Receptor-activated increases in intracellular calcium and protein tyrosine phosphorylation in vascular smooth muscle cells

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Abstract **We studied the effects of protein tyrosine kinase inhibitors (genistein and tyrphostin) on receptor-activated increases in cellular Ca^{2+} ($[Ca^{2+}]_i$), and protein tyrosine phosphorylation in cultured canine femoral arterial smooth muscle cells. Fura-2 imaging analysis showed that each agonist evoked a transient increase in ($[Ca^{2+}]_i$) followed by a sustained plateau phase. Experiments in Ca^{2+}-free medium showed that 70–80% of the transient increase in $[Ca^{2+}]_i$ evoked by either agonist is due to influx of extracellular Ca^{2+} whereas the plateau phase is only due to Ca^{2+} entry. Pre-incubation with genistein or tyrphostin markedly inhibited the transient rise in $[Ca^{2+}]_i$ evoked by serotonin or phenylephrine. Immunoblot analysis of cell extracts with anti-phosphotyrosine antibodies revealed that serotonin and phenylephrine also evoked an increase in tyrosine phosphorylation of several substrates. These increases were abolished by tyrosine kinase inhibitors. One of the major substrates was recognized by an antibody for *ras*GAP. These data suggest that receptor-activated increases in $[Ca^{2+}]_i$ in vascular smooth muscle cells may be coupled to receptor-activated increases in protein tyrosine phosphorylation.**

Key words: Ca^{2+} influx; Ca^{2+} release; Tyrosine kinase inhibitor; Protein tyrosine phosphorylation; Ratiometric fluorescence analysis; *ras*GAP; Vascular smooth muscle cell

1. Introduction

Since the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) is a major determinant of contractile force, much effort has been devoted to elucidating mechanisms which regulate $[Ca^{2+}]_i$ in smooth muscle [1,2]. It is now clear that multiple and complex signaling pathways participate in these mechanisms. Recently, we suggested that phosphorylation of proteins on tyrosine residues may be an important mechanism for regulating $[Ca^{2+}]_i$ in vascular smooth muscle cells (VSMC; [3–5]). This hypothesis is based on evidence showing that: (a) tyrosine kinase activity, such as that due to the proto-oncogene product, $pp^{60c\text{-}src}$, is 500- to 700-fold greater in extracts from smooth muscle that in extracts from cardiac or skeletal muscle [6,7]; (b) structurally diverse inhibitors of tyrosine kinase activity such as genistein and synthetic tyrphostins inhibit receptor-activated contraction of vascular and visceral smooth muscle [4,5,8,9]; and (c) vanadate, a known contractile agent which also inhibits protein tyrosine phosphatase activity, induces contraction of visceral smooth muscle that is dependent on extracellular Ca^{2+} and is associated with enhanced protein tyrosine phosphorylation of several substrates [8].

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To more directly test the hypothesis that a functional link exists between receptor-activated increases in $[Ca^{2+}]_i$ and protein tyrosine phosphorylation in smooth muscle cells, we studied interrelationships among receptor-activation of cultured VSMC, increases in $[Ca^{2+}]_i$, and enhanced protein tyrosine phosphorylation. We report that increases in $[Ca^{2+}]_i$ evoked by stimulation of α_1-adrenergic receptors with phenylephrine or serotoninergic receptors with serotonin are associated with enhanced protein tyrosine phosphorylation of several substrates. One of the substrates is *ras*GAP, the GTPase Activating Protein for the small G-protein called *ras*. We also report that genistein, a potent inhibitor of tyrosine kinases [10] reversibly inhibits receptor-activated increases in $[Ca^{2+}]_i$ and enhanced tyrosine phosphorylation of *ras*GAP. These observations suggest that a signaling pathway for increasing $[Ca^{2+}]_i$ in VSMC probably involves receptor activation, enhanced tyrosine phosphorylation of *ras*GAP and the participation of a small G-protein such as *ras*.

2. Materials and methods

2.1. Cell culture

Experiments were performed with VSMC cultures from canine femoral arteries using methods adapted from Seidel et al. [11] and Thyberg's laboratory [12]. Cells were seeded in T-25 cm^2 flasks and incubated at 37°C in humidified air and 5% CO_2. Growth medium (DMEM, pH 7.4, 10 mM MES, 10 mM TES, 10% fetal calf serum, 1 mM glutamine, 50 mg/ml ascorbic acid, and antibiotics) was changed every third day and cells were passaged 1:3 when confluent. Each culture was from a single animal. Only passages 2–4 were used for experiments.

