MINIREVIEW

Protein Tyrosine Phosphorylation in Smooth Muscle: A Potential Coupling Mechanism between Receptor Activation and Intracellular Calcium (44097)

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Abstract. **This review addresses a rapidly growing body of evidence suggesting that enhanced protein tyrosine phosphorylation may be a previously unrecognized mechanism for coupling receptor activation of vascular smooth muscle cells to increases in the intracellular concentration of Ca^{2+} and contraction. The hypothesis proposes that activation of diverse types of receptors that are not tyrosine kinase promotes stimulation of a cytosolic tyrosine kinase. In turn, the activated kinase induces tyrosine phosphorylation of substrates that are linked to regulatory mechanisms for release of intracellular Ca^{2+} stored in the sarcoplasmic reticulum and to regulatory mechanisms for influx of extracellular Ca^{2+}. Within this framework, we examine some relevant functional aspects of receptor and nonreceptor tyrosine kinases in different types of cells, the emerging relationships between tyrosine kinase activity and regulation of intracellular Ca^{2+}. We review studies of nonreceptor tyrosine kinase activity in vascular smooth muscle cells suggesting that a physiologically relevant kinase may be the enzyme called $pp60^{c\text{-}src}$. Data that appear to link tyrosine phosphorylation to contraction of smooth muscle are examined, particularly with respect to results obtained with tyrosine kinase inhibitors and measures of changes in tyrosine phosphorylation. Next, we review studies with cultured vascular smooth muscle cells that point to potential coupling between receptor activation, enhanced tyrosine phosphorylation of substrates such as the GTPase activating protein for *ras*, and the γ-1 isoform of phospholipase C, and mechanisms controlling Ca^{2+} influx and release. Emphasis is placed on examining the strengths and weaknesses of different experimental approaches. Lastly, a summary of the data is provided which calls attention to some major issues requiring resolution to permit acceptance or rejection of the underlying hypothesis, and we briefly address some of its possible pathophysiological implications.** [P.S.E.B.M. 1997, Vol 214]

Hypothesis

Recent studies in our laboratory have been guided by a novel hypothesis suggesting that receptor-activated increases in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in vascular smooth muscle cells (VSMC) are regulated by receptor-activated increases in tyrosine phosphorylation of one or more proteins (Fig. 1). A unique and critical feature of the hypothesis is that the term receptor activation is limited to receptors that are

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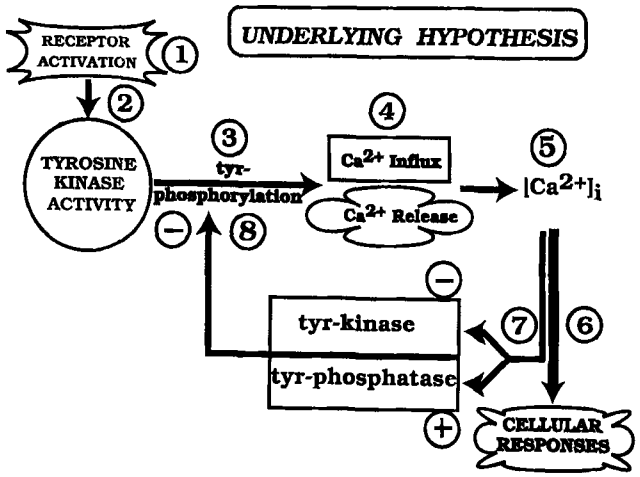

Figure 1. The underlying working hypothesis suggests that activation of receptors which are not tyrosine kinases in vascular smooth muscle cells (Step 1) initiates a series of events involving stimulation of protein tyrosine kinase activity and tyrosine phosphorylation (Steps 2 and 3). Substrates that are tyrosine phosphorylated are suggested to couple receptor activation to increases in $[Ca^{2+}]_i$ (Steps 4 and 5). The increase in $[Ca^{2+}]_i$ induces Ca^{2+}-dependent cellular responses and negative feedback regulation of the extent of tyrosine phosphorylation (Steps 6–8). Further details are given in the text.

not receptor tyrosine kinases. The hypothesis suggests that protein tyrosine phosphorylation is a regulatory mechanism that couples receptor activation to release of intracellular Ca^{2+} stored in the sarcoplasmic reticulum (SR) and to influx of extracellular Ca^{2+}. The resulting increase in $[Ca^{2+}]_i$ then triggers one or more cellular functions, such as contraction. In addition, however, the increase in $[Ca^{2+}]_i$ also is suggested to feed back negatively to limit or reduce receptor-activated protein tyrosine phosphorylation. A negative feedback loop of this kind may fine tune receptor-activated increases in $[Ca^{2+}]_i$ to levels that are commensurate with controlled cellular function. It also assists in avoiding deleterious effects that could result from excessive increases in $[Ca^{2+}]_i$.

The hypothesis does not imply that enhanced protein tyrosine phosphorylation is the only mechanism that couples receptor activation and increases in $[Ca^{2+}]_i$. Instead, it suggests that protein tyrosine phosphorylation is a previously unrecognized mechanism that functions in concert with other mechanisms (e.g., G-protein signaling) which orchestrate coupling between receptor-activation of smooth muscle cells and increases in $[Ca^{2+}]_i$.

We will review recent studies from this and other laboratories bearing on the hypothesis in VSMC. We will also compare and contrast relevant observations obtained in other cell types and offer a perspective aimed at identifying some important unanswered questions. Because of space constraints, we apologize to our colleagues for inadvertent omission of pertinent studies.

Introduction

Ca^{2+} is a key regulatory factor for diverse functions in a variety of cell types, including VSMC (1–5). Such diversity ranges from housekeeping functions involving multiple metabolic pathways, specialized functions including contraction, secretory activities, and cellular growth, proliferation, and death. Accordingly, it is not surprising that elucidation of mechanisms that regulate $[Ca^{2+}]_i$ has commanded extensive experimental study and intellectual effort.

It is now abundantly clear that complex and incompletely understood regulatory mechanisms exist in VSMC and related cell types (e.g., visceral and uterine smooth muscle cells, fibroblasts, platelets, etc.) for controlling receptor-activated increases in $[Ca^{2+}]_i$. Participating mechanisms include influx of extracellular Ca^{2+}, release of intracellular Ca^{2+} stored in the SR, extrusion of cytosolic Ca^{2+} by a sarcolemmal Ca^{2+}-ATPase, and several ion-exchange mechanisms allowing for bidirectional exchange of Ca^{2+} and another cation (e.g., Na^+) between the cytosolic and extracellular compartment (1–7).

The major regulatory mechanisms that we will address are modulation of Ca^{2+} influx and release. Influx of Ca^{2+} can occur through several types of voltage-dependent channels and through so-called receptor-operated and/or second messenger operated channels that appear to be voltage-independent. Moreover, influx channels may also be modulated by α and β subunits of trimeric G proteins, small monomeric G proteins such as *ras,* and protein kinases including cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). Comparable complexity is revealed among mechanisms that modulate Ca^{2+} release from intracellular stores (1–8). Currently, however, the extent to which any of these mechanisms is operative in VSMC is uncertain.

Protein Tyrosine Phosphorylation and Cellular Signaling. A rapidly growing database exists regarding the structure and functions of tyrosine kinases in different types of cells (9–12). In contrast, relatively little is known about the kinds of tyrosine kinases that are present in adult smooth muscle, and even less is known about their functions.

The vast majority of phosphorylated proteins in mammalian cells are phosphorylated on serine and threonine residues. The important role of serine-phosphorylation of the regulatory myosin light chains in control of smooth muscle contraction is well established (4, 13–17). Although less than 0.1% of the phosphorylated proteins are phosphorylated on tyrosine residues, protein tyrosine phosphorylation is a key mechanism for regulating diverse functions in different types of cells (9–12, 18–24). Such functions include mitogen-mediated stimulation of proliferation of VSMC, endothelial cells, fibroblasts, epithelial cells, and other cell types, long-

term potentiation in brain slices from the hippocampus, fertilization of sea-urchin eggs, antigen-mediated activation of lymphocytes, cellular responses elicited by platelet-activating factors, β-adrenergic stimulation of cAMP synthesis in cultured fibroblasts, differentiation of rat PC12 cells into sympathetic-like neuronal cells, release of catecholamines from cultured bovine chromaffin cells, downregulation of certain receptors on neuronal cells, and probably other cellular functions as well.

Receptor Tyrosine Kinases. Two major classes of tyrosine kinases are recognized. One class, the receptor tyrosine kinases, span the plasma membrane and are activated by specific ligands including growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and others (11, 12, 25). Generally, interaction of a specific ligand, like PDGF, with the extracellular domain of its receptor results in dimerization of the receptor, autophosphorylation of specific tyrosine residues at strategic sites along its cytoplasmic domain, and expression of tyrosine kinase activity against specific substrates such as *ras*GAP (i.e., the GTPase activating protein for the small monomeric G protein called *ras*), phospholipase C-γ1 (PLC-γ1), etc. Activation of a receptor tyrosine kinase by its specific ligand is required for expression of biological activity. For example, mutations in the PDGF receptor that prevent activation of tyrosine kinase activity abrogate the ability of PDGF to induce mitogenesis (11, 12). Similarly, tyrphostin, a synthetic inhibitor of tyrosine kinases, blocks EGF-induced mitogenesis in A431 cells (26) and PDGF-induced tyrosine phosphorylation in VSMC (27).

Tyrosine phosphorylation of the ligand-activated receptor or its substrates often occurs at specific sites that serve as targeting or recognition sites for other signaling molecules. Molecules such as *ras*GAP, PLC-γ1, pp60$^{c\text{-}src}$, and *src*-related kinases contain structural domains called SH2 (*src* homology) domains. These SH2 domains dock with specific tyrosine phosphorylated residues in a target molecule so that a protein-protein complex forms. In this fashion, molecules that participate in specific signaling cascades (*ras*GAP, PLC-γ1, pp60$^{c\text{-}src}$, etc.) are assembled in juxtaposition thereby permitting transfer of information along a desired signaling pathway (10, 11, 28, 29).

Activation of receptor tyrosine kinases in fibroblasts, platelets, some VSMC, and other cell types is usually associated with a 2- to 10-fold increase in $[Ca^{2+}]_i$. These Ca^{2+} responses are characterized by a rapid transient increase in $[Ca^{2+}]_i$ (1–4 min) that appears to be due to release of intracellular Ca^{2+} from the SR or endoplasmic reticulum. The transient is followed by a lower sustained increase in $[Ca^{2+}]_i$ (4–20 min) that appears to be due to influx of extracellular Ca^{2+} (11, 12, 30–32). The molecular mechanisms underlying these transient and sustained increases in $[Ca^{2+}]_i$ have not been completely elucidated.

Although receptors for PDGF, EGF, and bFGF are present in cultured VSMC, disparate results have been obtained regarding their presence in intact vascular smooth muscle (VSM) (33–35). This raises questions about the likelihood of these mitogens participating in minute-to-minute regulation of smooth muscle function. Whereas little expression of PDGF receptors is apparent in healthy intact blood vessels, expression of the receptors becomes more pronounced in VSMC that are located in atherosclerotic lesions. Conceivably, expression of these receptors may be related to the proliferative state of VSM so that receptor expression is minimal in healthy nonproliferative adult VSM, whereas expression is pronounced during early development where rapid growth is occurring and/or during pathological states associated with proliferative lesions in the vascular wall (e.g., atherosclerotic plaques, hypertension, etc.).

Nevertheless, earlier studies revealed that several mitogens which interact with receptor tyrosine kinases also contracted VSM and transiently increased $[Ca^{2+}]_i$. Both EGF and PDGF were shown to contract rat aortic strips (36–38). The response to PDGF was unaltered by α-adrenergic blockade with phentolamine or by inhibition of prostaglandin synthesis with indomethacin. In contrast, constriction of ileocolic artery produced in response to EGF was blocked by indomethacin, suggesting the response was due to EGF induced synthesis of prostaglandins (39). Further complexities were revealed by observations showing that both EGF and PDGF inhibited contractile responses in helical strips of mesenteric VSM, and both relaxed cerebral and coronary VSM (40). That is, the results showed that EGF and PDGF apparently contracted some segments of the arterial tree (e.g., aortic VSM), whereas the compounds relaxed or prevented contraction of other segments of the arterial tree (e.g., mesenteric VSM).

