Pervanadate Stimulates Amylase Release and Protein Tyrosine Phosphorylation of Paxillin and p125FAK in Differentiated AR4-2J Pancreatic Acinar Cells*

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We have studied the role of protein tyrosine phosphorylation in amylase secretion from differentiated AR4-2J cells. The secretagogue bombesin, the protein kinase C activator phorbol 12-myristate 13-acetate (PMA), and the protein-tyrosine phosphatase inhibitor pervanadate induced tyrosine phosphorylation of different proteins, including paxillin and p125FAK, which was reduced or blocked by the tyrosine kinase inhibitors genistein and tyrphostin B56, respectively. Both PMA and pervanadate continuously increased amylase secretion with a similar time course, reaching the level of bombesin-induced amylase release after 60 min. Their effects were not additive and could be inhibited by pre-incubation of AR4-2J cells with genistein or tyrphostin B56, respectively. Inhibition of protein kinase C with Ro 31-8220 nearly abolished the effects of PMA, but had no effect on either pervanadate-induced protein tyrosine phosphorylation or amylase secretion. An increase in cytosolic free Ca\(^{2+}\) concentration by thapsigargin or A23187 caused a rapid increase in amylase release within the initial 5 min. In the presence of PMA or pervanadate, amylase secretion was further stimulated to levels comparable to those induced by bombesin after 30 min of stimulation. Inhibition of PMA-induced amylase secretion by Ro 31-8220 was less at elevated cytosolic free Ca\(^{2+}\) concentrations than without Ca\(^{2+}\). Furthermore, an increase in cytosolic free Ca\(^{2+}\) concentration had no effect on protein tyrosine phosphorylation in either the absence or presence of PMA or pervanadate. We therefore conclude that in the cascade of events that lead to bombesin-induced protein secretion from AR4-2J cells, protein tyrosine phosphorylation occurs downstream of protein kinase C activation. A further step in secretion that is Ca\(^{2+}\)-dependent occurs distal to protein tyrosine phosphorylation.

In a variety of different cell types, cellular responses to hormones and/or growth factors are mediated by the activation of tyrosine kinases (1). Phosphorylation of tyrosine residues in specific proteins is due to the activity of not only tyrosine kinases, but also phosphotyrosine phosphatases. Specific phosphatase inhibitors, such as sodium vanadate (2), are useful tools in the study of hormone- and growth factor-induced tyrosine phosphorylation. Vanadium salts have been shown to have “insulin-like” effects both in intact animals and in cell cultures (3). H\(_2\)O\(_2\), like vanadate, partially mimics the effects of insulin in different cell culture systems (4, 5). Furthermore, it has been reported that pervanadate, which is generated by vanadate peroxidation in the presence of H\(_2\)O\(_2\), is a new powerful insulin-like agent that exceeds the sum of both vanadate and H\(_2\)O\(_2\) effects (6). Intraperitoneal injection of pervanadate into mice leads to tyrosine phosphorylation of numerous proteins in the liver and kidney within minutes (7). Thus, pervanadate may be a useful tool to study the role of tyrosine-phosphorylated proteins involved in signaling pathways.

In rat pancreatic acinar cells, enzyme secretion is stimulated in response to secretagogues such as cholecystokinin or bombesin (8). The signal cascade involves activation of receptor-coupled G-proteins, leading to the stimulation of phospholipase C activity, which is followed by production of inositol 1,4,5-trisphosphate and diacylglycerol. Whereas inositol 1,4,5-trisphosphate releases Ca\(^{2+}\) from intracellular stores (9), diacylglycerol leads to activation of protein kinase C (PKC) (10). Furthermore, tyrosine phosphorylation of a number of proteins could be observed following stimulation of the cells with cholecystokinin (11). It was suggested that tyrosine phosphorylation could amplify the secretory response rather than provide an obligate signal for enzyme secretion. On the other hand, tyrosine kinase inhibitors such as genistein and tyrphostin 25 were shown to inhibit agonist-induced amylase secretion and phospholipase C activation in rat pancreatic acinar cells (12, 13), indicating that tyrosine kinases are involved in receptor-mediated stimulation of amylase secretion.

