A Tyrosine Kinase Signaling Pathway, Regulated by Calcium Entry and Dissociated from Tyrosine Phosphorylation of Phospholipase Cγ-1, Is Involved in Inositol Phosphate Production by Activated G Protein-Coupled Receptors in Myometrium

BRUNO PALMIER, MONIQUE VACHER, SIMONE HARBON, and DENIS LEIBER
Signalisation et Réglations Cellulaires, Centre National de la Recherche Scientifique, Université Paris-Sud, Orsay Cedex, France

ABSTRACT
Our experiments were conducted to evaluate, in rat myometrium, the potential contribution of a protein tyrosine kinase (PTK) pathway in the hydrolysis of phosphatidylinositol-4,5-bisphosphate mediated by bombesin, endothelin-1 (ET-1), and carbachol. The production of inositol phosphates (InsP) by agonists and AlF₄⁻ was partly inhibited (35–40%) by genistein and tyrphostins, two PTK inhibitors. Genistein attenuated uterine contractions elicited by the stimulation of muscarinic and bombesin receptors, whereas pervanadate, a protein tyrosine phosphatase inhibitor, potentiated receptor-mediated contraction. Tyrosine-phosphorylated proteins were detected in detergent extracts from agonist- and pervanadate-stimulated myometrium. The amount of InsP produced in response to pervanadate was related to the tyrosine phosphorylation status of phospholipase C-γ1. In contrast, with ET-1 and bombesin, phosphorylated phospholipase C-γ1 made a minor contribution. Additional findings were rather consistent with a role for Ca²⁺. In fura-2-loaded cells, genistein partly decreased both the transient and sustained intracellular Ca²⁺ concentration phases induced by bombesin. The removal of extracellular Ca²⁺ or the addition of nifedipine inhibited (35%) InsP production due to bombesin and ET-1. The inhibitory effects of genistein and tyrphostins were abolished in Ca²⁺-depleted medium, were not additive with that of nifedipine, and (as for nifedipine) were counteracted by the Ca²⁺ channel agonist Bay K 8644. The data are consistent with a PTK-mediated process in the activation of the voltage-gated Ca²⁺ influx that is involved in the production of InsP by stimulated G protein-coupled receptors.

Signaling pathways associated with the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) play a key role in the regulation of cell function (Berridge, 1993). We have previously shown that in myometrium, PIP₂ breakdown mediated by various contractile agonists is associated with the stimulation of phospholipase C (PLC) via the activation of specific G protein-coupled receptors (Marc et al., 1988; Leiber et al., 1990; Amiot et al., 1993; Dokhac et al., 1994). PLC stimulation is insensitive to pertussis toxin, suggesting that a member of the Gq family is involved (Lajat et al., 1996). There is evidence that in the myometrium, at least two distinct mechanisms underlie the activation of PIP₂-PLC in response to carbachol and oxytocin (Dokhac et al., 1992). One mechanism concerns the well recognized agonist-induced activation of receptor-G₁₂ protein-PLCβ3 cascade (Lajat et al., 1996), which is insensitive to elevation of intracellular Ca²⁺ and contributes predominantly to the increased production of inositol phosphates. A second Ca²⁺-dependent pathway is responsible for the additional, although modest (35%), receptor- and G protein-mediated stimulation of a PLC activity through an increased influx of Ca²⁺ after the activation of voltage-operated Ca²⁺ channels (Dokhac et al., 1992, 1996).

Protein tyrosine kinases (PTKs) play a critical role in regulating various cellular processes, including PIP₂ hydrolysis, through tyrosine phosphorylation and the activation of PLC-γ in a number of cell systems and tissues (van der Geer and Hunter, 1994; Malarkey et al., 1995; Post and Heller Brown, 1996). We recently demonstrated (Palmier et al.,

Received for publication May 14, 1998.

This work was supported by grants from the Centre National de la Recherche Scientifique (ERS 0570 and EP1088) and by a contribution from the Association de la Recherche contre le Cancer (1355).

ABBREVIATIONS: ET-1, endothelin-1; InsP₃, inositol triphosphate; InsP₂, inositol bisphosphate; InsP₁, inositol monophosphate; PIP₂, phosphatidylinositol-4,5-bisphosphate; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; PLC, phospholipase C; Tyr, tyrphostin; Fura-2/AM, acetoxy methyl ester (AM) form of fura-2.
(Wistar, 4 weeks old) were treated with 30 g of estradiol for 2 days and used on the next day. Animals were sacrificed by decapitation, their uteri were removed immediately, and the myometrium was prepared free of endometrium as previously described (Marc et al., 1988; Amiot et al., 1993).

