Insulin Secretagogues Activate the Secretory Granule Receptor-like Protein-tyrosine Phosphatase IAR*

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To investigate the potential role of protein-tyrosine phosphatases (PTPs) in regulated secretion, cellular PTP activity was measured in pancreatic β cell lines after exposure to insulin secretagogues. A peak of elevated PTP activity was detected in whole cell lysates after 15–20 min of treatment of the cells with high KCl, glucose, or TPA, which did not appear upon treatment with control compounds. Neither was it detected in cells that do not undergo regulated secretion. The PTP activation was transient, SDS-resistant, and localized to the cytoskeleton fraction of cells. The cytoskeletal localization of IAR, a receptor-like PTP associated with secretory granules of neuroendocrine cells, suggested the possibility that IAR is the secretagogue-activated PTP. The transient expression of human IAR in βTC3 and HIT-T15 β cells, followed by treatment with secretagogues or control compounds and immunoprecipitation of human IAR, showed that immunoprecipitates from the secretagogue-treated cells contained an elevated PTP activity. The secretagogue-induced activation of IAR had identical kinetics to that of the endogenous PTP. Although ectopic IAR was present in membrane and cytoskeletal fractions from the cells, only the cytoskeleton-associated IAR could be activated. Thus IAR represents the endogenous secretagogue-responsive PTP, or at least a component of it, and is one of the few receptor-like PTPs for which enzymatic activation has been demonstrated. Insulin secretion is detected prior to IAR activation, suggesting that IAR is not required for immediate secretion but likely plays a role in events downstream of insulin secretion or in another pathway related to the specialized function of secretory cells.

In addition to the constitutive secretory pathway, endocrine, neuronal, and exocrine cells also possess a regulated secretory pathway in which particular stimuli induce the release of secretory granule contents. Although reversible phosphorylation is the predominant mechanism of regulation of cellular processes, little is known of phosphorylation and dephosphorylation events, especially those involving the modification of tyrosine residues, in regulated exocytosis.

In the pancreatic β cell, insulin secretion can be effected by a variety of stimulants, with high plasma glucose being the major physiological stimulus (reviewed in Refs. 1 and 2). Intracellular metabolism of glucose closes ATP-dependent K⁺ channels, inducing membrane depolarization and activating voltage-dependent Ca²⁺ channels (reviewed in Refs. 3 and 4). The ensuing rise in intracellular Ca²⁺, together with poorly understood events involving heterotrimetric and monomeric GTP-binding proteins (4–6), phosphatidylinositol metabolism (7), and protein phosphorylation (7–9), triggers exocytosis. Glucose and/or other insulin secretagogues activate several serine/threonine protein kinases, including Ca²⁺/calmodulin-dependent protein kinase II (10–12), myosin light chain kinase (13), protein kinase C (14–18), cAMP-dependent protein kinase (11, 19, 20), and MAP kinase (21). However the functions of these kinases in secretory events are unclear. The stimulation of insulin secretion by okadaic acid (11, 22), an inhibitor of certain serine/threonine protein phosphatases, is in agreement with a requirement for protein serine/threonine phosphorylation. In contrast, very little is known of protein tyrosine phosphorylation events in regulated secretion, particularly in islet cells. Protein-tyrosine kinases are activated, because MAP kinase activation requires tyrosine phosphorylation and is observed as an early event following stimulation. Nevertheless, blocking MAP kinase activation does not inhibit glucose-stimulated insulin secretion (23). Glucose and carbachol induce tyrosine phosphorylation of a 125-kDa protein and insulin secretion, and both are inhibited by a tyrosine kinase inhibitor (24). Vanadate, a protein-tyrosine phosphatase (PTP) inhibitor, potentiates glucose-stimulated insulin release from normal rat or mouse islets and affects Ca²⁺ influx, phosphoinositide metabolism, and protein tyrosine phosphorylation (25–27).