2.2. Measurement of intracellular Ca^{2+} with fura-2 [13,14]

Cells were grown on 22 mm coverslips for 24–48 h, serum deprived for 24 h, then incubated with 4 mM fura-2/AM for 1 h at room temperature, rinsed, and incubated with serum-free DMEM for 30 min to permit intracellular esterases to cleave fura-2/AM to fura-2. They were rinsed with physiological salt solution (PSS) and placed in a Bellco chamber at room temperature. PSS contained (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1.6, $MgCl_2$, 1.2; Na_2HPO_4, 1.2; and glucose, 5.6 (pH was adjusted to 7.54). Agonist induced increases in $[Ca^{2+}]_i$, evidenced by an increase in 340/380 fluorescence ratio, were monitored using a Nikon Diaphot TMD microscope and an Image-1 analysis system. Cells were excited at 340 nm and 380 nm and relative fluorescence was measured at 510 nm. We used changes in the 340/380 fluorescence ratio as an index of changes in $[Ca^{2+}]_i$ rather than routinely converting ratios into absolute $[Ca^{2+}]$. However, in situ calibrations in 16 cells showed that a ratio of 1 corresponds to 140 nM Ca^{2+} and a ratio of 3 corresponds to 965 nM, values which agree with reported levels for resting and stimulated $[Ca^{2+}]_i$ [13,14].

2.3. Determination of protein tyrosine phosphorylation

Parallel cell cultures were treated with serotonin or phenylephrine for 0–60 sec, and lysed in buffer containing 0.5% SDS, 5 mM EDTA, 1.5 mM sodium *ortho*-vanadate, 20 mM Tris, 150 mM NaCl, 100 nM okadaic acid, and a mixture of protease inhibitors. Lysates were heated at 95°C/5 min, centrifuged 5 min at 15,000 rpm, and supernatant pro-

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tein was adjusted to 1 mg protein/ml. Proteins were separated by SDS gel electrophoresis (4–15% gradient, 10 μg protein/lane), and electrophoretically transferred to nitrocellulose membranes. Non-specific binding sites were blocked by incubating in Tris-buffered saline + 0.5% Tween 20 (TBST) containing 2% bovine serum albumin (BSA) for 4 h at room temperature. Tyrosine phosphorylated proteins were detected with a monoclonal anti-phosphotyrosine antibody (UBI) diluted 1 : 5000 (0.2 μg/ml) in TBST + 2% BSA overnight at 4°C. The membranes were incubated with horseradish peroxidase conjugated anti-mouse antibody (Amersham), and washed in 5 changes of TBST. Substrates were visualized with chemiluminescense reagents (Amersham), exposure to autoradiographic film, and quantitative densitometry. In several experiments, membranes were stripped of phosphotyrosine antibody, and then probed with a polyclonal antibody for *ras*GAP (GTPase Activating Protein, UBI).

3. Results

3.1. Effects of serotonin and phenylephrine on $[Ca^{2+}]_i$ in the presence and absence of tyrosine kinase inhibitors

Stimulation of femoral arterial VSMC with serotonin or phenylephrine evoked a pronounced transient increase in $[Ca^{2+}]_i$ (Fig. 1). That is, stimulation with 100 nM serotonin resulted in a rapid 6.6-fold increase in the 340/380 ratio which then declined to a sustained level of 1.7-fold (Fig. 1A). Similarly, 100 μM phenylephrine produced a rapid 2.7-fold increase in the ratio which declined to a sustained level of about 1.4-fold (Fig. 1B). To determine the extent to which the transient and sustained increases in $[Ca^{2+}]_i$ were dependent on extracellular Ca^{2+}, responses were elicited in Ca^{2+}-free medium containing 0.5 mM EGTA. Stimulation of 104 cells with 100 nM serotonin in normal Ca^{2+}-containing PSS increased the 340/380 ratio from a basal ratio of 0.61 ± 0.03 to a maximal value of 5.11 ± 0.69, which then declined to a sustained level of 1.02 ± 0.09. However, after incubation in Ca^{2+}-free PSS containing EGTA for 5 min, the maximal increase in the ratio evoked by serotonin was only 1.76 ± 0.19 ($P < 0.005$ relative to control) and the sustained phase was virtually abolished. Therefore, about 80% of the early transient response was dependent on extracellular Ca^{2+}, whereas essentially all of the later sustained response was dependent on extracellular Ca^{2+}. Similar results were obtained with phenylephrine.