In Swiss 3T3 cells, bombesin-induced tyrosine phosphorylation of two proteins, the cytosolic protein kinase p125FAK and the cytoskeletal-associated protein paxillin, has been described as an early event in hormone-mediated cell growth (14, 15). The increase in tyrosine phosphorylation of both p125FAK and paxillin is accompanied by profound changes in the organization of the actin cytoskeleton and in the assembly of the focal adhesion plaques, which represent the sites of cell attachment to the extracellular matrix (16, 17). Both p125FAK and paxillin are localized in these distinct areas. They are assumed to be regulatory components of the complex of cytoskeletal proteins that link the actin cytoskeleton to the plasma membrane (reviewed in Ref. 18).

It was the purpose of this study to investigate if protein tyrosine phosphorylation is a step in the signal cascade of stimulus-secretion coupling in rat pancreatic acinar cells. We have used the tumor cell line AR4-2J, which has been described as a model system for long-term studies on pancreatic acinar cell function (19, 20).

Our results suggest that protein tyrosine phosphorylation...
downstream of protein kinase C activation is involved in stimulation of enzyme secretion from pancreatic AR4-2J cells. An increase in cytosolic free Ca\textsuperscript{2+} concentration accelerated amylase secretion, but had no effect on either PMA- or pervanadate-induced tyrosine phosphorylation. We therefore assume that Ca\textsuperscript{2+} is involved in protein secretion distal to protein tyrosine phosphorylation in such a way that priming of target proteins by tyrosine phosphorylation is a prerequisite for Ca\textsuperscript{2+}-dependent activation of secretion.

**EXPERIMENTAL PROCEDURES**

**Materials**—Genistein, tyrphostin B56, phorbol 12-myristate 13-acetate, Ro 31-8220, and thapsigargin were obtained from Calbiochem (Bad Soden, Germany) and were prepared in dimethyl sulfoxide as stock solutions. The anti-phosphotyrosine antibodies (clone PY20) were purchased from Santa Cruz (Heidelberg, Germany), anti-p125\textsuperscript{FAK} antibody (clone 2A7) from Biomol (Hamburg, Germany), and anti-phosphatase antibody (clone 349) and protein G PLUS/protein A-agarose from Di-anova (Hamburg). Peroxide of vanadate (pervanadate) was prepared by mixing vanadate (Sigma, Deisenhofen, Germany) with H\textsubscript{2}O\textsubscript{2} (Merck, Darmstadt, Germany) for 15 min at room temperature, followed by addition of catalase (Sigma) to remove residual H\textsubscript{2}O\textsubscript{2} as described by Fantus et al. (21). Cal celf serum was obtained from PAA Laboratories (Colbe, Germany), and penicillin/streptomycin from Life Technologies, Inc. (Eggenstein, Germany). Leupeptin was purchased from Serva (Heidelberg), and trypsin inhibitor (hen egg white) from Boehringer (Mannheim, Germany). Dulbecco’s modified Eagle’s medium and all other reagents (of analytical grade) were from Sigma.

**Cell Culture**—AR4-2J cells were obtained from American Type Culture Collection (ATCC CRL1429; Rockville, MD). The cells were seeded at 750,000 cells/35-mm Petri dish and were routinely grown for 72 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 \mu g/ml streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO\textsubscript{2}. Differentiation of the cells was induced by addition of 100 nM dexamethasone. After 72 h, the cells were washed with the appropriate buffer and used for the assays.

**Amylase Release**—For measurement of amylase release, AR4-2J cells were washed three times with KRH buffer (130 mM NaCl, 5 mM KCl, 2 mM MgCl\textsubscript{2}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1 mM CaCl\textsubscript{2}, 20 mM Hepes, 10 mM glucose, and 0.1 mg/ml trypsin inhibitor, pH 7.4) and preincubated for 10 min with or without kinase inhibitors at 37 °C. The cells were stimulated by addition of agonists with or without pervanadate, and at the indicated times aliquots of the supernatants were removed for the determination of amylase released by the cells. For measurement of the total amount of amylase, the cells were broken and scraped off in a buffer containing 0.1% Triton X-100, 5 mM Hepes, pH 7.0, 280 mM mannitol, 10 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM benzamidine, 1 \mu M leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, and 20 \mu g/ml trypsin inhibitor. Amylase activity was determined using the Phadebas amylase test kit (Pharmacia, Freiburg, Germany) and is expressed as a percent of the total cellular content of amylase.

