Contraction of vascular smooth muscle induced by phorbol 12,13 dibutyrate in human and rat pulmonary arteries

¹Jean-Pierre Savineau, Roger Marthan & Huguette Crevel

Laboratoire de Physiologie, Université de Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux, France

1 The effect of phorbol 12,13 dibutyrate (PDB) on vascular tone was studied in both human and rat isolated pulmonary arterial strips (HPA and RPA, respectively).

2 PDB (1 nM to 2 μ M) produced slowly developing, sustained and concentration-dependent contractions in HPA (mean $EC_{50} = 3.5$ nM, $n = 5$) and RPA (mean $EC_{50} = 120$ nM, $n = 5$). The maximal response was $185.6 \pm 25\%$ and $207 \pm 27.5\%$ ($n = 5$) of that induced by K^+ -rich (80 mM) solution, and $223 \pm 34.5\%$ and $176.5 \pm 38.6\%$ of the noradrenaline (10 μ M)-induced contraction in HPA and RPA, respectively.

3 PDB-induced contractions were not altered either by the presence of atropine (10 μ M), propranolol (5 μ M), phentolamine (5 μ M) or tetrodotoxin (10 μ M) in the bathing solution, or by the removal of endothelium from pulmonary arteries.

4 In HPA, the amplitude of PDB-induced contractions was significantly reduced by removal of external calcium ions, addition of verapamil (10 μ M) or trifluoperazine (TFP, 5 μ M) and significantly increased by Bay K 8644 (0.5 μ M). In contrast, in RPA, calcium-free solution and verapamil had only a moderate effect on the maximal PDB-induced contraction (approximately 20% reduction), whereas Bay K 8644 and TFP had no significant effect. In both HPA and RPA, PDB-contractions in calcium-free solutions were not modified by ryanodine (25 μ M) or by 8-(N,N diethylamino)octyl-3,4,5, trimethoxybenzoate hydrochloride (TMB-8, 50 μ M).

5 PDB-induced contractions were inhibited by protein kinase C (PKC) antagonists. The maximal response was decreased by $60 \pm 10.5\%$ and $35 \pm 11.5\%$ ($n = 5$) by 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H_7 , 50 μ M), $70.5 \pm 12.2\%$ and $56 \pm 18\%$ ($n = 5$) by phloretin (100 μ M) and $80.7 \pm 8.4\%$ and $71 \pm 14\%$ ($n = 5$) by staurosporine (25 nM) in HPA and RPA, respectively.

6 Long term treatment (15–20 h) of arterial strips with phorbol esters (phorbol 12,13 didecanoate, or PDB) abolished the contractile response to subsequent addition of PDB.

7 These results show that PDB is a potent vasoconstrictor agent in human and rat pulmonary arteries. Unlike the rat, part of the PDB response depends on calcium influx in human preparations. PDB action appears mainly mediated by the activation of protein kinase C. PKC could thus play a major role in the control of vascular pulmonary reactivity.

Keywords: Smooth muscle; human tissue; pulmonary arteries; contraction; phorbol ester; protein kinase C

Introduction

Smooth muscle of large arteries (e.g. aorta, pulmonary artery) is able to develop and maintain maximal contraction in response to hormone and neuromediator stimulation. It has been demonstrated that the onset of the contraction is linked to an increase in cytosolic calcium concentration ($[Ca^{2+}]_i$) and subsequent formation of a calcium-calmodulin-myosin light chain kinase (MLCK) complex leading to the phosphorylation of myosin light chains (MLC) (Adelstein & Hataway, 1979; Murphy *et al.*, 1983). In contrast, the mechanism responsible for the maintenance of tone which occurs together with a decrease in both $[Ca^{2+}]_i$ and phosphorylation of MLC (Kamm & Stull, 1985; Himpens *et al.*, 1988; Jiang & Morgan, 1989), remains unclear. Among the different hypotheses that have been proposed to explain this maintained component of tension, the one which ascribes a major role to the activation of protein kinase C (PKC) has received considerable attention (Chatterjee & Tejada, 1986; Rasmussen *et al.*, 1987; Jiang & Morgan, 1987). PKC is present in high concentrations in vascular smooth muscle (Kuo *et al.*, 1980; Kariya & Takai, 1987), and specifically activated by diacylglycerol (DAG) under physiological conditions. PKC can also be directly activated by tumour promoting agents, the so-called phorbol esters (Nishizuka, 1984; Abdel-Latif, 1986).

