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Increase in tone and intracellular Ca^{2+} in rabbit isolated ear artery by platelet-derived growth factor

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1 The effect of platelet-derived growth factor (PDGF-AB) on tone and intracellular Ca^{2+} ($[Ca^{2+}]_i$) was examined in rabbit isolated ear arteries. Arteries were mounted in a myograph and loaded with the Ca^{2+}-sensitive fluorescent indicator, fura-2, for concurrent measurements of isometric force and $[Ca^{2+}]_i$.

2 PDGF-AB contracted rabbit ear artery in a concentration-dependent manner. PDGF-AB induced tone was associated with a rise in $[Ca^{2+}]_i$. In the presence of noradrenaline, PDGF-AB induced a similar rise in $[Ca^{2+}]_i$ but contraction in response to PDGF-AB in the presence of noradrenaline was increased compared with PDGF-AB alone.

3 PDGF-AB-induced rise in $[Ca^{2+}]_i$ and tone were abolished by removal of extracellular Ca^{2+} (with addition of BAPTA, a Ca^{2+} chelator), and by preincubation with a dihydropyridine calcium channel blocker, (−)-202 791. Bistyrphostin, a selective inhibitor of tyrosine kinases, also inhibited PDGF-AB-induced tone, but had no effect on noradrenaline- or potassium-induced tone.

4 PDGF-AB contracts rabbit ear artery by increasing Ca^{2+} entry through voltage-operated calcium channels. This effect involves activation of a tyrosine kinase.

Keywords: Platelet-derived growth factor; vascular smooth muscle; calcium; tyrosine kinase

Introduction

Platelet-derived growth factor (PDGF) is a 28–32 kDa peptide originally described in platelets (Kohler & Lipton, 1974), which may have important physiological and pathological roles in the vasculature (Ross *et al.*, 1990). PDGF is now known to be produced by many cell types including endothelial cells, vascular smooth muscle cells and macrophages/monocytes (reviewed in Bobik & Campbell, 1993). PDGF exists in multiple molecular weight isoforms which are composed of two chains (A and B) which share considerable homology. All possible dimeric forms of PDGF have been described (PDGF-AA, PDGF-BB, PDGF-AB), although the dominant isoform produced varies, depending on cell type and experimental conditions (Bobik & Campbell, 1993).

There is also heterogeneity of PDGF receptors. PDGF receptors are 170–180 kDa peptides formed from two subunits (α and β). PDGF receptors may exist in three dimeric forms; αα, αβ and ββ (Hart *et al.*, 1988). These receptor dimers differ in their affinity for the PDGF isoforms; PDGF-AA binds to αα, PDGF-AB binds to αα and αβ, while PDGF-BB binds to αα, αβ and ββ (Seifert *et al.*, 1989). Consequently the different isoforms of PDGF may have different effects depending on which receptor isoforms are expressed by a particular cell type.

All three isoforms of PDGF are reported to be potent mitogens for vascular smooth muscle cells (Ross *et al.*, 1990; Bobik & Campbell, 1993). PDGF-AB is the dominant isoform in human platelets and may account for 50% of the mitogenic effect of platelet activation (Heldin *et al.*, 1981). PDGF also contracts vascular smooth muscle in some (Berk *et al.*, 1986; Berk & Alexander, 1989), but not all sites (Basset *et al.*, 1988). PDGF-induced vasoconstriction is reported to be unaffected by antagonists of α-adrenoceptors, $5\text{-}HT_2$ receptors or cyclo-oxygenase (Berk *et al.*, 1986) and presumably involves a direct action on the PDGF receptor, although the mechanism of this effect is unclear. In the case of all three isoforms the consequence of the interaction of PDGF with the membrane associated receptor is to induce receptor dimerization and autophosphorylation on multiple tyrosine sites of the intracellular domain of the receptor (Pazin & Williams, 1992). These phosphotyrosine residues can then activate a range of intracellular signalling pathways by a process involving binding to peptide domains in the effector proteins bearing homology with src protein (SH2 domains). Phospholipase C-γ, phosphatidyl-inositol-3 kinase, GTPase activating protein for $p21^{ras}$ (GAP) and $p21^{ras}$ all appear to be activated by PDGF in this way (Pazin & Williams, 1992). The role of one or any of these systems in PDGF-induced contraction of intact vascular smooth muscle is unclear, however, changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) play a key role in regulating vascular tone (Somlyo & Himpens, 1989). This study examined the possible role of changes in $[Ca^{2+}]_i$ in the contractile action of PDGF in rabbit isolated ear artery by use of the fluorescent Ca^{2+} indicator fura-2.