Taken together, these studies are consistent with the view that protein tyrosine kinase activity might be an important mechanism for modulating $[Ca^{2+}]_i$ in VSMC and for modulating the contractile state of VSM. However, caution is required in interpreting several aspects of the data and assessing their physiological relevance. First, most of the experiments were performed with mitogens purified from biological sources. The possibility that some of the preparations contained contaminants that contract or relax smooth muscle cannot be discounted. The presence of biologically active contaminants in the PDGF and EGF preparations may have contributed to some of the apparent disparities that were reported. Second, no tests were included to confirm that the responses were ascribable directly to PDGF or EGF (e.g., experiments performed in the presence and absence of neutralizing antibodies that should

block the biological activity of PDGF or EGF). Third, no tests were included to confirm that the responses were due to interactions between the mitogens and their receptors (e.g., experiments performed in the presence and absence of antibodies that block PDGF receptors or antibodies that block EGF receptors). Fourth, no measures of protein tyrosine phosphorylation were included to confirm that the responses were dependent on tyrosine kinase activity. Lastly, given the uncertainty of the expression of PDGF receptors in healthy adult VSM described earlier, the physiological significance of this potential mode of vascular activation is also uncertain. Such mechanisms, again as noted earlier, may be important during early development and during pathologies of the vascular wall (33–35).

Nonreceptor Tyrosine Kinases. The second group of tyrosine kinases are cytoplasmic enzymes. Many of these nonreceptor tyrosine kinases are encoded by proto-oncogenes such as c-*src*, *lck*, *fyn*, *yes*, etc. (9, 10, 19, 22–24). Some, like $pp60^{c\text{-}src}$, the tyrosine kinase encoded by c-*src*, are lipidated and bound to the cytoplasmic face of the plasma membrane, whereas others are not lipidated and appear to be located in the cytosol. Relatively little is known about mechanisms underlying activation of cytoplasmic tyrosine kinases. However, several of these enzymes are negatively regulated by phosphorylation of specific tyrosine residues. The kinase activity of $pp60^{c\text{-}src}$ is suppressed when tyrosine-527 is phosphorylated, whereas the activity of $p56^{lck}$ (i.e., the tyrosine kinase encoded by *lck*) is suppressed when tyrosine-505 is phosphorylated. A pronounced increase in enzymatic activity occurs following dephosphorylation of tyrosine-527 in $pp60^{c\text{-}src}$ or of tyrosine-505 in $p56^{lck}$. Each of these tyrosine residues is subject to phosphorylation and dephosphorylation *in vivo*. This suggests that cycles of phosphorylation and dephosphorylation probably participate in regulating the activity of these enzymes in intact cells. When phosphorylated, the region containing tyrosine-527 (or tyrosine-505) is thought to behave as a pseudo-substrate that is recognized and engaged by the SH2 domain of the enzyme. In this state, the activity of the kinase is suppressed.

Interestingly, recent evidence suggests that activation of antigen-specific receptors in B and T lymphocytes from various species is dependent on enhanced protein tyrosine phosphorylation that is mediated by $p56^{lck}$ and/or $p59^{fyn}$ (19, 41–45). The evidence also suggests that the enhanced tyrosine phosphorylation may be linked to increases in $[Ca^{2+}]_i$ that also occur during lymphocyte activation (35, 36). For example, herbimycin A, a benzoquinoid ansamycin antibiotic that inhibits tyrosine kinase activity (35), suppressed increases in protein tyrosine phosphorylation and $[Ca^{2+}]_i$ that occur during antigen or lectin-induced activation of cultured T cells (16, 19).

Related studies suggest that tyrosine kinase activity may directly regulate resting levels of and increases in $[Ca^{2+}]_i$ that occur during T-cell receptor activation (19). Retroviral expression of $pp60^{v\text{-}src}$, the viral oncogenic homolog of $pp60^{c\text{-}src}$, in T-cell hybridomas was associated with a significant increase in resting $[Ca^{2+}]_i$. Moreover, cells that expressed $pp60^{v\text{-}src}$ also exhibited a 2-fold greater increase in $[Ca^{2+}]_i$ when the T-cell receptor was activated. This enhanced responsiveness was not due to increased production of inositol 1,4,5-triphosphate (IP_3) or to an increased number of T-cell receptors. Instead, the increase in $[Ca^{2+}]_i$ resulting from receptor activation was ascribable to an apparent increase in influx of extracellular Ca^{2+}, and it was markedly suppressed by inhibition of tyrosine kinase activity with herbimycin A. However, these studies raise some concerns which should be addressed. First, the relevant tyrosine kinase in lymphocyte signal transduction is apt to be $p56^{lck}$ or $p59^{fyn}$. Therefore, it is important to determine how overexpression of these enzymes influences lymphocyte activation. Second, the specific activity of $pp60^{v\text{-}src}$ is about 50-fold greater than the specific activity of its cellular homolog $pp60^{c\text{-}src}$: this means the tyrosine kinase activity of the lymphocytes expressing $pp60^{v\text{-}src}$ was exaggerated markedly. Third, significant structural differences exist between $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ which impinge on how the enzymes are apt to be regulated (9, 10, 22). For example, $pp60^{v\text{-}src}$ lacks tyrosine-527, a negative regulatory site that exists in $pp60^{c\text{-}src}$. As noted earlier, phosphorylation of tyrosine-527 suppresses the kinase activity displayed by $pp60^{c\text{-}src}$; however, this kind of downregulation cannot occur with $pp60^{v\text{-}src}$. Accordingly, expression of $pp60^{v\text{-}src}$ may tell us what *can* happen in response to increased tyrosine kinase activity in certain biological settings, but it may not tell us what *does* happen when tyrosine kinase activity is activated under regulated physiological conditions. Nevertheless, these studies encouraged us to pursue evaluation of the working hypothesis illustrated in Figure 1.

Tyrosine Kinase Activity: Smooth Muscle Function

$pp60^{c\text{-}src}$ Tyrosine Kinase Activity Is Unusually High in Mammalian Smooth Muscle. Our interest in elucidating potential functions of nonreceptor tyrosine kinase activity in smooth muscle peaked when we discovered the tyrosine kinase activity ascribable to $pp60^{c\text{-}src}$ was surprisingly and unusually high in fully differentiated adult smooth muscle (46–48). Simon *et al.* (49) had shown that the expression of mRNA for $pp60^{c\text{-}src}$ was developmentally regulated in the visceral smooth muscle of drosophilia.

Unexpectedly, our experiments with extracts prepared from adult bovine tissues showed that the apparent activity of $pp60^{c\text{-}src}$ was 500- to 800-fold greater in

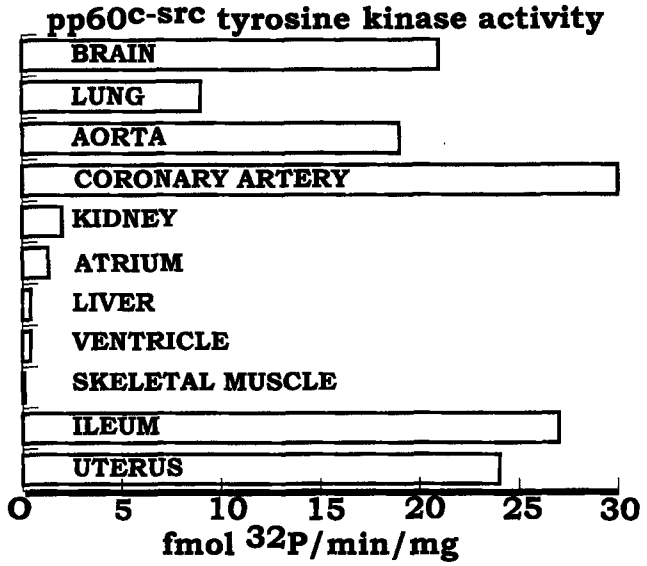

Figure 2. Tyrosine kinase activity ascribable to the enzyme called pp60$^{c\text{-}src}$ is unusually high in extracts from smooth muscle. Extracts were prepared from adult bovine tissues, subjected to immunoprecipitation with a noninhibitory monoclonal antibody for pp60$^{c\text{-}src}$ and assayed for tyrosine kinase activity using [γ-^{32}P]-ATP and enolase as a substrate (see Refs. 46–48). Note that the activity of pp60$^{c\text{-}src}$ in smooth muscle preparations (aorta, coronary artery, ileum, uterus) is 300- to 800-fold greater than in extracts from cardiac (atrium, ventricle) or skeletal muscle. The length of each bar is the mean value for enzyme activity in assays of 14–22 preparations.

extracts from vascular, uterine, and visceral smooth muscle than it was in extracts from either cardiac or skeletal muscle, and many times greater than it was in extracts from kidney or liver (Fig. 2). The enormous difference in activity detected between smooth and striated muscle suggested that tyrosine kinase activity might function in regulating one or more specialized functions exhibited by smooth muscle. Since the mechanisms for regulating stimulus-dependent increases in $[Ca^{2+}]_i$ are different in smooth and striated muscle (4, 13–17), we suspected the high tyrosine kinase activity that is present in smooth muscle might be linked to mechanisms that regulate $[Ca^{2+}]_i$ (46–49).

Tyrosine Kinase Inhibitors Suppress Contraction of Smooth Muscle. The relatively recent availability of structurally diverse compounds that inhibit tyrosine kinase activity (26, 27, 50–53) made it possible to assess the potential functional role(s) of tyrosine kinase activity during contraction of smooth muscle. An array of tyrosine kinase inhibitors, including genistein, tyrphostins, lavendustin, geldanomycin, and herbimycin, markedly and reversibly suppressed contractile responses resulting from stimulation of vascular and gastrointestinal smooth muscle (54–69). For example, a number of putative tyrosine kinase inhibitors suppressed increases in isometric force that were evoked by stimulation of (i) α_1-adrenergic receptors in mesenteric microvessels and in canine carotid arterial smooth muscle strips (54, 55); (ii) stimulation of histaminergic receptors in porcine carotid arterial smooth muscle (60); (iii) stimulation of muscarinic receptors in guinea pig taenia coli (54); and (iv) stimulation of other nontyrosine kinase receptors in different smooth muscle preparations (58, 59). Because the inhibitors are structurally diverse, it is likely that their efficacy in suppressing contraction is ascribable to inhibition of tyrosine kinase activity rather than to nonspecific effects on VSMC. Moreover, the concentration of genistein that inhibited contraction virtually abolished pp60$^{c\text{-}src}$ tyrosine kinase activity in carotid arterial extracts, whereas the activities of myosin light chain kinase and PKA were essentially unaltered (54, 55).

However, unlike results obtained with intact smooth muscle, tyrosine kinase inhibitors did not alter increases in isometric force that were evoked by direct activation of the contractile apparatus by Ca^{2+} in detergent-skinned smooth muscle (54). Taken together, these observations show that the compounds do not inhibit actin-myosin interactions, and they implied that genistein-mediated suppression of smooth muscle contraction was probably due to inhibition of tyrosine kinase activity. They also suggested that the apparent requirement for tyrosine kinase activity may be linked to membrane-dependent signaling pathways, or perhaps to one or more cytosolic signaling molecules that are leached from the muscle during detergent-skinning.

Enhanced Protein Tyrosine Phosphorylation Is Associated with Contraction of Intact Smooth Muscle: Relationship to Calcium Mobilization. Based on the hypothesis that protein tyrosine kinase activity might participate in regulation of smooth muscle contraction (Fig. 1) (46–48, 54–60), we reasoned that inhibition of tyrosine phosphatase activity should increase protein tyrosine phosphorylation in resting intact smooth muscle and thereby induce contraction (54, 55). Because vanadate, a known inhibitor of tyrosine phosphatase activity (61–63), also induces contraction of smooth muscle (64, 65), we suspected that vanadate-induced contraction of smooth muscle might involve inhibition of endogenous tyrosine phosphatase activity and consequent increases in protein tyrosine phosphorylation. To test this possibility, we studied the effects of vanadate on protein tyrosine phosphorylation and isometric force in intact strips of guinea-pig taenia coli (54, 55, 66). The data showed that (i) progressive increases in tyrosine phosphorylation of several substrates occurred when taenia coli was incubated in physiological salt solution containing vanadate, (ii) enhanced tyrosine phosphorylation is associated with a monotonic increase in isometric force that is maintained at a sustained level in the continued presence of vanadate, (iii) transfer of the strips to vanadate-free salt solution resulted in concomitant relaxation and dephosphorylation of the tyrosine phosphorylated substrates, (iv) addition of genistein to the medium after a sustained level of isometric

force was attained resulted in significant relaxation and a decrease in tyrosine phosphorylation, and (v) preincubation with genistein almost completely inhibited both vanadate-induced tyrosine phosphorylation and vanadate-induced increases in force. Direct evidence is now available showing that stimulation of receptors for neurohumoral agents in intact or permeabilized smooth muscle evokes protein tyrosine phosphorylation (54, 57–59). Collectively, these data are consistent with the view that vanadate-induced contraction of smooth muscle was, at least in part, due to vanadate-induced inhibition of tyrosine phosphatase activity, and that protein tyrosine phosphorylation could be a significant mechanism for regulating smooth muscle contraction.