Pre-incubation with 110 μM genistein for 45 min virtually abolished the transient rise in $[Ca^{2+}]_i$ evoked by serotonin or phenylephrine. Genistein also inhibited the later sustained response to phenylephrine, but it did not alter the sustained response to serotonin. Tyrphostin, a synthetic tyrosine kinase inhibitor that is structurally different from genistein and has a different mechanism of action [15], also inhibited the serotonin-activated Ca^{2+} transient. Before incubation with 80 μM tyrphostin (70 cells), 100 nM serotonin increased the 340/380 ratio from its basal value (0.47 ± 0.08) to a maximal value of 2.53 ± 0.72 ($P = 0.002$). However, incubation with tyrphostin for 45 min inhibited the response by 28.8% ($P = 0.012$), while incubation with tyrphostin for 2 h inhibited the response by 87.5% ($P = 0.007$).

3.2. Effects of serotonin, phenylephrine, and tyrosine kinase inhibitors on protein tyrosine phosphorylation in VSMC

Stimulation with 100 nM serotonin (Fig. 2A) or 100 μM phenylephrine (Fig. 3A) induced a rapid transient increase in protein tyrosine phosphorylation of a similar set of substrates.

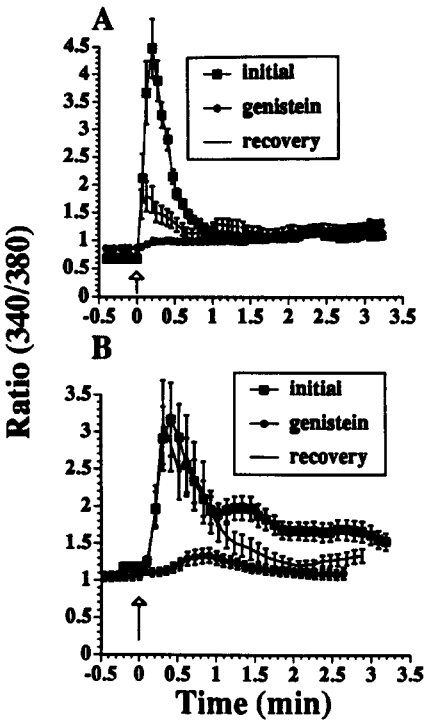

Fig. 1. Receptor-activated changes in $[Ca^{2+}]_i$ (340/380 fluorescence ratio; see section 2) in canine VSMC loaded with fura-2. Stimulation with either 100 nM serotonin (Panel A, 159 cells) or 100 μM phenylephrine (Panel B, 67 cells) produced a rapid transient increase in the 340/380 fluorescence ratio (159 cells). Pre-incubation with genistein (110 μM, 45 min) markedly reduced the transient response evoked by serotonin (67 cells) or phenylephrine (42 cells). However, the later sustained phase evoked by serotonin was unaffected by pre-incubation with genistein, whereas genistein completely abolished the later sustained phase evoked by phenylephrine. Complete recovery of responsiveness to phenylephrine occurred when cells were bathed in genistein-free PSS for 45 min. Recovery of responsiveness to serotonin, though apparent after 45 min in genistein-free PSS, was not complete until after 2 h without genistein. Blockade of serotonergic receptors with 100 nM methysergide (23 cells) eliminated responses to serotonin, and blockade of α_1-adrenergic receptors with 100 μM prazosin (18 cells) eliminated responses to phenylephrine.

Pre-incubation with 110 μM genistein or 80 μM tyrphostin, the same concentrations which inhibited receptor-activated increases in $[Ca^{2+}]i$ (Fig. 1), inhibited receptor-activated increases in protein tyrosine phosphorylation (Fig. 2).