**Measurement of[^2H]Inositol Phosphates.** Myometrial strips (about 25 mg) were allowed to equilibrate at 37°C for 25 min in 5 ml of Krebs-Ringer-bicarbonate buffer (pH 7.4) containing (117 mM NaCl, 4.7 mM KCl, 1.1 mM MgSO₄, 1.2 mM KH₂PO₄, 24.7 mM NaHCO₃, 0.8 mM CaCl₂, and 1 mM glucose; gas phase O₂/CO₂, 19:1) under constant agitation. Tissues were then incubated with 5 μCi of myo-[^2H]inositol (0.4 μM) in 0.8 ml of fresh buffer for 4 h, by which time the incorporation of[^2H] into inositol lipids has reached a plateau (Amiot et al., 1993). Myometrial strips were washed 3 times with nonradioactive Krebs’ buffer and transferred into 1 ml of fresh buffer and incubated for 20 min before the addition of 10 mM LiCl. After 10 min, the agents to be tested were added at the indicated concentrations, and incubation was further continued for the time indicated for the specific experiment. Reactions were stopped by immersing the tissue strips in 1.5 ml of cold 7% (w/v) trichloroacetic acid, followed by homogenization and centrifugation at 3000g for 15 min at 4°C. The trichloroacetic acid-soluble supernatants were extracted with diethyl ether, neutralized with Tris base, and applied to a column of the union exchange resin (AG 1-X8; formate form; 200–400 mesh) for the separation of the individual inositol phosphates as described previously (Marc et al., 1988; Amiot et al., 1993). Alternatively, total inositol phosphates [i.e., inositol triphosphate (InsP₃) plus inositol bisphosphate (InsP₂) plus inositol monophosphate (InsP₁)] were eluted together in a single step with 12 ml of 1 M ammonium formate plus 0.1 M formic acid. The[^2H] content of the fractions was determined by scintillation counting in QuickSafe A (Zinsser analytic). Results were expressed as cpm/100 mg of tissue or, alternatively, as a percentage of stimulation over the basal values obtained before the addition of the stimulatory agonist.

**Methods for Recording Uterine Contractile Responses.** The contractile activity of isolated myometrial strips was measured with an isometric transducing device. The segments were loaded at a basal tension of 0.2 to 0.3 g and were bathed at 37°C in 10 ml of Krebs’ buffer (95% O₂/5% CO₂) of the same salt composition as used for the above incubations. Contractile activity was integrated during a 2-min exposure to the indicated agent (Marc et al., 1988; Amiot et al., 1993; Palmier et al., 1996).

**Fura-2/AM Loading and Ca²⁺ Imaging in Isolated Uterine Myocytes.** The enzymatic dispersion procedure for isolating single myometrial cells from estrogen-treated rats was performed as described previously (Amédée et al., 1986; Dokhac et al., 1996). Uterine myocytes (8 × 10⁵ cells/ml) suspended in minimal essential medium with Earle’s balanced salts containing 10% (w/v) FCS were plated on collagen-coated glass coverslips and were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air for 20 to 24 h. Fura-2/AM loading and Ca²⁺ imaging of cells were carried out essentially as detailed elsewhere (Sauvadet et al., 1996). Briefly, cells attached to collagen were loaded for 20 min at 25°C with 2 μM Fura-2/AM in balanced salt solution (130 mM NaCl, 5.0 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose and 50 mM HEPES, pH 7.4) containing 1 mg/ml BSA. Cells were then rinsed twice with the balanced salt solution and allowed to incubate in the same buffer for 15 min at 25°C to facilitate hydrolysis of intracellular Fura-2/AM. For Ca²⁺ imaging, light from a 100-W xenon lamp was filtered alternately through 360- and 380-nm filters to determine the fluorescence ratio (F₃₆₀/F₃₈₀). Fura-2 fluorescence (Nikon UV-fluor × 40 objective) was filtered at 510 nm and recorded by an intensified CCD Photonic Science camera (Sauvadet et al., 1996). Each fluorescence image was the average of two images to improve the signal-to-noise ratio, and one average image was recorded every 3 s. Data are reported as the fluorescence ratio (F₃₆₀/F₃₈₀) after subtraction of the respective backgrounds. Tracings of fluorescence ratio are representative of at least six cells and were performed on two different cell isolations.

