Intracellular Calcium Regulates the Tyrosine Kinase Receptor Encoded by the MET Oncogene*

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Previous work (Gandino, L., Di Renzo, M. F., Giordano, S., Bussolino, F., and Comoglio, P. M. (1990) Oncogene 5, 721–725) has shown that the tyrosine kinase activity of the receptor encoded by the MET protooncogene is negatively modulated by protein kinase C (PKC). We now show that an increase of intracellular Ca^{2+} has a similar inhibitory effect in vivo, via a PKC-independent mechanism. In GTL-16 cells the p145^{MET} kinase is overexpressed and constitutively phosphorylated on tyrosine. A rapid and reversible decrease of p145^{MET} tyrosine phosphorylation was induced by treatment with the calcium ionophores A23187 or ionomycin. Experiments performed with the ionophores in absence of extracellular calcium showed that a rise in cytoplasmic Ca^{2+} concentration to 450 nM (due to release from intracellular stores) resulted in a similar effect. These Ca^{2+} concentrations had no effect on p145^{MET} autophosphorylation in an in vitro kinase assay. This suggests that the effect of Ca^{2+} on p145^{MET} tyrosine phosphorylation is not direct but may be mediated by Ca^{2+}-activated protein(s). Involvement of Ca^{2+}-dependent tyrosine phosphatases was ruled out by experiments carried out in presence of Na_3VO_4. In vivo labeling with [^{32}P]orthophosphate showed that the rise of intracellular Ca^{2+} induces serine phosphorylation of p145^{MET} on a specific phosphopeptide. This suggests that Ca^{2+} negatively modulates p145^{MET} kinase through the phosphorylation of a critical serine residue by a Ca^{2+}-activated serine kinase distinct from PKC.

The protein encoded by the MET protooncogene is an α-β chain heterodimer of 190 kDa (p190^{MET}; Giordano et al., 1989a). The α-chain (50 kDa) is exposed at the cell surface and is linked by disulfide bonds to the β-chain (145 kDa; p145^{MET}). The latter spans the plasma membrane and is endowed with tyrosine kinase activity (Tempest et al., 1988; Gonzatti-Haces et al., 1988; Giordano et al., 1989b). These structural and functional features suggest that p190^{MET} is a

receptor. In GTL-16 cells, due to the amplification of the MET oncogene (Ponzetto et al., 1991) the p190^{MET} receptor is overexpressed and the p145^{MET} tyrosine kinase is active in the absence of a ligand (Giordano et al., 1988; Giordano et al., 1989a). These cells provide a useful model to study positive and negative regulation of the p145^{MET} kinase activity. We have shown previously that p145^{MET} kinase is activated by autophosphorylation on tyrosine (Naldini et al., 1991b) and inhibited by protein kinase C (PKC)[1]-induced phosphorylation on serine (Gandino et al., 1990). The normal endogenous cellular activator of PKC is diacylglycerol which is produced transiently in the cell membrane as result of the breakdown of phosphatidylinositol phosphate by phospholipase C. This enzyme concomitantly produces inositol 1,4,5-triphosphate, a soluble cytoplasmic second messenger causing the release of calcium ions from intracellular stores (for a review, see Berridge, 1987). In this paper we investigated whether both arms of the signal transduction pathway triggered by phospholipase C modulate the p145^{MET} kinase. The results indicate that the increase in intracellular Ca^{2+} concentration is inhibitory. This negative regulation takes place independently from PKC, via activation of another serine kinase.

EXPERIMENTAL PROCEDURES

Reagents and Cells—Phorbol 12-myristate 13-acetate (TPA) and the calcium ionophores A23187 were purchased from Sigma and fura-2/AM and ionomycin from Behring Diagnostics, Triton X-100 was from Pierce Chemical Co. [γ-^{32}P]ATP (specific activity 7000 Ci/mmol), [^{32}P]orthophosphate (10 mCi/ml), and ^{125}I-protein A were obtained from Amersham Corp. Nitrocellulose filters for Western blots were from Bio-Rad. The molecular mass markers used in SDS-PAGE were ^{14}C-methylated myosin (200 kDa), phosphorylase b (92 kDa), bovine serum albumin (69 kDa), egg albumin (46 kDa), and carbonic anhydrase (30 kDa) (Amersham Corp.). Phosphotyrosine (Tyr(P)) antibodies were raised in rabbits immunized with p-amino-benzenephosphonate and affinity-purified as described previously (Comoglio et al., 1984). Anti-MET antibodies were raised in rabbits immunized against the synthetic peptide Val-Asp-Thr-Arg-Pro-Ala-Ser-Phe-Trp-Glu-Thr-Ser corresponding to the amino acid sequence at the C-terminal end of the predicted MET gene product and kindly provided by M. F. Di Renzo (University of Torino). GTL-16 cells are a clonal cell line derived from a poorly differentiated gastric carcinoma line (Motoyama et al., 1984). The cells were cultured at 37 °C, under a humidified atmosphere of 95% air-5% CO_2, in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml). Cells were used at approximately 90% confluence. The standard solution employed

[1] The abbreviations used are: PKC, protein kinase C; TPA, phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; TPCK, L-tosylamido-2-phenylethyl chloromethyl ketone; HPLC, high performance liquid chromatography; EGF, epidermal growth factor.

in the fluorimetric measurements was a Krebs-Ringer HEPES buffer (KRH) containing (in millimolar/l): NaCl, 140; KCl, 5; CaCl₂, 2; MgSO₄, 1.2; glucose, 6; HEPES-NaOH, 25, pH 7.4.