However, these data do not identify the step(s) in the signaling pathway between receptor activation and contraction that may be regulated by protein tyrosine phosphorylation. Based on studies of vanadate-induced contraction, we suggested that protein tyrosine phosphorylation may participate in regulating (i) mechanisms that couple receptor activation to increases in $[Ca^{2+}]_i$, and/or (ii) mechanisms that couple receptor activation to modulation of Ca^{2+} sensitivity for contraction (54, 55, 66). We observed that vanadate-induced contraction was dependent on extracellular Ca^{2+} (Fig. 3). That is, chelation of extracellular Ca^{2+} with EGTA during steady-state isometric force induced with vanadate resulted in prompt and complete relaxation (Fig. 3A, Points 2 and 3). However, even though isometric force declined to baseline, tyrosine phosphorylation was maintained at a high level (Fig. 3B, Points 2 and 3). Surprisingly, replacement of the medium with fresh salt solution that did not contain vanadate or EGTA resulted in a spontaneous transient contraction. Peak force attained during the transient was comparable to the high force attained during carbachol-induced contraction and was associated with persistent tyrosine phosphorylation (Fig. 3A, Point 4, and Fig. 3B, Lane 3). However, the decline of force to baseline during the spontaneous transient contraction was associated with dephosphorylation of the tyrosine phosphorylated substrates. In like fashion, blockade of Ca^{2+} entry with La^{3+} relaxed smooth muscle strips contracted with vanadate (Fig. 3D). In contrast to the relaxation induced with EGTA, replacement of the medium with fresh salt solution that was devoid of La^{3+} and vanadate did not induce a spontaneous contraction.

How can we reasonably account for the persistence of tyrosine phosphorylation following relaxation induced by chelation of extracellular Ca^{2+} with EGTA or by blockade of Ca^{2+} entry with La^{3-}? Probably, the persistence of tyrosine phosphorylation is simply ascribable to the continued presence of vanadate in the medium and continued inhibition of tyrosine phosphatase activity. How can we reasonably account for the spontaneous transient contraction that occurs after replace-

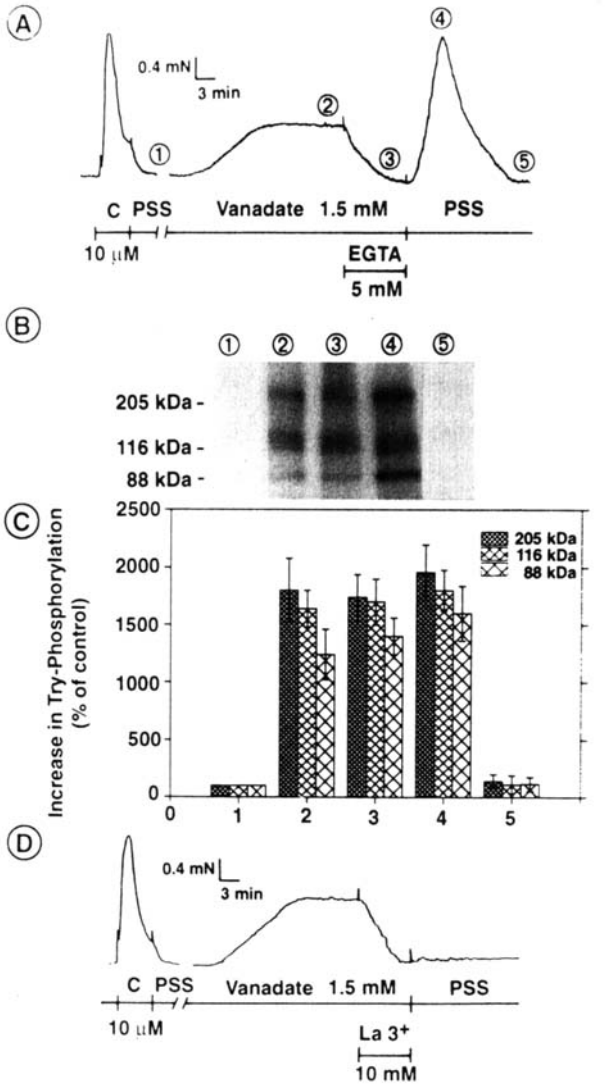

Figure 3. Vanadate-induced contraction of taenia coli is dependent on extracellular Ca^{2+}. (A) A segment of taenia coli was contracted with 10 μM carbachol (left), washed in normal PSS, and contracted with 1.5 mM vanadate. Addition of 5 mM EGTA to chelate Ca^{2+} resulted in complete relaxation. Replacement of the medium with fresh PSS (vanadate and EGTA free) elicited a rapid transient contraction. (B) Vanadate-induced contraction was associated with the usual pronounced increase in protein tyrosine phosphorylation (Lane 2, Point 2). This high level of phosphorylation persisted even after relaxation occurred by halting Ca^{2+} influx with EGTA (Lane 3, Point 3), and it was still present at the peak of the spontaneous transient contraction which occurred in fresh PSS (vanadate and EGTA free, Lane 4, Point 4). Dephosphorylation of the substrates occurred when force returned to baseline (Lane 5, Point 5). (C) Summary of changes in tyrosine phosphorylation which occurred in six experiments. (D) A segment of taenia coli from the same animal as in Panel A was contracted with 10 μM carbachol (left), washed in normal PSS, and contracted with 1.5 mM vanadate (middle). Addition of 10 mM $LaCl_3$ to the medium resulted in complete relaxation. Unlike results obtained with EGTA (A), replacement of the medium with fresh PSS did not elicit a spontaneous transient contraction. However, in accordance with results obtained in the presence of EGTA, high levels of phosphorylation were maintained after relaxation with $LaCl_3$ and dephosphorylation occurred within 15 min after relaxation (data not shown). Similar results were obtained in four experiments. (From Ref. 56.)

ment of the vanadate EGTA medium with fresh medium that is devoid of vanadate EGTA? We suggested (54, 55, 66) that the tyrosine phosphorylated substrates might poise the smooth muscle cells to permit influx of Ca^{2+} if extracellular Ca^{2+} was made available. Such influx could also promote release of intracellular Ca^{2+} through calcium-induced calcium release (2, 4–6). Accordingly, replacing the bathing medium with fresh salt solution that contained the usual concentration of Ca^{2+} (i.e., 1.6 m*M* $CaCl_2$) could have promoted a rapid increase in $[Ca^{2+}]_i$, which in turn would induce a rapid contraction of the smooth muscle cells. Since the fresh medium did not contain vanadate, inhibition of endogenous tyrosine phosphatase activity was likely to be relieved so that dephosphorylation of the substrates occurred. Therefore, the increase in $[Ca^{2+}]_i$ may be short-lived and result in only a transient contraction. In addition, the tyrosine-phosphorylated substrates may also participate in up-modulation of the Ca^{2+} sensitivity for actin-myosin interaction such that a relatively small increase in $[Ca^{2+}]_i$ occurring when the strips are transferred to fresh normal bathing medium could result in a large increase in isometric force (57). How can we reasonably account for the absence of a spontaneous contraction after replacement of the vanadate-La^{3+} medium with fresh medium that is vanadate and La^{3+} free? Because La^{3+} irreversibly blocks diverse types of Ca^{2+} influx channels (5) we interpreted the results to suggest that the apparent persistent relaxation exhibited in fresh medium simply reflected persistent blockade of Ca^{2+} entry sites. Stated differently, no Ca^{2+} entry, no increase in $[Ca^{2+}]_i$, no spontaneous contraction.

When these suggestions were offered, we recognized that they were inferential and we noted that additional experiments were required to warrant their acceptance or rejection. Nevertheless, when taken together and viewed within the framework of studies addressing tyrosine kinase activity, protein tyrosine phosphorylation, and potential regulation of cellular Ca^{2+} in other cell types (see Nonreceptor Tyrosine Kinases above), we were further encouraged to continue testing the working hypothesis (Fig. 1).

Receptor-Activated Increases in Tyrosine Phosphorylation and Increased $[Ca^{2+}]_i$ in VSMC

The development of relatively nontoxic Ca^{2+}-sensitive fluoroprobes, such as fluo-3 or fura-2, greatly stimulated study of agonist-induced changes in $[Ca^{2+}]_i$ in a wide range of cell types, including VSMC (1, 67–73). These compounds permit quantitative evaluation of changes in $[Ca^{2+}]_i$ that are induced in either large populations of cells or in single cells. Our studies were performed with VSMC loaded with fura-2. The intensity of the fluorescence signal of fura-2 excited at 340 nm increases progressively in the presence of increasing Ca^{2+}, whereas the intensity of the signal at 380 nm decreases as Ca^{2+} increases. Therefore, as amply documented by others (67–71), monitoring changes in the 340/380 ratio allows for rapid recording of changes in $[Ca^{2+}]_i$ that are independent of changes in cell volume or photobleaching of the fluoroprobe that might occur during the period of study.

Receptor-Activated Increases in $[Ca^{2+}]_i$. Increases in $[Ca^{2+}]_i$ evoked by receptor activation in VSMC and other cell types loaded with fura-2 are characterized by a transient increase in the 340/380 ratio that is rapid in onset (5–10 sec), quickly attains a maximal value (10–30 sec), and declines to a lower level (30–90 sec) that is sustained in the continued presence of agonist (1, 2, 4, 67–71). This response pattern is seen in primary VSMC from different segments of the arterial tree and in different clonal VSMC lines such as A10 and A7r5 embryonic rat aortic cells. The pattern is similar for responses evoked by activation of α_1-adrenergic receptors, muscarinic receptors, serotoninergic receptors, receptors for other vasoactive agents including thrombin, platelet activating factors, etc., and receptors for peptides such as angiotensin II, endothelin 1, and vasopressin.

Yet, in spite of these consistent similarities, single cell imaging analysis reveals that pronounced cell-to-cell heterogeneity exists among individual cells stimulated with the same agonist at the same time. As shown in Figure 4, such heterogeneity is reflected by cell-to-cell differences in the time of onset of the Ca^{2+} transient, its rate of rise, the maximal increase in $[Ca^{2+}]_i$ during the transient, its rate of decline, the magnitude of the lower sustained increase in $[Ca^{2+}]_i$, and the presence or absence of Ca^{2+} oscillations. Since all of the cells were studied in the growth-arrested state, these heterogeneities are not due to differences among the cells with respect to their stage in the cell cycle. Although the physiological significance of cell-to-cell heterogeneity, if any, is unknown, it is demonstrable with a variety of agonists in both clonal and primary VSMC. Similarly, whether or not heterogeneity reflects cell-to-cell differences in receptor number, differences in receptor activated signaling pathways, differences in the number and kind of Ca^{2+} influx pathways, the relative contribution of influx and release mechanisms, the kinds and relative sizes of intracellular Ca^{2+} stores, and/or other factors are unknown. However, it is noteworthy that cell-to-cell heterogeneity has been implicated as a contributing factor to the pattern of contractile responses elicited in intact VSM (74, 75). Although cell-to-cell heterogeneity is easily recognized during single cell imaging analyses, the phenomenon cannot be recognized in studies of cell populations because the fluorescence signals are recorded as an average of all the cells in the viewed population.

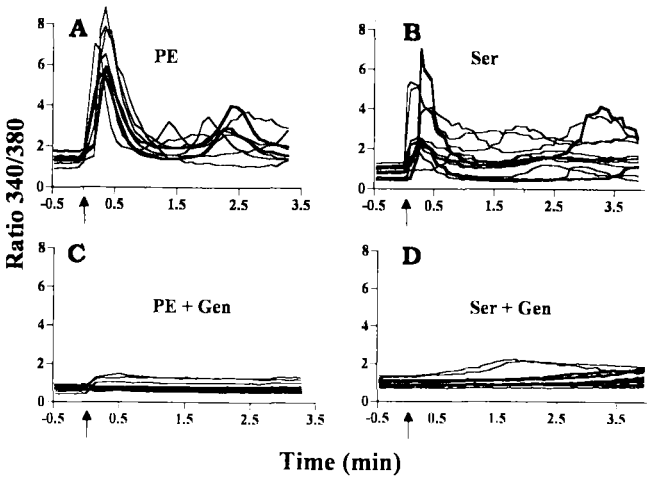

Figure 4. Canine femoral arterial smooth muscle cells were grown on coverslips and loaded with fura-2 AM (67). At zero time (abscissa), they were stimulated with either 100 μM phenylephrine (PE) (A) or 100 nM serotonin (Ser) (B). Changes in $[Ca^{2+}]_i$ were monitored in real time by recording changes in the 340/380 fluorescence ratio of fura-2 ratio (ordinate) using single cell imaging technology (Universal Imaging). Each tracing shows the receptor-activated changes in the 340/380 ratio in a single cell. In all cells, receptor activation induced a pronounced transient increase in $[Ca^{2+}]_i$, which was followed by either (a) one or more Ca^{2+} oscillations of varying magnitude and duration, or (b) a lower sustained increase in Ca^{2+}. Pre-incubation of the cells with 110 μM genistein (Gen; 45 min) virtually abolished the Ca^{2+} response induced by both phenylephrine (C) and serotonin (D). *In situ* calibration of fluorescence in 16 cells studied showed that a ratio of 1 represents 140 nM Ca^{2+}, and a ratio of 3 represents 965 nM Ca^{2+}. (From Ref. 73.)