Methods

Branches of rabbit ear artery were mounted in a myograph (Mulvany & Halpern, 1977) containing physiological saline at 37°C and bubbled with 95% O_2, 5% CO_2 for simultaneous measurement of force and $[Ca^{2+}]_i$ as previously described (Jensen *et al.*, 1992; 1993). The composition of the physiological saline was (in mM): NaCl 118, KCl 4.7, $CaCl_2.6H_2O$ 2.5, $MgSO_4.7H_2O$ 1.17, $NaHCO_3$ 25.0, $NaH_2PO_4.2H_2O$ 1.0, Na_2EDTA 0.03 and glucose 5.5. Arteries were loaded with the Ca^{2+}-sensitive fluorescent dye, fura-2 (Tsien, 1981; 1989), by incubation with physiological saline containing fura-2AM (5 μM) and 1 mg ml^{-1} bovine serum albumin for 2 h at 37°C. After thorough washing the myograph was mounted on the stage of an Axiovert 35 microscope (Carl Zeiss, Germany) coupled to a Deltascan D101 high intensity dual wavelength excitation source (Photon Technology International Inc., N.J., U.S.A.) which provided dual excitation fluorescent illumination at 340 and 380 nm. The output beam from the Deltascan was conducted to the microscope by quartz fibre optics. Excitation wavelengths were then reflected up by a dichroic mirror (FT 395 nm, Carl Zeiss, Germany) and focused onto the tissue with a quartz objective

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(Ultrafluor × 10 magnification, 0.20 numerical aperture, Carl Zeiss). Epifluorescence was collected via the objective and passed through the dichroic mirror. The emitted light was then filtered by a band pass filter (BP 500–530 nm, Carl Zeiss, Germany) *en route* to the photomultiplier tube (PMT). A camera output port with an adjustable aperture allowed the field of view to be adjusted so that only the vessel was included. Isometric force and fluorescence signal were captured at 4–8 Hz using a A/D interface (Photon Technology International Inc., N.J., U.S.A.) and an IBM AT microcomputer using commercially available software (Delta, Photon Technology International Inc., N.J., U.S.A.). During the period of the experiments (<90 min), there was no significant leakage or photobleaching of dye.

Measurements of $[Ca^{2+}]_i$ were not calibrated to absolute values using the technique outlined by Grynkiewicz and colleagues (1985) and previously used by us (Jensen *et al.*, 1993) since the effect of the calcium ionophore, ionomycin, was found to be inconsistent in these vessels and was often associated with leakage of dye leading to spurious values for R_{min} and R_{max}. Consequently data have been presented as the ratio of the signal at 340 and 380 nm excitation wavelengths with background fluorescence subtracted. Changes in $[Ca^{2+}]_i$ in response to a stimulant were normalised by expressing them as percentage change in the ratio of 340/380 nm signal induced by depolarization with a high potassium solution similar in composition to physiological saline except for equimolar substitution of KCl for NaCl (KPSS).

Drugs and chemicals

Bistyrphostin (Calbiochem, Nottingham, UK), bovine serum albumin (essentially fatty acid free) (Sigma, Poole, UK), cremaphor-EL (Sigma, Poole, UK), dimethylsulphoxide (DMSO) (Sigma, Poole, UK), fura-2AM (1-[2-(5-carbxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid acetoxymethyl (AM) ester (Molecular Probes Ltd., Eugene, U.S.A.), platelet-derived growth factor (PDGF-AB) (Calbiochem, Nottingham, UK), pluronic F-127 (Calbiochem, Nottingham, UK), (−) 202 791 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1, 4-dihydro-2,6-dimethyl-5-nitro-3-pyridene-carboxylate) (a gift from Sandoz AG, Basel). 1 mM stock fura-2AM was made up in 25 µl of DMSO/cremaphor-EL/pluronic F-127. The final concentration of DMSO (0.5%), cremophor-EL (0.1%) and pluronic F-127 (0.02%) in physiological saline did not affect basal tone or the contractile response of vessels. Other chemicals and buffers were obtained from Sigma (Poole, UK).