**Immunoblotting and Immunoprecipitation.** Myometrial strips (about 50 mg wet weight) were allowed to equilibrate for 20 min at 37°C in 5 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4 (gas...
phase O₂/CO₂, 19:1) under constant agitation. Tissue strips were then transferred into 1 ml of fresh buffer and further allowed to equilibrate for 10 min. The agents to be tested were added at the indicated concentration, and incubation was continued for the time indicated for the specific experiment. Reactions were stopped by immersion of the myometrial strips in liquid nitrogen. Frozen tissues were extracted in 600 μl of cold solubilization buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 100 mM NaF, 10 mM Na₂HPO₄, 200 mM Na₂VO₄, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin in 50 mM HEPES, pH 7.4), with an Ultra Turrax homogenizer as described previously (Palmier et al., 1996), with minor modifications. After 30 min at 4°C, the lysates were clarified by centrifugation (10,000g, 20 min at 4°C), and the protein content of the supernatant was determined (Lowry et al., 1951). In some experiments, detergent-extracted proteins (50 μg) were treated with Laemmli’s sample buffer (Laemmli, 1970) and resolved by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane for immunoblotting.

For immunoprecipitation experiments, detergent-extracted proteins (500 μg) were incubated with 5 μl of anti-PLC-γ1 rabbit polyclonal antibody overnight at 4°C and then with protein A-Sepharose (20 mg) for 2 h at 4°C. Immune complexes were collected by centrifugation at 10,000g for 60 s and washed five times with cold solubilization buffer and then once in cold PBS. Immunoprecipitated proteins were dissolved in 20 μl of 5% SDS and 25 mM dithiothreitol, heated for 10 min at 95°C, treated with Laemmli’s sample buffer, and subjected to 7.5% SDS-polyacrylamide gel electrophoresis. The separated proteins were then transferred to nitrocellulose for immunoblotting.

The nitrocellulose membranes were blocked for 1.5 h at 37°C with 3% BSA in TBS (Tris-buffered saline: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, containing 0.1% Tween 20). The sheets were then washed with TTBS. The blocked nitrocellulose sheets were blotted with the monoclonal anti-phosphotyrosine antibody (1:7500, 0.13 mM NaCl, containing 0.1% Tween 20). The sheets were then washed with TTBS. The immunoreactive bands were visualized by enhanced chemiluminescence system after sequential incubation with horseradish peroxidase-conjugated goat anti-mouse IgG for 60 min at room temperature. In certain experiments, the blots were reprobed after stripping in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol for 60 min at 57°C. Nitrocellulose sheets were rinsed in TBS and then reblocked with 3% BSA in TTBS and reprobed with monoclonal anti-PLC-γ1 antibody (0.5 μg/ml). Quantification of the developed blots was performed using a densitometer (Molecular Dynamics).

Data Analysis. The results are expressed as mean ± S.E.M. and were analyzed statistically using Student’s t test. P ≤ .05 was considered to be significant.

Results

Effect of Protein Tyrosine Kinase Inhibitors Genistein and Tyrphostins on Accumulation of Inositol Phosphates Triggered by Bombesin. Results in Table 1 show that the production of [³H]inositol phosphates, triggered by bombesin (Amiot et al., 1993), was attenuated (35–40%) in the presence of genistein, a PTK inhibitor (Akiyama et al., 1987). In experiments not reported (n = 3), it was found that genistein did not affect the potency of bombesin (EC₅₀ values for bombesin were 8.8 ± 0.9 and 9 ± 1 nM in control and genistein-treated tissues, respectively). Data in Fig. 1A show the inhibitory curve to increasing concentrations of genistein against bombesin-mediated inositol phosphate generation. Inhibition by genistein was dose dependent (IC₅₀ = 5 μM; maximal 40% inhibition at 50 μM). Pretreatment of the myometrium with 50 μM genistein resulted in an attenuation in the inositol phosphate response elicited by AIF₄⁻, a direct activator of G proteins.

Effects of Genistein on Oxytocin-, Carbachol-, ET-1-, and Fluoroaluminate-Mediated Inositol Phosphate Accumulation. Genistein inhibited not only the production of inositol phosphates triggered by bombesin (Table 1) but also that caused by oxytocin, carbachol, and ET-1, which activate the PLC pathway in the myometrium via their respective G protein-coupled receptors (Marc et al., 1988; Leiber et al., 1990; Amiot et al., 1993; Dokhae et al., 1994). The attenuation by 50 μM genistein of the generation of inositol phosphates for oxytocin, carbachol, and ET-1 averaged 32%, 37%, and 39%, respectively. Genistein (50 μM) was similarly able to inhibit by 44% the inositol phosphate response elicited by AIF₄⁻, a direct activator of G proteins.