**Protein Tyrosine Phosphorylation Induced by Pervanadate and Bombesin**

**RESULTS**

**Amylase Secretion Induced by Pervanadate and Bombesin**—In the presence of pervanadate, amylase secretion from AR4-2J cells increased with time. However, the kinetics of amylase secretion induced by different concentrations of pervanadate were different compared with those for bombesin-induced amylase secretion (Fig. 1A). Whereas bombesin stimulated amylase secretion mostly during the initial 10 min, pervanadate-stimulated amylase release above basal levels was detectable after 15 min and increased continuously within 60 min of observation. After 60 min of incubation, maximal amylase release was obtained with 100 \mu M pervanadate, which was comparable to that obtained by a maximally effective bombesin concentration. In Fig. 1B, the dose-response relationship of pervanadate-stimulated amylase release in the absence and presence of bombesin is shown. After 30 min of incubation, secretion was stimulated by the maximally effective concentration of 100 \mu M pervanadate from 7.0 ± 0.7% of total amylase in control cells to 13.3 ± 1.2%. Half-maximal stimulation occurred at 20 \mu M pervanadate. At a maximally effective concentration of bombesin, amylase secretion over 30 min (19.4 ± 1.8%) was not significantly increased by pervanadate (23.1 ± 2.3%). After 60 min of incubation, maximal amylase secretion was obtained with 100 \mu M pervanadate, which was in the same range as the amylase release induced by a maximally effective bombesin concentration (31.6 ± 0.2%). Bombesin-stimulated amylase release was not further increased by any of the tested pervanadate concentrations. Taken together, these results indicate that bombesin-stimulated amylase secretion is probably mediated by a tyrosine kinase. As shown in Table I, the single components that are necessary to prepare pervanadate did not increase basal or bombesin-stimulated amylase secretion. H\textsubscript{2}O\textsubscript{2} alone had an inhibitory effect on both unstimulated and bombesin-stimulated amylase secretion. This effect is probably due to free oxygen radicals because no inhibition of amylase secretion was observed when H\textsubscript{2}O\textsubscript{2} was preincubated with catalase (Table I).

**Protein Tyrosine Phosphorylation Induced by Pervanadate and Bombesin**—To further assess the role of tyrosine phosphorylation in stimulus-secretion coupling, we compared both pervanadate- and bombesin-induced protein tyrosine phosphorylation in AR4-2J cells by immunoblot analysis using a monoclonal antibody to phosphotyrosine. Fig. 2A shows that treatment of cells with 100 \mu M pervanadate leads time-dependently to a strong tyrosine phosphorylation of multiple proteins. The earliest phosphorylation of proteins migrating with apparent molecular masses of 145, 120–140, 82, and 70 kDa was observed already after 3 min of incubation, but the cells with pervanadate and increased during the observed period of 25 min. During this time, more and more proteins became tyrosine-phosphorylated in a dose-dependent manner (Fig. 2B).

At pervanadate concentrations of 2 and 5 \mu M, a protein band of 145 kDa and one of 82 kDa appeared, respectively. After 25 min of incubation of cells with 50 \mu M pervanadate, protein bands of apparent molecular masses of 145, 120–140, 82, and 70 kDa were observed. The band at 145 kDa increased already after 3 min of incubation, but the cells with pervanadate and increased during the observed period of 25 min. During this time, more and more proteins became tyrosine-phosphorylated in a dose-dependent manner (Fig. 2B).
were detected that migrated with apparent molecular masses of 180, 145, 120–140, 98, 82, 70, 62, 58, 55, 51, 42, and 37 kDa (Fig. 2B). Bombesin-induced protein tyrosine phosphorylation, which was maximally stimulated after 15 min of incubation with 10 nM bombesin, showed a different pattern (Fig. 2B, bn). The most prominent bands observed migrated with apparent molecular masses of 120–140 and ~70 kDa. These bands probably correspond to the focal adhesion proteins p125FAK and paxillin, respectively, known to be tyrosine-phosphorylated after bombesin treatment in Swiss 3T3 cells (14, 15). Immunoprecipitation and immunoblotting showed that previous treatment of AR4-2J cells with pervanadate increased tyrosine phosphorylation of p125FAK and paxillin ~5- and 39-fold, respectively, compared with basal phosphorylation in control cells (Fig. 3). Whereas tyrosine phosphorylation of p125FAK induced by bombesin was also ~3-fold of the control, tyrosine phosphorylation of paxillin was increased only by ~12-fold above basal phosphorylation. In the anti-paxillin immunocomplexes of bombesin- and pervanadate-stimulated cells, anti-phosphotyrosine immunoreactivity migrated as broad diffuse bands with an apparent molecular mass of 66–75 kDa. After pervanadate stimulation, the anti-paxillin antibody detected a shift from 63 kDa to the phosphorylated form at 64–70 kDa (see Fig. 5B, lower panel). This indicates that tyrosine phosphorylation of paxillin by the physiological stimulus bombesin is lower compared with the nonspecific stimulus pervanadate. Furthermore, the finding that the anti-paxillin antibody detected bands with a lower molecular mass of 63 kDa after pervanadate treatment (Fig. 5B, upper panel) indicates that the nonspecific stimulus pervanadate increases the phosphorylation of paxillin at a lower molecular mass.