Measurement of Intracellular Ca²⁺ Concentration—Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with the fluorescent probe fura-2 as described by Grynkiewicz *et al.*, (1985). Monolayers of two to four dishes (100-mm diameter) were washed twice with phosphate-buffered saline (PBS) and then treated at 37 °C with 2 ml of the same buffer containing trypsin (0.01%) and EDTA (0.9 mM). Detachment of the cells from the dish was completed within 5 min. Trypsin was neutralized by resuspending the cells in growth medium for 3 h at 37 °C under continuous stirring. Cells were then centrifuged at 800 rpm for 5 min, resuspended in RPMI 1640 plus 1% fetal calf serum (cell concentration, 5×10^6 cell/ml) and loaded with fura-2 by a 30-min incubation period at 37 °C with 3 µM fura-2 pentacetoxymethylester (fura-2/AM). For the fluorimetric measurement of $[Ca^{2+}]_i$, 10^6 cells were placed in the cuvette of a thermostatically controlled (37 ± 1 °C) cell holder. Fluorescence records were taken at an excitation wavelength of 345 nm and emission of 490 nm, with slits of 5 nm. The calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was done according to Grynkiewicz *et al.* (1985).

Cell Treatments and Western Blotting—Subconfluent cultures of GTL-16 cells were incubated for the indicated times with TPA (160 nM) A23187(10 µM if not otherwise indicated), ionomycin (10 µM, if not otherwise indicated), or Na₃VO₄ (100 µM). The treatment was terminated by placing the cells on ice. Monolayers were washed twice with ice-cold PBS and cells solubilized in boiling Laemmli buffer (Laemmli, 1970). Samples were adjusted to a protein concentration of 300 µg/well, run in 8% SDS-PAGE, and transferred to nitrocellulose sheets. Blots were probed with 10 µg/ml of purified Tyr(P) antibodies or with anti-*MET* serum (1:500) followed by ¹²⁵I-labeled protein A as described in details elsewhere (Di Renzo *et al.*, 1986). Filters were subjected to autoradiography for 24 h at −70 °C using intensifying screens.

Protein Kinase C and Ca²⁺ Depletion—For PKC depletion, subconfluent cultures of GTL-16 cells were incubated for 20 h with 160 nM of TPA as described previously (Gandino *et al.*, 1990). To deplete extracellular calcium, cells were washed and incubated in fresh RPMI medium, containing 4 mM EGTA, 2 min before the addition of ionophores. To deplete intracellular calcium, cells were left in the presence of 4 mM EGTA and 10 µM A23187 for different times as indicated in the legend of the figures.

Immunoprecipitation and in Vitro Autophosphorylation Assay—GTL-16 cells were washed twice with ice-cold PBS and lysed in DIM buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 5 mM MgCl₂, 300 mM sucrose, 5 mM EGTA) plus 1% Triton X-100 and a mixture of protease inhibitors. The cell lysates were centrifuged at 10,000 rpm at 4 °C for 30 min and incubated with anti-*MET* serum coupled to Sepharose-protein A. Bound proteins were washed several times in DIM buffer without EGTA and MgCl₂ and incubated in the same buffer with different concentration of MgCl₂, CaCl₂, and 10 µM of $[\tau\text{-}^{32}P]ATP$. The kinase reaction was performed on ice for 2 min and stopped with 1 ml of Tris-buffered saline with 10 mM EDTA and 100 µM Na₃VO₄. After a brief centrifugation proteins were eluted in boiling Laemmli buffer and subjected to 8% SDS-PAGE followed by autoradiography for 12 h at −70 °C using intensifying screens. The relative amount of phosphate incorporated in p145^{MET} was estimated by measuring the optical density of the corresponding autoradiography band with a laser densitometer (Pharmacia LKB 2202 Ultroscan).

[³²P]Orthophosphate Labeling and Immunoprecipitation—Subconfluent cultures of GTL-16 cells were incubated for 4 h at 37 °C with 1 mCi/ml of [³²P]orthophosphate in phosphate-free RPMI medium in the absence of serum. The cultures were placed on ice, washed twice with cold PBS, and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 100 µM Na₃VO₄, 50 mM NaF) and a mixture of protease inhibitors. Cell lysates were clarified at 30,000 rpm for 30 min at 4 °C and immunoprecipitated with anti-*MET* serum coupled to Sepharose-protein A. Bound proteins were washed several times in RIPA buffer and eluted in boiling Laemmli buffer. Eluted proteins were subjected to 8% SDS-PAGE followed by autoradiography for 1 h at −70 °C using intensifying screens.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described by Cooper *et al.* (1983). Briefly, ³²P-labeled proteins were excised from the dried gel and eluted in 0.05 M ammonium bicarbonate, pH 8, in the presence of 0.1% SDS and 0.716 M β-mercaptoethanol. Trichloroacetic acid-precipitated proteins were hydrolyzed in 50 µl of 6 N HCl and placed in a 110 °C bath for 1 h.

Samples were washed in water, lyophilized, and resuspended in 5% acetic acid, 0.5% pyridin, 5 mM EDTA, pH 3.5 (running buffer) containing 1 mg/ml of each unlabeled phosphoserine, phosphothreonine, and phosphotyrosine. Samples were run on cellulose plate for 1 h at 1.2 kV in running buffer. The plate was dried, and phosphoamino acids were localized by spraying the plate with 0.25% ninhydrin in acetone. The dried plate was then subjected to autoradiography at −70 °C using intensifying screens for 7 days.

Phosphopeptide Mapping—For phosphopeptide mapping the ³²P-labeled bands were excised from the polyacrylamide gel, washed twice with 10% methanol in order to remove SDS, minced, and dried in a lyophilizer. The gel slices were rehydrated with 50 mM NH₄CO₃, pH 7.8, containing 50 µg/band of TPCK (L-tosylamido-2-phenylethyl chloromethyl ketone)-trypsin and incubated for 2 h at 37 °C. The tryptic digestion was repeated once, and the gel slices were further eluted with 50 mM NH₄CO₃, pH 7.8. Eluates were then lyophilized and further incubated with 100 µg/ml trypsin for 2 h at 37 °C. The essicated sample was then resuspended in buffer A (100% water containing 0.1% trifluoroacetic acid) and filtered on a 0.2-µm Acrodisc filter (Gelman Sciences). The phosphopeptides were analyzed on a reverse phase C₂-C₁₈ Superpack Pep-S column (Pharmacia LKB Biotechnology Inc.), resolved on a gradient of acetonitrile in buffer A of 0.46%/min (from 0 to 32% acetonitrile in 82 min) with a flow rate of 1 ml/min. The eluted radioactivity was monitored by a Radiomatic A-100 radioactive flow detector (Packard). The fractions collected from radio-HPLC runs were counted in a β-counter (Packard), and the fractions corresponding to the largest radioactive peaks were pooled, lyophilized, and subjected to phosphoamino acid analysis.