Effects of Genistein on Myometrial Contractions Triggered by Bombesin and Carbachol. Treatment of myometrial strips with 20 μM genistein caused a rightward shift in the bombesin dose-response curve (Fig. 2), with an EC₅₀ value of 2 ± 0.3 and 7 ± 0.6 nM in the absence and presence of genistein, respectively. Maximal tension was achieved by increasing bombesin concentration, consistent with previous observations (Marc et al., 1988; Leiber et al., 1990; Amiot et al., 1993) that a suboptimal generation of InsP₃ is sufficient to cause maximal contraction. Data in Fig. 3A show the inhibitory curve to increasing concentration of genistein against carbachol-elicted contraction. The muscarinic agonist was used at 6 μM, a concentration that triggers almost maximal contractile activity but no more than 30% of the inositol phosphate response (Marc et al., 1988; Leiber et al., 1990). Genistein antagonized carbachol-elicted contrac-

| TABLE 1 Inhibitory effect of genistein on agonist- and fluoroaluminate-stimulated generation of inositol phosphates |
|---------------------------------|-----------------|
|                                  | Genistein        | + Genistein 50 μM |
| [³H]Inositol-phosphate values    |                 |                 |
| cpm/100 mg tissue × 10⁻³        |                 |                 |
| Control                         | 20 ± 2          | 20 ± 12         |
| Bombesin                        | 308 ± 30        | 208 ± 21* (35%) |
| Oxytocin                        | 355 ± 32        | 245 ± 25* (32%) |
| Carbachol                       | 160 ± 13        | 109 ± 8* (37%)  |
| ET-1                            | 290 ± 28        | 186 ± 17* (39%) |
| AIF₄⁻                          | 205 ± 19        | 124 ± 11* (44%) |

* P < .01 versus agonist alone.
Tyrosine Kinase Activation and Ca\(^{2+}\) Influx by GPCRs

Effects of Fluoroaluminates and Pertussis Toxin on Protein Tyrosine Phosphorylation. As shown in Fig. 5, the increase in tyrosine phosphorylation triggered by bombesin was totally insensitive to pertussis toxin, similar to the inability of the toxin to affect bombesin-mediated PLC activation (Amiot et al., 1993). The contribution of a G protein to the protein tyrosine phosphorylation process is illustrated in Fig. 5. AlF\(_4\) was able to induce enhanced protein tyrosine phosphorylation, which was potentiated by 3 \(\mu\)M pervanadate. The profile of tyrosine phosphorylated proteins was strikingly similar to that observed with agonists acting through seven transmembrane receptors.

Effects of Bombesin and ET-1 on Tyrosine Phosphorylation of PLC-\(\gamma\)1 in Rat Myometrium. To investigate whether PLC-\(\gamma\)1 was among the proteins that undergo tyrosine phosphorylation, myometrial strips were incubated in the presence of 100 nM bombesin added alone or in combination with 3 \(\mu\)M pervanadate. Equal amounts of protein from detergent-extracted myometrium were immunoprecipitated with anti-PLC-\(\gamma\)1, and Western blots of the precipitated proteins were probed with both the anti-phosphotyrosine and anti-PLC-\(\gamma\)1 antibodies. Immunoblot analysis with anti-PLC-\(\gamma\)1 antibodies (Fig. 6, B and D) showed that similar amounts of PLC-\(\gamma\)1 were present in the control and in the differentially stimulated preparations. Bombesin alone induced a transient increase in tyrosine phosphorylation of...
PLC-\(\gamma_1\) that peaked (less than 4-fold) at 30 s and then declined and reached basal levels after 1 min (Fig. 6A). In additional experiments, it was found that the increase in tyrosine-phosphorylated PLC-\(\gamma_1\) was similar in bombesin-treated myometrial strips, either unloaded or loaded under the conditions used for recording tension. A 3.6- and 2-fold stimulation of tyrosine-phosphorylated PLC-\(\gamma_1\) was obtained for loaded strips versus 3.2- and 1.2-fold stimulation for unloaded strips exposed to bombesin for 30 s and 1 min, respectively. Notably, the phosphotyrosine content of PLC-\(\gamma_1\) was augmented 5-fold when myometrial strips were stimulated for 30 s with bombesin in the presence of 3 \(\mu\)M pervanadate and was maintained for at least 10 min (Fig. 6C). The phosphorylation of PLC-\(\gamma_1\) was strongly reduced by the two protein tyrosine kinase inhibitors genistein at 50 \(\mu\)M and Tyr47 at 100 \(\mu\)M (not shown). Similar results were obtained for myometrial strips stimulated by ET-1 (100 nM), in which a 6- to 7-fold increase in tyrosine phosphorylation of PLC-\(\gamma_1\) was observed.