Relative Contribution of Ca^{2+} Influx and Release during Receptor-Activated Increases in $[Ca^{2+}]_i$. As noted earlier, increases in $[Ca^{2+}]_i$ are ascribable to influx of extracellular Ca^{2+} and/or release of intracellular Ca^{2+} stored in the SR. A frequently used approach for assessing the relative contribution of influx and release to the rise in $[Ca^{2+}]_i$ evoked by receptor activation is to compare changes in $[Ca^{2+}]_i$ that are evoked in the presence and absence of extracellular Ca^{2+} (1–4, 67–73, 76). Responses evoked in the presence of extracellular Ca^{2+} are reflective of both influx and release. In contrast, responses elicited in the absence of extracellular Ca^{2+} are only reflective of release.

Many studies in VSMC and other cell types reveal that the early transient increase in $[Ca^{2+}]_i$ is due to release and influx of Ca^{2+}, whereas the lower sustained component of the response is due to influx only. Our studies with primary and clonal VSMC show that (i) pronounced cell-to-cell heterogeneity exists with respect to the relative contribution of influx and release during the transient component, and (ii) the sustained component is due to influx only (55, 72, 73, 76).

Two populations of A7r5 cells were seen during single cell imaging analysis of preparations stimulated with [arginine^8]-vasopressin (AVP) (64, 72, 73). Combining results from all of the cells studied and expressing the data as average values revealed a profile indicating that the magnitude of the transient attained in the presence of extracellular Ca^{2+} was very similar to the magnitude of the response attained in the absence of extracellular Ca^{2+} (Fig. 5, A and B). Shown in this fashion, the result illustrates how the data would be seen if the cells were studied as a population of cells monitored as a suspension or on a coverslip in a fluorimeter. In either case (i.e., the average of ratios seen in a large number of single cells viewed individually versus the ratio seen in a large group of cells viewed together), the result would suggest that the transient increase in $[Ca^{2+}]_i$ induced by AVP is almost entirely due to release of intracellular Ca^{2+}. However, comparison of the results among all of the cells studied showed that this inference is incorrect. Surprisingly, in 36% of the cells the transient was greater in the absence of extracellular Ca^{2+} (Fig. 5C; ratio: 6.12 ± 0.71) than in the presence of extracellular Ca^{2+} (Fig. 5A; ratio: 3.11 ± 0.22; $P < 0.0005$). In most of the cells (64%), the transient was significantly and markedly smaller in absence of extracellular Ca^{2+} (Fig. 5D; ratio: 1.12 ± 0.06) than in the presence of

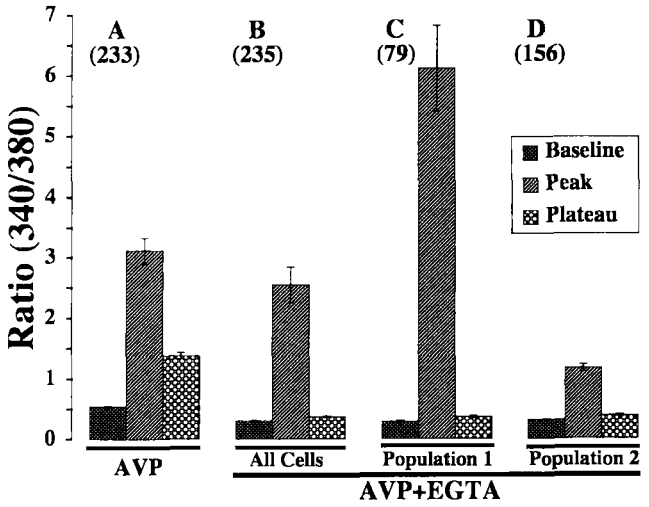

Figure 5. Activation of A7r5 VSMC with AVP in the absence of extracellular Ca^{2+} shows that two populations exist with respect to receptor-activated release of intracellular Ca^{2+}. Coded bars show the mean baseline values of 340/380 ratio, as well as the maximal increase in the ratio during the rapid transient response and the magnitude of the sustained lower increase (plateau) for cells activated with AVP in the presence (A) and absence of extracellular Ca^{2+} (C–D). Small vertical bars show ±SEM, and the number of cells studied is shown above each panel in parentheses. (A) The Ca^{2+} response evoked by AVP in the presence of extracellular Ca^{2+}. (B) The mean response evoked in all cells studied in the absence of extracellular Ca^{2+}. Note that the transient increase in Ca^{2+} (340/380 ratio) is slightly suppressed whereas the sustained component is virtually abolished in the absence of extracellular Ca^{2+}. However, careful examination of the responses elicited by AVP in each cell represented in Panel A showed that the cells could be partitioned into two populations. In about 1/3 of the cells (C), the transient response was enhanced more than 2-fold following activation with AVP in the absence of extracellular Ca^{2+}. In contrast, in 2/3 of the cells (D) the transient was decreased by about 60%. In all cells studied in the absence of extracellular Ca^{2+} (C–D), the baseline was suppressed and the sustained response to AVP was eliminated (see text and Refs. 72 and 73).

extracellular Ca^{2+} (Fig. 5A; ratio: 3.11 ± 0.22; $P < 0.0001$). Therefore, in these cells about 60% of the transient probably was due to influx of extracellular Ca^{2+}, whereas 30% was due to release of intracellular Ca^{2+}. Though unknown, the mechanism(s) underlying this unexpected result may be related to the complex relationships known to exist among the intraluminal concentration of Ca^{2+} in the SR, the cytosolic concentration of Ca^{2+}, the sensitivity of Ca^{2+} channels in the SR to second messengers and G proteins, and interactions between the extent of filling of the stores and influx of extracellular Ca^{2+} (2, 4, 6).

In canine femoral or carotid arterial smooth muscle cells studied in primary culture, about 80% of the transient increase in $[Ca^{2+}]_i$ evoked by stimulation of either α_1-adrenergic or serotoninergic receptors was due to influx of extracellular Ca^{2+}. About 20% was due to release of intracellular Ca^{2+} (76). That is, the magnitude and duration of the transient evoked in the presence of extracellular Ca^{2+} (1.6 m*M*) was reduced by 80% when the same cells are stimulated in the absence of extracellular Ca^{2+} (i.e., media containing 0.2 m*M* EGTA but with no added Ca^{2+}). However, in a significant fraction of the cells studied (ca. 25%), the transient was completely abolished in the absence of extracellular Ca^{2+}, an observation suggesting that all of the transient was due to influx of Ca^{2+}. The heterogeneity in the relative contribution of influx and release to the Ca^{2+} transient observed in these primary cells is similar to that observed in A7r5 cells in that both influx and release of Ca^{2+} participate in the response. However, the results differ from each other in that the transient is entirely due to release in a significant fraction of A7r5 cells, whereas the transient is entirely due to influx in a significant fraction of the primary VSMC.

Unlike the transient component of receptor-activated increases in $[Ca^{2+}]_i$, the lower sustained component of the response was always eliminated in the absence of extracellular Ca^{2+} (Fig. 5). Elimination of the sustained component was apparent in both clonal and primary VSMC and in responses that were evoked with all agonists tested (i.e., AVP, phenylephrine, serotonin, endothelin 1, angiotensin, and carbachol). Therefore, the delayed component appears to be invariably and entirely dependent on influx of extracellular Ca^{2+}.

Tyrosine Kinase Inhibitors Suppress Receptor-Activated Influx and Release of Ca^{2+}. Both the transient and the sustained components of receptor-activated increases in $[Ca^{2+}]_i$ are virtually abolished by genistein (Figs. 4 and 5). The inhibitory effects of genistein were reversible and evident at concentrations that block $pp60^{c\text{-}src}$ tyrosine kinase activity. These concentrations did not alter the activity of either myosin light chain kinase, phosphorylase kinase, PKA (46), or several other serine/threonine kinases (18). Therefore, the inhibitory effects of genistein on Ca^{2+} responses are probably due to inhibition of tyrosine kinase activity. Since the transient component of the response reflects both influx and release of Ca^{2+}, and the sustained component reflects influx only, the results imply that tyrosine kinase activity is involved in mechanisms that regulate influx of Ca^{2+} and release of Ca^{2+}. However, these results do not imply that the Ca^{2+} influx pathways utilized during the transient are the same as the influx pathways activated during the sustained component of the response.

Recently, Rembold *et al.* (60) confirmed that genistein inhibits contraction induced by activation of receptors for histamine in porcine carotid arterial smooth muscle. Their results also showed that genistein-mediated inhibition of contraction was associated with inhibition of the rise in $[Ca^{2+}]_i$ that normally occurs during histamine receptor activation. More recently, a preliminary communication reported by Liu and Sturek (77) confirmed that genistein inhibited receptor-activated increases in $[Ca^{2+}]_i$ in still another type of VSM, namely, coronary VSMC. Their results, like our earlier studies with a variety of agonists in different VSMC (55, 72, 73, 76), showed that endothelin 1–induced activation of both Ca^{2+} influx and release of Ca^{2+} were inhibited by genistein.

On the other hand, two recent reports claim that the inhibitory effects of genistein and other tyrosine kinase inhibitors may involve mechanisms that are independent of their ability to inhibit tyrosine kinases. Toma *et al.* observed that analogs of tyrosine kinase inhibitors which purportedly did not inhibit tyrosine kinase activity were nevertheless effective inhibitors of receptor-activated contraction of mesenteric microvessels (78). They also reported that certain compounds that purportedly do inhibit tyrosine kinase activity did not inhibit receptor-activated contraction. In like fashion, Smirnov and Aaronson (79) reported an apparent dissociation among the efficacy of putative tyrosine kinase inhibitors on K^- channels in VSMC. An important assumption implicit in both of these studies is that the compounds are or are not *in vivo* inhibitors of tyrosine kinase activity in the preparations studied. Surprisingly, however, and unlike our earlier and more recent studies (55–57, 72–77), neither of the studies cited included measures of changes in protein tyrosine phosphorylation or tyrosine kinase activity before and after treatment with the compounds. Therefore, we cannot discount the possibility that compounds that did not inhibit contraction or K^+ channels also did not inhibit endogenous tyrosine kinase activity or tyrosine phosphorylation (78, 79).

Receptor-Activated Tyrosine Phosphorylation and Increases in $[Ca^{2+}]_i$. If receptor-activated tyrosine phoshorylation is coupled to receptor-activated increases in $[Ca^{2+}]_i$, then (Criterion A) receptor activation must be associated with enhanced protein tyrosine phosphorylation of one or more substrates; (Criterion B) the

time course for tyrosine phosphorylation should precede, or be coincident with, the time course for increases in $[Ca^{2+}]_i$; and (Criterion C) compounds that inhibit tyrosine kinase activity must suppress both tyrosine phosphorylation and the increase in $[Ca^{2+}]_i$.

If experimental observations are inconsistent with these minimal criteria, the suspected coupling between tyrosine kinase activity and receptor-activated increases is unlikely, and the hypothesis can probably be rejected. In contrast, if observations are consistent with the criteria, the hypothesis is probably likely but is not established. Further criteria must be defined in order to establish that the relationship between tyrosine phosphorylation and increases in $[Ca^{2+}]_i$ is a causative one. Thus, compliance between experimental observations and the stated minimal criteria is necessary but insufficient for validation of the hypothesis. To what extent, then, is the available evidence consistent with the underlying hypothesis?

