Part 1
Soft tissue

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Signal transduction pathways in vascular cells exposed to cyclic strain

The importance of external physical forces in influencing the biology of cells is just being realised. Recent reports demonstrate that exposure of endothelial cells (EC) to a flowing culture media or to repetitive elongation can result in changes in morphology, proliferation and secretion of macromolecules (Dewey *et al.*, 1981; Davies *et al.*, 1984; Frangos, Eskin & McIntire, 1985; Sumpio *et al.*, 1987; Diamond, Eskin & McIntire, 1989; Sumpio & Widmann, 1990; Iba & Sumpio, 1991, 1992). Now, the major impetus in the field is to define the 'mechanosensor(s)' on the cells that are sensitive to the different external forces, the coupling intracellular pathways and the subsequent nuclear events which precede the cell response.

Mechanosensors

Cell surface sensors

The cell's plasma membrane, besides serving as a barrier to protect the cell interior, is the site of action and translation of external to internal signals. Although no 'strain-receptor' as such has been identified, it is clear that endothelial cells can 'sense' changes in pressure and strain. Furthermore, it is likely that this 'sensor' is located on the cell surface. The endothelial cell surface consists of multiple projections covered by a thin layer of glycocalyx (consisting mainly of glycoproteins, proteoglycans and derived substances). In an effort to characterise possible cell surface sensors, Suarez & Rubio (1991) perfused isolated guinea pig hearts with concavalin A or heparinase (agents which modify endothelial cell surface glycoproteins) and attenuated both the flow and pressure stretch induced rise in glycolytic flux normally seen in guinea pig hearts, while having no effect on basal glycolytic values. In contrast,

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infusion with hyaluronidase and chondroitinase (agents which modify surface proteoglycans) had no effect on flow-activated glycolysis, while modifying basal glycolytic values. Their data suggest that cell surface glycoproteins might play a role in 'sensing' changes in blood vessel flow and pressure.

Ion channels

Ion channels maintain the electrochemical balance, pH and osmolarity of the interior milieu of the cell. These channels span the cell membrane and are therefore subject to the mechanical stresses which affect the membrane. It would seem reasonable to presume that any force which affects the membrane tension may affect the channels within.

One model of mechanotransduction involving ion channels pictures the channel as a 'cylindrical plug' of protein embedded in the membrane (Kirber, Walsh & Singer, 1988) and predicts that in order for energy to activate the channel, it needs to be transmitted by cytoskeletal strings. Experiments utilising cytochalasins to disrupt the cytoskeleton have demonstrated an increase in channel sensitivity. The cytochalasins are postulated to act by cleaving *either* non-channel attachment sites in the membrane, thereby increasing the lattice spacing, or the parallel elastic elements in the membrane. The cell shape of capillary endothelial cells has also been shown to be determined by a counterbalance between the contractile forces of the microfilaments and the compression resistance of the microtubules (Ingber & Folkman, 1989). It suggests that membrane tension, and hence the state of the mechanosensitive ion channels, may be a function of the cytoskeletal elements.

Recent studies have also suggested the presence of mechanosensitive ion channels in vascular cells (for review see Davies, 1989). Endothelial cells have been shown to have 'stretch-activated' calcium-permeable channels using the cell attached patch technique. Using porcine aortic endothelial cells Lansman, Hallam & Rink (1987) found that by applying suction (10–20 mm Hg or 1.3–2.7 dynes cm^{-2}) to a cell attached membrane patch, a cation selective current (with a slope conductance of 19.1 pS and reversal potential of 17 mV when the patch electrode contained 110 mM $CaCl_2$) was observed which they speculated could carry sufficient calcium to serve as the second messenger in prostacyclin and EDRF (endothelium-derived relaxing factor) release. Stretch-activated ion channels have been described in other cell types (Guharay & Sachs, 1984; Morris, 1990; Davis *et al.*, 1992), including rat ventricular myocytes (Craelius, Chen & El-Sherif, 1988) and arterial baroreceptors (Schreiber *et al.*, 1971; Singh, 1982). The demonstration of a cation-selective channel

that is permeable to Ca^{2+} is not a universal finding since others have failed to confirm these channels in cultured endothelial cells from either bovine pulmonary artery, human umbilical vein or rabbit aorta (Adams *et al.*, 1989). In addition, Morris & Horn (1991) have recently suggested that, at least in the snail neurone growth cones, the single channel recordings of a potassium-selective stretch-activated channel may be an irrelevant artefact. Since they were unable to correlate their single-channel recordings with any macroscopic currents, it is as yet unclear whether these findings will pertain exclusively to snail neurones. In contrast, Davis *et al.* (1992) have reported that they were able to observe activation of a cation channel ($K^+ > Na^+ > Ba^{2+} > Ca^{2+}$; with a slope conductance of 7 pS when there was 110 mM $CaCl_2$ in the patch electrode) in both whole cell and single-channel recordings when 10–15% stretch (above control) was applied to porcine coronary artery smooth muscle cells.