RESULTS

Calcium Ionophores Treatment Inhibits Tyrosine Phosphorylation of p145^{MET} in Intact Cells—GTL-16 cells express a high amount of the p190^{MET} receptor whose 145-kDa β-subunit is autophosphorylated on tyrosine *in vivo*, as assessed by Western blot analysis of total cellular proteins probed with Tyr(P) antibodies (Giordano *et al.*, 1988). It has been shown previously (Gandino *et al.*, 1990) that treatment of GTL-16 cells with TPA for 1 h at 37 °C inhibits the tyrosine phosphorylation of p145^{MET}. We now show that a 5-min treatment with 10 µM calcium ionophore A23187 also reduced the extent of tyrosine phosphorylation of the p145^{MET} subunit. The same effect was observed using a different calcium ionophore, ionomycin (Fig. 1A). The amount of receptor in samples was identical as assessed in Western blots probed with antibodies against a synthetic peptide derived from the predicted *MET* sequence (anti-*MET*: Fig. 1B). Both in the case of A23187 and ionomycin, the extent of tyrosine phosphorylation of the
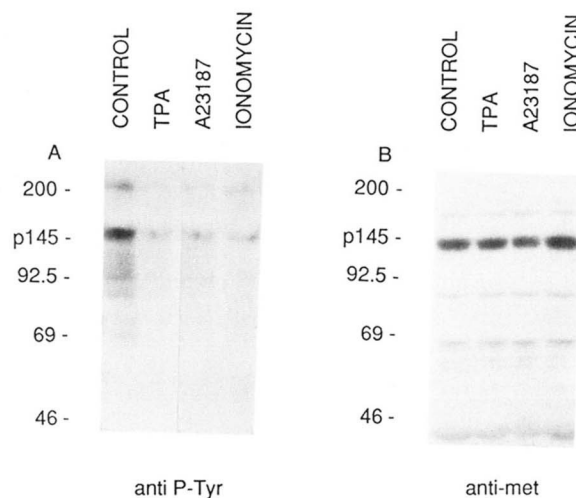

FIG. 1. **Inhibition of tyrosine phosphorylation of p145^{MET}.** Western blot of proteins solubilized from GTL-16 cells exposed to 100 ng/ml of TPA for 1 h or to calcium ionophores A23187 (10 µM) or ionomycin (10 µM) for 5 min. *A*, Western blot probed with Tyr(P) antibodies; *B*, Western blot probed with anti-*MET* serum.
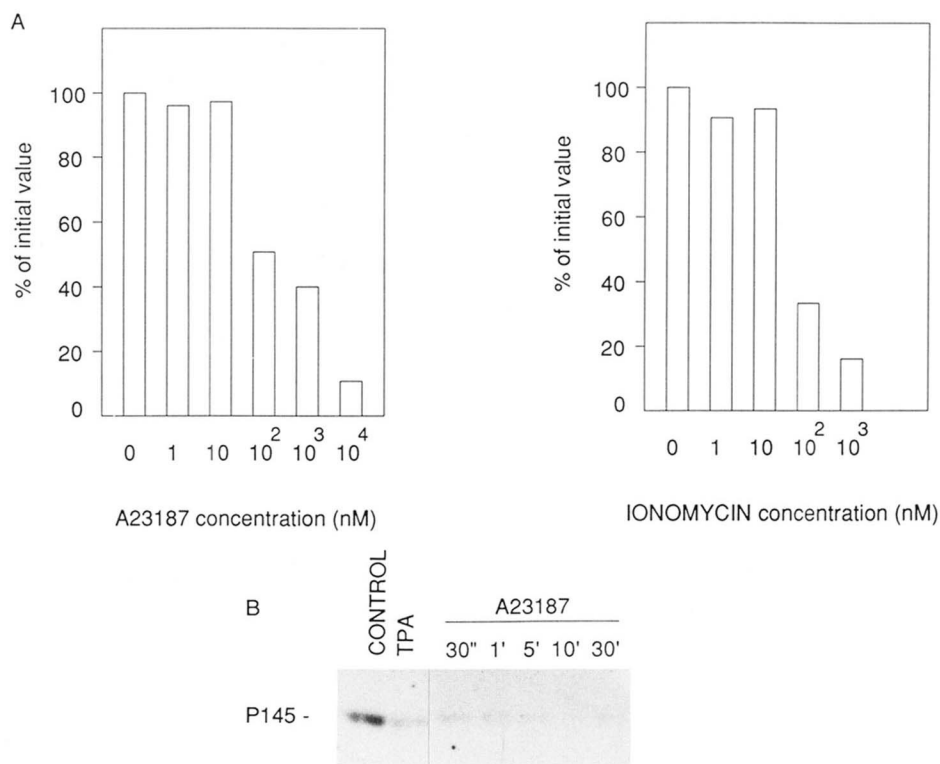
FIG. 2. **Dose dependence and time course of the inhibition of p145^{MET} tyrosine phosphorylation.** *A*, relative amount of phosphotyrosine present in p145^{MET}, estimated by measuring the optical density of the corresponding band in radiograms of Western blots probed with Tyr(P) antibodies. Values were measured after 5-min treatment with increasing concentration of A23187 or ionomycin. 100% refers the amount of phosphotyrosine present in untreated samples. *Bars* indicate the mean of triplicate values in a representative experiment (standard deviations never exceeded ± 6%). *B*, time course of tyrosine phosphorylation of p145^{MET} after cell treatment with A23187 (10 μM). Proteins were solubilized after the indicated times and analysed in Western blot probed with Tyr(P) antibodies.

p145^{MET} decreased as a function of the concentration of the ionophore added; the half-maximal effect, measured after 5 min, was observed at a concentration close to 100 nM (Fig. 2A). Time course experiments showed that the inhibitory effect of A23187 (10 μM) was already detectable after 30 s of treatment and was maximal after 5 min (Fig. 2B).