Tsuda *et al.* (80) and Molloy *et al.* (81) showed that treatment of cultured VSMC with different receptor agonists resulted in enhanced tyrosine phosphorylation of a set of substrates ranging from 40 to 205 kDa. The significance of such phosphorylation, if any, was uncertain. However, several preliminary communications from this laboratory reported that structurally diverse tyrosine kinase inhibitors suppressed receptor-activated increases in $[Ca^{2+}]_i$ in primary and clonal VSMC (55, 72, 73, 76). These results, like our earlier studies of vanadate-induced contraction of smooth muscle (55, 56), suggested that tyrosine kinase activity might be involved in mechanisms that regulate changes in $[Ca^{2+}]_i$. In studies to test this possibility, we observed that activation of either α_1-adrenergic or serotoninergic receptors in canine primary VSMC induced a transient increase in tyrosine phosphorylation of a set of substrates that were remarkably similar to the substrates reported with different types of VSMC (Criterion A; Figs. 6 and 7) (55, 73, 76). Additionally, we observed that the time course for receptor-activated tyrosine phosphorylation was similar to the time course for receptor-activated increases in $[Ca^{2+}]_i$ (Criterion B; cf., Fig. 4). Further, apparent inhibition of tyrosine kinase activity with genistein or tyrphostin suppressed both tyrosine phosphorylation and the Ca^{2+} response (Criterion C; Fig. 6) (55, 73, 76). Collectively, these data are consistent with the stated minimal criteria to assert a potential functional link between receptor-activated tyrosine phosphorylation and receptor-activated increases in $[Ca^{2+}]_i$.

As noted earlier, however, at least two reports suggested that the effects of tyrosine kinase inhibitors might be due to mechanisms other than inhibition of tyrosine kinase activity (78, 79). As also noted, neither of these studies included measures of tyrosine phosphorylation or measures of changes in $[Ca^{2+}]_i$. Thus, the possibility that responses which were unaltered by putative inhibitors was due to a failure of the compounds to inhibit endogenous tyrosine kinase activity could not be excluded. To examine this issue we extended our studies of AVP-activated tyrosine phosphorylation and increases in $[Ca^{2+}]_i$ in A7r5 cells (73).

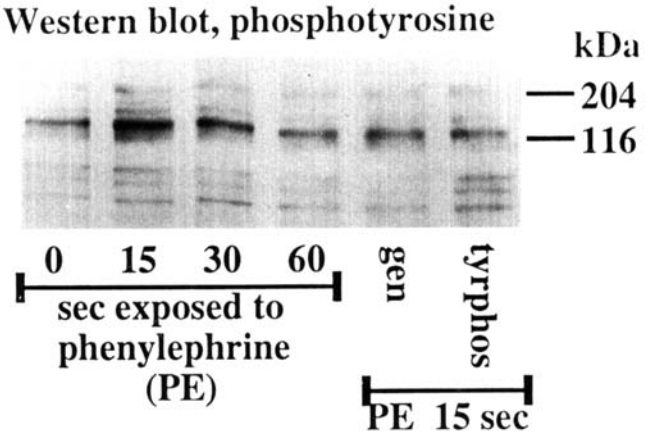

Figure 6. Activation of α_1-adrenergic receptors in VSMC with phenylephrine enhances protein tyrosine phosphorylation: receptor-activated tyrosine phosphorylation is suppressed by tyrosine kinase inhibitors. Cells were exposed to 100 μM phenylephrine (PE) for 0–60 sec and processed for immunoblot analysis of changes in protein tyrosine phosphorylation. A pronounced transient increase in tyrosine phosphorylation was apparent for a major substrate of 116 kDa and several other polypeptides. The increase in tyrosine phosphorylation observed after 15 sec of exposure to phenylephrine (PE) was markedly inhibited in cells that had been pre-incubated with 110 μM genistein or 80 μM tyrphostin for 45 min. Similar results were obtained in four experiments. (From Ref. 76.)

Genistein virtually abolished both the transient increase in $[Ca^{2+}]_i$ and the sustained increase in $[Ca^{2+}]_i$ that is evoked by AVP (72, 73). These results are consistent with results obtained during receptor activation of canine primary VSMC (55, 73, 76). In contrast, tyrphostin 47, another putative tyrosine kinase inhibitor, did not alter either the transient or sustained component of the Ca^{2+} response. Further, lavendustin A, a third inhibitor, suppressed the transient component by 30%–40% but did not alter the sustained component of the response. The results obtained with tyrphostin 47 and lavendustin A are provocative. First, both compounds reportedly inhibit receptor-activated contraction in several different smooth muscle preparations (54). Second, tyrphostin 47 markedly inhibits increases in $[Ca^{2+}]_i$ evoked by receptor activation of primary VSMC (73, 76). Yet the minimal effects of tyrphostin 47 and lavendustin A on AVP-activated increases in $[Ca^{2+}]_i$ appear to be consistent with the reports showing that some tyrosine kinase inhibitors do not alter receptor-activated contraction of mesenteric VSM, or the activation of certain ion channels in VSMC (78, 79). Can these apparently disparate results be reconciled? Is it possible that different tyrosine kinase inhibitors do not always inhibit tyrosine kinase activity in all smooth muscle preparations studied?

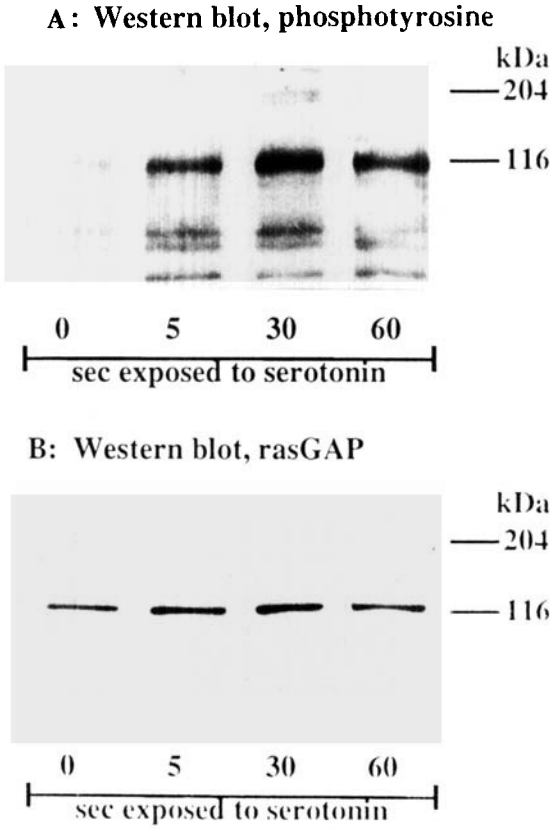

Figure 7. Activation of serotoninergic receptors evokes tyrosine phosphorylation of *ras*GAP (*ras*GTPase-activating protein). (A) Cells were stimulated with 100 n*M* serotonin and processed for immunoblot analysis (Western blot) of tyrosine phosphorylated substrates. Note that the set of substrates phosphorylated in response to serotonin is similar to the substrates phosphorylated in response to phenylephrine shown in Figure 6. (B) The immunoblotted nitrocellulose membrane photographed in Panel A was stripped of phosphotyrosine antibody and probed with a polyclonal antibody for *ras*GAP. Only the 116-kDa substrate was recognized by the *ras*GAP antibody. Similar results were obtained in six experiments. (From Ref. 76.)

To study these issues with AVP-activated A7r5 cells as a model requires assessment of AVP-activated changes in tyrosine phosphorylation. That is, are AVP-activated increases in $[Ca^{2+}]_i$ associated with enhanced tyrosine phosphorylation? Does genistein block both responses? Do tyrphostin 47 and lavendustin A also block both responses, is only one response blocked, or is neither response blocked? Unexpectedly, using the same analytical procedures we used previously, we were unable to document changes in tyrosine phosphorylation in response to AVP (Fig. 8A, Lanes 1–5). In contrast, vanadate-induced increases in tyrosine phosphorylation were easily demonstrated (Fig. 8A, Lane 6). We suspected that A7r5 cells might possess high endogenous tyrosine phosphatase activity. If so, dephosphorylation of phosphorylated substrates could have occurred during the time required to process cell extracts for analysis of tyrosine phosphorylated proteins. In support of this idea, we observed that an apparent increase in tyrosine phosphorylation occurred when the cells were pre-incubated with vanadate to inhibit endogenous phosphatase activity (Fig. 8A, Lanes 1 and 6). This result was consonant with results reported from this and other laboratories showing that vanadate enhances tyrosine phosphorylation (55, 56, 63). Importantly, AVP-induced tyrosine phosphorylation became apparent in cells pre-incubated with vanadate (Fig. 8B). That is, the extent of phosphorylation attained during AVP activation was greater than that attained in the presence of vanadate alone. Moreover, the time course for AVP-induced tyrosine phosphorylation was consistent with the time course for AVP-induced increases in $[Ca^{2+}]_i$, again sug-

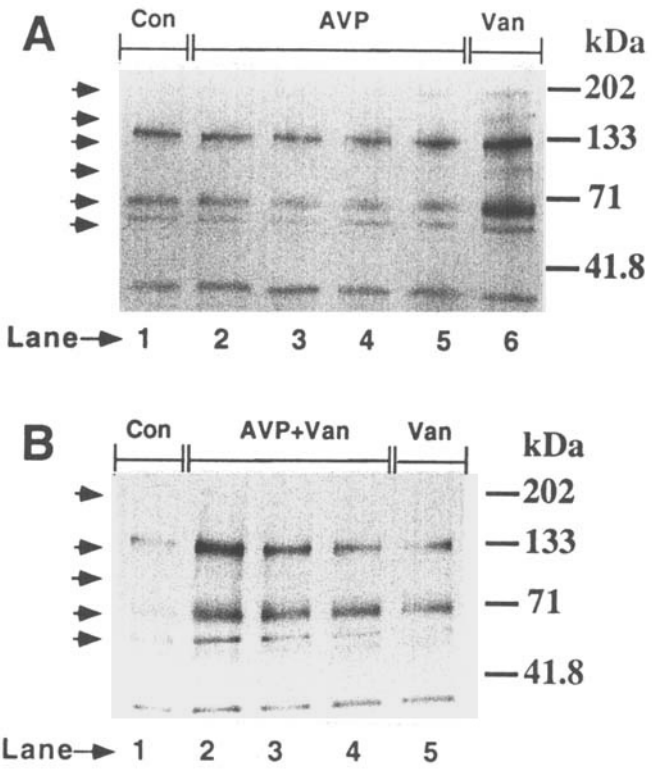

Figure 8. AVP induces a transient increase in tyrosine phosphorylation in A7r5 cells after pre-incubation with 10 m*M* vanadate. In this and subsequent photographs of immunoblots, arrows at left point to tyrosine phosphorylated substrates. The apparent M_r and mobility of marker proteins are shown at right, incubation conditions are given above the photograph, and electrophoretic lanes are identified by numbers at the bottom of the photographs. (A) No change in tyrosine phosphorylation, relative to untreated control cells (Lane 1, Con), occurred during exposure to 20 n*M* AVP for 15, (Lane 2), 30 (Lane 3), 60 (Lane 4), or 120 sec (Lane 5). Though not shown, similar results were obtained with cells fixed in 20% trichloroacetic acid, or with cells homogenized in the presence of 10 m*M* vanadate. However, pre-incubation of the cells in 10 m*M* vanadate alone (Lane 6) induced a pronounced increase in tyrosine phosphorylation of several substrates of 202, 150–155, 116, 95, 65, and 55 kDa. (B) In subsequent experiments, cells were either untreated (Lane 1, Con), pre-incubated with 10 m*M* vanadate alone (Lane 5, Van), or activated with 20 n*M* AVP after pre-incubation with vanadate (Lanes 2–4, AVP + Van). Under these conditions, AVP always induced pronounced transient increases in tyrosine phosphorylation of a similar set of substrates (55–202 kDA) that were greater than the increase produced by vanadate alone. Similar results were obtained in five experiments with maximal phosphorylation occurring 10–20 sec after AVP activation. (From Ref. 72.)

