be rapidly and transiently activated, particularly if it is an early signalling event, as in bombesin stimulated Src activation in Swiss 3T3 cells (Rodriguez-Fernandez & Rosengurt, 1996). If this is the case, measuring Src activity at the maximal contraction may not be appropriate. An alternative explanation is that PP2 does not just inhibit Src tyrosine kinase, but also inhibits other tyrosine kinases within the Src family. If Src tyrosine kinase itself is not activated, however, it is likely that a related tyrosine kinase is involved.

UK14304 increased intracellular calcium in segments of palmar lateral vein, with a maximum of around 60% of the response to 60 mM KCl. This increase was inhibited by removing calcium from the Krebs–Henseleit buffer. This is in line with a previous study in which it was shown that α_2 adrenoceptor-mediated vasoconstriction is dependent upon extracellular calcium (Roberts, 2001). The increase in intracellular calcium in the absence of extracellular calcium was virtually undetectable suggesting that all the increase in intracellular calcium is due to calcium influx and not released from intracellular stores. Interestingly, 50 μM nifedipine had no significant effect on the α_2 adrenoceptor-mediated calcium response suggesting that the increase is not due to the influx of calcium through voltage-sensitive calcium channels. Nifedipine inhibits α_2 adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein and also inhibits ERK activation (Roberts, 2001). Although lower concentrations of nifedipine (0.1 and 1 μM) inhibited the UK14304-induced contractions in the palmar lateral vein, the degree of inhibition was only small (10–20%). Greater inhibition (70–80%) was obtained at 10–50 μM nifedipine. At these higher concentrations nifedipine may well be acting at other transmembrane channels such as potassium channels (Teramoto & Brading, 1998), or other sites (Kalsner, 1997). The fact that 50 μM nifedipine had no effect on the UK14304-induced calcium response suggests that this could well be the case. Further studies are required to determine the site of action of nifedipine.

Although nifedipine did not inhibit the calcium influx, LY294002 did, suggesting that PI 3-kinase is upstream of this calcium response. Confusingly, removal of extracellular calcium inhibits Akt activation. These data could suggest that PI 3-kinase stimulates calcium influx, which then leads to Akt activation which is downstream of PI 3-kinase. In vascular smooth muscle cells, PI 3-kinase can activate L-type calcium channels (Seki *et al*., 1999; Viard *et al*., 1999), although L-type calcium channels do not seem to be involved in this present study as nifedipine failed to inhibit the calcium response. In Chinese hamster ovary (CHO) cells, PI 3-kinase causes calcium influx through a non-L-type calcium channel, independent of Akt activation. In fact, Akt activation in these cells is not dependent on influx of calcium (Kansra *et al*., 2001). On the other hand, Akt can be activated in rat vascular smooth muscle cells by calcium influx (Takahashi *et al*., 1999). In T cells, PI 3-kinase stimulates calcium influx through a pathway that is not inhibited by nifedipine (Hsu *et al*., 2000). Taking these data together, it would not seem unreasonable to suggest that α_2 adrenoceptors in porcine palmar lateral vein stimulate calcium influx through a non-L-type calcium channel by activation of PI 3-kinase. This influx of calcium is necessary for activation of Akt.

Time-dependency

α_2 Adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein occurs rapidly, with the maximum contraction occurring at around 3–4 min. By 5 min the tension is already dropping, and by 20 min the tension is half the maximum response. ERK2 activity is raised at 5 min, which is in line with the maximum contraction, but unlike the tension, remains constant over the 20 min. As ERK2 activity remains raised, it is possible that ERK2 is involved in the maintenance of the contraction. On the other hand, the MEK inhibitor PD98059 inhibits the initial phase of contraction as well as the sustained phase indicating that ERK2 is involved in both phases. Akt activity is also raised after 5 min, suggesting that PI 3-kinase is involved at this early stage. However, Akt activity continues to rise in spite of the fact that the tension is dropping. This would suggest that, although PI 3-kinase is involved in the vasoconstriction (as LY294002 inhibits contractions), Akt, which is downstream of PI 3-kinase, is not. However, as Akt activity is continuing to rise, it may be involved in other, long-term effects of α_2 adrenoceptor stimulation. To understand what these long-term effects could be requires further experimentation.