We suspected that the tyrosine-phosphorylated substrate of 116 kDa might be *ras*GAP, a known tyrosine-phosphorylated substrate of 116–120 kDa which participates in diverse signaling pathways [16,17]. Accordingly, nitrocellulose membranes which were immunoblotted for phosphotyrosine proteins were stripped of antibody and reprobed with a polyclonal antibody for *ras*GAP (Fig. 3B). The single band recognized by the *ras*GAP specific antibody was coincident with the tyrosine phosphorylated substrate of 116 kDa. None of the other tyrosine-phosphorylated substrates were recognized by the *ras*GAP antibody. Moreover, when cellular extracts were immunoprecipitated with rasGAP antibody before electrophoresis, the 116 kDa tyrosine phosphorylated substrate was recovered in the precipitate (not shown).

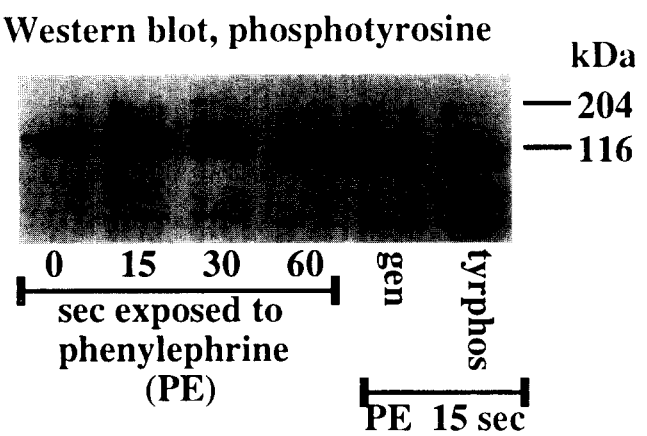

Fig. 2. Activation of α_1-adrenergic receptors in VSMC with phenylephrine enhances protein tyrosine phosphorylation:receptor-activated tyrosine phosphorylation that is suppressed by tyrosine kinase inhibitors. Cells were exposed to 100 μM phenylephrine for 0–60 sec and processed for immunoblot analysis 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 s exposure to phenylephrine (PE) was markedly inhibited in cells which had been pre-incubated with 110 μM genistein or 80 μM tyrphostin for 45 min. Similar results were obtained in 4 experiments.

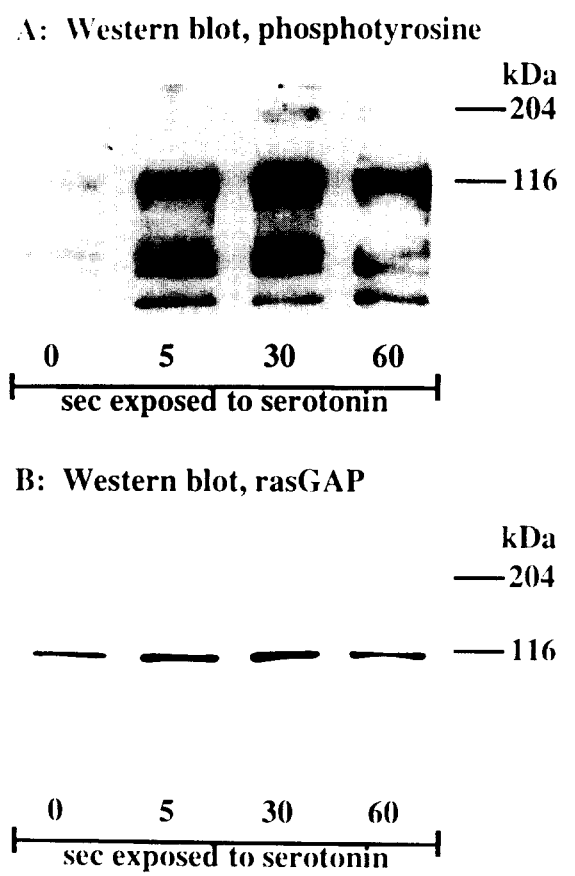

Fig. 3. Activation of serotoninergic receptors evokes tyrosine phosphorylation of *ras*GAP (*ras*GTPase Activating Protein). (Panel A) Cells were stimulated with 100 nM serotonin and processed for immunoblot analysis (Western blot) of tyrosine phosphorylated substrates as in Fig. 2. Note that the set of substrates phosphorylated in response to serotonin is similar to the substrates phosphorylated in response to phenylephrine shown in Fig. 2. (Panel 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 6 experiments.