In a variety of arteries from the systemic circulation, phorbol esters induce slowly-developing and sustained contractions, the calcium requirement of which varies from one

vessel to another (Spedding, 1987; Sawamura *et al.*, 1987; Chiu *et al.*, 1988; Jiang & Morgan, 1989). In contrast, the effect of phorbol esters in the pulmonary circulation has been less extensively studied. Information about the involvement of protein kinase C in the control of pulmonary vascular tone especially in human tissue could be relevant to a better understanding of pathophysiological processes such as hypoxic vasoconstriction or pulmonary hypertension.

We therefore designed the present study to investigate the effect of phorbol 12,13 dibutyrate (PDB), a potent PKC activator (Castagna *et al.*, 1982), on vascular tone in both human and rat isolated pulmonary arteries. The sensitivity of PDB-induced contractile responses to calcium ions, calmodulin antagonists and PKC inhibitors was studied in the two types of preparations. A brief account of some of these results has been published previously (Savineau *et al.*, 1991).

Methods

Tissue preparations

Experiments were performed on small strips (200–300 μ m in diameter, 8–10 mm in length) isolated from human lobar pulmonary artery (HPA) and the rat main pulmonary artery (RPA). Human preparations were dissected from lungs obtained at thoracotomy from patients aged from 52 to 66 years undergoing resection for lung carcinoma. From a macroscopically tumour-free part of the specimen, arteries

¹ Author for correspondence.

(3–4 mm in internal diameter) were dissected out free of surrounding parenchyma, then transferred to physiological solution and opened along the longitudinal axis. Animal preparations were obtained from male Wistar rats aged from 15–18 weeks, after intraperitoneal injection of 40 mg sodium pentobarbitone and intracardiac administration of 100 iu heparin. After removal of connective tissue, the pulmonary artery was opened and strips (2–3 for each artery) were cut transversely.

Mechanical recording

Isometric contraction was measured in an experimental chamber (Savineau *et al.*, 1988) by means of a highly sensitive force transducer (Akers 801, Horten, Norway).

At the beginning of the experiments, K^+ -rich (80 mM) solution or noradrenaline (10 μ M) was repeatedly applied in order to obtain at least three contractions similar in both amplitude and kinetics before the addition of phorbol ester. Concentration-response curves to PDB were then constructed by adding cumulative concentrations of PDB (from 1 to 25 nM in HPA, and from 25 nM to 2 μ M in RPA). A concentration increment was made once the maximal contractile effect of the preceding concentration had been recorded (usually 10–12 min). Generally two or three cumulative concentration-response curves (CCRC) were constructed in the same preparation. It was verified in control experiments that three or even four identical CCRC to PDB could be obtained from the same arterial strip. The response to each concentration of PDB alone or of PDB in the presence of another tested substance was expressed as a percentage of the maximal response obtained in the first CCRC.

Experiments were also performed on endothelium-free vascular preparations. Endothelium-free arteries were prepared by gently rubbing the luminal surface with a stainless steel wire. Strips dissected from these arteries were precontracted by noradrenaline (1 μ M) and then exposed to acetylcholine (ACh, 10 μ M): a percentage of ACh-induced relaxation, less than 10%, was accepted as evidence of endothelium removal. CCRCs to PDB were compared between intact and endothelium-free arteries. In this case, the response to an individual concentration of PDB was expressed as a percentage of the contraction evoked by 80 mM K^+ .