Although the calcium imaging was done in segments in which tension was not recorded, it is still possible to get an indication of how this response fits in with the other signalling pathways. The calcium response in palmar lateral vein segments was rapid, reaching a maximum 1–2 mins after addition of UK14304. The response then dropped, reaching 50% of the maximum response after 5 min, and then remained constant at this level for the next 5 min. This would suggest that calcium is involved at an early stage of the signalling pathway, and fits in with the theory that it is upstream of ERK activation. The calcium response drops by 50% by 5 min when the contraction has just reached maximum, and ERK2 activation is at maximum.

Role of EGF receptor transactivation

This present study has demonstrated that the EGF receptor tyrosine kinase inhibitor AG1478 inhibits α_2 adrenoceptor-mediated vasoconstriction and ERK2 activation in the porcine palmar lateral vein. This suggests that α_2 adrenoceptors cause transactivation of the EGF receptor, which in turn leads to activation of the ERK signal transduction cascade resulting in vasoconstriction. Similar transactivation of the EGF receptor has been shown in thromboxane and AII-induced activation of ERK in smooth muscle cells (Miggins & Kinsella, 2001; Gao *et al*., 2001; Eguchi *et al*., 2001). Transactivation of the EGF receptor is believed to occur through tyrosine phosphorylation of the receptor, leading to activation of the associated tyrosine kinase in the absence of EGF itself (Zwick *et al*., 1999). The thromboxane-mimetic U46619 is believed to transactivate the EGF receptor in vascular smooth muscle cells through Src-mediated phosphorylation (Gao *et al*., 2001) which is thought to increase catalytic activity (Biscardi *et al*., 1999). There was a slight, but significant increase in the level of phosphotyrosine labelling in EGF receptor immunoprecipitates from UK14304-stimulated samples compared to controls suggesting that there is an increase in EGF receptor phosphorylation.

Transactivation of the EGF receptor by α_2 adrenoceptors in the porcine palmar lateral vein appears to be downstream of PI 3-kinase. AG1478 failed to inhibit the activation of Akt, and so transactivation cannot be upstream of PI 3-kinase. As PI 3-kinase appears to mediate calcium influx, it is likely that the EGF receptor transactivation is dependent upon calcium, as is the case in AII-mediated transactivation of the EGF receptor in rat vascular smooth muscle cells (Eguchi *et al*., 1998). Because there is only a small increase in EGF receptor phosphorylation it would be difficult to determine the effects of the various inhibitors on this phosphorylation. As such the role of EGF receptor transactivation in the signalling cascade can only be inferred from the data using the EGF receptor tyrosine kinase inhibitor.

The data presented in this study would suggest that α_2 adrenoceptor-mediated vasoconstriction occurs through activation of a Src family tyrosine kinase leading to activation of PI 3-kinase. PI 3-kinase stimulates calcium influx resulting in EGF receptor transactivation. This EGF receptor transactivation then results in activation of the ERK-MAP kinase cascade leading to vasoconstriction. The challenge for future research is to determine the mechanisms of ERK-mediated vasoconstriction.

This study was supported by The Wellcome Trust. Porcine tissues were obtained from G. Woods & Sons Ltd. abattoir (Clipstone, Nottinghamshire, U.K.).

References

BISCARDI, J., MAA, M.-C., TICE, D.A, COX, M.E., LEU, T.-H. & PARSONS, S.J. (1999). c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr^{845} and Tyr^{1101} is associated with modulation of receptor function. *J. Biol. Chem.,* **274,** 8335–8343.