Statistics and data analysis

All data are presented as means ± s.e.means of n observations. Statistical comparison of data were made by Student's paired t test. $P<0.05$ was considered significant.

Concentration-response data were fitted to a logistic function:

$$y = \frac{A + (B - A)}{1 + \left\{\frac{10^c}{10^x}\right\}^D}$$

Where y = effect at a given concentration of drug; A = minimum effect; B = maximum effect; c = log EC_{50}; x = log [drug]; D = 'Hill slope', by non-linear regression using Excel 5.0 (Microsoft, U.S.A.) and a macro written by the author on an IBM compatible PC.

Results

Cumulative addition of PDGF-AB (1 pM–1 nM) caused a concentration-dependent increase in tone in rabbit isolated ear artery (Figure 1). The pD_2 for PDGF-AB-induced contraction was calculated to be 10.50 ± 0.08 ($n = 4$) which corresponds to an EC_{50} value of around 30 pM. Addition of a supramaximal concentration of PDGF-AB (1 nM) caused a

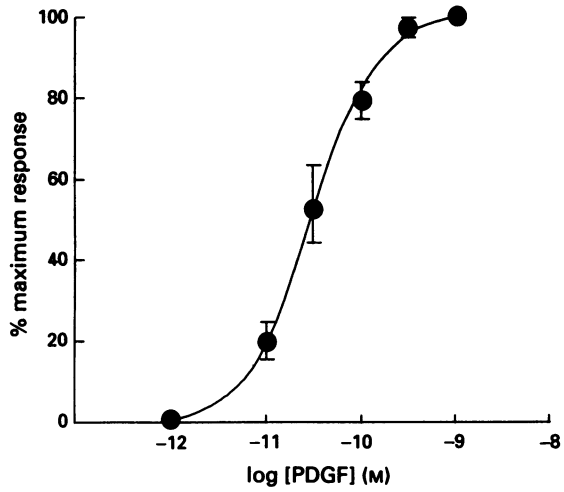

Figure 1 Concentration-response relationship for the contractile effects of platelet-derived growth factor (PDGF-AB). PDGF-AB was added cumulatively to arteries in physiological saline. Contraction was calculated as % maximum response to PDGF. Points represent means ± s.e.means of 4 separate experiments. The solid line represents the line of best fit obtained by non-linear regression to a logistic function as described in Methods.

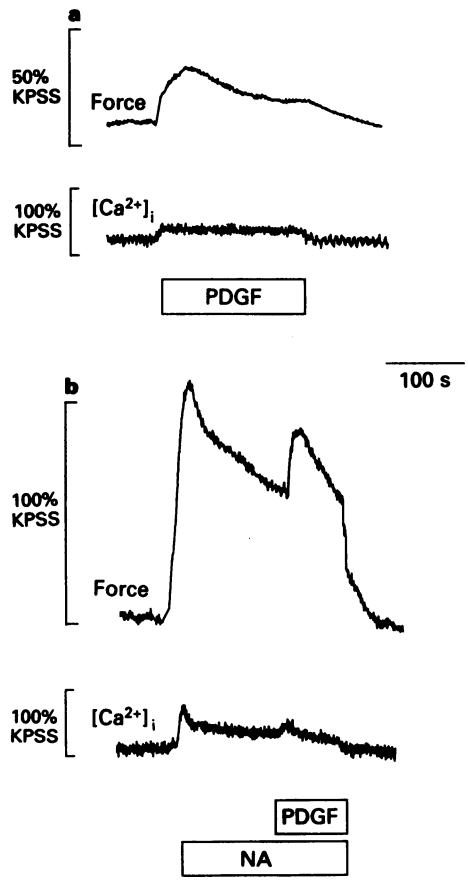

Figure 2 The effect of platelet-derived growth factor (PDGF-AB) on isometric force and $[Ca^{2+}]_i$ in rabbit isolated ear artery. Figure shows traces from individual experiments showing the effect of PDGF-AB (PDGF; 1 mM) on force (upper trace) and $[Ca^{2+}]_i$ (lower trace) (a) in physiological saline and (b) in physiological saline after precontraction with noradrenaline (NA; 10 µM). Period of exposure to the drugs is indicated by the bars, calibration bars are also shown. Traces are representative of 4 similar experiments.