Data in Fig. 7 attempt to further correlate the level of tyrosine-phosphorylated PLC-\(\gamma_1\) with the extent of inositol phosphate production mediated by pervanadate, bombesin, and ET-1. There seems to be a close correlation between the gradual increase in tyrosine-phosphorylated PLC-\(\gamma_1\) with increasing pervanadate concentration (3, 10, and 25 \(\mu\)M) and

---

**Fig. 3.** Opposing effects of genistein and pervanadate on carbachol-mediated myometrial contraction. A, contractions were induced by an almost maximal concentration (6 \(\mu\)M) of carbachol, and increasing concentrations of genistein were added at 2-min intervals. B, Carbachol-mediated contraction in the presence of 0.11% dimethyl sulfoxide, which is the concentration of the vehicle used at the maximal dose of genistein. The degree of contraction is expressed as a percentage of the response due to carbachol alone (100%). C, contractile activity displayed by 1 \(\mu\)M carbachol (CB), 0.8 \(\mu\)M pervanadate (PV), or 1 \(\mu\)M carbachol plus 0.8 \(\mu\)M pervanadate without (open bars) or with (dotted bars) a 5-min prior treatment with 20 \(\mu\)M genistein. Where indicated (hatched bars), myometrial strips were exposed for 2 min to pervanadate (PV) before incubation with 20 \(\mu\)M genistein for 5 min, followed by the addition of carbachol or buffer. The degree of the contractile response is expressed as a percentage of the response to a maximally effective concentration (25 \(\mu\)M) of carbachol (=100%). Values are mean ± S.E.M. for three independent experiments.

---

**Fig. 4.** Effect of bombesin on protein tyrosine phosphorylation in rat myometrium. A, myometrial strips were treated with 100 nM bombesin (Bn) for the times indicated (lanes a–h). B, tissues were incubated for 20 min in the absence or presence of 3 \(\mu\)M pervanadate (PV), without or with the addition of 100 nM bombesin (Bn), for the times indicated (lanes a–e). When used, the specific antagonist \(\text{[D-phe}^6\text{]bombesin-(6–13) methyl ester (1 \(\mu\)M)}\) was added 10 min before the agonist. Phosphorylated proteins in detergent extracts were detected by immunoblotting with anti-phosphotyrosine antibodies. The positions of molecular mass markers (\(\times 10^{-3}\)) are shown. Bottom, densitometric quantification of tyrosine-phosphorylated proteins in the 120- to 130-kDa range (hatched bars) and in the 70- to 80-kDa range (open bars), determined with a densitometer (Molecular Dynamics). Absorbance is expressed in arbitrary units relative to control (= 1). Data represent one of four similar experiments.

---

**Fig. 5.** Effects of AlF\(_4^–\) and pertussis toxin on protein tyrosine phosphorylation in rat myometrium. Left, myometrial strips were incubated in the absence or presence of 3 \(\mu\)M pervanadate (PV) for 10 min and then were treated for an additional 10 min without or with 20 mM NaF plus 10 \(\mu\)M AlCl\(_3\). Right, tissues were incubated for 6 h in the absence or presence of 400 ng/ml pertussis toxin (PTX). Tissues were then washed with fresh buffer. Rechallenge incubations were conducted with 3 \(\mu\)M PV plus 100 nM bombesin (Bn) as described in the legend to Fig. 4. In both cases, phosphorylated proteins in detergent extracts were detected by immunoblotting with anti-phosphotyrosine antibodies. The antibodies were used alone or in combination with 0.5 mM phosphotyrosine (pY). Positions of molecular mass markers (\(\times 10^{-3}\)) are shown Data represent one of three similar experiments.
the corresponding ability of the protein tyrosine phosphatase inhibitor to gradually increase the production of inositol phosphates. For both bombesin- and ET-1-stimulated myometrium, the production of inositol phosphates which was sensitive to genistein was associated with a rather modest increase in the amount of tyrosine-phosphorylated PLC-γ1. Thus, for a PTK-dependent increase in inositol phosphate production higher to that achieved with 25 μM pervanadate, a small (5- to 6-fold) augmentation of tyrosine-phosphorylated PLC-γ1 was obtained with both bombesin and ET-1 versus a 56-fold increase in the level of tyrosine-phosphorylated PLC-γ1 associated with the PTP inhibitor. The data suggested that factors other than PLC-γ1 are probably involved in the genistein-sensitive production of inositol phosphates triggered by G protein-coupled receptors.