4. Discussion

The major point of this study is that receptor-activated increases in $[Ca^{2+}]_i$ in cultured VSMC may be coupled to receptor-activated increases in protein tyrosine phosphorylation. This conclusion is supported by our observations showing that: (a) stimulation of α-1-adrenergic or serotonergic receptors results in a transient increase in $[Ca^{2+}]_i$ (Fig. 1); (b) receptor-activation also enhances tyrosine phosphorylation of the same or similar set of substrates (Figs. 2 and 3); and (c) both the increase in $[Ca^{2+}]_i$ and the increase in protein tyrosine phosphorylation are inhibited by compounds which inhibit tyrosine kinase activity. The differential effect of tyrosine kinase inhibitors on the delayed sustained Ca^{2+} response to serotonin and phenylephrine may reflect differences between Ca^{2+} influx pathways activated by different vasoactive agents [1,2,18]. In contrast, the consistent inhibition of transient increases in $[Ca^{2+}]_i$ evoked by serotonin or phenylephrine suggest that both the influx and release components of the transient are modulated by tyrosine phosphorylation.

Previous studies showed that stimulation of cultured rat aortic smooth muscle cells with vasoactive agents increased tyrosine phosphorylation of several substrates [19,20]. The pattern of protein tyrosine phosphorylation observed in this study was remarkably similar to that observed in the earlier studies. Our data also indicate that one of the major tyrosine phosphorylated substrates, a polypeptide of 116 kDa is *ras*GAP. However, Molloy et al. did not observe changes in tyrosine phosphorylation of *ras*GAP during receptor-activation of rat aortic smooth muscle cells [20]. This disparity may reflect procedural differences. We used canine femoral VSMC stimulated with serotonin or phenylephrine, and single label immunoblot technology for assessing changes in phosphorylation of *ras*GAP. Molloy et al. [20] studied rat aortic cells stimulated with angiotensin II, and used immunoprecipitation technology for assessing changes in phosphorylation of *ras*GAP.

*ras*GAP is known to physically associate with the tyrosine kinase $pp60^{c\text{-}src}$. Tyrosine phosphorylation of *ras*GAP appears to decrease its ability to enhance the GTPase activity of its target, *ras* [16,17]. Therefore, *ras* is maintained in its activated GTP-bound state. Moreover, *ras* stimulates Ca^{2+} channels in neuronal cells [21]. Since $pp60^{c\text{-}src}$ [6,7], *ras* [22], and *ras*GAP are present in VSMC (Fig. 3B), it is tempting to speculate that they may participate in a signalling pathway which modulates receptor-activated increases in $[Ca^{2+}]_i$ required for smooth muscle contraction. An attractive feature of this hypothesis is that it is testable. In accordance with results from other laboratories (reviewed in [1,2,18], the initial Ca^{2+} transient evoked by either phenylephrine or serotonin is due to both (a) influx of Ca^{2+} which is evident in the presence of extracellular Ca^{2+}, and (b) release of intracellular Ca^{2+} which is evident in the absence of extracellular Ca^{2+} (see section 3). As noted earlier, tyrosine phosphorylation probably participates in both the influx and release components of the Ca^{2+} transient because both genistein

and tyrphostin markedly suppress phosphorylation and virtually abolish the Ca^{2+} transient. However, it is likely that mechanisms in addition to protein tyrosine phosphorylation participate in modulation of receptor-activated increases in $[Ca^{2+}]_i$ in VSMC [1,5,18]. It is also possible that that the inhibitory effects of genistein and tyrphostin involve mechanisms in addition to inhibition of tyrosine kinase activity.

In summary, the new evidence reported in this study (Figs.1–3) strongly suggests that receptor-activation of VSMC enhances tyrosine phosphorylation of several substrates, including *ras*GAP, and that such phosphorylation may be coupled to receptor-activated increases in $[Ca^{2+}]_i$. Future studies aimed at elucidating underlying mechanisms which may couple receptor- activated increases in protein tyrosine phosphorylation and increases in $[Ca^{2+}]_i$ promise to enhance our understanding of how smooth muscle function is regulated.

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