contraction associated with a rise in $[Ca^{2+}]_i$ which was usually sustained (Figure 2). In general, force and $[Ca^{2+}]_i$ changes in response to supramaximal concentrations of PDGF-AB (1 nM) were smaller than those induced by 10 μM noradrenaline (NA) or high potassium solution (KPSS) (Figure 3). Following precontraction with NA, PDGF-AB induced increases in $[Ca^{2+}]_i$ which were similar to those seen in the absence of NA. However in the presence of NA, PDGF-AB induced significantly greater contractions than in the absence of NA (Figure 3). Preincubation of arteries for 15 min with 5 μM bistyrphostin, a selective inhibitor of receptor-linked tyrosine kinases (Levitzki & Golin, 1991), inhibited PDGF-AB-induced contraction but had no effect on contraction in response to NA or KPSS (Figure 4). In addition both increases in tone and $[Ca^{2+}]_i$ in response to PDGF-AB (1 nM) were abolished by removal of extracellular Ca^{2+} ($n = 3$) or by preincubation for 15 min with 5 μM (−)-202 791, a dihydropyridine calcium antagonist ($n = 3$); in contrast responses to NA were only partially attenuated by these procedures (Figure 5).

Discussion

PDGF-AB contracts rabbit ear artery in a concentration-dependent manner. The EC_{50} value observed in these studies is similar to that previously reported in rat aortic strips (Berk & Alexander, 1989). PDGF-AB also increases $[Ca^{2+}]_i$ and it is likely that this rise in $[Ca^{2+}]_i$ is the major signal responsible for PDGF-AB-induced contraction in this tissue. The rise in $[Ca^{2+}]_i$ brought about the PDGF-AB in rabbit isolated ear artery appears to depend entirely on influx of extracellular Ca^{2+} and does not appear to involve release of intracellular Ca^{2+} stores. In contrast, noradrenaline does transiently increase tone and $[Ca^{2+}]_i$ under the same conditions indicating that intracellular stores are not completely depleted by re-

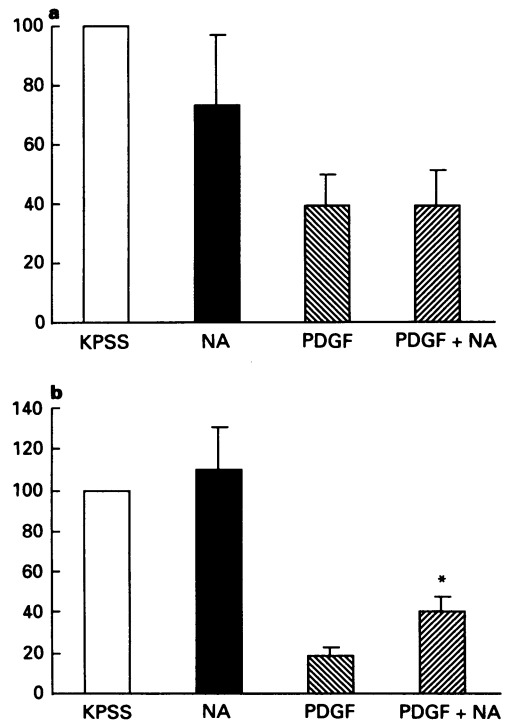

Figure 3 The effect of contractile stimulants on (a) $[Ca^{2+}]_i$ and (b) isometric force in rabbit ear artery. KPSS = potassium solution, NA = noradrenaline (10 μM), PDGF = platelet derived growth factor-AB (1 nM), PDGF + NA = PDGF-AB (1 nM) added after induction of stable increase in tone and $[Ca^{2+}]_i$ by noradrenaline (10 μM). Data are means ± s.e.means of 4 observations. $^*P < 0.05$ compared with PDGF alone by Student's paired t test.

