The signal transduction cascade which initiates transmembrane influx of Ca\textsuperscript{2+} into endothelial cells in response to the discharge of intracellular Ca\textsuperscript{2+} stores is thought to involve a step sensitive to tyrosine kinase inhibition. We investigated the interrelationship between Ca\textsuperscript{2+} signaling and protein tyrosine phosphorylation following cell stimulation with either the receptor-dependent agonist, bradykinin, or the protein-tyrosine phosphatase inhibitor, phenylarsine oxide. In cultured human endothelial cells phenylarsine oxide instigated a concentration-dependent increase in the intracellular concentration of free Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}). This increase in [Ca\textsuperscript{2+}]\textsubscript{i} was not associated with the tyrosine phosphorylation of phospholipase C\gamma, enhanced formation of inositol 1,4,5-trisphosphate, or the rapid depletion of intracellularly stored Ca\textsuperscript{2+} but was coincident with the enhanced and prolonged tyrosine phosphorylation of a marked number of cytoskeletal proteins. In bradykinin-stimulated cells the tyrosine phosphorylation of the same cytoskeletal proteins (most notably 85- and 100-kDa proteins) was transient when cells were stimulated in the presence of extracellular Ca\textsuperscript{2+}, was maintained under Ca\textsuperscript{2+}-free conditions, and was reversed following readuction of extracellular Ca\textsuperscript{2+}. These data suggest that the tyrosine phosphorylation of 2 cytoskeletal proteins is determined by the level of Ca\textsuperscript{2+} present in intracellular stores thus indicating a critical role for tyrosine phosphorylation in the control of capacitative Ca\textsuperscript{2+} entry in endothelial cells.

There is a certain amount of evidence to suggest that the tyrosine phosphorylation of, as yet unidentified, cellular proteins may be involved in the control of store-regulated or "capacitative" Ca\textsuperscript{2+} entry following the agonist-induced depletion of intracellular stores in non-excitable cells. The first evidence for such a role of tyrosine kinases in intracellular Ca\textsuperscript{2+} signaling was obtained in platelets, in which the tyrosine phosphorylation of a group of proteins was found to be transiently elevated following stimulation with thrombin (1–3). This thrombin-induced protein tyrosine phosphorylation could be mimicked by the depletion of intracellular Ca\textsuperscript{2+} stores following inhibition of the Ca\textsuperscript{2+}-ATPase and was sensitive to the chelation of intracellular Ca\textsuperscript{2+} by Ryanodine. Repletion of Ca\textsuperscript{2+} stores was, on the other hand, associated with a return to basal phosphorylation levels (1). These observations inferred that the depletion of intracellular Ca\textsuperscript{2+} stores favors tyrosine phosphorylation whereas store refilling and the restoration of homeostatic levels of [Ca\textsuperscript{2+}]\textsubscript{i} favors tyrosine dephosphorylation of specific proteins (1). In support of these findings, protein-tyrosine kinase inhibitors such as genistein were reported to inhibit both the thrombin-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i}, as well as the subsequent aggregation (2). Since genistein has been found to attenuate Ca\textsuperscript{2+} influx following cell stimulation with both receptor-dependent and receptor-independent agonists, it would appear that the tyrosine kinase substrate protein is likely to be intimately involved in the regulation of Ca\textsuperscript{2+} entry processes rather than being linked to specific cell receptors. Recently we have demonstrated that the bradykinin as well as the thapsigargin-induced Ca\textsuperscript{2+} influx in endothelial cells is also mediated by a tyrosine kinase inhibitor-sensitive mechanism (4). Although the chain of events which result in the enhanced membrane permeability to Ca\textsuperscript{2+} remain largely unexplained, these observations suggest that a tyrosine-phosphorylated protein might be involved in the regulation of Ca\textsuperscript{2+} influx. Therefore the aim of the present study was to address the hypothesis that protein tyrosine phosphorylation controls capacitative Ca\textsuperscript{2+} influx in human endothelial cells. To this end we investigated the effects of agonist stimulation on tyrosine phosphorylation as well as the effects on [Ca\textsuperscript{2+}]\textsubscript{i}, of altering the balance between tyrosine kinase and phosphatase activity.

**EXPERIMENTAL PROCEDURES**

Materials—Genistein, ionomycin, and fura-2/AM were obtained from Calbiochem-Novabiochem GmbH, thapsigargin was obtained from Research Biochemicals International, and BAPTA/AM\textsuperscript{1} and Pluronic F-127 were from Molecular Probes. HEPES was from Serva, M-119 medium was from Life Technologies, Inc., and penicillin, streptomycin, L-glutamine, glutathione, and L-(+)-ascorbic acid (Biokt protection medium) were from Biochrom. Bradykinin, phenylarsine oxide, 2,3-dimercaptopropanol, orthovanadate, and all other substances were obtained from Sigma.