**Table I**

| Condition                      | Control | 10 nM bombesin |
|--------------------------------|---------|----------------|
| None                           | 8.3 ± 0.5 | 21.6 ± 1.2    |
| 1 mM vanadate                  | 7.4 ± 0.4 | 21.8 ± 0.6    |
| 1 mM H2O2                      | 4.9 ± 1.7 | 7.3 ± 0.7     |
| 120 milliunits catalase         | 7.7 ± 0.3 | 23.0 ± 3.1    |
| 1 mM H2O2 + 120 milliunits catalase | 7.5 ± 0.3 | 21.6 ± 1.3    |
| 1 mM vanadate + 1 mM H2O2      | 12.0 ± 2.6 | 14.2 ± 1.8    |
| 1 mM vanadate + 1 mM H2O2 + 120 milliunits catalase | 15.6 ± 0.1 | 23.8 ± 2.0    |

**Fig. 1.** Time and dose dependence of pervanadate-stimulated amylase secretion from AR4-2J cells. A, time course of unstimulated and pervanadate- and bombesin-stimulated amylase release. AR4-2J cells were incubated in KRH buffer for up to 60 min in the absence and presence of pervanadate (pv) at the indicated concentrations or with 10 nM bombesin (bn). At the indicated time points, aliquots were removed from the medium, and amylase secreted into the medium was quantified. Amylase release is expressed as a percent of total amylase content of the cells present at the beginning of the incubation (n = 4). B, effect of various concentrations of pervanadate on basal and bombesin-stimulated amylase release. AR4-2J cells were incubated in KRH buffer for 30 or 60 min at 37 °C in the absence or presence of bombesin-stimulated amylase secretion. AR4-2J cells were incubated in KRH buffer for up to 60 min in the absence or presence of pervanadate (pv) at the indicated concentrations without (control (c)) and with 10 nM bombesin (bn). Amylase secreted into the medium after 30 and 60 min of incubation was quantified and is expressed as a percent of total amylase content of the cells present at the beginning of the incubation (n = 4).

**Fig. 2.** Effect of pervanadate on protein tyrosine phosphorylation in AR4-2J cells. AR4-2J cells were incubated in KRH buffer with 100 μM pervanadate for the indicated times (A) or at different pervanadate concentrations for 25 min or with 10 nM bombesin (bn) for 15 min at 37 °C (B). The different incubation times for pervanadate and bombesin correspond to the time at which each agent had its maximal effect on tyrosine phosphorylation. Cell lysates were subjected to SDS-PAGE, followed by electrotransfer and immunoblotting with anti-phosphotyrosine antibodies as described under “Experimental Procedures.” The experiment shown is representative of two separate experiments.

**Pervanadate Stimulates Amylase Secretion**
molecular mass compared with the anti-phosphotyrosine antibody (see Fig. 5B, compare lower and upper panels) suggests that these bands represent minor phosphorylated or unphosphorylated forms of paxillin.