Adragna (1991) studied the effect of cyclic stretch on sodium and potassium transport in bovine aortic EC (BAEC). He found an increase in Na^+ and K^+ content in cells stretched at 3 cycles min^{-1} and 24% strain for 7 days. The effect of ouabain and of ouabain plus furosemide suggested that cyclic stretch stimulated entry, or inhibited exit of Na^+ and K^+, or both. Further experiments using bradykinin suggested that cyclic stretch and bradykinin act on endothelial cells via an increase in intracellular calcium through release from intracellular pools and calcium entry into the cell. The intracellular calcium then behaves as a second messenger activating various ion channels to produce the observed ion shifts.

Further studies are required to investigate the importance and effect of mechanosensitive ion channels in vascular cells, which are subject to a continuously changing mechanical environment.

Intracellular coupling pathways

Once the endothelial cell has 'sensed' a change in the applied strain, the signal is 'transduced' to the cell interior. Multiple signal transduction pathways exist in vascular endothelial cells. This is not unexpected since the endothelium is metabolically active and many different inputs are processed simultaneously. However, it would be unlikely that one could activate a single second messenger pathway in an isolated manner, without this having important effects on the other pathways. For example, a rise in intracellular calcium can activate the calcium/calmodulin-dependent enzyme constitutive nitric oxide synthase, which in turn will stimulate the soluble guanylate cyclase and increase cGMP levels; this in turn may