Cell Culture—Human umbilical vein endothelial cells, isolated from umbilical cords as described (5), were seeded either on quartz coverslips or in culture dishes (35 mm, Falcon) containing medium M-119 and 20% heat-inactivated fetal calf serum (Vitromex) supplemented with penicillin (50 units/ml), streptomycin (50 \mu g/ml), L-glutamine (10 mM), glutathione (5 mM), and L-(+)-ascorbic acid (5 mg/ml). [Ca\textsuperscript{2+}]\textsubscript{i} was estimated in cells grown on coverslips for 24 h.

Measurement of [Ca\textsuperscript{2+}]\textsubscript{i}—For the measurement of [Ca\textsuperscript{2+}]\textsubscript{i}, endothelial cells were loaded with the fluorescent Ca\textsuperscript{2+}-sensitive dye fura-2 by incubation with 3 \mu M fura-2/AM and 0.025% (w/v) Pluronic F-127 at 37°C for 90 min. Thereafter the coverslips were washed in HEPES-modified Tyrode solution of the following composition (mM): NaCl, 132; KCl, 4; CaCl\textsubscript{2}, 1; MgCl\textsubscript{2}, 0.5; HEPES, 9.5; glucose, 5. [Ca\textsuperscript{2+}]\textsubscript{i} was determined by guest on July 25, 2018http://www.jbc.org/Downloaded from

\* This study was supported by the Deutsche Forschungsgemeinschaft (Bu 436/4–3) and the Deutsche Gesellschaft für Herz- und Kreislaufforschung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\dag To whom correspondence should be addressed: Zentrum der Physiologie, Klinikum der J. W. Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt/Main, Germany. Tel.: 49-69-6301-6052; Fax: 49-69-6301-7668; E-mail: busse@merlin.add.uni-frankfurt.de.

\textsuperscript{1}The abbreviations used are: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; IP3, inositol 1,4,5-trisphosphate; MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; GTP\textsubscript{S}, guanosine 5’-3-O-thiotriphosphate.
determined fluorometrically in a thermostatically controlled cuvette as described previously (6), and calculated as recommended by Grynkiewicz et al. (7). Preliminary experiments demonstrated that incubation of endothelial cells with the membrane permeable Ca²⁺ chelator BAPTA-AM (10 μM, 30 min) was sufficient to abrogate the bradykinin (100 nM)-induced Ca²⁺ response (data not shown).

In fura-2-loaded confluent primary cultures of human umbilical vein endothelial cells were washed twice in HEPES-Tyrode solution and incubated at 37 °C with or without various receptor-dependent and -independent stimuli as described under "Results." Thereafter, cells were washed with ice-cold HEPES buffer containing sodium fluoride (100 mM), Na₄P₂O₇ (15 mM), Na₃VO₄ (2 mM), leupeptin (2 μg/ml), pepstatin A (2 μg/ml), trypsin inhibitor (10 μg/ml), and phenylmethylsulfonyl fluoride (44 μg/ml) and harvested by scraping. The cell suspension was centrifuged at 13,000 × g for 60 s, cells contained in the pellet were then lysed in buffer containing 1% (w/v) Triton X-100, on ice for 5 min, and centrifuged at 10,000 × g for 10 min. Approximately 30 μg of protein from the resulting supernatant or from the Triton X-100 insoluble fraction was separated by 10% or 7% SDS-polyacrylamide gel electrophoresis, respectively, as described (4). Tyrosine-phosphorylated proteins were detected with a mouse monoclonal anti-phosphotyrosine antibody (1 μg/ml) (Upstate Biotechnology Inc.) and were visualized by enhanced chemiluminescence using a commercially available kit (Amersham). Prestained molecular weight marker proteins (Bio-Rad) were used to identify markers for the SDS-polyacrylamide gel electrophoresis.

Immunoprecipitation of Tyrosine-phosphorylated Proteins—The Triton X-100-soluble 10,000 × g supernatant from control and stimulated endothelial cells was prepared as described and 100 μg of protein was diluted in 500 μl of radioimmunoprecipitation assay (RIPA) buffer containing Tris-HCl, pH 7.5 (50 mM), NaCl (150 mM), EGTA (1 mM), sodium deoxycholate (0.25%), Nonidet P-40 (1%), Na₃VO₄ (1 mM), sodium fluoride (1 mM), leupeptin (2 μg/ml), pepstatin A (2 μg/ml), trypsin inhibitor (10 μg/ml), and phenylmethylsulfonyl fluoride (44 μg/ml). Tyrosine-phosphorylated proteins were precipitated by shaking gently overnight at 4 °C with 50 μl of an anti-phosphotyrosine antibody covalently linked to agarose (Upstate Biotechnology Inc.). The agarose was recovered by centrifugation, washed 5 times with RIPA buffer, resuspended in SDS sample buffer, and boiled for 10 min. Phospho- lase Cγ, PLCγ present in the resulting supernatant was analyzed by Western blotting and detected with an anti-bovine monoclonal antibody (Upstate Biotechnology Inc.).