Two related receptor-like PTPs, called IA-2 (or ICA 512) and IAR (islet cell antigen-related PTP) (or IA-2β, PTP-NP, phogrin, ICAAR, NE-6), have been identified and cloned and exhibit high expression in neuroendocrine tissues such as pancreas and brain (28–35). These PTPs have highly homologous intracellular regions (74% amino acid identity) and less closely related extracellular regions (24% amino acid identity). Within neurons and β-islet cells, IA-2 is an intrinsic membrane protein of secretory granules that undergo regulated exocytosis (36). The extracellular region of IA-2 is extended into the granule lumen and is thus more appropriately termed the luminal region, with the intracellular region facing into the cytosol. Stimulation of exocytosis in RIN cells results in exposure of IA-2 at the cell surface, from which it is internalized and resorted in the Golgi complex to nascent secretory granules. Likewise IAR is localized to the membrane of insulinoma cell secretory granules with the same N-terminal and cytosolic C-terminal orientation as IA-2 (31). Both IA-2 and IAR have a conserved PTP catalytic domain in the intracellular region, and

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§ The abbreviations used are: MAP, mitogen-activated protein kinase; PTP, protein-tyrosine phosphatase; Chaps, 3-[3-cholamidopro- pyl]dimethylammoniomopropane-1-sulfonate; TPA, 12-O-tetradecanoylphorbol-13-acetate; hIAR, human IAR.

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the recombinant intracellular region of IAR has been demonstrated to have PTP activity in vitro toward two artificial substrates, pNPP and phosphotyrosyl-casein (32). This activity is extremely low compared with that of similar forms of other PTPs and may indicate that IAR has high substrate selectivity or other cellular requirements for activity. Nevertheless, the catalytic capability of IAR, in conjunction with its subcellular localization as a membrane protein of secretory granules, suggests a potential enzymatic role for this PTP in regulated secretion.

To investigate the role of PTPs in regulated secretion, we have examined secretagogue-induced alterations in PTP activity in the pancreatic β cell lines βTC-3, HIT-T15, and RIN. We provide evidence that cytoskeleton-associated PTP activity, a component of which is IAR, is stimulated by insulin secretagogues. The kinetics of PTP activation suggest that this phosphatase is unlikely to be required for secretion but is involved in later, secretion-stimulated processes.

EXPERIMENTAL PROCEDURES

Plasmids—The cDNA encoding the full-length human IAR protein (GenBank accession number AF007555) and with the 5′ untranslated region replaced with the Kozak consensus sequence CCACC (32) was cut out from pBluescript SK(+) and cloned into the mammalian expression vector pXJ41-neo (37) to create pXJ41-hIAR-neo. A fragment containing the IAR active site sequence was released from pXJ41-hIAR-neo using EcoRV and NheI and replaced with the corresponding fragment from pGEX-3C-hIAR(C945S) (32) to form pXJ41-hIAR(C945S)-neo.

Cell Culture and Transfections—The RIN 5Aβ and βTC3 cells were gifts from H. DeAizpurua. The RIN 5Aβ cells were subcloned by limiting dilution to reduce the observed cell-to-cell variation in insulin secretion, and the subclone RIN 5Aβ-12 was chosen for experimental work. The HIT-T15 cells were purchased from the ATCC (passages 66–72), as were COS-1 and NIH 3T3 cells. The RIN 5Aβ cells were cultured in RPMI (10 mM Hepes) supplemented with 10% fetal calf serum and penicillin/streptomycin, and COS-1 cells were cultured in F12K medium containing 10% dialyzed horse serum, 2.5% fetal calf serum and penicillin/streptomycin. The βTC-3 and NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose, 10% fetal calf serum, and penicillin/streptomycin, and COS-1 cells were maintained in the same medium but with 10 g/liter glucose. Transient transfections of the above cells were carried out with plasmid DNA (10 μg/10-cm dish or 30 μg/15-cm dish) and LipofectAMINE™ reagent (Life Technologies, Inc.) with the amount of total DNA equalized between transfections by the addition of the empty plasmid pXJ41-neo. The cells were maintained in medium with serum for 24 h prior to harvesting.