Solutions

Physiological solutions had the following composition: (a) reference solution (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. The solution was aerated with 5% CO_2 in O_2 . (b) Calcium-free solution was prepared by omitting $CaCl_2$ and adding EGTA (0.2 mM). (c) K^+ -rich (80 mM) solution was prepared by substituting an equimolar amount of KCl for NaCl.

Chemicals

Chemicals used in the present study were: acetylcholine chloride, atropine, ethyleneglycol-bis(β -amino-ethylether) N,N,N' , N' -tetraacetic acid (EGTA), 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7), 8-(N,N -diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), noradrenaline, phentolamine, phloretin, propranolol, phorbol 12,13 dibutyrate, phorbol 12,13 didecanoate (PDD), 4 α -phorbol 12,13 didecanoate (4 α PDD), tetrodotoxin, trifluoperazine (TFP), verapamil, N -(6-aminoethyl)-5-chloro-1-naphthalene-sulphonamide (W7) all from Sigma (La Verpillère, France); methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644) from Bayer (Leverkusen, Germany); staurosporine from Fluka (Mulhouse, France); ryanodine from Reserach Biomedical Inc. (Wayland, MA, U.S.A.).

Bay K 8644, phorbol esters, phloretin and staurosporine were dissolved in dimethyl sulfoxide (DMSO). The

maximum concentration of DMSO was <0.1% and had no effect on the contractility of pulmonary arteries.

Analysis of results

The amplitude of contraction is expressed as mean $\pm$ s.d. for n , number of experiments. Significance was assessed by a paired Student's t -test. A difference between means was considered significant when $P < 0.05$. Values for EC_{50} (concentration producing 50% of the maximal response) are given as geometric means with 95% confidence limits (CL).

Results

Contractile responses induced by phorbol 12,13 dibutyrate in pulmonary arteries

Figure 1 shows a typical example of the effect of cumulatively applied concentrations of PDB on the tone of human and rat isolated pulmonary arteries. PDB induced a slowly developing and sustained contraction (the mean time for the maximal effect for each concentration was: 6 ± 1.2 min and 8.2 ± 2.4 min, $n = 10$, in HPA and RPA, respectively). The geometric mean EC_{50} was 3.5 nM, $n = 5$, (95% CL: 2.7 nM and 4.8 nM) in HPA, and 120 nM, $n = 5$, (95% CL: 80 nM and 220 nM) in RPA. The maximal response was $185.6 \pm 25\%$ and $207 \pm 27.5\%$ ($n = 5$) of the K^+ -rich (80 mM) solution-evoked contraction, and $223 \pm 34.5\%$ and $176.5 \pm 38.6\%$ ($n = 5$) of the noradrenaline (10 μ M)-evoked contraction in HPA and RPA, respectively. Removal of PDB after recording the maximal response was followed by a slow relaxation of tissues which returned to their basal tone in 46 ± 3.7 min and 36.7 ± 3.2 min ($n = 5$) in HPA and RPA, respectively (not shown).

CCRC to PDB was not significantly modified by the presence in the bathing solution of any of the following substances: atropine (10 μ M), phentolamine (5 μ M), propranolol (5 μ M), tetrodotoxin (10 μ M) ($n = 6$ in HPA and RPA). Removal of endothelium produced no significant change of the PDB-induced contractions in either HPA or RPA ($P > 0.05$, $n = 5$) (not shown). The inactive phorbol 4 α 12,13 didecanoate (1 nM to 5 μ M) had no contractile effect in HPA ($n = 3$) or in RPA ($n = 4$).