Assay of Inositol 1,4,5-Trisphosphate—Confluent cultures of human umbilical vein endothelial cells were washed twice with HEPES-Tyrode solution and allowed to equilibrate for 20 min. Thereafter, cells were stimulated with either bradykinin (100 nM) or phenylarsine oxide (10 μM) for the times indicated under "Results." The incubation was stopped by the aspiration of the HEPES-Tyrode and addition of 400 mM ice-cold 6% (v/v) trichloroacetic acid. Samples were then left on ice for 30 min, cells and supernatant were then harvested by scraping and the precipitation centrifuged at 2000 × g (15 min, 4 °C). The supernatants were then extracted 4 times with 5 volumes of water-saturated diethyl ether and were neutralized by titration with NaHCO₃. Inositol 1,4,5-trisphosphate (IP₃) in the samples was then assayed using a commercially available kit (Biotrak, Amersham).

Protein-tyrosine Phosphatase Assay—Protein-tyrosine phosphatase activity was assayed in whole cell lysates from cells stimulated as described under "Results." Activity was determined by monitoring the dephosphorylation of the phosphopeptide RRLIEDAEYARG using a commercially available kit (Upstate Biotechnology Inc.).

Statistical Analysis—Unless otherwise indicated data are expressed as mean ± S.E. Statistical evaluation was performed using Student's t test for unpaired data, one-way analysis of variance (ANOVA) followed by a Bonferroni t test, or ANOVA for repeated measures where appropriate. Values of p < 0.05 were considered statistically significant.

RESULTS

Effect Phenylarsine Oxide on [Ca²⁺]i—In fura-2-loaded human endothelial cells the protein-tyrosine phosphatase inhibitor, phenylarsine oxide induced a slightly delayed and concentration-dependent (1–10 μM) increase in [Ca²⁺]i (Fig. 1A). Concentrations of phenylarsine oxide lower that 1 μM failed to have any effect on [Ca²⁺]i, while the addition of 3 μM phenylarsine oxide resulted in a gradual increase in [Ca²⁺]i, and consistently produced low frequency [Ca²⁺]i oscillations which were synchronized throughout the entire cell population. Preliminary evidence indicates that this phenomenon is associated with a concomitant oscillation in membrane potential attributed to the activation of Ca²⁺-activated K⁺ channels (not shown). The highest concentration of phenylarsine oxide used (10 μM) produced a rapid increase in [Ca²⁺]i, which in 40% of the cells tested were biphasic in nature, consisting of an initial Ca²⁺ peak followed by a second more delayed Ca²⁺ increase. Oscillations in [Ca²⁺]i were rarely observed in the presence of the highest concentration of phenylarsine oxide (10 μM). The tyrosine kinase inhibitor genistein (100 μM), which has previously been shown to attenuate bradykinin-stimulated Ca²⁺-influx in endothelial cells (4), markedly reduced the Ca²⁺ response to phenylarsine oxide, [Ca²⁺]i, as measured 10 min after the addition of phenylarsine oxide (10 μM), was 522 ± 30 nM in solvent-treated cells compared with 197 ± 14 nM in cells preincubated with genistein (p < 0.01, Fig. 1B).

The phenylarsine oxide-induced increase in [Ca²⁺]i, was completely reversed by the diithiole reagent 2,3-dimercaptopropanol (50 μM) which binds to vicinal sulfhydryl groups (Fig. 2). In these experiments, the subsequent addition of bradykinin (100 nM) resulted in a normal Ca²⁺ response suggesting that the effects of phenylarsine oxide were completely reversible and that the inhibitor did not lead to the permanent uncoupling of the agonist-induced signal transduction pathway.

In the absence of extracellular Ca²⁺, phenylarsine oxide (10 μM) induced a small, slowly developing, increase in [Ca²⁺]i, (after 10 min [Ca²⁺]i, had increased from 69.7 ± 12 nM to 106.5 ± 23 nM, n = 8, p < 0.001; Fig. 3). Subsequent addition of the Ca²⁺-ATPase inhibitor, thapsigargin (0.3 μM), resulted in an immediate further increase in [Ca²⁺]i, ([Ca²⁺]i, increased from 104 ± 24 to 218 ± 15 nM; n = 4, p < 0.01) demonstrating that the protein-tyrosine phosphatase inhibitor did not deplete intracellular Ca²⁺ stores. In cells stimulated with phenylarsine oxide in the absence of extracellular Ca²⁺, the readdition of Ca²⁺ was associated with an immediate increase in [Ca²⁺]i (Fig. 3).

Effects of Phenylarsine Oxide and Bradykinin on PLCγ Phosphorylation and IP₃ Production—In order to establish that the...
Tyrosine Phosphorylation and Ca\(^{2+}\) Influx in Endothelial Cells

The dithiol agent 2,3-dimercaptopropanol (DMP) reverses the increase in intracellular Ca\(^{2+}\) elicited by phenylarsine oxide (PAO). The effects of DMP (50 \(\mu\)M) were tested on fura-2-loaded human endothelial cells exposed to PAO (10 \(\mu\)M) for 10 min. Following the return of [Ca\(^{2+}\)]\(_i\) to baseline levels, cells were stimulated with the receptor-dependent agonist, bradykinin (100 nM). The results are presented as the mean \(\pm\) S.E. of data obtained in five separate experiments.