BYLUND, D.B., EIKENBURG, D.C., HIEBLE, J.P., LANGER, S.Z., LEFKOWITZ, R.J., MINNEMAN, K.P., MOLINOFF, P.B., RUFFOLO, R.R. & TRENDELENBURG, U. (1994). International Union of Pharmacology nomenclature on adrenoceptors. *Pharmacol. Rev.,* **46,** 121–136.

DATTA, K., BELLACOSA, A., CHAN, T.O. & TSICHLIS, P.N. (1996). Akt is a direct target of the phosphatidylinositol 3-kinase. Activation by growth factors, v-Src and v-Ha-ras, in Sf9 and mammalian cells. *J. Biol. Chem.,* **271,** 30835–30839.

DAULHAC, L., KOWALSKI-CHAUVEL, A., PRADAYROL, L., VAYSSE, N. & SEVA, C. (1999). Src-family tyrosine kinases in activation of ERK-1 and p85/p110-phosphatidylinositol 3-kinase by G/CCK_B receptors. *J. Biol. Chem.,* **274,** 20657–20663.

EGUCHI, S., DEMPSEY, P.J., FRANK, G.D., MOTLEY, E.D. & INAGAMI, T. (2001). Activation of MAPKs by angiotensin II in vascular smooth musle cells. Metalloprotease-dependent EGF receptor activation is required for activation of Erk and p38 MAPK but not for JNK. *J. Biol. Chem.,* **276,** 7957–7962.

EGUCHI, S., NUMAGUCHI, K., IWASAKI, H., MATSUMOTO, T., YAMAKAWA, T., UTSUNOMIYA, H., MOTLEY, E.D., KAWAKATSU, H., OWADA, K.M., HIRATA, Y., MARUMO, F. & INAGAMI, T. (1998). Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J. Biol. Chem.,* **273,** 8890–8896.

GAO, Y., TANG, S., ZHOU, S. & WARE, A. (2001). The thromboxane A_2 receptor activates mitogen-activated protein kinase via protein kinase C-dependent Gi coupling and Src-dependent phosphorylation of the epidermal growth factor receptor. *J. Pharmacol. Exp. Therap.,* **296,** 426–433.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A generation of Ca^{2+} indicators with greatly improved fluoresence properties. *J. Biol. Chem.,* **260,** 3440–3450.

HAWES, B.E., LUTTRELL, L.M., VAN BIESEN, T. & LEFKOWITZ, R.J. (1996). Phosphatidylinositol 3-kinase is an early intermediate in the $G\beta\gamma$-mediated mitogen-activated protein kinase signaling pathway. *J. Biol. Chem.,* **271,** 12133–12136.

HSU, A.-L., CHING, T.-T., SEN, G., WANG, D.-S., BONDADA, S., AUTHI, K.S. & CHEN, C.-S. (2000). Novel function of phosphoinositide 3-kinase in T cell Ca2+ signaling. *J. Biol. Chem.,* **275,** 16242–16250.

IBITAYO, A.I., TSUNODA, Y., NOZU, F., OWYANG, C. & BITAR, K.N. (1998). Src kinase and PI 3-kinase as a transduction pathway in ceramide-induced contraction of colonic smooth muscle. *Am. J. Physiol.,* **275,** G705–G711.

KALSNER, S. (1997). Vasodilator action of calcium antagonists in coronary arteries *in vitro*. *J. Pharmacol. Exp. Therap.,* **281,** 634–642.

KANSRA, V., GROVES, C., GUTIERREZ-RAMOS, J.C. & POLAKIEWICZ, R.D. (2001). Phosphatidylinositol 3-kinase-dependent extracellular calcium influx is essential for CX_3CR1-mediated activation of the mitogen-activated protein kinase cascade. *J. Biol.Chem.,* **276,** 31831–31838.