Calcium-dependence of phorbol 12,13 dibutyrate-induced contractions

Effect of calcium-free solutions Removal of external calcium ions significantly reduced the amplitude of PDB-responses in

Figure 1 Typical experiment showing the effect of cumulatively-applied phorbol 12,13 dibutyrate (PDB) concentrations in human pulmonary artery (HPA) and rat pulmonary artery (RPA). PDB concentrations were: 2.5 (a), 5 (b), 10 (c) and 25 nM (d) in HPA; 50 (a'), 100 nM (b'); 0.5 (c') and 2 μ M (d') in RPA. The maximum PDB-induced contraction was 190 and 200% of that induced by the potassium-rich (80 mM K^+) solution in HPA and RPA, respectively.

Figure 2 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the presence (▲, ■) and in the absence (△, □) of external calcium ions, in human (HPA, ▲, △) and rat (RPA, ■, □) pulmonary arteries, respectively. The calcium-free solution contained 0.2 mM EGTA. Abscissa scale: $-\log$ concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB—25 nM in HPA, or 2 μ M in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. * $P < 0.05$ and ** $P < 0.01$ indicate a response in the absence of external Ca-ions which is significantly different from the corresponding response in the control experiment.

HPA, especially those evoked by low concentrations (1–5 nM) of PDB, (contractions evoked by 2.5 and 25 nM PDB were reduced by $85.5 \pm 17.8\%$ and $28 \pm 7.7\%$, $n = 5$, respectively). In RPA, this effect was less important (the maximal response was reduced by only $17.6 \pm 11\%$, $n = 5$) (Figure 2). In another set of experiments, CCRC were performed in calcium-free solution containing either ryanodine (25 μ M) or TMB-8 (50 μ M). No significant difference was observed between the CCRC obtained in the presence and in the absence of ryanodine ($n = 3$) and TMB-8 ($n = 4$) in both types of arteries.

Effect of drugs acting at the voltage-dependent calcium channel We tested the effect of a calcium-channel agonist, Bay K 8644, and of a calcium-channel antagonist, verapamil, on PDB-induced contractions. Bay K 8644 (0.5 μ M) added 10 min before the beginning of the CCRC had *per se* no effect on the vascular tone in both types of arteries, but increased the amplitude of PDB-induced contractions. This increase was significant only in HPA. Addition of verapamil (10 μ M) 20 min before PDB significantly reduced the contractile responses in HPA (70 $\pm$ 8.4% and 25 $\pm$ 12.5% reduction for 2.5 and 25 nM PDB, respectively), but had only a slight effect on the maximal responses in RPA (Figure 3).

Effect of calmodulin-antagonists

Pretreatment of pulmonary arterial strips for 15 min with trifluoperazine (TFP, 5 μ M) significantly decreased PDB-induced contractions in HPA (especially those evoked by low PDB concentrations, 1–5 nM). This effect of TFP was not significant in RPA (Figure 4). Similar results were obtained in the presence of W7 (10 μ M) ($n = 3$ in HPA and RPA, not shown).

Effects of protein kinase C inhibitors

In the subsequent series of experiments, we tested the effect of three substances commonly accepted as PKC-inhibitors: H7, phloretin and staurosporine. Each substance was added to the bathing solution for 15 min before the beginning of the CCRC. H7 (50 μ M), phloretin (100 μ M) and staurosporine (25 nM) inhibited in a potent manner PDB contractile responses (Figures 5, 6, 7) in the two types of arteries. In HPA, the maximal response was decreased by $60 \pm 10.5\%$, $70.5 \pm 12.2\%$ and $80.5 \pm 8.4\%$ ($n = 5$) for H7, phloretin and staurosporine, respectively. Similarly in RPA the maximal response was

Figure 3 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) alone (▲, ■), in the presence of either 0.1 μ M Bay K 8644 (●, ○) or 10 μ M verapamil (△, □) in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa scale: $-\log$ concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB—25 nM in HPA, or 2 μ M in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. * $P < 0.05$ and ** $P < 0.01$ indicate a response in the presence of Bay K 8644 or verapamil which is significantly different from the corresponding response in the control experiment.

decreased by $35 \pm 11.5\%$, $56 \pm 18\%$ and $71 \pm 14\%$ ($n = 5$) for H7, phloretin and staurosporine, respectively.