Effects of the tyrosine phosphatase inhibitor on [Ca\(^{2+}\)]\(_i\) were not related to the depletion of intracellular Ca\(^{2+}\) stores. The effects of phenylarsine oxide on the tyrosine phosphorylation of PLC\(_Y\) and production of IP\(_3\) were compared with those of the receptor-dependent agonist, bradykinin.

When tyrosine-phosphorylated proteins were immunoprecipitated from endothelial cells treated with phenylarsine oxide (10 \(\mu\)M) and blotted with anti-PLC\(_Y\), a clear signal was apparent in control cells but could not be detected in cells incubated with the tyrosine phosphatase inhibitor for up to 5 min (Fig. 4A). Stimulation of endothelial cells with bradykinin (100 nM), on the other hand, resulted in a rapid increase in the tyrosine phosphorylation of PLC\(_Y\), with a 3.5-fold increase in tyrosine-phosphorylated protein being detected within 30 s (Fig. 4B). The bradykinin-induced tyrosine phosphorylation of PLC\(_Y\) was relatively transient and tyrosine phosphorylation of the PLC\(_Y\) returned to near basal levels within 2 min.

In accordance with its effect on the tyrosine phosphorylation of PLC\(_Y\), phenylarsine oxide (10 \(\mu\)M) failed to precipitate an increase in intracellular levels of IP\(_3\) at any of the time points measured. In bradykinin-treated endothelial cells IP\(_3\) levels increased 7-fold within 10 s and had returned to baseline values within 1 min (Fig. 4C).

These observations demonstrate that the phenylarsine oxide-induced increase in [Ca\(^{2+}\)]\(_i\) is not due to the rapid depletion of intracellular stores and that activation of a transmembrane influx accounts for most of the tyrosine phosphatase inhibitor-induced increase in [Ca\(^{2+}\)]. As a consequence of its effects on fura-2 fluorescence it was not possible to repeat these experiments using a second widely used tyrosine phosphatase inhibitor, sodium orthovanadate.

Effect of Tyrosine Phosphatase Inhibitors on Triton-soluble Proteins—Since the overall cellular level of tyrosine phosphorylation is determined by the delicate balance between the activity of tyrosine kinases and tyrosine phosphatases, inhibition of dephosphorylation would be expected to result in a net increase in detectable phosphotyrosine-containing proteins. In cultured human endothelial cells, phenylarsine oxide (10 \(\mu\)M) induced a clear time-dependent increase in the tyrosine phosphorylation of a triplet of bands centered at \(-80\) kDa as well as a 42/44-kDa doublet (Fig. 5A). The latter proteins were identified in immunoprecipitation experiments as the 42- and 44-kDa isoforms of the mitogen-activated protein kinase (MAP kinase; not shown). The enhanced tyrosine phosphorylation of MAP kinase was evident 2 min after addition of the inhibitor and was maximal after 5–10 min. These effects of phenylarsine oxide were not observed in endothelial cells pretreated with the tyrosine kinase inhibitor genistein (100 \(\mu\)M, 10 min: not shown).

In order to evaluate the role of [Ca\(^{2+}\)]\(_i\) in phenylarsine oxide-stimulated tyrosine phosphorylation, cells were either incubated in a nominally Ca\(^{2+}\)-free buffer or pretreated with the intracellular Ca\(^{2+}\) chelator BAPTA (10 \(\mu\)M, 30 min). Phenylarsine oxide-induced tyrosine phosphorylation of the 42/44-kDa doublet was largely Ca\(^{2+}\)-dependent since a slight increase in tyrosine phosphorylation was observed in cells stimulated in the absence of Ca\(^{2+}\) but no increase was observed in BAPTA-treated cells. The tyrosine phosphorylation of the \(-80\)-kDa triplet was, on the other hand, largely Ca\(^{2+}\)-independent (Fig. 5B).

A second protein-tyrosine phosphatase inhibitor, sodium orthovanadate (0.3 \(\mu\)M), also resulted in a genistein-sensitive, time-dependent increase in the tyrosine phosphorylation of the 42- and 44-kDa isoforms of MAP kinase as well as the \(-80\)-kDa...
Effect of Tyrosine Phosphatase Inhibitors on Triton-insoluble Cytoskeletal Proteins—Protein-tyrosine phosphatase inhibition had much more rapid and pronounced effects on levels of phosphotyrosine containing proteins in the Triton X-100-insoluble (cytoskeletal) fraction. Within 30 s phenylarsine oxide induced phosphorylation of 5 distinct bands, corresponding to estimated molecular masses of 70, 75, 77, and a doublet of 220 kDa, while a series of bands of approximately 100, 130, and 265 kDa were apparent after 1 to 2 min (Fig. 7A).