**Dihydropyridine-Sensitive Ca^{2+} Channels as Targets for Inhibitory Effect of Genistein and Tyr47.** Data in Fig. 8 show that omission of Ca^{2+} from the incubation medium resulted in a decrease (35%) in the amount of inositol phosphates generated by bombesin. The addition of nifedipine 1 min before bombesin caused a similar reduction in the agonist-mediated inositol phosphate response. The degree of inhibition caused by Ca^{2+} withdrawal or the addition of nifedipine was similar (40%) to that elicited by the tyrosine kinase inhibitors genistein and Tyr47 at their maximum effective concentrations. Both genistein and Tyr47 could no longer attenuate the inositol phosphate response when incubations were carried out in a Ca^{2+}-poor medium. Similarly, the simultaneous addition of both nifedipine and genistein or of nifedipine and Tyr47 gave inhibition no greater than that obtained with either agent alone. In addition, the inhibitory effects of genistein and Tyr47 on the increase in the generation of inositol phosphates due to bombesin were prevented by Bay K 8644, similar to the antagonistic effect exerted by the Ca^{2+} channel agonist on the nifedipine-induced inositol phosphate response. These data imply that the ability of the PTK inhibitors to attenuate the generation of inositol phosphates resulted from an inhibition of bombesin-mediated Ca^{2+} influx via nifedipine-sensitive Ca^{2+} channels. Similar findings were obtained with ET-1 (data not shown).

The importance of a PTK-regulated, voltage-gated Ca^{2+}-entry process in the contractile effect of bombesin is illustrated in Table 2. Inhibition caused by 20 μM genistein (Fig. 2 and Table 2) was comparable to that of 10 nM nifedipine. Contractions triggered by 0.5 and 2.5 nM bombesin were attenuated in the presence of nifedipine by 76 ± 8% and 57 ± 6%, respectively, and in the presence of genistein by 90 ± 9% and 64 ± 6%, respectively. Of interest, inhibitions by both nifedipine and genistein were abrogated by the Ca^{2+} channel agonist Bay K 8644.

As illustrated in Fig. 9A, the application of bombesin (100 nM) caused a transient intracellular Ca^{2+} concentration ([Ca^{2+}]_i) peak followed by a lower but sustained increase in [Ca^{2+}]_i (“plateau phase”). When genistein (20 μM) was applied 5 min before bombesin, the peptide evoked a smaller...
The data are consistent with the inhibition of Ca\(^{2+}\) influx by the PTK inhibitor.

**TABLE 2**

<table>
<thead>
<tr>
<th>Nifedipine</th>
<th>Genistein</th>
<th>Tyrophostin 47</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 nM</td>
<td>2.5 nM</td>
<td>2.5 nM</td>
</tr>
<tr>
<td>% of maximal contractile response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>21 (\pm) 2</td>
<td>75 (\pm) 7</td>
</tr>
<tr>
<td>Nifedipine (10 nM)</td>
<td>5 (\pm) 0.5(^a)</td>
<td>32 (\pm) 3(^a)</td>
</tr>
<tr>
<td>Genistein (20 (\mu)M)</td>
<td>2 (\pm) 0.2(^a)</td>
<td>27 (\pm) 4(^a)</td>
</tr>
<tr>
<td>Bay K 8644 (10 nM)</td>
<td>ND</td>
<td>83 (\pm) 9</td>
</tr>
<tr>
<td>Bay K 8644 (10 nM) + nifedipine (10 nM)</td>
<td>18 (\pm) 3(^a)</td>
<td>68 (\pm) 7(^a)</td>
</tr>
<tr>
<td>Bay K 8644 (10 nM) + genistein (20 (\mu)M)</td>
<td>16 (\pm) 2(^a)</td>
<td>70 (\pm) 8(^a)</td>
</tr>
</tbody>
</table>