Effect of the Tyrosine Kinase Inhibitors Genistein and Tyrphostin B56 on Pervanadate- and Bombesin-stimulated Amylase Secretion and Protein Tyrosine Phosphorylation—The effects of pervanadate on amylase secretion and tyrosine phosphorylation indicate that a tyrosine kinase is involved in these processes. We therefore tested the effects of tyrosine kinase inhibitors on pervanadate- and bombesin-stimulated amylase secretion and on protein tyrosine phosphorylation. As shown in Fig. 4, 100 μM genistein reduced both pervanadate- and bombesin-stimulated amylase secretion to basal levels, whereas 100 μM tyrphostin B56 nearly completely inhibited basal and pervanadate- and bombesin-stimulated amylase secretion. Similarly, both genistein and tyrphostin B56 also reduced pervanadate-induced protein tyrosine phosphorylation by −60 and 90%, respectively (Fig. 5A), whereas bombesin-induced protein tyrosine phosphorylation was reduced to basal phosphorylation by genistein and was completely blocked by tyrphostin B56. As shown in Fig. 5B (upper panel), pervanadate-stimulated tyrosine phosphorylation of paxillin was −39-fold of the control and was reduced by genistein and tyrphostin B56 by −70 and 90%, respectively, whereas bombesin-induced tyrosine phosphorylation was −14-fold of the control and was reduced to basal phosphorylation by genistein and was completely blocked by tyrphostin B56. The apparently lower content of paxillin, as detected with the anti-paxillin antibody, in immunoprecipitates of bombesin- and pervanadate-stimulated cells compared with that of control cells is likely due to the shift of paxillin (Fig. 5B, lower panel). Densitometric scanning showed that the total amount of paxillin in the immunoprecipitates of stimulated cells was 82.5 and 135.7% after bombesin and pervanadate stimulation, respectively, compared with that of control cells. This indicates that the differences in the phosphotyrosine content of paxillin are due to different levels of phosphorylation and not to various amounts of protein (Fig. 5B, lower panel). In the samples from genistein-treated cells, the amount of precipitated paxillin was about half of the control and was probably due to degradation of paxillin. Fig. 5C shows that pervanadate-induced tyrosine phosphorylation of p125FAK was 4-fold of the control and was reduced to basal levels by genistein and by −90% by tyrphostin B56. Bombesin-stimulated tyrosine phosphorylation was 5-fold of the control and was also reduced to basal levels by genistein and was completely inhibited by tyrphostin B56. Since the anti-p125FAK antibody is not useful for immunoblot analysis, it was not possible to control the amounts of protein precipitated with this antibody. However, equal amounts of immunoglobulins in each lane detected by immunoblot analysis suggested that equal amounts of p125FAK had been precipitated.

We compared the ability of various concentrations of genis-
tein to inhibit bombesin-induced amylase secretion with its ability to decrease tyrosine phosphorylation of p125\textsubscript{FAK} and paxillin. As shown in Fig. 6, inhibition of bombesin-stimulated amylase release was detectable at \( \sim 30 \) \( \mu \)M, half-maximal at 60 \( \mu \)M, and maximal at \( \sim 150 \) \( \mu \)M. The dose-response relationship for inhibition of tyrosine phosphorylation of p125\textsubscript{FAK} and paxillin by genistein was similar. Inhibition was detectable at 20 \( \mu \)M, half-maximal at \( \sim 50 \) \( \mu \)M, and maximal at 200 \( \mu \)M.

Effect of Pervanadate on Phospholipase C Activity—Previous reports on rat pancreatic acinar cells suggest that tyrosine kinases are involved in the activation of phospholipase C induced by different secretagogues such as cholecystokinin, bombesin, or carbachol (12, 13). To test if pervanadate-stimulated amylase secretion is induced by pervanadate-mediated activation of phospholipase C, AR4-2J cells were treated with 100 \( \mu \)M or 1 \( \text{nM} \) pervanadate for 10 s to 50 min and with 10 \( \text{nM} \) bombesin for 15 s for comparison. Phospholipase C activity was determined by measurement of inositol 1,4,5-trisphosphate production. At no time could any significant change in inositol 1,4,5-trisphosphate production be observed in response to pervanadate compared with the control, whereas bombesin increased inositol 1,4,5-trisphosphate levels to \( \sim 5 \)-fold of the control (data not shown).