Pretreatment of endothelial cells with the tyrosine kinase inhibitorgenisteintended to attenuate the phenylarsine oxide-induced tyrosine phosphorylation although the effects were by no means prevented by the inhibitor (Fig. 7B). Almost complete reversal of the phenylarsine oxide-induced increase in tyrosine phosphorylation was achieved by subsequent addition of 2,3-dimercaptopropanol (50 μM; Fig. 7C). The removal of extracellular Ca²⁺ or the chelation of intracellular Ca²⁺ failed to alter the phenylarsine oxide induced increase in tyrosine phosphorylation of cytoskeletal proteins (Fig. 8).

Effect of Bradykinin on Triton-soluble and -insoluble Proteins—In order to investigate the putative link between Ca²⁺ signaling and the tyrosine phosphorylation of specific proteins, we studied the effect of extracellular Ca²⁺ removal and read- dition on bradykinin-stimulated tyrosine phosphorylation.

In the presence of extracellular Ca²⁺, bradykinin (100 nM) induced an immediate increase in the tyrosine phosphorylation of 3 Triton-soluble proteins (60, 77, and 86 kDa). Phosphorylation of these proteins was maximal after 30 s and returned to control levels over 5 min (Fig. 9A). In the same cells bradykinin also induced tyrosine phosphorylation of the 42/44-kDa MAP kinase doublet which was detectable 2 min after agonist stimulation and was maximal after 5 min, as described previously (4). Stimulation of endothelial cells in the absence of extracellular Ca²⁺ failed to alter phosphorylation of the 60-, 77-, or 86-kDa proteins but resulted in only a transient increase in the

**Fig. 5.** Phenylarsine oxide enhances the tyrosine phosphorylation of Triton X-100-soluble proteins, including the p42 and p44 MAP kinases, in human endothelial cells. Triton-soluble proteins from human endothelial cells were (A) incubated in the presence or absence of phenylarsine oxide (PAO; 10 μM) for the indicated times or (B) incubated with PAO (10 μM, 2 min) in the presence or absence of extracellular Ca²⁺, following pretreatment or not with the intracellular Ca²⁺ chelator BAPTA (10 μM; 30 min). Triton X-100-soluble proteins were separated by SDS-PAGE and tyrosine-phosphorylated proteins were detected using a specific antiphosphotyrosine antibody as described under “Experimental Procedures.” The results presented are representative of experiments performed using seven different cell batches.

**Fig. 6.** Orthovanadate time dependently enhances the tyrosine phosphorylation of Triton X-100-soluble proteins, including the p42 and p44 MAP kinases, in human endothelial cells. Human endothelial cells were incubated in the presence or absence of sodium orthovanadate (0.3 mM) for the times indicated. Proteins were separated by SDS-PAGE and tyrosine-phosphorylated proteins were detected using a specific antiphosphotyrosine antibody as described under “Experimental Procedures.” The results presented are representative of experiments performed using two different cell batches.

**Fig. 7.** The phenylarsine oxide-induced increase in the tyrosine phosphorylation of Triton X-100-insoluble (cytoskeletal) proteins is attenuated in the presence of the tyrosine kinase inhibitor genistein and following administration of the dithiol agent 2,3-dimercaptopropanol (DMP). Triton-soluble proteins from human endothelial cells were: A, incubated in the presence or absence of phenylarsine oxide (PAO; 10 μM) for the indicated times; B, incubated with PAO (10 μM, 10 min) in the presence or absence of genistein (100 μM); or C, incubated or not with DMP (50 μM) following exposure to PAO (10 μM) for 10 min. Cytoskeletal proteins were separated by SDS-PAGE and tyrosine-phosphorylated proteins were detected using a specific antiphosphotyrosine antibody as described. The results presented are representative of experiments performed using four different cell batches.
Tyrosine Phosphorylation and Ca\(^{2+}\) Influx in Endothelial Cells

with estimated molecular masses of 85, 100, 110, and 125 kDa which returned to baseline levels after 5–10 min (Fig. 10A). In the absence of extracellular Ca\(^{2+}\), bradykinin induced a distinct increase in the phosphorylation of the 85- and 100-kDa proteins which was immediately reversed upon readdition of extracellular Ca\(^{2+}\) (Fig. 10B).

Effect of Thapsigargin on Triton-soluble and -Insoluble Proteins—Thapsigargin (100 nM) induced tyrosine phosphorylation of the 42- and 44-kDa isoforms of the MAP kinase. Tyrosine phosphorylation of the 42/44-kDa doublet was not evident in cells stimulated in the absence of extracellular Ca\(^{2+}\) but a distinct tyrosine phosphorylation of both bands was detected 5 min after the readdition of Ca\(^{2+}\) to the incubation medium (not shown). These findings are in line with previously published results (4).

Thapsigargin (100 nM) induced the tyrosine phosphorylation of 4 proteins in the Triton-insoluble fraction corresponding to molecular masses of 85, 100, 110, and 125 kDa. However, the tyrosine phosphorylation of these bands was not transient, as was observed following cell stimulation with bradykinin, but was maintained for up to 10 min. Removal of extracellular Ca\(^{2+}\) did not influence the pattern of tyrosine phosphorylation which emerged following stimulation with thapsigargin, and the readdition of extracellular Ca\(^{2+}\) to depleted cells was not associated with a visible change in the phosphorylation pattern (Fig. 11).