Secretagogue Treatments and Insulin Assay—Cells at 50–60% confluence were treated with modified Krebs-Ringer buffer containing 10 mM NaCl, 5 mM NaHCO3, 2 mM CaCl2, 1 mM MgCl2, 1.15 mM Na2HPO4, 0.4 mM KH2PO4, 0.1 mM MgSO4, 20 mM Hepes, 0.1% bovine serum albumin, 0.001% phenol red) before preincubation in the same buffer containing 0.1 mM Na3VO4 and followed by rabbit anti-mouse IgG conjugated to peroxidase (Sigma) (1:5000). Immunoblots were developed using the ECL system (Amer sham). For MAP kinase analysis by Western blotting, whole cell lysates were prepared in harvesting buffer containing 0.1 mM Na3VO4 and resolved by SDS-polyacrylamide gel electrophoresis. After transfer to membranes, sequential immunoblotting was carried out with anti-phosphotyrosine antibody (PY-20) and anti-pan-ERK antibody (both from Transduction Laboratories).

Cell Fractionation—Cells were scraped into harvesting buffer, sonicated, and centrifuged at 100,000 × g for 30 min. The supernatant was washed three times with harvesting buffer, and a portion was used for duplicate assays of associated PTP activity, whereas another portion was used for immunoprecipitation.

RESULTS

Insulin Secretagogues Induce PTP Activation in βTC-3 and HIT-T15 Cells—In accord with the known activity of high extracellular KC1 as an insulin secretagogue on pancreatic islets and β cell lines, insulin secretion was observed after the addition of high (55 mM) but not low (1 mM) KC1 to βTC3 cells (Fig. 1A). The phosphorylation and activation of MAP kinase have been reported to occur in response to insulin secretagogues (21). Treatment of the cells with high KC1 followed by probing of cell lysates with anti-phosphotyrosine antibody demonstrated the rapid appearance of a phosphotyrosine-containing band of about 42 kDa that was not detected after treatment with low KC1 (Fig. 1B, top panel). Reprobing of the membrane with anti-MAP kinase antibody demonstrated that the novel phosphotyrosine-containing band comigrated with MAP kinase, which was present in all lanes (Fig. 1B, bottom panel). Thus, although these particular βTC3 cells do not secrete insulin in response to glucose (data not shown), they do respond as expected to high KC1, another insulin secretagogue. We examined whether high or low KC1 induced any alterations in cellular PTP activity by assaying the ability of whole cell lysates to dephosphorylate the peptide substrate phosphotyrosyl-RR-sre. Over a period of 60 min of treatment with low KC1, a small and insignificant increase was initially observed (0–15 min) in cellular PTP activity, which subsequently decreased to basal level (Fig. 1C). High KC1 treatment elicited a more pronounced increase in PTP activity, with a peak of elevated PTP activity after 20 min (Fig. 1C). This peak represented a 2.8-fold increase in PTP activity compared with that measured at 20 min of low KC1 treatment and rapidly disappeared; it was undetectable by 25 min after high KC1 treatment.

The effects of insulin secretagogues on another β cell line, HIT-T15, were examined. The secretagogues 15 mM glucose, 55 mM KC1, or TPA (in MeSO) all induced insulin secretion, with a 30-min treatment resulting in at least a 2-fold higher amount of insulin released than treatment with the respective control compounds (1 mM glucose, 1 mM KC1, and MeSO) (Fig. 2, A, C, and D).
Activation of IAR in Secretory Cells

Fig. 1. High KCl induces insulin secretion, MAP kinase, and PTP activation in βTC3 cells. A, cells were incubated in Krebs-Ringer buffer for 20 min and then either maintained in fresh Krebs-Ringer buffer (basal buffer) or in high (55 mM, ) or low (1 mM, ) KCl buffer. Aliquots of the buffers were removed at the times shown, and the insulin content was measured. The points represent the fold increase in insulin amount over that in the basal buffer and are the means ± S.D. of four measurements from two independent experiments. B, cells were incubated in basal buffer for 30 min (time 0) and then in high (H) or low (L) KCl buffers for the times shown. Cell lysates were prepared as described under “Experimental Procedures” and probed with anti-phosphotyrosine antibody (top panel). The membrane was stripped and reprobed with anti-pan-ERK antibody (bottom panel). The anti-pan-ERK signal comigrated with the signal indicated by the arrow in the top panel. C, cells were incubated as described for B in high ( ) or low ( ) KCl buffers. Whole cell lysates were prepared at the indicated times and assayed in duplicate for PTP activity as described under “Experimental Procedures.” The points are the means ± S.D. of the results of three independent experiments. The mean PTP activities measured after 20 min of treatment with high and low KCl buffers are significantly different (p < 0.05).