Role of Protein Kinase C in Pervanadate- and Bombesin-stimulated Amylase Secretion and Protein Tyrosine Phosphorylation—It is well established that secretagogue-stimulated amylase secretion from rat pancreatic acinar cells is mediated by PKC (24, 25). To test if pervanadate-induced amylase release is also mediated by PKC activity and if a tyrosine kinase is involved in the PKC-activated pathway, the specific PKC inhibitor Ro 31-8220 (26, 27) was tested on amylase secretion and tyrosine phosphorylation. As shown in Fig. 7A, the time course of PMA-stimulated amylase secretion was nearly identical to that induced by pervanadate. Incubation of the cells with both PMA and pervanadate showed no higher stimulatory effect than with each component alone. Preincubation of AR4-2J cells with Ro 31-8220 inhibited PMA- and bombesin-stimulated amylase secretion by \( \sim 80 \) and 50\%, respectively, whereas secretion induced by pervanadate alone or in combination with PMA was not significantly inhibited (Fig. 7B). These data suggest that PKC and the responsible tyrosine kinase are acting in the same signaling pathway and that PKC functions upstream of this tyrosine kinase.

Previous reports on Swiss 3T3 cells suggest that bombesin-induced protein tyrosine phosphorylation is also mediated by PKC (28). As shown in Fig. 8, in AR4-2J cells, the PMA-induced protein tyrosine phosphorylation pattern was nearly identical to that induced by bombesin. However, the degree of tyrosine phosphorylation of proteins differed. PMA-induced tyrosine phosphorylation of proteins with an apparent molecular mass of \( \sim 70 \) kDa was about one-third of that induced by bombesin. Preincubation of the cells with the PKC inhibitor Ro 31-8220 inhibited both PMA- and bombesin-induced protein tyrosine phosphorylation. Protein tyrosine phosphorylation induced by pervanadate was not significantly affected by Ro 31-8220 (Fig. 8). These results indicate that bombesin-stimulated tyrosine phosphorylation of 120–140- and 70-kDa proteins is mediated by PKC and that the responsible tyrosine kinase functions downstream of PKC activity.

Effect of Thapsigargin on PMA- and Pervanadate-stimulated Amylase Secretion and Protein Tyrosine Phosphorylation—The slower time course of amylase release induced by pervanadate or PMA compared with bombesin indicates that Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} entry, by which the initial rapid phase of bombesin-induced amylase secretion is mediated (29), were not affected.
by tyrosine kinase activity. Therefore, we tested if an increase in cytosolic free Ca$^{2+}$ concentration could enhance amylase secretion within the first minutes in the presence of pervanadate or PMA using the Ca$^{2+}$-ATPase inhibitor thapsigargin (30) or the calcium ionophore A23187. As shown in Fig. 9A, thapsigargin increased PMA-stimulated amylase release to levels comparable to those induced by bombesin, whereas thapsigargin alone increased amylase release only slightly within the first 5–10 min and thereafter showed no higher secretion rate than the unstimulated control cells. Amylase release induced by the combination of pervanadate and thapsigargin was increased within the first 10 min to the same extent as by thapsigargin alone (Fig. 9B). Thereafter, the rate of stimulation was similar to that with pervanadate alone, indicating that both pervanadate and thapsigargin have additive effects on amylase secretion (Fig. 9B). Similarly, the increase in cytosolic free Ca$^{2+}$ concentration by the ionophore A23187 increased amylase secretion within the first 10 min. It was then additive with pervanadate and stimulated amylase secretion to levels comparable to those induced by bombesin within 30 min (Fig. 9B). An increase in cytosolic free Ca$^{2+}$ concentration by thapsigargin or the calcium ionophore A23187 had no effect on basal or PMA- or pervanadate-induced protein tyrosine phosphorylation (data not shown), indicating that an increase in cytosolic free Ca$^{2+}$ concentration, which leads to the initial increase in amylase secretion, affects the secretory mechanisms downstream of tyrosine phosphorylation. We therefore expected that the PKC inhibitor Ro 31-8220 would not inhibit PMA- or thapsigargin-induced amylase secretion to the same extent as that induced by PMA alone. As shown in Table II, PMA- or thapsigargin-stimulated amylase secretion after 60 min of incubation was inhibited by Ro 31-8220 by $\sim$50% and was comparable to the inhibition of bombesin-stimulated amylase release by Ro 31-8220 (Fig. 7B and Table II).