Effects of Phenylarsine Oxide and Bradykinin on Tyrosine Phosphatase Activity—Human endothelial cells were found to express a basal tyrosine phosphatase activity which was attenuated in the presence of both phenylarsine oxide and orthovanadate. Bradykinin induced a 2-fold increase in phosphatase activity which was inhibited in cells pretreated with either phenylarsine oxide or sodium orthovanadate (Table I).

DISCUSSION

Over the last few years there have been several reports that the transmembrane influx of Ca\(^{2+}\) is selectively attenuated in a number of cell types following inhibition of tyrosine kinases (1, 8–10), thus suggesting that cellular levels of tyrosine phosphorylation play a determinant role in regulating Ca\(^{2+}\) entry in non-excitable cells. In the present study the protein-tyrosine phosphatase inhibitor, phenylarsine oxide, induced a concentration-dependent increase in [Ca\(^{2+}\)]\(i\), and elicited the tyrosine phosphorylation of a number of endothelial proteins with the most marked effects being apparent in the Triton X-100-insoluble, or cytoskeletal, fraction. Both the increase in [Ca\(^{2+}\)]\(i\), and the enhanced tyrosine phosphorylation were attenuated in cells pretreated with the tyrosine kinase inhibitor, genistein, supporting the hypothesis that a tyrosine-phosphorylated protein may be involved in the regulation of [Ca\(^{2+}\)]\(i\), in endothelial cells.

In principle there are two ways by which an agonist can induce capacitative Ca\(^{2+}\) entry. The activation of the transmembrane Ca\(^{2+}\) influx pathway could be attributed to an indirect, i.e. instigation of capacitative Ca\(^{2+}\) entry following the mobilization of intracellularly stored Ca\(^{2+}\), or a direct effect, i.e. activation of a Ca\(^{2+}\) influx regulatory protein. In contrast to the receptor-dependent agonist, bradykinin, phenylarsine oxide failed to tyrosine phosphorylate PLC\(\gamma\) or increase cellular levels of IP\(_3\). The tyrosine phosphatase inhibitor therefore appeared unable to mobilize intracellular Ca\(^{2+}\) via the classical signaling pathway associated with agonist-induced activation of endothelial cells. Inhibition of the Ca\(^{2+}\)-ATPase is also unable to account for the phenylarsine oxide-induced Ca\(^{2+}\) response. In the absence of extracellular Ca\(^{2+}\), phenylarsine oxide had no immediate effect on [Ca\(^{2+}\)]\(i\), but when incubated with cells for longer periods did induce a slight elevation. Subse-
Fig. 10. Bradykinin induces the tyrosine phosphorylation of cytoskeletal proteins which demonstrate sensitivity to the filling state of the intracellular Ca\textsuperscript{2+} store. Human endothelial cells were incubated in the presence or absence of extracellular Ca\textsuperscript{2+}. Following cell stimulation for 2 min, Ca\textsuperscript{2+} (1 mM) was added for the times indicated. The results presented are representative of experiments performed using seven different cell batches.

Table I

| Phosphate (pmol/µg cell lysate) | Control | Bradykinin-stimulated |
|--------------------------------|---------|-----------------------|
| Solvent                        | 364     | 754                   |
| Phenylarsine oxide (10 µm)     | 106     | 122                   |
| Orthovanadate (0.3 mM)         | 289     | 220                   |

Influx in Endothelial Cells

Fig. 11. The Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin induces the maintained tyrosine phosphorylation of several cytoskeletal proteins. Human endothelial cells were incubated with either solvent or thapsigargin (TG; 100 nM) for 5 min in the presence or absence of extracellular Ca\textsuperscript{2+}. Following cell stimulation in Ca\textsuperscript{2+}-free buffer for 5 min, Ca\textsuperscript{2+} (1 mM) was added and the incubation stopped after a further 2 min (Ca\textsuperscript{2+} readdd.). The results presented are representative of experiments performed using six different cell batches.

Effect of the tyrosine phosphatase inhibitors phenylarsine oxide and sodium orthovanadate on protein-tyrosine phosphatase activity

Tyrosine-phosphatase activity was assessed as phosphate (pmol) liberated from a substrate peptide per µg of whole cell lysates from control and bradykinin (100 nM; 2 min)-stimulated human endothelial cells. The data presented are representative of results obtained in three separate experiments.

The constitutively active tyrosine kinase v-Src results in elevated basal levels of [Ca\textsuperscript{2+}], as well as in the exaggeration of agonist-stimulated Ca\textsuperscript{2+} responses (13).