Calcium ionophores alter the permeability to ions of both the cell plasma membrane and the membranes of intracellular stores. Experiments were performed to assess whether the release of Ca^{2+} from these internal stores was sufficient to determine a decrease of p145^{MET} tyrosine phosphorylation. In these experiments, the extracellular calcium in the culture medium was removed by the addition of 4 mM EGTA before addition of A23187 to the cells. Under these conditions the ionophore A23187 could still release the Ca^{2+} stored in the intracellular compartment. The intracellular Ca^{2+} concentrations reached were effective in inhibiting the kinase activity of p145^{MET} (Fig. 3). The inhibition was reversible. If cells were left in the presence of EGTA and A23187 for 30 min or more, the ensuing calcium efflux resulted in lowering the intracellular concentration to the pretreatment values. Under these conditions the tyrosine phosphorylation of p145^{MET} was restored (Fig. 3).

Tyrosine Phosphorylation of p145^{MET} Is Inhibited at Physiological Concentration of Intracellular Ca^{2+}—The actual intracellular calcium concentrations ([Ca^{2+}]$_i$) reached under the experimental conditions described above were measured by the fluorescent probe fura-2. As expected, in GTL-16 cells treated with ionomycin, [Ca^{2+}]$_i$ began to increase immedi-
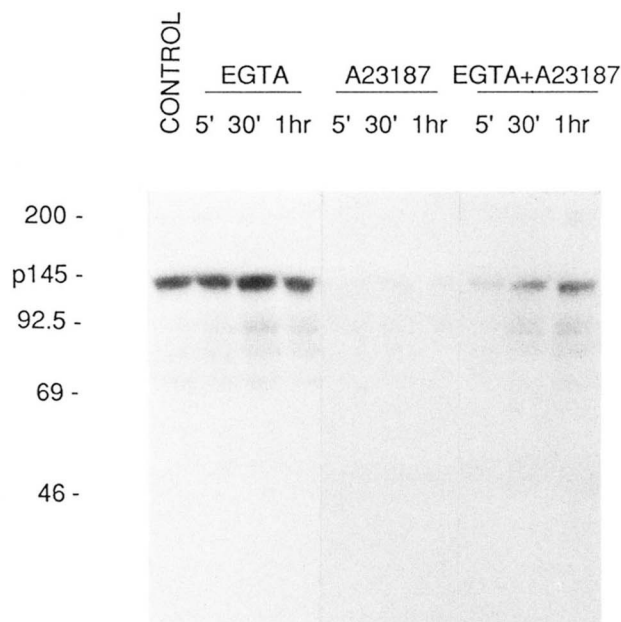

FIG. 3. **Inhibition of p145^{MET} tyrosine phosphorylation by Ca^{2+} mobilized from intracellular stores.** Western blot analysis with Tyr(P) antibodies of proteins solubilized after the indicated times from cells untreated (control) or incubated with 4 mM EGTA to remove the extracellular calcium. *Center* and *right panels* show the effect of the ionophore A23187 (10 μM) in the presence (*center*) or in the absence (*right*) of extracellular calcium.

ately, reaching a maximum after a few seconds. On the average, the $[Ca^{2+}]_i$ increase was from 226 (±46) to 733 (±41) nM (means ± S.D.; $n = 3$ and 6, respectively). Thereafter, $[Ca^{2+}]_i$ remained elevated at a plateau for at least 25 min, when measurements were interrupted (Fig. 4, *trace A*). On the other hand, when this experiment was carried out on cells incubated in the same medium containing excess (4 mM) EGTA to buffer Ca^{2+} to very low values (<1 nM), the initial peak was reduced of about 50% ($[Ca^{2+}]_i$. The intracellular concentration increased from 210 (±35) to 450 (±20) nM (means ± S.D., $n = 6$) and returned to resting value in about 7 (±2) min (Fig. 4, *trace B*). Finally, dose-response experiments showed that detectable increases of $[Ca^{2+}]_i$ were observed beginning with ionomycin concentration as low as 100 pM (15% of maximal response, not shown). Half-maximal effect was observed at 1 µM and maximal at 10 µM; however at ionophore concentration <100 nM, these responses became more transient, even if the plateau phase persisted for at least 15 (±2) min. These measurements show that the observed inhibition of the p190^{MET} tyrosine kinase activity takes place at intracellular calcium concentrations falling within the range of physiological responses (Swope and Standbrunn, 1988; Corpe *et al.*, 1989).

Protein Kinase C and Ca²⁺ Inhibit p145^{MET} by Independent Mechanisms—As mentioned above (Fig. 1), TPA-induced PKC activation inhibits the kinase activity of p146^{MET}. It is known that in some cells PKC interferes with calcium channels affecting the intracellular Ca^{2+} concentration (for a review see Nishizuka, 1986). This is not the case in GTL-16 cells where TPA treatment did not result in any detectable variation of the steady-state level of $[Ca^{2+}]_i$ (Fig. 4, *trace C*) nor did it affect the response to ionomycin (not shown). The fact that PKC and Ca^{2+} inhibit p146^{MET} kinase by two independent mechanisms was proved by two mirror experiments. GTL-16 cells were depleted of PKC by 20-h treatment with 100 ng/ml of TPA. In these conditions, TPA or other phorbol esters were unable to exert any regulatory effect on the extent of tyrosine phosphorylation of p145^{MET}. On the contrary, in PKC-depleted cells, the calcium ionophores A23187 or ionomycin were still able to exert their inhibitory effect on p145^{MET} tyrosine phosphorylation (Fig. 5A). On the other hand, when cells were depleted of intracellular calcium by incubation for 1.5 h with EGTA and A23187, subsequent TPA treatment, for 30 min, was still able to inhibit the autophosphorylation on tyrosine of p146^{MET} (Fig. 5B).