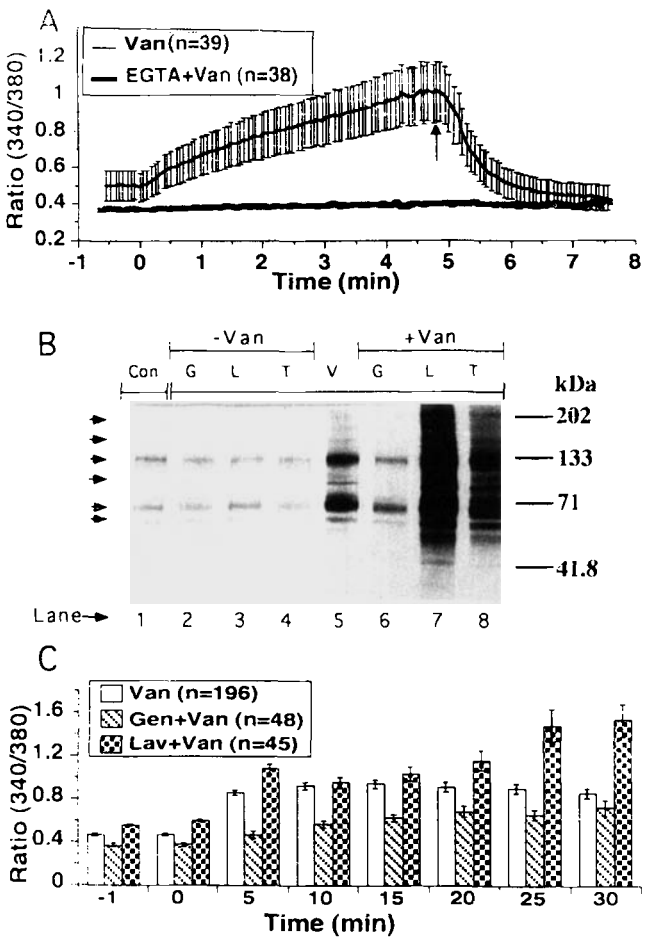

Figure 9. Vanadate-induced tyrosine phosphorylation is associated with a small increase in $[Ca^{2+}]_i$ that is dependent of extracellular Ca^{2+}, inhibited by genistein, and unexpectedly enhanced by lavendustin A and tyrphostin 47, two different tyrosine kinase inhibitors. (A) Exposure of A7r5 cells to vanadate induced a slow progressive rise in $[Ca^{2+}]_i$ that was reversed when extracellular Ca^{2+} was chelated by 5 m*M* EGTA and blocked by pre-incubation with 0.5 m*M* EGTA. (B) Pre-incubation with 148 μ*M* genistein (Lane 2), 105 μ*M* lavendustin A (Lane 3), or 80 μ*M* tyrphostin 47 (Lane 4) did not significantly alter basal tyrosine phosphorylation (Lane 1). However, like the results shown in Figure 6, genistein inhibited vanadate-induced phosphorylation (Lanes 5 and 6). Surprisingly, pre-incubation with lavendustin (Lane 7) or tyrphostin (Lane 8) markedly enhanced vanadate-induced phosphorylation. (C) Genistein inhibited the vanadate-induced increase in $[Ca^{2+}]_i$ during early exposure to vanadate (5–15 min) that became less pronounced after longer times (20–30 min). In contrast, lavendustin did not alter the early increase in the 340/380 ratio induced by vanadate (5–15 min) but progressively increased the ratio after longer exposure to vanadate (20–30 min). (From Ref. 72.)

gesting that the two events probably are functionally coupled (Fig. 8B). Further, AVP-activated increases in tyrosine phosphorylation and $[Ca^{2+}]_i$ were both blocked by genistein (72).

However, because tyrosine phosphorylation was enhanced by vanadate alone, it was necessary to assess its effects on $[Ca^{2+}]_i$. As shown in Figure 9A, vanadate induced a slow monotonic rise in $[Ca^{2+}]_i$ that was dependent on extracellular Ca^{2+}. This increase (340/380 ratio: 0.94 ± 0.04; n = 196) was only about 15%–25% of the maximal increase that occurred in response to AVP (340/380 ratio: 3.11 ± 0.22; n = 233; $P \leq 0.0001$). What about the effects of tyrosine kinase inhibitors on vanadate-induced increases in tyrosine phosphorylation and $[Ca^{2+}]_i$? Can the effects observed help us to reconcile the apparent disparities described earlier?

Neither genistein, lavendustin A, nor tyrphostin 47 exerted a significant effect on basal levels of tyrosine phosphorylation (Fig. 9B, Lanes 1–4). In contrast, genistein markedly inhibited vanadate-induced tyrosine phosphorylation (Fig. 9B, Lanes 5 and 6). Genistein also inhibited the vanadate-induced increase in $[Ca^{2+}]_i$ that was dependent on extracellular Ca^{2+} (Fig. 9C). Surprisingly, the effects of lavendustin A and tyrphostin 47 were more complex. Unlike the effect of genistein, both lavendustin A and tyrphostin 47 greatly stimulated vanadate-induced phosphorylation (Fig. 8B, Lanes 5, 7, and 8). Indeed, the increase in vanadate-induced phosphorylation produced by these compounds precluded assessment of their effects on AVP-activated tyrosine phosphorylation. Moreover, lavendustin A did not inhibit the increase in $[Ca^{2+}]_i$ that occurred during 10–15 min of exposure to vanadate (Fig. 9C). However, after 20 min, the response to vanadate was greater in cells treated with lavendustin A. We suspect that the potentiation of vanadate-induced increases in $[Ca^{2+}]_i$ produced by lavendustin A may reflect increased Ca^{2+} influx resulting from the stimulation of vanadate-induced tyrosine phosphorylation. Thus, an increase in $[Ca^{2+}]_i$ occurred when vandate-induced phosphorylation was enhanced either by receptor activation with AVP (Fig. 8B) or by treatment with lavendustin A (Fig. 9, B and C). Though more difficult to interpret, potentiation of vanadate-induced tyrosine phosphorylation by lavendustin A and tyrphostin 47 may be reflective of complex and reciprocal regulatory relationships between protein tyrosine kinases and tyrosine phosphatases (82, 83). Since *src*-related kinases are negatively regulated by phosphorylation of specific tyrosine residues, dephosphorylation of these sites by tyrosine phosphatases results in increased tyrosine kinase activity (see Nonreceptor Tyrosine Kinases above). Similarly, tyrosine phosphatase activity can be regulated by tyrosine phosphorylation. The need for pre-incubation of A7r5 cells with vanadate to demonstrate AVP-activated tyrosine phosphorylation (Fig. 8), compared with the ease of demonstrating receptor-activated tyrosine phosphorylation in primary VSMC without pre-incubation with vanadate, suggests that the complex regulatory interplay between tyrosine kinase and phosphatase activity may differ in different cells.

The unexpected results obtained with lavendustin A and tyrphostin 47 underscore the need for measuring changes in tyrosine phosphorylation when tyrosine kinase inhibitors are used to assess the potential role of tyrosine kinase activity during a biological response. Accordingly, the disparate conclusions regarding the

involvement of tyrosine kinases in contraction of mesenteric microvessels (78) or in channel regulation (79) may be due to peculiarities of the inhibitors used rather than the participation of tyrosine kinase activity. The key point is that when inhibition of receptor-activated tyrosine phosphorylation can be documented, inhibition of receptor activated increases in $[Ca^{2+}]_i$ also occurs (Figs. 6 and 7).

Tyrosine Phosphorylated Substrates in VSMC. Multiple substrates are tyrosine phosphorylated during receptor activation of VSMC (Figs. 6–9). Most of the substrates are yet to be identified. Which of the substrates may participate in coupling receptor-activation and increases in $[Ca^{2+}]_i$ is unknown. It is also important to note that not all phosphotyrosine proteins are recognized by phosphotyrosine antibodies, and that relevant substrates might escape detection because they may be low abundance proteins. Therefore much work needs to be done to identify, characterize, and elucidate the function(s) of substrates that are tyrosine phosphorylated during receptor activation of VSMC.

Nevertheless, one of the substrates, a protein of 116–120 kDa, is recognized by antibodies for *ras*GAP, the GTPase-activating protein for the small G protein called *ras* (Fig. 7). Interestingly, the activity of *ras*GAP is downregulated when it is tyrosine phosphorylated (84, 85). Therefore, its ability to enhance the GTPase activity of *ras* is suppressed, a situation implying that *ras* can be maintained in its activated GTP-bound state. Activated *ras* stimulates neuronal Ca^{2+} channels (86), a condition that should result in an increase in $[Ca^{2+}]_i$. Moreover, *ras*GAP is tyrosine phosphorylated by $pp60^{c\text{-}src}$ (87). Since VSMC contain *ras* (88), *ras*GAP (55, 73, 76), and high levels of $pp60^{c\text{-}src}$ (46–48), it is tempting to suggest that they function in a signaling pathway coupling receptor activation to influx of Ca^{2+}. In this context, Wijetunge *et al.* (89) showed that voltage-activated Ca^{2+} currents in rabbit ear artery smooth muscle cells were inhibited by tyrosine kinase inhibitors. In more recent studies, Wijetunge and Hughes reported that $pp60^{c\text{-}src}$ can enhance voltage-dependent Ca^{2+} currents in VSMC and that the effects of the enzyme were blocked by a synthetic peptide that inhibits the enzyme and by tyrosine kinase inhibitors (90). Preliminary studies in this laboratory showed that Ba^{2+} influx occurs through L-type Ca^{2+} channels in A10 VSMC, and that such influx was virtually abolished by genistein (91). However, further studies are required to determine whether the inhibitory effect of genistein on Ba^{2+} is mediated (i) at the level of the L channel itself, (ii) by an unidentified auxiliary protein, and/or (iii) by an upstream regulatory mechanism, such as inhibition of a K^+ channel. Additionally, it is also possible that $pp60^{c\text{-}src}$, either directly or indirectly, can regulate Ca^{2+} release. Interestingly, Harnick *et al.* (92) demonstrated that the type 1 IP_3 receptor in T lymphocytes was subject to tyrosine phosphorylation. A subsequent preliminary communication by Ondrias *et al.* (93) reported that IP_3-mediated Ca^{2+} release in lymphocytes was regulated by tyrosine kinase activity. These results are exciting because the type 1 IP_3 receptor is also present in VSMC (94), and receptor-activated Ca^{2+} release in VSMC appears to be regulated by tyrosine kinase activity (Figs. 4 and 9) (55, 60, 72, 73, 76, 77).

Available evidence also suggests that formation of IP_3 can be regulated by tyrosine phosphorylation. Marrero *et al.* (95) reported that stimulation of receptors for angiotensin II in rat VSMC induced tyrosine phosphorylation and activation PLC-γ1, another known substrate for $pp60^{c\text{-}src}$ (96). The time course for phosphorylation of PLC-γ1 was commensurate with the time course for angiotensin II stimulation of IP_3 formation. Both events, activation of PLC-γ1 and formation of IP_3, were inhibited by genistein. In subsequent studies, Marrero *et al.* (92) reported that electroporation of antibodies for $pp60^{c\text{-}src}$ virtually abolished angiotensin II–mediated phosphorylation of PLC-γ1 and markedly suppressed formation of IP_3. Since IP_3 has been assigned a major role in Ca^{2+} release in VSMC and other cell types (1, 2, 4–6), these studies again point to an important potential function of $pp60^{c\text{-}src}$ and tyrosine phosphorylation in VSMC (46–49, 97). However, because Ca^{2+} release or changes in $[Ca^{2+}]_i$ were not included in these experiments, further studies are required to directly link activation of PLC-γ1 and Ca^{2+} release in VSMC. This is particularly important because evidence suggests that the generation of IP_3 in VSM is dependent on a trimeric G protein, and that the relevant PLC is apt to be the β isoform (4). Therefore, additional studies are required to resolve this issue.

Summary and Perspectives

In summary, results obtained in this and other laboratories show the following:

1. Tyrosine kinase activity of $pp60^{c\text{-}src}$ is about 500- to 800-fold greater in smooth muscle than it is in skeletal or cardiac muscle (46–48).
2. Structurally diverse inhibitors of tyrosine kinase activity suppress smooth muscle contraction induced by activation of α_1-adrenergic receptors, muscarinic receptors, and receptors for other vasoactive agents, such as serotonin, vasopressin, endothelin 1, or angiotensin II; yet none of these receptors is a tyrosine kinase (54, 55, 58–60).
3. Vanadate, a known inhibitor of tyrosine phosphatase activity, induces contraction of smooth muscle; this contraction is dependent on extracellular Ca^{2+} and is associated with enhanced protein tyrosine phosphorylation (55, 56).
4. Incubation of VSMC with vanadate induces an increase in $[Ca^{2+}]_i$ that is dependent on extracellular

Ca^{2+} and associated with enhanced protein tyrosine phosphorylation (72, 73).