**Discussion**

In the present study, we demonstrated in estradiol-treated rat myometrium that the stimulation of the PIP\(_2\)-PLC pathway by activated G protein-coupled receptors, as assessed by the increased production of InsP\(_3\), InsP\(_2\), and InsP\(_1\), and the attendant increase in muscle tension (Marc et al., 1988; Leiber et al., 1990; Amiot et al., 1993; Dokhac et al., 1994), were in part regulated by a tyrosine phosphorylation-dependent process. The production of inositol phosphates stimulated by bombesin was decreased in a dose-dependent manner by two sets of PTK inhibitors, genistein and active tyrphostins, with a maximal inhibition of 35% to 40%. Genistein similarly reduced inositol phosphate generation triggered by oxytocin, carbachol, and ET-1, acting via their respective receptors (Marc et al., 1988; Leiber et al., 1990; Dokhac et al., 1994), as well as by AlF\(_4^{-}\), a direct G protein activator (Marc et al., 1988). This is consistent with a PTK regulatory process operating downstream from receptor activation. A potential PTK-linked signal transduction pathway for the regulation of smooth muscle contraction has been suggested (Hollenberg, 1994) and was demonstrated to operate for uterine contraction triggered by perversanate, a potent PTP inhibitor (Palmier et al., 1996). The contractile action of G protein-coupled receptors such as angiotensin, histamine, and \(\alpha\)-adrenergic receptors in various smooth muscle systems is also inhibited by PTK inhibitors (Di Salvo, 1994; Hollenberg, 1994; Gould et al., 1995). Our data are in line with these observations because they illustrate the opposing effects (i.e., inhibitory and stimulatory) for genistein and perversanate, respectively, on agonist-mediated myometrial contraction.

An increase in cellular PTK activities triggered by bombesin was demonstrated by the ability of the peptide to cause a transient increase in the tyrosine phosphorylation of several proteins in the 70- to 80-kDa and 120- to 130-kDa range. The potentiated increase in tyrosine phosphorylation observed if perversanate was also present is consistent with a potential role for PTPs (Fischer et al., 1991) in controlling phosphotyrosine levels in the myometrium. Pharmacological evidence was further provided that both PLC activation and enhanced protein tyrosine phosphorylation were triggered by the same subclass of bombesin receptors. Our finding that AlF\(_4^{-}\) gave a similar pattern of enhanced tyrosine protein phosphorylation revealed the involvement of heterotrimeric...
G proteins. The tyrosine phosphorylation of proteins triggered by bombesin was insensitive to pertussis toxin. The toxin also has no effect on PLC activation, indicating that the putative G protein that is involved in both receptor-mediated effects is, at least in part, represented by Gq/G11 (Lajat et al., 1996).

It is well known that PLC-γ isoforms are activated by phosphorylation on specific tyrosine residues (Carpenter et al., 1993; van der Geer and Hunter, 1994). PLC-γ has been identified as a potential substrate for several tyrosine kinases, as well as for nonreceptor tyrosine kinases (Liao et al., 1993; van der Geer and Hunter, 1994; Marrero et al., 1995). It has also been shown that the increase in PIP2 hydrolysis triggered by G protein-coupled receptors such as the M1 muscarinic (Gusovsky et al., 1993) or the angiotensin AT1 receptor (Marrero et al., 1994, 1995) is in part regulated via a PTK pathway that appears to be concomitant with phosphorylation of tyrosinases in PLC-γ1. We recently identified myometrium PLC-γ1 as one of the proteins that is tyrosine phosphorylated on stimulation with pervanadate in association with both increased generation of inositol phosphates and enhancement of muscle tension (Palmier et al., 1996). In this study, we provide further evidence for a close correlation between the tyrosine phosphorylation status of PLC-γ1 and the level of inositol phosphate production triggered by different concentrations of pervanadate, supporting the idea that the two events may be causally related. Stimulation by bombesin and ET-1, particularly if combined with a low concentration of pervanadate, led to an increase in the phosphotyrosine content of PLC-γ1. However, compared with phosphorylation obtained with high doses of pervanadate, a very low level of tyrosine-phosphorylated PLC-γ1 was associated with activation of G protein-coupled receptors and accounted at best for no more than 5% to 10% of the PTK-dependent production of inositol phosphates. Our data strongly suggest that in the rat myometrium, factors other than phosphorylated PLC-γ1 are involved in the PTK-dependent production of inositol phosphates mediated by G protein-coupled receptors.