Tyrosine Kinase Activity of p145^{MET} Is Not Inhibited in Vitro by Physiological Ca²⁺ Concentration—Tyrosine kinase activity is known to be sensitive to the concentrations of divalent ions (Pritchard *et al.*, 1989). In order to study the possible direct effect of Ca^{2+} on the activity of p145^{MET}, we performed an *in vitro* kinase assay in the presence of increasing concentrations of Ca^{2+}. The reaction was carried out at physiological concentration of Mg^{2+} (0.6 mM), using the p145^{MET} kinase immunoprecipitated by specific anti-*MET* antibodies. Fig. 6 shows that no inhibitory effect was measured within the physiological range of Ca^{2+} concentrations.
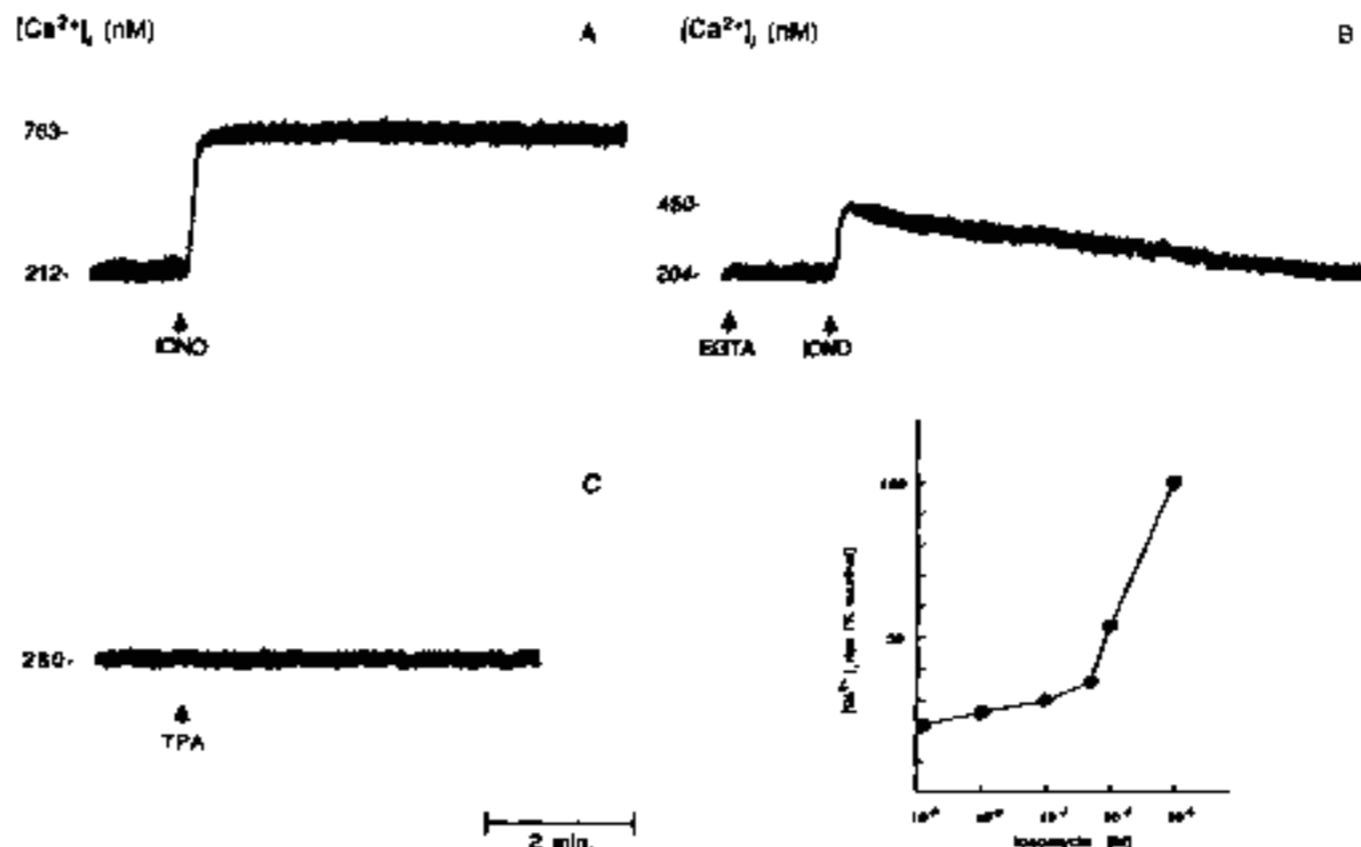

FIG. 4. $[Ca^{2+}]_i$ increase induced by ionomycin in GTL-16 cells. GTL-16 cells were loaded with the fluorescent Ca^{2+} indicator fura-2 and changes in fluorescence continuously monitored. Where indicated by the arrows, ionomycin (*IONO*: 10 µM, *traces A* and *B*), EGTA (4 mM, *trace B*), or TPA (100 ng/ml, *trace C*) were added to the cell suspensions. The numbers to the left of each trace refer to the $[Ca^{2+}]_i$ expressed as nanomolar (nM). Values shown in the dose-response curve are expressed as percentage of the maximum value observed after treatment with 10 µM ionomycin (standard deviations never exceeded ±5%).
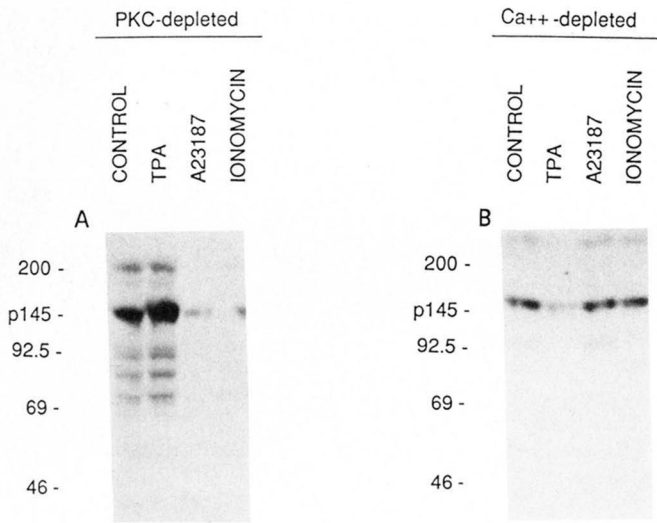
Ca^{2+} Inhibition of the MET-tyrosine Kinase Receptor