5. Receptor activation of intact smooth muscle or cultured VSMC induces tyrosine phosphorylation of a set of substrates that includes proteins of 40–205 kDa; one of the substrates is *ras*GAP, and another is PLC-γ1 (55, 73, 76, 95, 97).
6. Receptor activation of primary and clonal VSMC induces an increase in $[Ca^{2+}]_i$ that is characterized by a transient increase in $[Ca^{2+}]_i$ which usually is due to influx of extracellular Ca^{2+} and release of Ca^{2+} from the SR; the transient is followed by a lower sustained increase in $[Ca^{2+}]_i$, which is due to only influx of Ca^{2+} (1, 5, 6, 55, 68–73, 76, 77).
7. When receptor-activated tyrosine phosphorylation in VSMC is blocked by compounds that inhibit tyrosine kinase activity, receptor-activated increases in Ca^{2+} influx and release are also blocked (55, 72, 73, 76).
8. Activation of voltage-dependent Ca^{2+} currents in VSMC is promoted by $pp60^{c\text{-}src}$ and blocked by genistein; similarly, influx of Ba^{2+} appears to occur through L-type Ca^{2+} channels that are directly or indirectly modulated by tyrosine kinase activity (89–91).
9. Activation of receptors for angiotensin II in VSMC involves $pp60^{c\text{-}src}$-dependent tyrosine phosphorylation of PLC-γ1 and formation of IP_3.

In addition, preliminary studies indicate that increased $[Ca^{2+}]_i$ may stimulate tyrosine phosphatase activity or inhibit tyrosine kinase activity such that receptor-activated tyrosine phosphorylation may be regulated through a negative feedback loop (Kaplan and Di Salvo, unpublished observations). Collectively, these results lend strong support to the hypothesis that receptor-activated increases in protein tyrosine phosphorylation of one or more substrates, such as *ras*GAP and PLC-γ1, may be coupled to receptor-activated increases in influx of extracellular Ca^{2+} and release of intracellular Ca^{2+} stored in the SR (Fig. 1).

Yet, in spite of the rapidly growing body of supporting data, further studies are needed to establish a causal link between receptor-activated tyrosine phosphorylation and changes in $[Ca^{2+}]_i$. In this context, an attractive feature of the working hypothesis is that it generates relevant questions that can be pursued to delineate specific signaling pathways that couple receptor-activated tyrosine phosphorylation to release and to influx of Ca^{2+}. Testable questions that require resolution include the following:

1. How does interaction between agonists and receptors that are not tyrosine kinases induce stimulation of tyrosine kinase activity?
2. Is $pp60^{c\text{-}src}$ the relevant tyrosine kinase that is activated following agonist-receptor interaction?
3. How is tyrosine phosphorylation coupled to release of intracellular Ca^{2+} from the SR? Which of the tyrosine phosphorylated substrates is coupled to release? Does tyrosine phosphorylation regulate formation of second messengers that induce Ca^{2+} release? Do multiple redundant signaling pathways exist between receptor activation and Ca^{2+} release? Is tyrosine phosphorylation and activation of PLC-γ1 involved in coupling agonist-receptor interaction and release, are *ras*GAP and *ras* also involved, and does another signaling pathway exist that involves PLC-β? Does tyrosine phosphorylation modulate the sensitivity of IP3 receptors, ryanodine receptors, and/or other receptors that mediate Ca^{2+} release?
4. How is tyrosine phosphorylation coupled to influx of extracellular Ca^{2+}? Which of the tyrosine phosphorylated substrates is coupled to Ca^{2+} influx? Is tyrosine phosphorylation of *ras*GAP a prominent mechanism for coupling agonist-receptor interaction and influx, is *ras* involved, and are other monomeric or trimeric G proteins involved? What types of Ca^{2+} influx pathways are modulated by tyrosine phosphorylation? Is MAP kinase involved?
5. To what extent are release and influx pathways in communication with each other? Does tyrosine phosphorylation participate in the communication mechanism? How do tyrosine phosphorylation-dependent pathways communicate with Ca^{2+} signaling pathways that are independent of tyrosine phosphorylation?
6. How are the Ca^{2+} signaling pathways that are initiated by tyrosine phosphorylation terminated? Is there more than one relevant tyrosine phosphatase? Is the phosphatase(s) regulated?

One can easily recognize that this is only a partial list of relevant questions that require resolution. Some of the listed questions are descriptive in nature, but all are amenable to experimental study and offer the potential for developing new insight into underlying mechanisms.

Establishing a causal link between receptor-activated tyrosine phosphorylation and receptor-activated increases in $[Ca^{2+}]_i$ has significant pathophysiologic implications. Conceivably, unscheduled upregulation of one or more sites in the signaling pathway evoked by receptor activation may produce undesired increases in $[Ca^{2+}]_i$ that could result in vascular spasm, perhaps culminating in myocardial infarction or stroke. Prolonged upregulation may also be a contributing factor to certain forms of progressive hypertension. Similarly, inappropriate downregulation may contribute to failed peripheral vasoconstriction resulting in circulatory failure as in shock and/or limit neurohumoral regulation of blood pressure. Because protein tyrosine phosphory-

lation is also an important regulatory mechanism for cell growth, and because certain vasoactive neurohumoral agents such as serotonin, angiotensin II, AVP, and endothelin 1 can modulate VSMC growth (33–35), the possibility that defects in the signaling pathway may exacerbate, or contribute to, diseases such as atherosclerosis or peripheral vascular disease associated with diabetes merits investigation. In like fashion, smooth muscle diseases involving the genitourinary tract or the gastrointestinal system may also involve defects in the signaling pathway(s) coupling receptor activation, enhanced protein tyrosine phosphorylation, and increased $[Ca^{2+}]_i$. Moreover, identification of defects in the signaling pathway(s) should offer insight into developing new pharmacologic interventions to correct the defect. Accordingly, future studies that are aimed at establishing causal relationships among receptor activation, enhanced protein tyrosine phosphorylation, and increases in $[Ca^{2+}]_i$ promise to yield new understanding of mechanisms that couple receptor activation and $[Ca^{2+}]_i$ in vascular and other types of smooth muscle.

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1. Tsein RW, Tsein RY. Calcium channels, stores, and oscillations. Annu Rev Cell Biol **6:**715–760, 1990.
2. Pozzan T, Rizzuto R, Volpe P, Meldolesi J. Molecular and cellular physiology of intracellular calcium stores. Physiol Rev **74:**595–636, 1994.
3. McDonald TF, Pelzer S, Trautwein W, Pelzer DJ. Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. Physiol Rev **74:**365–507, 1994.
4. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. Nature **372:**231–236, 1994.
5. van Breemen C, Saida K. Cellular mechanisms regulating Ca^{2+} in smooth muscle. Annu Rev Physiol **51:**129–145, 1989.
6. Himpens B, Missiaen L, Casteels R. Ca^{2+} homeostasis in vascular smooth muscle. J Vasc Res **32:**207–219, 1995.
7. O'Donnell ME, Owen NE. Regulation of ion pumps and carriers in vascular smooth muscle. Physiol Rev **74:**683–721, 1994.
8. Wickman K, Clapham DE. Ion channel regulation by G proteins. Physiol Rev **756:**865–885, 1995.
9. Parsons JT, Weber MJ. Genetics of src: Structure and functional organization of a protein tyrosine kinase. Curr Top Microbiol Virol **147:**80–127, 1990.
10. Cantley LG, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soitaff S. Oncogenes and signal transduction. Cell **64:**281–302, 1991.
11. Fantl WJ, Johnson DE, Williams LT. Signalling by receptor tyrosine kinases. Annu Rev Biochem **62:**453–481, 1993.
12. Williams LT. Signal transduction by the platelet-derived growth receptor. Science **243:**1564–1570, 1989.
13. Hartshorne DJ, Matsushima S, Ito M, Guerriero V, Kawamura T Jr. Myosin light chain kinase: Structure-function relationships. Jpn J Pharmacol **58:**17P–22P, 1992.
14. Barron JT, Barany M, Barany K, Storti RV. Reversible phosphorylation and dephosphorylation of the 20,000-dalton light chain of myosin during the contraction cycle of arterial smooth muscle. J Biol Chem **255:**6238–6244, 1980.
15. Walker JS, Wingard CJ, Murphy RA. Energetics of crossbridge phosphorylation and contraction in vascular smooth muscle. Hypertension **23:**1106–1112, 1994.
16. Kamm KE, Stull JT. Regulation of smooth muscle contractile elements by second messengers. Annu Rev Physiol **51:**299–314, 1989.
17. Di Salvo J, Gruenstein E, Silver P. Ca^{2++} dependent phosphorylation of Ca^{2++} sensitive bovine aortic actomyosin. Proc Soc Exp Biol Med **158:**410–414, 1978.
18. O'Dell TJ, Kandel ER, Grant GN. Long term potentiation hippocampus is blocked by tyrosine kinase inhibitors. Nature **353:**558–560, 1991.
19. Zenner G, Dirk zur Hausen J, Burn P, Mustelin T. Towards unraveling the complexity of T-cell signal transduction. Bioessays **17:**967–975, 1995.
20. Vostal JG, Jackson WL, Shulman NR. Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. J Biol Chem **266:**16911–16916, 1991.
21. Sargeant P, Farndale RW, Sage SO. ADP- and thapsigargin-evoked Ca^{2+} entry and protein-tyrosine phosphorylation are inhibited by the tyrosine kinase inhibitors genistein and methyl-2,5-dihydroxycinnamate in fura-2-loaded human platelets. J Biol Chem **268:**18151–18156, 1993.
22. Hunter T. Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signalling. Cell **80:**225–236, 1995.
23. Ely CM, Oddie K, Litz JS, Rossomando AJ, Kanner SB, Sturgill TW, Parsons SJ. A 42-kD tyrosine kinase substrate linked to chromaffin cell secretion exhibits an associated MAP kinase activity and is highly related to a 42-kD mitogen-stimulated protein in fibroblasts. J Cell Biol **110:**731–742, 1990.
24. Wilson LK, Luttrell DK, Parsons JT, Parsons SJ. $pp60^{c\text{-}src}$ tyrosine kinase, myristylation, and modulatory domains are required for enhanced mitogenic responsiveness to epidermal growth factor seen in cells overexpressing c-src. Mol Cell Biol **9:**1536–1544, 1989.
25. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell **61:**203–212, 1990.
26. Margolis B, Bellot F, Honegger AM, Ullrich A, Schlessinger J, Zilberstein A. Tyrosine kinase activity is essential for the association of phospholipase C-γ with the epidermal growth factor receptor. Mol Cell Biol **10:**435–441, 1990.
27. Bilder GE, Krawiec JA, McVety K, Gazit A, Gilon C, Lyall R, Zilberstein A, Levitzki A, Perrone MH, Schreiber AB. Tyrphostins inhibit PDGF-induced DNA synthesis and associated early events in smooth muscle cells. Am J Physiol **260:**C721–C730, 1991.
28. Koch CA, Anderson D, Moran MF, Ellis C, Pawson T. SH2 and SH3 domains: Elements that control interactions of cytoplasmic signalling proteins. Science **252:**668–674, 1991.
29. Heldin CH. SH2 domains: Elements that control protein interactions during signal transduction. TIBS **16:**450–452, 1991.
30. Morgan KG, DeFeo TT, Wenc K, Weinstein R. Alterations of excitation contraction coupling by platelet-derived growth factor in enzymatically isolated and cultured vascular smooth muscle cells. Pflugers Arch **405:**77–79, 1985.
31. Yarden Y, Ullrich A. Growth factor receptor tyrosine kinases. Annu Rev Biochem **57:**443–478, 1988.
32. Ozaki Y, Yatomi Y, Jinnai Y, Kume S. Effects of genistein, a tyrosine kinase inhibitor, on platelet functions. Biochem Pharmacol **46:**395–403, 1993.
33. Thyberg J, Hedin U, Sjolund M, Palmberg L, Bottger BA. Regulation of differentiated properties and proliferation of arterial smooth muscle cells. Arteriosclerosis **10:**966–990, 1990.
34. Schwartz SM, Heimark RL, Majesky MW. Developmental mechanisms underlying pathology of arteries. Physiol Rev **70:**1177–1209, 1990.
35. Ross R. Growth, regulatory mechanisms, and formation of the lesions of atherosclerosis. Ann NY Acad Sci **748:**1–6, 1995.
36. Berk BC, Brock TA, Webb RC, Taubman MB, Atkinson WJ, Gimbrone MA, Alexander RW. Epidermal growth factor, a vascular smooth muscle mitogen, induces rat aortic contraction. J Clin Invest **75:**1083–1086, 1985.