Since it had been reported that PMA-mediated amylase secretion was not inhibited by genistein (11, 13), it had been suggested that tyrosine kinase activity was not involved in this stimulatory pathway. Our data show, however, that amylase secretion induced by PMA alone or by a combination of PMA and thapsigargin as well as bombesin was inhibited by genistein (Fig. 10). This confirms our conclusion that a tyrosine kinase downstream of PKC is involved in amylase secretion.

**DISCUSSION**

We have examined the role of protein tyrosine phosphorylation in amylase secretion from differentiated AR4-2J rat pancreas. Previous evidence had suggested that both an increase in cytosolic free Ca$^{2+}$ concentration and activation of protein kinase C are critical steps in stimulus-secretion coupling (25, 29, 31). Whereas an initial rapid phase of hormone-induced enzyme secretion is mediated by production of inositol 1,4,5-trisphosphate followed by Ca$^{2+}$ release from in-
tracellular stores and \( \text{Ca}^{2+} \) influx into the cell, a second slower sustained phase is associated with production of diacylglycerol and activation of protein kinase C (25, 29). Both the \( \text{Ca}^{2+} \) and the protein kinase C-induced pathways act synergistically. Artificial activation of these pathways by elevation of cytosolic \( \text{Ca}^{2+} \) concentration in the presence of stimulators of protein kinase C, such as phorbol esters, could mimic the effect of secretagogues on enzyme secretion (29, 31, 32). Although different proteins have been shown to be phosphorylated in response to secretagogues of enzyme secretion, key targets in the \( \text{Ca}^{2+} \)–diacylglycerol-dependent cascade of stimulatory events have not yet been identified.

**A Protein-tyrosine Kinase Acts Downstream of Protein Kinase C in Bombesin-stimulated Amylase Secretion**—This study gives strong evidence that distal to the activation of protein kinase C, tyrosine kinase activation and tyrosine phosphorylation of one or more proteins play a crucial role in bombesin-induced amylase secretion. Our results show that two proteins, p125*FAK* and paxillin, are mainly tyrosine-phosphorylated by stimulation of AR4-2J cells with bombesin (Fig. 3). The striking correlation of the dose-response curves for genistein-induced inhibition of both bombesin-stimulated amylase secretion and tyrosine phosphorylation of p125*FAK* and paxillin indicates that cytosolic free \( \text{Ca}^{2+} \) elevation by phorbol esters, as described for adherent cells, is the same as that in AR4-2J cells (Fig. 1). However, the time courses of amylase release induced by maximally effective concentrations of bombesin and pervanadate are quite different. Whereas amylase secretion rapidly increases within the first minutes after addition of bombesin, the second phase continues at a lower rate for the duration of agonist stimulation (Fig. 1A). In contrast, stimulation by pervanadate does not lead to a significant amylase release earlier, but 10–15 min after addition, and the level of bombesin-stimulated amylase release is reached after 60 min. This time course resembles that of phorbol ester-stimulated amylase secretion (Fig. 7A). The effects of both pervanadate and PMA are not additive. Since bombesin-stimulated sustained amylase secretion and pervanadate-stimulated amylase release are also not additive (Fig. 1B), it can be concluded that a common mechanism is involved in stimulation of amylase secretion by these substances. Furthermore, Ro 31-8220, a specific inhibitor of protein kinase C, inhibits bombesin- and PMA-induced amylase secretion (Fig. 7B) as well as protein tyrosine phosphorylation (Fig. 8), but has no effect on both pervanadate-induced amylase secretion and protein tyrosine phosphorylation. In addition, secretion induced by PMA or pervanadate is inhibited by the tyrosine kinase inhibitor genistein (Figs. 4 and 10). These observations are compatible with the interpretation that stimulation of protein kinase C precedes tyrosine kinase activation. This is in contrast to previous studies on freshly isolated rat pancreatic acinar cells that showed that genistein had no effect on the secretory response to bombesin (11, 13). A possible explanation for this difference could be the use of freshly isolated pancreatic acinar cells in those studies compared with our cells kept in tissue culture.