Since the inhibition of protein-tyrosine phosphatases resulted in an increase in [Ca\textsuperscript{2+}], it would appear likely that phosphatase activity plays a crucial role in damping Ca\textsuperscript{2+} influx both in unstimulated cells and following agonist stimulation. Indeed, the observation that protein-tyrosine phosphatase inhibitors can themselves induce cellular responses implies that there is a significant basal activity of protein-tyrosine phosphatases in cultured human endothelial cells. This was confirmed by determination of tyrosine phosphatase activity in whole cell lysates. Moreover, the observation that the tyrosine kinase inhibitor genistein attenuated both the increase in [Ca\textsuperscript{2+}] and tyrosine phosphorylation initiated by phenylarsine oxide suggests that a certain basal phosphatase activity is required to counteract phosphorylation by constitutively active protein-tyrosine kinases. Thus the dynamic balance between tyrosine kinase and phosphatase activity may play a central role in the maintenance of homeostatic levels of [Ca\textsuperscript{2+}] in unstimulated cells.

Based on current knowledge, the hypothetically ideal Ca\textsuperscript{2+}-influx-regulatory protein in non-excitable cells should be “activated” immediately after agonist-induced emptying of intracellular Ca\textsuperscript{2+} stores, even in the absence of extracellular Ca\textsuperscript{2+}, and to remain so until store filling is accomplished. Ideally this protein should be membrane-associated, either permanently or temporarily, and preferably linked by some manner or means to the cation channel by which Ca\textsuperscript{2+} enters the cell. To identify proteins which conform with these criteria the tyrosine phosphorylation of proteins from cells stimulated with bradykinin, thapsigargin, and the tyrosine phosphatase inhibitors was monitored in Triton X-100-soluble and -insoluble fractions. In the soluble fraction, bradykinin induced the rapid and transient phosphorylation of 3 proteins which contrasted with the relatively slow tyrosine phosphorylation of the 42- and 44-kDa isoforms of MAP kinase, as described previously (4). Cell stimulation in the absence of extracellular Ca\textsuperscript{2+} was without effect on the phosphorylation of the -60-, 77-, and 86-kDa proteins whereas agonist-induced phosphorylation of the 42- and 44-kDa isoforms of the MAP kinase was critically dependent on an increase in [Ca\textsuperscript{2+}]. The Ca\textsuperscript{2+}-ATPase inhibitor, thapsigargin, elicited essentially the same effects on Triton-soluble proteins. Sodium orthovanadate and phenylarsine oxide, however, induced the maintained, Ca\textsuperscript{2+}-independent phosphorylation of a protein triplet of ~80 kDa.

In the cytoskeletal fraction bradykinin also evoked a rapid and transient tyrosine phosphorylation of 4 proteins (85, 100, 110, and 125 kDa) which appeared identical to proteins permanently tyrosine phosphorylated following application of phenylarsine oxide. In contrast to the effects seen in the presence of
extracellular Ca^{2+}, a maintained tyrosine phosphorylation of these proteins was observed in cells stimulated following the removal of extracellular Ca^{2+}. Readdition of extracellular Ca^{2+} to these Ca^{2+}-depleted cells was associated with the transient dephosphorylation and maintained rephosphorylation of the 110- and 125-kDa proteins and the sustained dephosphorylation of the 85- and 100-kDa proteins. In similar experiments using thapsigargin, the reapplication of extracellular Ca^{2+} to depleted cells did not affect tyrosine phosphorylation of the 125-kDa protein or result in the dephosphorylation of the 85- and 100-kDa proteins. This observation was, however, not unexpected since, in the continued presence of the Ca^{2+}-ATPase inhibitor refilling of intracellular Ca^{2+} stores is antagonized, thus the store remains empty although the signal for refilling, is sustained. In bradykinin-stimulated cells, however, store refilling could be accomplished following Ca^{2+} readdition and the tyrosine phosphorylation of the 85- and 100-kDa proteins was transient. It would therefore appear that the tyrosine phosphorylation of the 85- and 100-kDa proteins mirrors the filling state of intracellular Ca^{2+} stores, thus these two cytoskeletal proteins fit the requirements of the hypothetical Ca^{2+} influx regulatory protein. Although similar experiments involving intracellular Ca^{2+} depletion and refilling in platelets have also demonstrated the existence of tyrosine-phosphorylated proteins apparently sensitive to the filling state of the Ca^{2+} store (1, 14), the reported apparent molecular weights of these proteins differs from that of proteins displaying similar characteristics in the present study.