37. Berk BC, Alexander RW, Brock TA, Gimbrone MA, Webb RC. Vasoconstriction: A new activity for platelet-derived growth factor. Science **232:**87–90, 1986.
38. Berk BC, Alexander RW. Vasoactive effects of growth factors. Biochem Pharmacol **38:**219–225, 1989.
39. Gan BS, MacCannell KL, Hollenberg MD. Epidermal growth factor urogastrone causes vasodilatation in the anesthetized dog. J Clin Invest **80:**199–206, 1987.
40. Bassett JE, Bowen-Pope DF, Takayusi M, Dacey RG. Platelet-derived growth factor does not constrict rat intracerebral arterioles in vitro. Microvasc Res **35:**368–373, 1988.
41. June CH, Fletcher MC, Ledbetter JA, Schieven GL, Siegel JN, Phillips AF, Samelson LE. Inhibition of tyrosine phosphorylation prevents T-cell receptor–mediated signal transduction. Proc Natl Acad Sci **97:**7722–7726, 1990.
42. Niklinska BB, Yamada H, O'Shea JJ, June CH, Ashwell JD. Tyrosine-kinase-regulated and inositol phosphate-independent Ca^{2+} elevation and mobilization in T cells. J Biol Chem **267:**7154–7159, 1992.
43. Lane PJL, Ledbetter JA, McConnell FM, Draves K, Deans J, Schievers GL, Clark A. The role of tyrosine phosphorylation in signal transduction through surface Ig in human B cells. Inhibition of tyrosine phosphorylation prevents intracellular calcium release. J Immunol **146:**715–722, 1991.
44. Mustelin T, Coggeshall KM, Altman A. Rapid activation of the T-cell tyrosine protein kianse $pp56^{lck}$ by the CD45 phosphotyrosine phosphatase. Proc Natl Acad Sci U S A **86:**6302–6306, 1989.
45. Mustelin T, Coggeshall KM, Isakov N, Altman A. T-cell antigen receptor–mediated activation of phospholipase C requires tyrosine phosphorylation. Science **274:**1584–1587, 1990.
46. Di Salvo J, Gifford D, Kokkinakis A. $pp60^{c\text{-}src}$ kinase activity in bovine conary extracts is stimulated by ATP. Biochem Biophys Res Commun **153:**388–394, 1988.
47. Di Salvo J, Gifford D, Kokkinakis A. ATP- and polyphosphate mediated stimulation of $pp60^{c\text{-}src}$ kinase activity in extracts from vascular smooth muscle. J Biol Chem **264:**10773–10778, 1989.
48. Di Salvo J, Gifford D, Kokkinakis A. A novel tissue-specific mechanism for modulating the specific activity of $pp60^{c\text{-}src}$ kinase. Adv Prot Phosphatases **5:**93–104, 1989.
49. Simon M, Dress B, Kornberg T, Bishop JM. The nucleotide sequence and the tissue-specific expression of drosophila c-src. Cell **42:**831–840, 1985.
50. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem **262:**5592–5595, 1987.
51. Onoda T, Iinuma H, Sasaki Y, Hamada M, Issiki K, Naganawa H, Takeuchi T. Isolation of a novel tyrosine kinase inhibitor, lavendustin A, from Streptomyces griseolavendus. J Natl Prod **52:**1252–1257, 1989.
52. Gazit AP, Yaish C, Gilon C, Levitzki A. Tyrphostins I: Synthesis and biological activity of protein tyrosine kinase inhibitors. J Med Chem **32:**2344–2352, 1989.
53. Yamaki H, Iguchi-Ariga SM, Ariga H. Inhibition of c-myc gene expression in murine lymphoblastoma cells by geldandmycin and herbimycin, antibiotics of benzoquinoid ansamycin group. J Antibiot **42:**604–610, 1989.
54. Di Salvo J, Steusloff A, Semenchuk LA, Satoh S, Kolquist K, Pfitzer G. Tyrosine kinase (TK) inhibitors suppress agonist-induced contraction in smooth muscle. FASEB J **6:**A26, 1992.
55. Di Salvo J, Pfitzer G, Semenchuk LA. Protein tyrosine-phosphorylation, cellular Ca^{2+}, and Ca^{2+} sensitivity for contraction of smooth muscle. Can J Physiol Pharmacol **72:**1434–1439, 1994.
56. Di Salvo J, Semenchuk LA, Lauer J. Vanadate-induced contraction of smooth muscle and enhanced protein tyrosine phosphorylation. Arch Biochem Biophys **304:**386–391, 1993.
57. Steusloff A, Paul E, Semenchuk LA, Di Salvo J, Pfitzer G. Modulation of Ca^{2+} sensitivity in smooth muscle by genistein and protein tyrosine phosphorylation. Arch Biochem Biophys **320:**236–242, 1995.
58. Yang S-G, Saifeddine M, Hollenberg MD. Tyrosine kinase inhibitors and the contractile action of epidermal growth factor, urogastrone and other agonists in gastric smooth muscle. Can J Physiol Pharmacol **70:**85–93, 1992.
59. Hollenberg MD. Tyrosine kinase pathways and the regulation of smooth muscle contractility. TIPS **15:**108–114, 1994.
60. Gould EM, Rembold CM, Murphy RA. Genistein, a tyrosine kinase inhibitor, reduces Ca^{2+} mobilization in swine carotid artery. Am J Physiol **268:**1425–1429, 1995.
61. Gressesr MJ, Tracey AS, Stankiewicz PJ. The interaction of vanadate with tyrosine kinases and phosphatases. Adv Prot Phosphatases **4:**35–57, 1987.
62. Tonks NK, Charbonneau H. Protein tyrosine dephosphorylation and signal transduction. TIBS **14:**497–500, 1990.
63. Hamaguchi M, Grandori C, Hanafusa H. Phosphorylation of cellular proteins in Rous Sarcoma virus-infected cells: Analysis use of anti-phosphotyrosine antibodies. Mol Cell Biol **8:**3035–3042, 1988.
64. Shimada T, Shimamura K, Sunano S. Effects of sodium vanadate on various types of vascular smooth muscles. Blood Vessels **23:**113–124, 1986.
65. Sanchez-Ferrer CF, Marin J, Lluch M, Valverde A, Salaices M. Actions of vanadate on vascular tension and sodium pump activity in cat isolated cerebral and femoral arteries. Br J Pharmacol **93:**53–60, 1988.
66. Di Salvo J, Kolquist K, Lauer J, Semenchuk LA. Vanadate-induced contraction of smooth muscle is associated with protein-tyr phosphorylation. FASEB **6:**A1545, 1992.
67. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem **260:**3440–3450, 1985.
68. Williams DA, Becker PL, Fay FS. Regional changes in calcium underlying contraction of single smooth muscle cells. Science **235:**1644–1648, 1987.
69. Berman DM, Sugiyama T, Goldman WF. Ca^{2+} stores in smooth muscle cells: Ca^{2-} buffering and coupling to AVP-evoked inositol phosphate synthesis. Am J Physiol **266**(Cell Physiol 35):C276–C283, 1994.
70. Goldman WF, Bova S, Blaustein MP. Measurement of intracellular Ca^{2+} in cultured arterial smooth muscle cells using Fura-2 and digital imaging microscopy. Cell Calcium **11:**221–231, 1991.
71. Hughes AD, Schacter M. Multiple pathways for entry of calcium and other divalent cations in a vascular smooth muscle cell line (A7r5). Cell Calcium **15:**317–330, 1994.
72. Kaplan N, Di Salvo J. Coupling between [arginine^8]-vasopressin-activated increases in protein tyrosine phosphorylation and cellular calcium in A7r5 aortic smooth muscle cells. Arch Biochem Biophys **326:**271–280, 1996.
73. Di Salvo J, Kaplan N, Semenchuk LA. Protein tyrosine phosphorylation and regulation of intracellular calcium in smooth muscle. In: Barany M, Ed. Biochemistry of Smooth Muscle Contraction. New York: Academic Press, pp 283–293, 1996.
74. Iino M, Yamazawa T, Miyashita Y, Endo M, Kasai H. Critical intracellular Ca^{2+} concentration for all-or-none Ca^{2+} spiking in single smooth muscle cells. EMBO J **12:**5287–5291, 1993.
75. Ohta T, Ito S, Nakazato Y. All-or-nothing responses to carbachol in single intestinal smooth muscle cells of rat. Br J Pharmacol **112:**972–976, 1994.
76. Semenchuk LA, Di Salvo J. Receptor-activated increases in intracellular calcium and protein tyrosine phosphorylation in vascular smooth muscle cells. FEBS Lett **370:**127–130, 1995.
77. Liu Y, Sturek M. Cellular mechanisms for tyrosine kinase inhibitor-induced attenuation of myoplasmic calcium response to endothelin. Biophys J **70:**A21, 1996.

78. Toma C, Jensen PE, Prieto D, Hughes A, Mulvany MJ, Aalkjaer C. Effects of tyrosine kinase inhibitors on the contractility of rat mesenteric arteries. Br J Pharmacol **114:**1266–1272, 1995.
79. Smirnov SV, Aaronson PI. Inhibition of vascular smooth muscle cell K^+ currents by tyrosine kinase inhibitors genistein and ST 638. Circ Res **76:**310–316, 1995.
80. Tsuda T, Kawahara Y, Shii K, Koide M, Ishida Y, Yokoyama M. Vasoconstrictor-induced protein-tyrosine phosphorylation in cultured vascular smooth muscle cells. FEBS Lett **285:**44–48, 1991.
81. Molloy CJ, Taylor DS, Weber H. Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells. J Biol Chem **268:**7338–7345, 1993.
82. Vogel W, Lammers R, Huang J, Ullrich A. Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. Science **259:**1611–1614, 1993.
83. Feng G-S, Hui C-C, Pawson T. SH2-containing phosphotyrosine phosphatase as a target of protein tyrosine kinases. Science **259:**1607–1611, 1993.
84. Moran MF, Polakis P, McCormick F, Pawson T, Ellis C. Protein-tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of p21ras GTPase-activating protein. Mol Cell Biol **11:**1804–1812, 1991.
85. Ellis C, Moran M, McCormick F, Pawson T. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature **343:**377–381, 1990.
86. Hescheler J, Klinz F-J, Schultz G, Wittinghofer A. Ras proteins activate calcium channels in neuronal cells. Cell Signal **3:**127–133, 1991.
87. Moran MF, Koch CA, Anderson D, Ellis C, England L, Martin GS, Pawson, T. Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc Natl Acad Sci U S A **87:**8622–8626, 1990.
88. Adam LP, Hathaway DR. Identification of mitogen-activated protein kinase phosphorylation sequences in mammalian *h*-caldesmon. FEBS Lett **32:**56–60, 1993.
89. Wijetunge S, Aalkjaer C, Schacter M, Hughes AD. Tyrosine kinase inhibitors block calcium channel currents in vascular smooth muscle cells. Biochem Biophys Res Commun **189:**1620–1623, 1992.
90. Wijetunge S, Hughes AD. $pp60^{c\text{-}src}$ increases voltage-operated calcium channel currents in vascular smooth muscle cells. Biochem Biophys Res Commun **217:**1039–1044, 1995.
91. Di Salvo J, Nelson SER, Chien T. Genistein and L-type Ca^{2+} channel antagonist inhibit Ba^{2+} entry in A10 vascular smooth muscle cells (VSMC). Biophys J **70:**A286, 1996.
92. Harnick DJ, Jayaraman T, Ma Y, Mulieri P, Go LO, Marks AR. The human type 1 inositol 1,4,5-trisphosphate receptor from T lymphocytes. J Biol Chem **270:**2833–2840, 1995.
93. Ondrias K, Jayaraman T, Marks AR. Functional association between Fyn kinase and the inositol 1,4,5-triphosphate receptor. Biophys J **70:**A20, 1996.
94. Chalovich JM, Greene LE, Eisenberg E. Crosslinked myosin subfragment-1: A stable analogue of the subfragment-1.ATP complex. Proc Natl Acad Sci U S A **80:**4909–4913, 1983.
95. Marrero MB, Paxton WG, Duff JL, Berk BC, Bernstein KE. Angiotensin II stimulates tyrosine phosphorylation of phospholipase C-γ1 in vascular smooth muscle cells. J Biol Chem **269:**10935–10939, 1994.
96. Nakanishi O, Shibasaki F, Hidaka M, Homma Y, Takenawa T. Phospholipase C-γ1 associates with viral and cellular src kinases. J Biol Chem **268:**10754–10759, 1993.
97. Marrero MB, Schieffer B, Paxton WG, Schieffer E, Bernstein KE. Electroporation of $pp60^{c\text{-}src}$ antibodies inhibits the angiotensin II activation of phospholipase C-γ1 in rat aortic smooth muscle cells. J Biol Chem **270:**15734–15738, 1995.