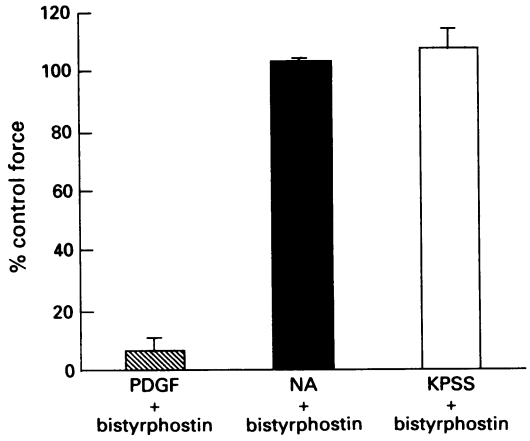

Figure 4 The effect of bistyrphostin, a selective inhibitor of tyrosine kinase, on contractile responses of rabbit ear artery to platelet-derived growth factor (PDGF; 1 nM), noradrenaline (NA; 10 μM) and potassium solution (KPSS). Tissues were preincubated with bistyrphostin (5 μM) for 15 min prior to application of contractile stimulant. Response were calculated as % of response to the same agonist prior to addition of bistyrphostin. Data are means ± s.e.means of 4 separate experiments.

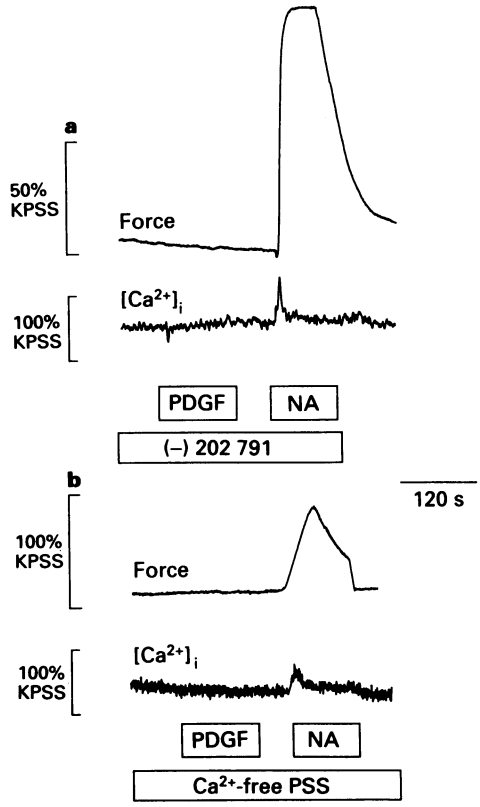

Figure 5 The role of extracellular Ca^{2+} in the action of platelet-derived growth factor (PDGF). Traces from individual experiments show (a) the effect of preincubation with dihydropyridine calcium channel blocker, (−)-202 791, on the effect of PDGF on force (upper trace) and $[Ca^{2+}]_i$ (lower trace). Period of exposure to drugs is indicated by the bars, PDGF = PDGF-AB (1 nM), NA = noradrenaline (10 μM), (−)-202 791 = (−)-202 791 (5 μM). Calibration bars are shown. (b) The effect of removal of extracellular Ca^{2+} on the effect of PDGF on force (upper trace) and $[Ca^{2+}]_i$ (lower trace). The artery was incubated in a Ca^{2+}-free physiological saline containing 1 mM BAPTA (Ca^{2+}-free PSS) for 3 min prior to addition of PDGF-AB and maintained in Ca^{2+}-free conditions until after washout of noradrenaline (NA). Period of exposure to drugs is indicated by the bars, PDGF = PDGF-AB (1 nM), NA = noradrenaline (10 μM). Calibration bars are shown. Traces are representative of 3 similar experiments.