and E). Cellular PTP activity increased during the first 5–10 min of treatment both with the secretagogues and with the respective control compounds. After this initial increase, PTP activity continued to increase to a peak at 15 min in the secretagogue-treated cells, whereas PTP activity plateaued at a lower level in the control compound-treated cells (Fig. 2, B, D, and F). At the 15-min time point, the peak PTP activity in the insulin-secreting cells was about 1.2–1.3-fold higher than the PTP activity in the control cells. Thus secretagogues induce a general profile and peak of PTP activation similar to that of insulin-secreting cells was about 1.2–1.3-fold higher than the

High KCl Induces PTP Activation in RIN but Not COS-1 or NIH 3T3 Cells—We also examined the effect of high KCl treatment on PTP activity in whole cell lysates of RIN 5AH-12 and COS-1 or NIH 3T3 cells. The RIN 5AH-12 cells are a subclone of a rat insulinoma cell line (RIN 5AH) that can undergo constitutive but not a regulated secretory pathway. The RIN 5AH-12 cells secrete insulin in response to high KCl treatment (not shown) and exhibit a KCl-stimulated and time-dependent increase in cellular PTP activity. High KCl elicits a peak of PTP activity at 15 min, which is about 2-fold elevated compared with PTP activity measured upon low KCl treatment at that time (Fig. 3A). Activation is transient and disappears by 20 min of high KCl treatment. In contrast, no differences in PTP activity were observed in lysates of low or high KCl-treated COS-1 or NIH 3T3 cells (Fig. 3, B and C), suggesting that the effect of high KCl on PTP activity is related to its ability to stimulate regulated secretion in certain cell types and not a nonspecific effect on all cells in culture. AtT-20 pituitary cells, which utilize the regulated secretory pathway to secrete adrenocorticotropic hormone, also exhibit increased PTP activity in response to high but not low KCl (1.6-fold increase at 20 min in high versus low KCl) (data not shown). Thus the effect of high KCl on PTP activity is not limited to or related to insulin secretion in particular.

Cellular Localization of the KCl-activated PTP—To determine the subcellular localization of the high KCl-activated PTP, βTC3 cells were preincubated in Krebs-Ringer (basal) buffer followed by high or low KCl buffers for 20 min. Cytosol and membrane fractions were prepared, as well as a fraction made by solubilization of the Triton X-100 insoluble material with either KCl/Chaps or SDS, and termed the cytoskeleton fraction (40, 41). The PTP activities of these fractions were then assayed. In terms of PTP specific activity measured in the fractions of cells treated with low KCl, the cytosol had much higher activity than the membrane and cytoskeletal fractions. To allow ready comparison and visualization of the data, the PTP activity measured in each type of fraction treated with low KCl was taken as 100%, and the percentage of activity in the respective fraction after high KCl treatment was calculated accordingly. A 20-min treatment with high KCl resulted in elevated PTP activity in the cytoskeletal fraction, whether this was prepared by solubilization with KCl/Chaps or with SDS (Fig. 4A). Because in other experiments the whole cell lysates were prepared in buffers with SDS, we used SDS in all later experiments. No significant differences in PTP activity were found in the membrane or cytosol fractions with low or high KCl treatment (Fig. 4A). It is possible that a secretagogue-responsive PTP activity in the cytosol or membrane fractions is masked by the activity of other PTPs. To check this, and for comparison with the activity of the total cell lysate independently prepared in SDS-containing buffer, the fractions were...
also assayed after the addition of 0.5% SDS, as the cytoskeletal secretagogue-responsive PTP is active in this concentration of detergent. The presence of SDS virtually abolished PTP activity in the membrane and cytosol fractions, and there was no difference in the remaining PTP activity measured in these fractions prepared from low or high KCl treated cells (data not shown). Thus the secretagogue-activated PTP appeared to be present only in the cytoskeleton-associated cellular component. The cytoskeleton fraction from high KCl-treated cells contained 41% of the total protein and 69% of the total PTP activity recovered in all fractions with a specific activity of $0.85 \text{ pmol/min/mg}$.