**Role of Ca\(^{2+}\) in Protein Tyrosine Phosphorylation and Amylase Secretion**—The slower time course of amylase release induced by pervanadate indicates that \( \text{Ca}^{2+} \) release and \( \text{Ca}^{2+} \) entry, by which the initial rapid phase of agonist-induced amylase secretion is mediated (29), are not affected by tyrosine kinase activity. This agrees with our observation that pervanadate affects neither unstimulated nor bombesin-stimulated inositol 1,4,5-trisphosphate production. An increase in cytosolic free \( \text{Ca}^{2+} \) concentration by addition of thapsigargin or A23187 rapidly increases amylase secretion within the first minutes after addition. This effect of elevated cytosolic free \( \text{Ca}^{2+} \) concentration is additive to those of PMA and pervanadate (Fig. 9) and indicates that both protein tyrosine phosphorylation and elevation of cytosolic free \( \text{Ca}^{2+} \) concentration act together in the stimulation of amylase secretion. Inhibition of PKC by Ro 31-8220 inhibits PMA-induced amylase secretion in the absence of elevated cytosolic \( \text{Ca}^{2+} \) concentration to a higher degree than in its presence (see Table II). This indicates that the initial rapid phase of agonist-stimulated amylase secretion is probably due to an effect of \( \text{Ca}^{2+} \) downstream of tyrosine kinase activity. In agreement with this, we did not observe any increase in protein tyrosine phosphorylation after elevation of intracellular \( \text{Ca}^{2+} \) concentration by thapsigargin or A23187 (data not shown). Moreover, PMA-induced protein tyrosine phosphorylation was neither increased nor accelerated within the initial 10 min by an increase in intracellular \( \text{Ca}^{2+} \) concentration. These data are consistent with the interpretation that tyrosine phosphorylation of target proteins in the secretory machinery is an essential requirement for exocytosis to occur. Both tyrosine kinase and \( \text{Ca}^{2+} \) could act in sequence on a common step in exocytosis in such a way that priming of target proteins by tyrosine phosphorylation facilitates \( \text{Ca}^{2+} \) activation of secretion. If tyrosine kinase is blocked by genistein, \( \text{Ca}^{2+} \)-dependent secretion is also abolished (see Fig. 10).

**Role of p125*FAK* and Paxillin in Cellular Functions**—p125*FAK* and paxillin had been originally described as components of the focal adhesions anchoring cultured cells to extracellular matrix proteins (18). There is evidence from a number of cell types that p125*FAK* and paxillin are regulatory components of cytoskeletal proteins that link the actin cytoskeleton to the plasma membrane. Paxillin binds to vinculin and is tyrosine-phosphorylated in adherent cells, presumably...
by one of the tyrosine kinases with which it associates (p125FAK, Csk, Src, or Lck) (18, 33, 34). Recent in vitro findings demonstrate that p125FAK phosphorylates paxillin at Tyr-118 (35). Furthermore, the time course of tyrosine phosphorylation for p125FAK in Swiss 3T3 and AR4-2J cells preceding that of paxillin (36) suggests that p125FAK might be the tyrosine kinase itself that phosphorylates paxillin.

Tyrosine phosphorylation of paxillin is associated with cytoskeletal reorganization. It has been shown that tyrosine phosphorylation of paxillin is involved in actin filament assembly in cultured cells spreading on an extracellular matrix substrate (37). In embryonic chick tissue, paxillin is highly phosphorylated at developmental stages when the turnover of actin filament interactions is the most rapid (38). In many cells, exocytosis coincides with disassembly of actin filaments at the terminal web, and blocking actin depolymerization at this site prevents exocytosis (39–41). Muallem et al. (42) showed that actin filament disassembly is indeed a final trigger for exocytosis in freshly prepared rat pancreatic acinar cells. A recent study on rat pancreatic acinar cells by García et al. (43) showed that p125FAK and paxillin are tyrosine-phosphorylated in a Ca2+- and PKC-dependent manner after cholecystokinin stimulation. However, in contrast to these observations, protein tyrosine phosphorylation is independent of Ca2+-independent manner downstream of PKC activation. Ca2+-dependent tyrosine phosphorylation of paxillin (36) coincides with disassembly of actin filaments at the exocytosis in freshly prepared rat pancreatic acinar cells. A recent study on rat pancreatic acinar cells (43).<ref>2 P. Feick, S. Gilhaus, and I. Schulz, unpublished observation.</ref>

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