LUTTRELL, L.M., DELLA ROCCA, G.J., VAN BIESEN, T., LUTTREL, D.K. & LEFKOWITZ, R.J. (1997). G $\beta\gamma$ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A Scaffold for G protein-coupled receptor-mediated Ras activation. *J. Biol. Chem.,* **272,** 4637 – 4644.

MIGGINS, S.M. & KINSELLA, B.T. (2001). Thromboxane A_2 receptor mediated activation of the mitogen activated protein kinase cascades in human uterine smooth muscle cells. *Biochim. Biophys. Acta,* **1539,** 147 – 162.

ROBERTS, R.E. (2001). Role of the extracellular regulated kinase (Erk) signal transduction cascade in α_2 adrenoceptor-mediated vasoconstriction in porcine palmar lateral vein. *Br. J. Pharmacol.,* **133,** 859 – 866.

RODRIGUEZ-FERNANDEZ, J.L. & ROZENGURT, E. (1996). Bombesin, bradykinin, vasopressin, and phorbol esters rapidly and transiently activate src family tyrosine kinases in Swiss 3T3 cells. Dissociation from tyrosine phosphorylation of p125 focal adhesion kinase. *J. Biol. Chem.,* **271,** 27895 – 27901.

SEKI, T., YOKOSHIKI, H., SUNAGAWA, M., NAKAMURA, M. & SPERELAKIS, N. (1999). Angiotensin II stimulation of Ca^{2+} channel current in vascular smooth muscle cells in inhibited by lavendustin-A and LY294002. *Pflügers Arch.,* **437,** 317 – 323.

TAKAHASHI, T., TANIGUCHI, T., KONISHI, H., KIKKAWA, U., ISHIKAWA, Y. & YOKOYAMA, M. (1999). Activation of Akt/protein kinase B after stimulation with angiotensin II in vascular smooth muscle cells. *Am. J. Physiol.,* **276,** H1927 – H1934.

TERAMOTO, N. & BRADING, A.F. (1998). The effects of nifedipine and other calcium antagonists on the glibenclamide-sensitive K^+ current in smooth muscle cells from pig urethra. *Br. J. Pharmacol.,* **123,** 1601 – 1608.

THOMAS, S.M. & BRUGGE, J.S. (1997). Cellular functions regulated by Src family tyrosine kinases. *Ann. Rev. Cell. Dev. Biol.,* **13,** 513 – 609.

VANHAESEBROECK, B. & ALESSI, D.R. (2000). The PI3K-PDK1 connection; more than just a road to PKB. *Biochem. J.,* **346,** 561 – 576.

VIARD, P., EXNER, T., MAIER, U., MIRONNEAU, J., NURNBERG, B. & MACREZ, N. (1999). G$\beta\gamma$ dimers stimulate vascular L-type Ca^{2+} channels via phosphoinositide 3-kinase. *FASEB J.,* **13,** 685 – 694.

VLAHOS, C.J., MATTER, W.F., HUI, K.Y. & BROWN, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.,* **269,** 5241 – 5248.

WRIGHT, I.K., HARLING, R., KENDALL, D.A. & WILSON, V.G. (1995). Examination of the role of inhibition of cyclic AMP in α_2-adrenoceptor mediated contractions of the porcine isolated palmar lateral vein. *Br. J. Pharmacol.,* **114,** 157 – 165.

YAMAGISHI, T., YANAGISAWA, T., SATOH, K. & TAIRA, N. (1994). Relaxant mechanisms of cyclic AMP-increasing agents in porcine coronary artery. *Eur. J. Pharmacol.,* **251,** 253 – 262.

ZWICK, E., HACKEL, P.O., PRENZEL, N. & ULLRICH, A. (1999). The EGF receptor as central transduder of heterologous signalling systems. *Trends Pharmacol. Sci.,* **20,** 408 – 412.

(Received May 16, 2002
Revised August 19, 2002)
Accepted October 1, 2002)