Effect of long-term treatment of preparation with phorbol esters

After recording K^+ -rich (80 mM)-induced contraction and constructing CCRC to PDB, pulmonary arterial strips were incubated for 15–20 h in the presence of phorbol 12,13 didecanoate or PDB itself at a concentration of 10 nM in HPA and 0.5 μ M in RPA. This treatment was followed by a 2 h washing period with the reference solution. K^+ -rich solution and PDB were then successively applied again. Potassium-induced contraction was reduced only by $21 \pm 7.2\%$ ($n = 4$; $P < 0.05$) and $17 \pm 5.6\%$ ($n = 4$, $P > 0.05$) in HPA and RPA, respectively. In contrast, contractile responses to PDB stimulation completely disappeared in HPA ($n = 4$). In RPA, 2 μ M of PDB produced only a small response, the amplitude of which was $15.6 \pm 6.4\%$ ($n = 4$, $P < 0.01$) of the maximal control response.

Figure 4 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the absence (▲, ■) and in the presence of 5 μ M trifluoperazine (TFP, △, □), in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa scale: $-\log$ concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB—25 nM in HPA, or 2 μ M in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. ** $P < 0.01$ indicate a response in the presence of TFP which is significantly different from the corresponding response in the control experiment.

Figure 5 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the absence ($\blacktriangle$, $\blacksquare$) and in the presence of $50\mu\text{M}$ 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7) ($\triangle$, $\square$) in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa scale: $-\log$ concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB— 25 nM in HPA, or $2\mu\text{M}$ in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. * $P < 0.05$ and ** $P < 0.01$ indicate a response in the presence of H7 which is significantly different from the corresponding response in the control experiment.

Figure 6 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the absence ($\blacktriangle$, $\blacksquare$) and in the presence of $100\mu\text{M}$ of phloretin ($\triangle$, $\square$) in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa scale: $-\log$ concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB— 25 nM in HPA, or $2\mu\text{M}$ in RPA. Data points are mean, $n = 5$. Vertical bars show s.d. * $P < 0.05$ and ** $P < 0.01$ indicate a response in the presence of phloretin which is significantly different from the corresponding response in the control experiment.

Figure 7 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the absence ($\blacktriangle$, $\blacksquare$) and in the presence of 25 nM staurosporine ($\triangle$, $\square$) in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa scale: $-\log$ concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB— 25 nM in HPA, or $2\mu\text{M}$ in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. * $P < 0.05$ and ** $P < 0.01$ indicate a response in the presence of staurosporine which is significantly different from the corresponding response in the control experiment.

Discussion

The present work has revealed that PDB is a potent constrictor agent in mammalian pulmonary arteries. Its efficacy is approximately twice that of potassium-rich solution or nor-adrenaline. Moreover, for the first time, it is shown that human pulmonary vascular tissue is very sensitive to phorbol esters, the potency of which is in the nanomolar range.