In addition to the putative tyrosine-phosphorylated Ca^{2+} influx regulatory protein, a number of other mechanisms have been proposed to regulate capacitative Ca^{2+} entry. Nonhydrolyzable analogues of GTP, such as GTPγS, have been shown to interfere with Ca^{2+} signaling in a number of cell types. The inhibitory effect of these analogues occurs at some point after the release of intracellular Ca^{2+} and prior to the activation of Ca^{2+} influx. These effects can be prevented by GTP thus implying that a small G protein is involved in communicating the empty state of intracellular Ca^{2+} stores to the plasma membrane (15–18). The role of the “calcium influx factor,” a small, phosphate-containing, non-protein factor termed originally isolated from Jurkat T lymphocytes (19), as an exclusive messenger for capacitative Ca^{2+} entry has recently been questioned since the lymphocyte-derived factor has also been demonstrated to mobilize Ca^{2+} from intracellular stores (20). Involvement of the cytochrome P-450 monooxygenase in the regulation of Ca^{2+} influx has also been proposed on the basis of observations that a number of chemically distinct P-450 inhibitors, such as the imidazole anti-fungal agents, potently inhibited Ca^{2+} influx in endothelial cells and platelets (21–23). This hypothesis is supported by the findings that the induction of P-450 by β-naphthoflavone, potentiated agonist-induced Ca^{2+} influx and that the P-450 product, 5,6-epoxyeicosatrienoic acid, activated Ca^{2+} entry into endothelial cells without prior depletion of intracellular Ca^{2+} (24). Such observations suggest that the regulation of capacitative Ca^{2+} entry into endothelial cells is a complex process likely to involve the activation of protein tyrosine kinases and phosphatases, small G proteins, serine/threonine phosphatases, and probably also the cytochrome P-450 monooxygenase.

In summary, in the present study we observed that treatment of endothelial cells with protein-tyrosine phosphatase inhibitors resulted in the prolonged tyrosine phosphorylation of 2 cytoskeletal proteins and an increased Ca^{2+} influx via a mechanism independent of intracellular Ca^{2+} store depletion. Our findings strongly suggest that the tyrosine phosphorylation of both cytoskeletal proteins mirrors the filling state of the intracellular Ca^{2+} store and that they play a central role in the regulation of capacitative Ca^{2+} entry.

Acknowledgments—We are indebted to Isabel Winter and Annette Kirsch for expert technical assistance.

REFERENCES
1. Vostal, J. G., Jackson, W. L., and Shulman, N. R. (1991) J. Biol. Chem. 266, 16911–16916
2. Asahi, M., Yanagi, S., Ohta, S., Sakai, K., Takeuchi, F., Taniguchi, T., and Yamamura, H. (1992) FEBS Lett. 309, 10–14
3. Yatomi, Y., Ozaki, Y., and Kume, S. (1992) Biochem. Biophys. Res. Commun. 186, 1480–1486
4. Fleming, I., Fisbøl, B., and Busse, R. (1995) Circ. Res. 76, 522–529
5. Busse, R., and Lamontagne, D. (1991) Naunyn-Schmiedebergs Arch. Pharmacol. 344, 126–129
6. Fleming, I., Hecker, M., and Busse, R. (1994) Circ. Res. 74, 1220–1226
7. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
8. Huckle, W. R., Dy, R. C., and Earp, H. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8837–8841
9. Lee, K. M., Tocas, K., and Villereal, M. (1993) J. Biol. Chem. 268, 6995–9948
10. Yule, D. I., Kim, E. T., and Williams, J. A. (1994) Biochem. Biophys. Res. Commun. 202, 1697–1704
11. Fletcher, M. C., Samelson, L. E., and June, C. H. (1993) J. Biol. Chem. 268, 23697–23703
12. Imbert, V., Peyron, J.-F., Farahi Far, D., Mari, B., Auberger, P., and Rossi, B. (1994) Biochem. J. 297, 163–173
13. Nikolisina, B. B., Yamada, H., O’Shea, J. J., June, C. H., and Ashwell, J. D. (1992) J. Biol. Chem. 267, 7154–7159
14. Jenner, S., Farndale, R. W., and Sage, S. O. (1994) Biochem. J. 303, 337–339
15. Bird, G. S. J., and Putney, J. W., Jr. (1993) J. Biol. Chem. 268, 21486–21488
16. Fasafalo, C., Hoth, M., and Penner, R. (1993) J. Biol. Chem. 268, 20737–20740
17. Berven, L. A., and Barritt, G. J. (1994) FEBS Lett. 346, 235–240
18. Hajnoczy, G., Lin, C., and Thomas, A. P. (1994) J. Biol. Chem. 269, 10280–10287
19. Randriamampita, C., and Tsien, R. Y. (1993) Nature 364, 809–814
20. Bird, G. S. J., Bian, X., and Putney, J. W., Jr. (1995) Nature 373, 481–482
21. Sargeant, P., Clarkson, W. D., Sage, S. O., and Heemskerk, J. W. M. (1992) J. Biol. Chem. 267, 23697–23703
22. Alvarez, J., Montero, M., and García-Sancho, J. (1991) Biochem. J. 274, 274–283
23. Alvarez, J., Montero, M., and García-Sancho, J. (1992) J. Biol. Chem. 267, 11789–11793
24. Graier, W. F., Simecek, S., and Sturek, M. (1995) J. Physiol. 482, 259–274
Interdependence of Calcium Signaling and Protein Tyrosine Phosphorylation in Human Endothelial Cells
Ingrid Fleming, Beate Fisslthaler and Rudi Busse

J. Biol. Chem. 1996, 271:11009-11015.
doi: 10.1074/jbc.271.18.11009

Access the most updated version of this article at http://www.jbc.org/content/271/18/11009

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 24 references, 13 of which can be accessed free at http://www.jbc.org/content/271/18/11009.full.html#ref-list-1