PKC-depleted

Ca++ -depleted

CONTROL TPA A23187 IONOMYCIN

CONTROL TPA A23187 IONOMYCIN

A

B

200 -
p145 -
92.5 -
69 -
46 -

200 -
p145 -
92.5 -
69 -
46 -

FIG. 5. Ca^{2+} and PKC inhibit p145^{MET} tyrosine phosphorylation by independent mechanisms. Proteins solubilized and analyzed in Western blot probed with Tyr(P) antibodies. *A*, cells were depleted of PKC, as described under "Experimental Procedure," washed, and incubated in the absence (control) or in the presence of 100 ng/ml TPA for 1 h or 10 μM A23187 or ionomycin for 5 min. *B*, cells were depleted of calcium by prolonged treatment with 4 mM EGTA and 10 μM A23187 to discharge intracellular stores and further incubated with TPA (100 ng/ml) for 30 min or A23187 or ionomycin for 5 min.

c - met tyrosine phosphorylation (A. U.)

6
5
4
3
2
1
0

| Mg^{2+} (mM) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 2.5 |
| Ca^{2+} (mM) | - | 10^{-5} | 10^{-4} | 10^{-3} | 10^{-2} | 0.1 | 0.1 |

FIG. 6. *In vitro* tyrosine kinase activity of p145^{MET}. The p145^{MET} was immunoprecipitated with anti-*MET* antibodies, and the kinase reaction was performed on ice for 2 min in the presence of [τ-^{32}P]ATP at the indicated concentrations of Mg^{2+} and Ca^{2+}. After SDS-PAGE and autoradiography, the kinase activity was assessed by measuring the optical density of the ^{32}P-labeled band with a laser densitometer. *A.U.*, absorbance units.

Inhibition was observed only at concentrations higher than 100 μM; the kinase activity was restored by increasing the concentration of Mg^{2+}, suggesting that the inhibitory effect of Ca^{2+} at nonphysiological concentrations was due to competition with Mg^{2+} for generation of ATP-divalent cation complexes.

The Ca^{2+}-dependent Inhibition of p145^{MET} Tyrosine Phosphorylation Is Not Mediated by Tyrosine Phosphatases—The amount of phosphotyrosine in proteins is due to the balanced activity of tyrosine kinases and tyrosine phosphatases. To

evaluate if tyrosine-specific phosphatase(s) were involved in the loss of phosphotyrosine from p145^{MET} after treatment *in vivo* with calcium ionophores, experiments were performed in the presence of sodium orthovanadate (Na_3VO_4), a powerful tyrosine phosphatases inhibitor with broad specificity (Jones *et al.*, 1989). GTL-16 cells were treated for 1 h with 100 μM Na_3VO_4 and then incubated for different length of time with the ionophore A23187 in the presence of the inhibitor. As expected, after Na_3VO_4 treatment the amount of phosphotyrosine in p145^{MET} was highly increased (Fig. 7). Under these conditions the tyrosine phosphorylation of a number of other proteins also increased.
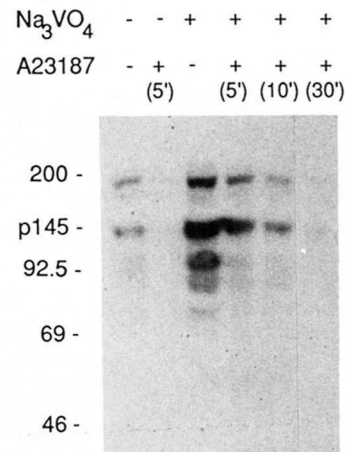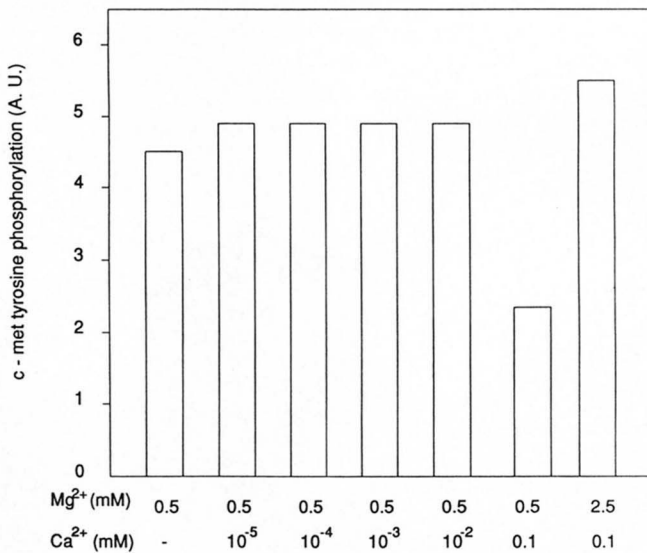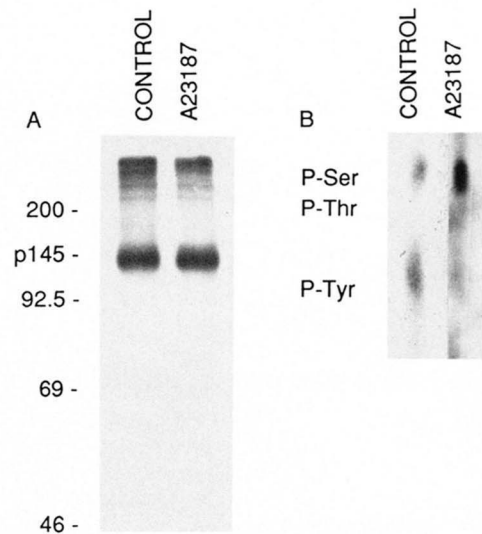
These may be considered candidate substrates of the