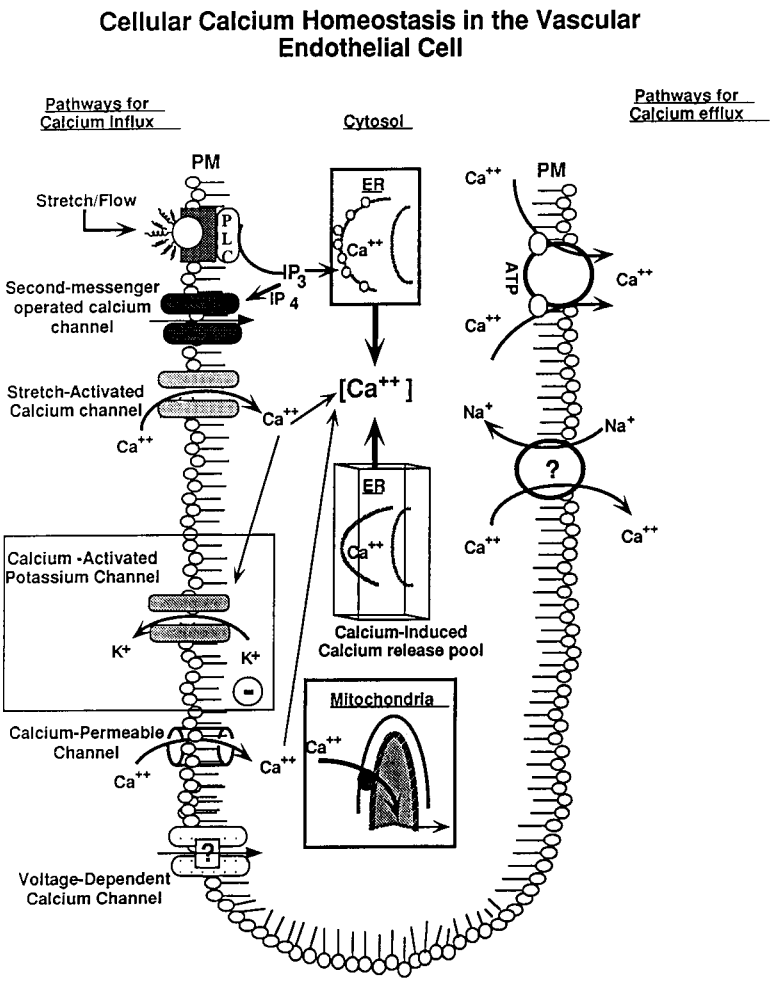

Fig. 1. Cytosolic calcium concentration is the result of a balance between calcium influx, intracellular calcium redistribution and calcium efflux. There are multiple pathways for calcium influx in endothelial cells, some or all of which may or may not be present depending on the type of endothelial cell studied. Possible pathways for influx include: (i) stretch or flow activated increases in inositol phosphate. IP_4 may then open a calcium permeable channel; (ii) there may be a stretch-activated calcium channel; (iii) a rise in $[Ca^{2+}]_i$ through mobilisation of intracellular stores may then activate a calcium-dependent potassium channel. By hyperpolarising the EC it favours calcium entry through a calcium-permeable channel, down its electrogenic gradient; (iv) voltage activated calcium channel, though present in many excitable cells does

feedback and modulate intracellular calcium and cAMP levels via the cGMP-dependent cAMP phosphodiesterase (Lincoln, 1989; Lugnier & Schini, 1990; Lewis & Smith, 1992; Schilling & Elliot, 1992). Thus, cellular responses could be viewed as the result of the integrated activation of a variety of signal transduction pathways; abnormalities in cellular response would then be viewed as a persistent or abnormal activation of one of these pathways, ultimately leading to disease (atherosclerosis, hypertension, etc.).

Intracellular calcium

In many tissues an elevation in intracellular calcium is an important second messenger system. However, since a persistent elevation of intracellular calcium is potentially toxic to the cell, a rise in cytosolic calcium (whether from intracellular or extracellular sources) is balanced by an increase in calcium efflux (Fig. 1). Since the cell membrane has a low calcium permeability, calcium must enter through specific pathways. In general, pathways for calcium entry include the membrane calcium-permeable channels which are receptor or second messenger operated, or voltage-dependent.

Initial reports (Singer & Peach, 1982; Rubanyi, Schwartz & Vanhoutte, 1985) suggested that the endothelium contained dihydropyridine-sensitive channels, implicating voltage dependent calcium channels as signal transducers in this tissue. However, multiple subsequent studies (Bossu *et al.*, 1989; Takeda & Klepper, 1990; Busse, Lückhoff & Pohl, 1992; Schilling & Elliott, 1992) using both whole cell or single channel patch-clamp studies have been unable to demonstrate that voltage dependent calcium channels are present in the vascular endothelium. The reason(s) for these discrepancies are unclear but may relate to differences in cell preparation. Interestingly, recent reports in renal tubule cells (Bacskai & Friedman, 1990; Gesek & Friedman, 1992) suggest the possibility that one can induce the appearance of latent dihydropyridine-sensitive calcium channels by pre-exposure to certain

Fig. 1 (*cont.*)

not appear to be present in EC. There seem to be at least two intracellular calcium pools an IP_3 mobilisable pool and a calcium-inducible pool. The efflux pathways for calcium efflux are not well characterised but calcium can be pumped out through a calcium ATPase pump or sodium–calcium exchanger. The latter has been shown to be involved in calcium influx in certain cells, although this does not seem to be the case for EC.

hormones, which may account for some of the different results reported in endothelial cells. While cell membrane potential plays a role in calcium entry, in endothelial cells it is membrane hyperpolarisation and not depolarisation that stimulates calcium influx. A rise in cytosolic calcium through mobilisation of intracellular stores activates a calcium-dependent potassium channel, which by hyperpolarising the cell favours calcium entry down its electrogenic gradient (Busse *et al.*, 1992). It has been reported that arterially derived endothelial cells have a more negative resting potential (−60 to −70 mV) while venous endothelial cells are more depolarised (−30 mV) and lack the inward rectifying K^+ current (Jacob, Sage & Rink, 1990). These differences, together with species differences, may partly account for some of the reported differences in calcium influx reported from the various endothelial cell types.

The pathways for calcium entry during cyclic strain include a 'stretch-activated' cation channel. In addition, we have observed changes in intracellular calcium in bovine aortic EC exposed to cyclic strain (O. Rosales, unpublished observations). Application of 24% strain at 60 cycles min^{-1} leads to a rapid rise in intracellular calcium through an increase in IP_3 (inositol 1,4,5-trisphosphate) with mobilisation of internal calcium stores and through stimulating extracellular calcium influx. The calcium influx is blocked by gadolinium and removal of extracellular calcium, suggesting entry through a cation channel. Endothelial cells have also been recently reported to have an inositol 1,3,4,5-tetrakisphosphate (IP_4) modulated low conductance channel (Lückhoff & Clapham, 1992). Its contribution to the changes we have observed is unclear although IP_4 must be generated during cyclic strain.