In both human and rat pulmonary arteries, PDB produced sustained contractions which were characterized as follows. First, the time course of the contraction was slow as in other vascular beds (Forder *et al.*, 1985; Spedding, 1987; Salices *et al.*, 1990). Second, the contraction was not altered by the presence of adrenoceptor or cholinergic antagonists or by removal of endothelium. Third, the contraction was reduced in a potent manner by inhibitors of protein kinase C activity such as H7, phloretin and staurosporine (Gschwendt *et al.*, 1984; Hidaka *et al.*, 1984; Tamaoki *et al.*, 1986). The rank order of efficacy of these inhibitors was staurosporine > phloretin > H7, this order being similar to that observed in the rat aorta (Auguet *et al.*, 1989). Fourth, the contraction disappeared after prolonged treatment of tissues with PKC activators. Such a treatment has been shown to induce a 'down-regulation' of PKC activity in vascular smooth muscle cells (Pfeilschifter *et al.*, 1989). It has been suggested that this 'down-regulation' could be related to a persistent state of membrane translocation of PKC leading to its hydrolysis by proteases (Wolf *et al.*, 1985; Secret *et al.*, 1991). Moreover, the inactive 4 phorbol 12,13 didecanoate (Castagna *et al.*, 1982) had no effect on the tone of these arteries. These results together with the facts that: (1) PDB directly binds to and activates PKC (Kikkawa *et al.*, 1983); (2) PKC is present in arterial smooth muscle (Kuo *et al.*, 1980; Kariya & Takai, 1987) strongly suggest that the PDB-effect in pulmonary arteries is mainly mediated by activation of smooth muscle cell PKC. Taking into account the kinetics of PDB-induced responses (Figure 1), it thus appears that activation of PKC could be an effective pathway in the maintenance of vascular tone during agonist stimulation in pulmonary arteries.

The mechanism by which activation of PKC leads to smooth muscle contraction is not fully elucidated and different cellular sites have been proposed (Abdel-Latif, 1986; Rasmussen *et al.*, 1987). One of them is the voltage-dependent calcium channel. Although PDB increases calcium channel current in some enzymatically dispersed vascular cells (Fish *et al.*, 1988; Loirand *et al.*, 1990), conflicting results have been reported regarding the external calcium-dependence of contractions induced by phorbol esters in blood vessels. In this respect, our results on the effect of calcium-free solutions (Figure 2), verapamil and Bay K 8644 (Figure 3), show a clear species difference: PDB-induced contraction was not dependent on calcium influx in RPA, whereas it exhibited a significant external calcium-dependence in HPA, especially at low concentrations. Since Bay K 8644 alone had no effect on pulmonary arterial tone, we suggest that in HPA, PDB depolarizes smooth muscle cells producing an influx of calcium ions through voltage-dependent channels. This calcium entry could then be responsible for part of the PDB response via a pathway involving the activation of MLCK by a Ca-calmodulin complex. This hypothesis is supported by the results of the experiments with TFP (Figure 4) and W7, two calmodulin-antagonists (Levin & Weiss, 1977; Asano *et al.*, 1982). The component of the tension independent of calcium influx in HPA as well as that more important in amplitude in RPA could be due to the action of PKC on other sites in the vascular smooth muscle cell. These sites include sarcoplasmic reticulum (SR), myosin light chains (MLC) and myofilaments. It has been shown that phorbol esters act on the calcium-release mechanism of SR in cardiac muscle (Rogers *et al.*, 1990). It is unlikely, however that such an action could have occurred in our experiments for the following reasons. PDB contractions in calcium-free solution were (i) unaffected by the presence of ryanodine or TMB8, both substances known to

alter the Ca release from smooth muscle SR (Chiou & Malagodi, 1975; Hwang & Van Breemen, 1987); (ii) not significantly different from those obtained in the presence of TFP in calcium-containing solution (compare Figures 2 and 4). These results suggest that the component of tension independent of calcium influx was not related to a variation in $[Ca^{2+}]_i$. Phorbol esters could also directly phosphorylate MLC on sites different from those phosphorylated by MLCK (Ikebe *et al.*, 1985; Singer, 1990). Lastly, phorbol esters could act at the site of myofilaments either by direct stimulation (Chatterjee & Tajeda, 1986) or by increasing their calcium sensitivity (Nishimura *et al.*, 1990). Additional experiments with skinned fibres as well as biochemical measurements are required to

determine precisely the mechanism of action of PKC in pulmonary smooth muscle cells.

In conclusion, the present work has shown that phorbol 12,13 dibutyrate is one of the most potent constrictor agents in pulmonary arteries. In human tissue, a part of this effect is dependent on calcium influx. Activation of PKC appears to be a primary step in the action of PDB. Our findings suggest that PKC activity could be an important factor in pulmonary vascular responsiveness.

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