Na_3VO_4	-	-	+	+	+	+
$A23187$	-	+	-	+	+	+
		(5')		(5')	(10')	(30')

200 -
p145 -
92.5 -
69 -
46 -

FIG. 7. Inhibition of p145^{MET} tyrosine phosphorylation by A23187 is independent from tyrosine phosphatases. Cells were treated with 100 μM Na_3VO_4 for 1 h and stimulated for 5, 10, or 30 min (*brackets*) with 10 μM A23187, as indicated. Proteins were solubilized and analyzed in Western blot probed with Tyr(P) antibodies.

CONTROL A23187

CONTROL A23187

A

200 -
p145 -
92.5 -
69 -
46 -

B

P-Ser
P-Thr

P-Tyr

FIG. 8. Analysis of the amino acids phosphorylated in p145^{MET} in response to A23187 treatment. *A*, SDS-PAGE of proteins solubilized from [^{32}P]orthophospate-labeled cells and immunoprecipitated with anti-*MET* antibodies. *Control*, unstimulated cells; *A23187*, cells treated with 10 μM A23187 for 5 min. *B*, high voltage electrophoresis of phosphoamino acids hydrolyzed from the p145 band eluted from the SDS-PAGE shown in *A*. *P-Ser*, phosphoserine; *P-Thr*, phosphothreonine; *P-Tyr*, phosphotyrosine.
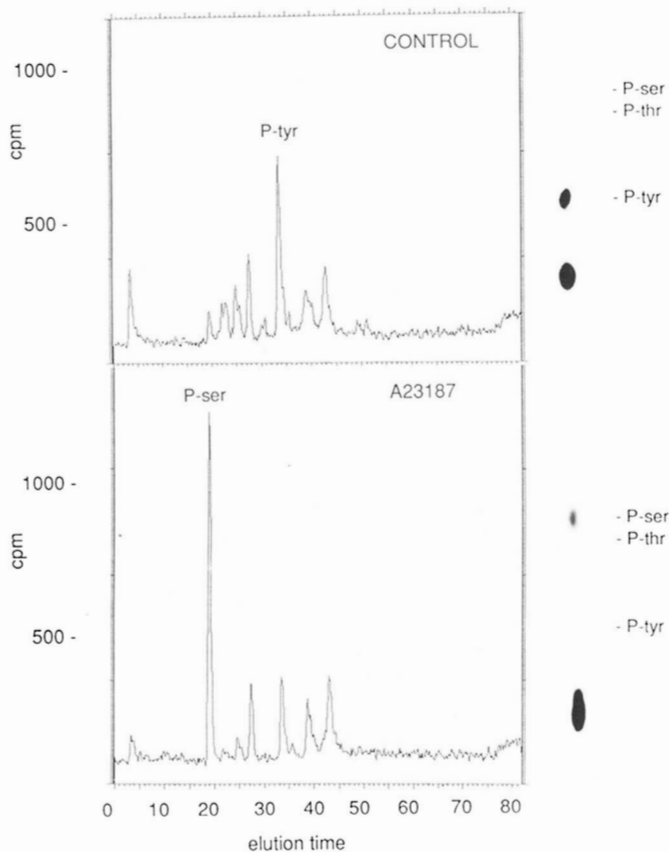
FIG. 9. **Analysis of tryptic pepdides phosphorylated in p145^MET in response to A23187 treatment.** [³²P]Orthophospate-labeled SDS-PAGE bands of p145^MET were subjected to TPCK-trypsin digestion in 50 mM ammonium carbonate, pH 7.8, for 4 h at 37 °C. The cleavage products were analyzed on a RP radio-HPLC PepS column developed with a 0–32% acetonitrile gradient in 82 min, as described under "Experimental Procedures." The *upper panel* (*CONTROL*) shows the elution profile of phosphopeptides originated from p145^MET immunoprecipitated from untreated GTL-16 cells. The *lower panel* (*A23187*) shows the profile of p145^MET immunoprecipitated from cells treated with 10 μM A23187 for 5 min. The phospho-amino acid analysis of the major HPLC peaks are shown on the *right*. *P-ser*, phosphoserine; *P-thr*, phosphothreonine; *P-tyr*, phosphotyrosine.

p145^MET kinase. This observation indicates that the phosphatases involved in the turnover of phosphate groups of the p145^MET tyrosine phosphorylation sites are indeed sensitive to vanadate. Fig. 7 also shows that in the presence of vanadate ions the amount of phosphotyrosine in p145^MET still decreased with time as a consequence of increased intracellular Ca²⁺ concentration. This experiments suggest that calcium ions regulate the steady-state level of tyrosine phosphorylation by affecting tyrosine kinase rather that tyrosine phosphatase activity.

Increased Intracellular Ca²⁺ Concentration Induces Serine Phosphorylation of p145^MET Kinase—After calcium ionophore treatment, the total amount of phosphate detectable in p145^MET was unaffected, although the level of tyrosine phosphorylation decreased (Fig. 8A). To identify the newly phosphorylated amino acids, [³²P]orthophosphate-labeled GTL-16 cells were treated with A23187, solubilized, and immunoprecipitated with anti-*MET* antibodies. Phosphoamino acid analysis of the protein immunoprecipitated from control cells revealed that phosphotyrosine was the most abundant phosphorylated amino acid. A limited amount of phosphoserine was also detectable. After A23187 treatment the protein was almost exclusively phosphorylated on serine residues (Fig.