Taken together these data suggest that in tissues subjected to cyclic strain (i.e. the blood vessel) calcium mobilisation from intracellular stores and calcium entry through stretch-activated channels may play an important second messenger role.

Inositol phosphates and diacylglycerols

The plasma membrane contains a pool of lipid precursor molecules which upon hormonal activation can serve as substrates for the generation of second messengers (Liscovitch, 1992). For instance, the phosphatidylinositols serve as precursors for a number of different messenger molecules. Stimulation of cell-surface receptors initiates hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), which produces at least two second messengers – IP_3 and diacylglycerol (DAG).

The effect of cyclic strain on the activation of inositol phosphates and DAG in bovine aortic EC has been a subject of active investigation in

our laboratory. Rosales & Sumpio (1992a) recently reported that the initiation of cyclic strain from 0 to 60 cycles min^{-1} (0.5 s elongation alternating with 0.5 s relaxation) induced a time-dependent, monophasic accumulation of IP_3 (peak at 10 s), inositol bisphosphate (IP_2), and inositol monophosphate (IP_1) on EC prelabelled with [^{3}H]myoinositol. Cyclic strain had a similar effect on radioimmunoactive IP_3 mass.

Changes in cyclic strain frequency promoted inositol phosphate hydrolysis as well. When bovine aortic EC were subjected to an acute variance in cyclic strain frequency from 60 to 100 cycles min^{-1}, a sequential increase in IP_3, IP_2 and IP_1 levels paralleled those triggered by the initiation of cyclic strain. An increase in IP_3 mass was documented at the same time. The kinetics of DAG formation nearly paralleled those of IP_3 production in both types of experiments, suggesting that the early changes in IP_3 and DAG levels were the direct result of PIP_2 hydrolysis mediated by phospholipase C. The increase in intracellular calcium from the IP_3-sensitive pool and higher levels of DAG provide for some of the substrates needed for PKC (protein kinase C) activation.

Although the effect of cyclic strain on the kinetics of IP_3 levels in BAEC was characterised by a single early-phase peak at 10 s and a rapid return to baseline levels after 35 s, the generation of DAG was biphasic. There was an early peak at 10 s followed by a sustained phase of activation after 100 s that persisted for up to 8 min. In a variety of cell types, DAG formation is biphasic with an initial peak as a result of PIP_2 hydrolysis by a phosphatidylinositol-specific phospholipase C (PI-PLC). This DAG is normally transient, and temporally corresponds to the formation of IP_3 which is frequently followed by a more sustained elevation of DAG. This later sustained phase of DAG formation is most likely due to hydrolysis of phosphatidylcholine (PC) in various stimulated cells.

The sustained formation of DAG in BAEC in response to cyclic strain is crucial because sustained PKC activation is a prerequisite essential for causing long-term physiological response such as cell proliferation and differentiation. Several mechanisms may be responsible for the signal-induced formation of DAG from PC (Griendling *et al.*, 1986; Liscovitch, 1992). There is a PC-reactive PLC which requires tyrosine phosphorylation for its activation. This type of PLC remains poorly defined. It is more likely that PC is hydrolised by phospholipase D (PLD) in a signal-dependent manner resulting in the formation of phosphatidic acid, which is converted to DAG by the removal of its phosphate. Phospholipase A_2 (PLA_2) has been shown to be activated by most of the signals which induce PI hydrolysis. Thus, the sustained phase accumulation emphasises the multiple sources of DAG in EC, including

PIP_2, and ensures that those cellular responses that are controlled by PKC can be maintained throughout agonist stimulation. Although phospholipases A_2, C and D are all present in the endothelial cell and all of them appear to be activated by cyclic strain, the initial rapid rise of IP_3 (the IP_3 peak is observed within 10 s of stretch) is secondary to PI-PLC activation (Evans, unpublished observations). The role of each of these signal-activated phospholipases in the generation of DAG is under current investigation in our laboratory.