moval of extracellular Ca^{2+} or a calcium channel blocker. The failure of PDGF-AB to abolish store release by noradrenaline (see Figure 5) and the similarity in the rise in $[Ca^{2+}]_i$ following PDGF-AB application in the presence of noradrenaline (when noradrenaline has already mobilized intracellular Ca^{2+} stores) is also consistent with this interpretation. This finding in intact isolated vascular smooth muscle differs from previous studies of cultured vascular smooth muscle (Berk & Alexander, 1989; Cirillo *et al.*, 1993; Kobayashi *et al.*, 1994), and is somewhat surprising in view of previous reports showing that PDGF induces inositol 1,4,5 trisphosphate (IP_3) production by activation of phospholipase C-γ in cultured vascular smooth muscle (Rosengurt, 1986; Kawahara *et al.*, 1988). However, a previous study has also reported that contractile responses to PDGF in rat isolated aorta were abolished by removal of extracellular Ca^{2+} (Berk *et al.*, 1986), so this difference may reflect difference between signal transduction mechanisms linked to PDGF receptors in intact and cultured vascular smooth muscle. The effect of PDGF-AB was blocked by bistyrphostin, a selective inhibitor of receptor-linked tyrosine kinases (Levitzki & Gilon, 1991). The tyrphostins are a group of low molecular weight compounds (Gazit *et al.*, 1989) which have been shown to inhibit the effects of tyrosine kinases in a number of systems (Gazit *et al.*, 1989), including vascular smooth muscle (Bilder *et al.*, 1991), and to have little or no significant effect on a number of serine-threonine kinases (Gazit *et al.*, 1989; Levitzki & Gilon, 1991). Recently Sauro & Thomas (1993) reported that a tyrphostin inhibited both the contractile action of PDGF and PDGF-induced tyrosine phosphorylation at similar concentrations in rat aorta. Our finding is consistent with this previous report and suggests that the action of PDGF is mediated through tyrosine phosphorylation. Di Salvo and colleagues (1993) have previously reported that tyrosine kinase inhibitors suppress noradrenaline-induced contraction, however we, like Sauro & Thomas (1993), saw no effect of a tyrphostin on α-adrenoceptor-induced tone at concentrations that blocked responses to PDGF. The reasons for this discrepancy are unclear but could reflect differences between the tyrphostins used in these studies.

Our findings with the dihydropyridine calcium antagonist, (−)-202 791, suggest that PDGF-AB-induced Ca^{2+} influx takes place largely or wholly as a result of activation of voltage-operated calcium channels. Recently we have also found that PDGF-AB increases voltage-dependent calcium channel currents in rabbit isolated single ear artery cells studied by whole cell voltage clamp techniques (Wijetunge & Hughes, 1994). Since both the contractile effect of PDGF-AB in isolated ear artery and the increase in calcium channel currents seen following PDGF-AB in rabbit single ear artery cells are blocked by inhibitors of tyrosine kinases this suggests that the action of PDGF-AB depends on tyrosine phosphorylation. However, whether tyrosine phosphorylation may directly modulate voltage-operated calcium channels in vascular smooth muscle as has been previously suggested (Wijetunge *et al.*, 1992), or whether some secondary signal transduction mechanism linked to the PDGF receptor is involved remains to be established. As mentioned above precontraction with noradrenaline did not affect the PDGF-AB-induced rise in $[Ca^{2+}]_i$ but the force generated in response to PDGF-AB was increased under these conditions. A number of contractile agents which act through receptors coupled to G proteins, including noradrenaline have been reported to increase the sensitivity of the contractile machinery of vascular smooth muscle to Ca^{2+} (Nishimura *et al.*, 1988; Somlyo & Himpens, 1989; Jensen *et al.*, 1992). Whilst the mechanism of this effect is still debated, it seems that such an effect is the most likely explanation of the increased force production by PDGF-AB in the presence of noradrenaline. Such interactions may have important physiological significance *in vivo*, when a number of contractile influences may be expected to act together on a blood vessel. Signalling systems linked to tyrosine kinases, such as $p21^{rho}$ and $p21^{ras}$ have also been proposed to modulate Ca^{2+}-sensitivity in vascular smooth muscle (Hirata *et al.*, 1992; Satoh *et al.*, 1993). Our data suggests that if PDGF-AB increases Ca^{2+}-sensitivity in vascular smooth muscle, then noradrenaline can induce an additional enhancement of Ca^{2+}-sensitivity, which may indicate that they act by distinct mechanisms. Alternatively PDGF-AB may have little effect on Ca^{2+}-sensitivity. Further studies will be required to resolve this issue.

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