8*B*). In order to assess if the increase in phosphoserine content was due to phosphorylation of specific site(s) induced by calcium, phosphopeptide analysis was performed. Reverse phase radio-HPLC separation of ³²P-labeled p145^MET tryptic peptides from control or ionophore-treated cells was performed. P145^MET from control cells yielded seven peaks: the major one, eluting at 35 min, contained only phosphotyrosine (Fig. 9). This represent the phosphopeptide containing the major tyrosine phosphorylation site.[2] P145^MET immunoprecipitated from ionophore-treated cells yielded the same peptides, but the amount of ³²P incorporated in the peak eluting after 35 min was drastically reduced. On the other hand, the amount of radioactivity incorporated in the peak eluting after 19 min was increased more than 20-fold. Phosphoamino acid analysis of this peak showed that phosphoserine was the only labeled amino acid (Fig. 9). The other peaks were virtually unchanged.

DISCUSSION

Structural and functional properties of the protein encoded by the *MET* oncogene (p190^MET) indicate that this transmembrane molecule is a tyrosine kinase receptor (Gonzatti-Haces, 1988; Giordano *et al.*, 1988). Its unique two-chain composition (Giordano *et al.*, 1989a; Giordano *et al.*, 1989b) suggests that p190^MET is the prototype of a new class of receptors distinct from the already known three classes that include epidermal growth factor (EGF), insulin, and platelet-derived growth factor receptors, respectively (reviewed by Yarden and Ullrich, 1988). In man, the p190^MET receptor is expressed in hepatocytes, in cells of epithelial origin, and in a variety of epithelial tumors (Prat *et al.*, 1991). Recently, hepatocyte growth factor (Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989; Zarnegar *et al.*, 1990) has been proposed as the putative ligand (Bottaro *et al.*, 1991; Naldini *et al.*, 1991a).

The intrinsic tyrosine kinase activity of the β subunit of the *MET* receptor (p145^MET) is positively regulated by autophosphorylation on tyrosine residues (Naldini *et al.*, 1991b). Previous work from this laboratory has also shown that p145^MET kinase is negatively regulated by PKC activation *in vivo*. This inhibition is mediated by serine phosphorylation of p145^MET (Gandino *et al.*, 1990). It is known that PKC negatively modulates several tyrosine kinase receptors, including EGF receptor, insulin receptor, and insulin-like growth factor 1 receptor. In all the above cases, inhibition is mediated by threonine and/or serine phosphorylation (Cochet *et al.*, 1984; Hunter *et al.*, 1984; Davis and Czech, 1985; Jacobs *et al.*, 1983; Takayama *et al.*, 1988; Koshio *et al.*, 1989). Colony-stimulating factor 1 receptor functions are also inhibited by PKC activation, although through a different mechanism involving activation of a specific protease (Downing *et al.*, 1989).

The data reported in this paper show that p145^MET kinase is negatively regulated also by the increase of intracellular Ca²⁺ concentration. The inhibitory effect was observed at [Ca²⁺]$_i$ levels in the physiological range (between 200 and 760 nM), as determined by fluorometric analysis. Inhibition was observed both after ionophore-induced calcium influx from the extracellular compartment and after release from intracellular stores. The Ca²⁺-induced inhibition was reversible and totally independent from PKC activation, as it took place also in PKC-depleted cells. On the other hand, PKC inhibits the p145^MET kinase by a mechanism which is totally independent from intracellular Ca²⁺.

A Ca²⁺-dependent negative regulation has been observed recently also in the case of the EGF receptor. Treatment with

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[2] R. Ferracini, P. Longati, L. Naldini, E. Vigna, and P. M.Comoglio, submitted for publication.

the ionophore A23187 resulted in inhibition of EGF induced receptor tyrosine phosphorylation and loss of high affinity binding sites (Friedman *et al.*, 1989). Also in this case the effect of Ca^{2+} appeared to be independent from PKC activation (Friedman *et al.*, 1989; Verheijden *et al.*, 1990).

The importance of $[Ca^{2+}]_i$ oscillation in cells is due to the fact that Ca^{2+} acts as second messenger to affect a large number of biochemical activities in the various subcellular compartments (for a review see Carafoli, 1987). The precise mechanism by which Ca^{2+} inhibits the kinase of $p145^{MET}$ is unknown. Ca^{2+} does not seem to directly affect the enzymatic activity of $p145^{MET}$. Although it is not possible to assess the local concentration of calcium at the membrane in response to ionophores, no inhibitory effect was measured within the physiological range of Ca^{2+} concentrations in an *in vitro* kinase assay; inhibition was observed only at concentrations higher than 100 μM. A calcium dependent activation of tyrosine specific phosphatase(s) could explain the observed decrease in tyrosine phosphorylation of $p145^{MET}$. Recently, the role of phosphotyrosine phosphatases in regulating the tyrosine kinase activity of receptors has been emphasized (Roome *et al.*, 1988; Lin *et al.*, 1988). However, all the tyrosine phosphatases known so far are inhibited by vanadate ions (Jones *et al.*, 1989), and such an inhibitor did not impair the calcium-dependent inhibitory effect. A calcium-dependent activation of a $p145^{MET}$-specific protease was also ruled out, since the amount of $p145^{MET}$ was not altered in cells treated with Ca^{2+} ionophores. Inhibition of the activity of $p145^{MET}$ by Ca^{2+} is associated to serine phosphorylation of a specific phosphopeptide. It is well known that calcium and calmodulin may activate a number of protein kinases that phosphorylate serine and/or threonine residues on a wide spectrum of different substrates (for a review, see Edelman *et al.*, 1987). Since $p145^{MET}$ is inhibited by Ca^{2+} concentrations within the physiological range, the signal transduced by the *MET* receptor is likely to be modulated by other receptor systems coupled to transient Ca^{2+} fluxes.

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