The underlying mechanisms that modulate this monophasic increase in IP_3 levels are yet to be determined. We have preliminary evidence that the transient rise in IP_3 formation is not modified by calcium-free media, nickel, an inhibitor of calcium influx, or the benzohydroquinone tBu-BHQ, a specific mobiliser of the IP_3 sensitive pool (O. Rosales, unpublished observations). Similarly, pretreatment with charybdotoxin, a non-selective K^+ channel blocker, or gadolinium, a stretch-activated calcium channel antagonist, failed to reduce the IP_3 response to cyclic strain. It is not blocked by pertussis toxin (1 μg ml^{-1} per 4 h) which would suggest that this response is not G_i-linked. One possibility, since G_q is linked to PI-PLC in other tissues, is that stretch is activating PI-PLC through this same G protein. These observations suggest that cyclic strain-mediated PIP_2 hydrolysis is controlled by phospholipase C, yet it is extracellular calcium and pertussis toxin independent. Further work, however, needs to be done in this area.

Protein kinase C (PKC)

PKC is a family of serine/threonine kinases characterised by a dependence on phospholipids and DAG for activation. Since their original description (Kawahara *et al.*, 1980; Kishimoto *et al.*, 1980) these kinases have been implicated in numerous cellular responses (see Rasmussen *et al.*, 1991 for review). The present dogma is that PKC appears to be predominantly in a cytosolic fraction and cell activation is associated with translocation of PKC to a membrane fraction where it is then in an active form (although to date no study has demonstrated that PKC translocation is synonymous with its activation and this sequence of events has been questioned). Part of the present confusion is a result of the multiple methods used to study its function: (i) one can examine its cellular redistribution into either cytosolic or membrane fractions (e.g. by Western blotting, where the fractions are separated by centrifugation, separated by SDS-PAGE and immunoblotted with specific antibodies); (ii) PKC translocation from cytosol to membrane can be evaluated by

immunocytochemistry where using electron or confocal microscopy with isoform specific PKC antibodies one can determine exactly where PKC is located; (iii) activity can be measured by phosphorylation of a PKC-specific substrate: the 80 kDa myristoylated, alanine-rich, C-kinase substrate or MARCKS (Albert *et al.*, 1986; Calle *et al.*, 1992); (iv) one can indirectly assess PKC's role in a particular response by use of 'specific' inhibitors. The usefulness of the last approach is seriously hampered, however, by the lack of selective PKC inhibitors (Rasmussen *et al.*, 1991).

At least nine isoforms of PKC have been identified with the α, β (1 and 2) and γ isoforms, being dependent on calcium for activation (Bell & Burns, 1991). What role an increase in cytosolic calcium plays in modulating PKC is still unclear but calcium probably facilitates the initial translocation of PKC to the membrane and, once translocated, continued calcium influx also appears to modulate the activity of the membrane-bound fraction. In contrast to the α, β and γ isoforms, the ε, δ and ξ forms appear to be calcium-independent. Both groups of kinases have two identifiable domains: a catalytic domain (the ATP binding site, blockable by staurosporine) and a regulatory domain (the phospholipid and DAG binding site, blockable by calphostin) (Bell & Burns, 1991; Rasmussen *et al.*, 1991).

Available data suggest that PKC is an important mediator of the adaptation of vascular EC to cyclic strain *in vitro* (Rosales & Sumpio, 1992b). PKC activity was documented by measuring the transfer of phosphate from [^{32}P]ATP to histone. Cyclic deformation (60 cycles min^{-1}, 24% maximum strain) resulted in a biphasic translocation of PKC from the cytosolic to the particulate fraction. There was an early increase in activity in the particulate fraction at 10 s which paralleled those of IP_3 and DAG, and a second rise which occurred after 100 s of cyclic strain and was sustained up to nearly 8 min. The activation of PKC is pivotal in the modulation of multiple EC responses to mechanical stretch, such as the secretion of prostacyclin, endothelin EDRF, tissue plasminogen activator, growth and proliferation. This relocation in PKC activity was confirmed by immunohistochemical staining and confocal microscopy. In resting BAEC, PKCα and β predominantly stained the cytosol (in contrast to HUVECS (human umbilical vein endothelial cells) where by Western blotting the α, ε and ζ isoforms are present). Cyclic strain caused an intense perinuclear and nuclear translocation of PKCβ and a marked perinuclear halo redistribution of PKCα (Fig. 2). In contrast, stimulation of BAEC with phorbol ester resulted in only PKCα redistribution to the perinuclear region, whereas PKCβ staining remained mostly cytosolic