Previous results from our laboratory (Dokhc et al., 1992, 1996) showed that at least two distinct mechanisms underlie the activation of PLC in myometrium by carbachol and oxytocin. One mechanism concerns the well recognized agonist-induced activation of the receptor-Gαi protein-PLCβ cascade (Lajat et al., 1996), which appears to be insensitive to increases in intracellular Ca2+. A second, Ca2+-dependent pathway involves the stimulation of PLC activity via an increase in Ca2+ influx, resulting from the activation of voltage-gated Ca2+ channels. Both the Ca2+-dependent and -independent processes are involved in the rapid breakdown of PIP2 with the concomitant production of active Ins(1,4,5)P3 (Dokhc et al., 1992). This study extends these observations to bombesin and ET-1 and provides evidence for PTK interference in the Ca2+ entry-dependent process involved in PLC activation. The increased generation of inositol phosphates and the uterine contractions triggered by activated bombesin receptors were attenuated to the same extent by either genistein or nifedipine, and both inhibitory effects were abolished by the Ca2+ channel agonist Bay K 8644 (Triggle and Rampe, 1989). Our findings that genistein decreased both the peak and sustained [Ca2+]i triggered by bombesin are consistent with a number of observations reporting the inhibitory effects of genistein on agonist-mediated Ca2+ mobilization (Di Salvo et al., 1994; Gould et al., 1995; Liu and Sturek, 1996). The possibility of a direct blocking action of genistein on Ca2+ channels cannot be excluded (Kusaka and Sperelakis, 1995), but the similarity, noted here, between the effects of genistein and another, structurally unrelated PTK inhibitor (Levitzki, 1992), Tyr47 (and Tyr25), provides evidence for a tyrosine phosphorylation-dependent effect. Collectively, the data support the contention that voltage-sensitive Ca2+ channel activity is the predominant target for the PTK-dependent regulatory process that contributes to agonist-mediated inositol phosphate production and contraction and that there is no major role for PTK regulation at a step distal to the channel. Our finding that phosphorylated PLC-γ1 palys a minor role in the agonist-mediated production of inositol phosphates is consistent with a recent report by Di Salvo and Nelson (1998) demonstrating in vascular smooth muscle cells that the tyrosine phosphorylation of PLC-γ1 is not required for PTK-dependent increases in intracellular calcium concentration triggered by the stimulation of diverse G protein-linked receptors.

PTK activity has been suggested to control Ca2+ entry induced by G protein-coupled receptors in various smooth muscles (Gusovsky et al., 1993; Liu and Sturek, 1996). Evidence has also been provided that PTKs exert a stimulatory modulation of L-type Ca2+ channels in pituitary GH3 cells (Cataldi et al., 1996) and in various smooth muscle cell preparations (Wijetunge and Hughes, 1995; Hatakeyama et al., 1996), including pregnant rat myometrial cells (Kusaka and Sperelakis, 1995). It is unclear whether the tyrosine residues of the channel itself become phosphorylated or whether some intermediate messenger regulates the activity of the channel.

In summary, the data presented here are consistent with two cascades of events for GRP-prefering bombesin receptors: 1) bombesin receptor stimulation—activation of Gαi/G11—stimulation of PLC-β3—stimulation of inositol phosphate production and 2) bombesin receptor stimulation—activation of Gαq/G11—opening of voltage-gated Ca2+ channels with an increase in the influx of Ca2+—stimulation of PIP2-PLC activity. Three isoforms of PIP2-PLC (PLC-β3, PLC-γ1, and PLCδ) have been identified in rat myometrium (Lu et al., 1995; Lajat et al., 1996; Palmier et al., 1996). The PLC isoform that is regulated via an increase in Ca2+ influx remains to be identified. Cascades 1 and 2 account for 65% and 35% of bombesin-mediated inositol phosphate production, respectively. Both cascades appear to operate for two other G protein-coupled receptors: endothelin and muscarinic receptors. Although it is well accepted that tyrosine phosphorylation events are induced by G protein activation (Lev et al., 1995; Malarkey et al., 1995; Post and Heller Brown, 1996), work mentioning are two recent studies (Liu et al., 1996; Umemori et al., 1997) that have shown that the tyrosine phosphorylation of Gαq/G11 subunits by protein tyrosine kinases may increase their ability to convey agonist-mediated activation of PLC. The possibility for a tyrosine phosphorylation step operating at the level of Gαq/G11 and its potential contribution to the PTK-dependent regulation have not been addressed in this report and would be worth considering. It will also be interesting to identify the PTK or PTKs that trigger the observed protein tyrosine phosphorylation and activation processes in the myometrium and to determine their mech-
anism of activation by G protein-coupled receptors. These and other concerns are the subject of our current studies.

Acknowledgments

We are grateful to Dr. Françoise Pecker for extremely helpful advice and assistance with the Ca²⁺ measurement studies and to Dr. Philippe Jourdon for critical discussions. We also thank Gisèle Thomas for scanning analysis and help with the figures.

References


Di Salvo JG, Pfitzer G and Semenchuck LA (1994) Protein tyrosine phosphorylation, and other concerns are the subject of our current studies.


Send reprint requests to: Dr. Denis Leiber, Laboratoire de Signalisation et Regulations Cellulaires, CNRS, EP 1088, Bâtiment 430, Université Paris-Sud, 91405 Orsay Cedex, France.