This localization was of interest because many secretory granule proteins associate with the cytoskeleton and because two related PTPs, IA-2 (ICA512) and IAR (phogrin, etc.), were recently identified as secretory granule transmembrane proteins (31, 36). Furthermore, IA-2 is reported to associate with the neuroendocrine cell cytoskeleton (36). We have examined the localization of the related secretory granule PTP IAR using an antibody that specifically recognizes the luminal domain of human but not rat or mouse IAR. Fractionation of βTC3 cells transiently expressing hIAR into cytosol, membrane, and cytoskeleton fractions, followed by probing with the anti-hIAR antibody, showed the presence of hIAR mainly in the cytoskeleton fraction, with some also detected in the membrane fraction (Fig. 4B). Consistent with observed proteolytic processing of the endogenous rat protein (31), two forms of hIAR were detected: a 125-kDa protein likely corresponding to the full-length protein after signal peptide cleavage and a 80-kDa protein likely corresponding to the mature protein following endoproteolytic cleavage at a candidate RKK site (amino acids 425–427) in the luminal region of the protein.

**High KCl Treatment Activates Ectopically Expressed hIAR**—The cytoskeleton association of IAR and IA-2 suggests that one or both of these PTPs might be responsible for the observed...
secretagogue-stimulated PTP activity. We examined whether IAR could be activated by high KCl treatment, and because we lack an antibody that recognizes the endogenous IAR of murine βTC3 cells, we used an approach involving ectopic expression of hIAR for which we have immunological reagents. The βTC3 cells were transfected with empty plasmid or plasmid containing wild-type hIAR or a catalytically inactive hIAR mutant (hIAR-C945S) in which the essential cysteine residue in the active site had been mutated to serine (32). After 24 h, the cells were harvested in Krebs-Ringer (basal) buffer, and the immunoprecipitates from these cells treated with high KCl contained elevated PTP activity compared with that measured after low KCl treatment (open bars). The results are shown as the means ± S.D. from at least four independent experiments (lanes 1, 2, and 3a), and the asterisk denotes a significant difference from the PTP activity of low KCl-treated cells (p < 0.001). B, the βTC3 cells were transiently transfected with pXJ41-neo (mock) or pXJ41-hIAR-neo (hIAR). The cells were harvested, and cytosol (lane 1), membrane (lane 2), and 0.5% SDS-solubilized cytoskeleton (lanes 3) fractions were prepared. Fractions were probed with anti-hIAR antibody. The arrows show the two forms of hIAR, and the positions of molecular mass markers (kDa) are shown on the right.

form of the mutant hIAR appeared to be proteolytically processed slightly differently from wild-type hIAR, resulting in the appearance of an 80- and 82-kDa doublet, although the reason for this is unclear.

Similar experiments were carried out with HIT-T15 cells transiently transfected with hIAR. Treatment of the cells with low or high glucose was followed by immunoprecipitation of hIAR from total cell lysates prepared at different times of treatment. Assay of the immunoprecipitates revealed a small peak of PTP activity after 15 min of exposure of the cells to high glucose and essentially unchanged PTP activity throughout the treatment with low glucose (Fig. 6A). Western blotting showed equivalent levels of hIAR in the immunoprecipitates (Fig. 6B). In other experiments, HIT-T15 cells transiently expressing hIAR were treated with low or high KCl. Elevated PTP activity was present in hIAR immunoprecipitates from the cells exposed to high KCl, with a peak of activity (about 2-fold over unstimulated or low KCl treated cells) detected at 15 min (Fig. 6C). Thus hIAR activation by two different secretagogues and in two different neuroendocrine cell lines is consistently observed. Also, the time dependence of hIAR activation mirrors that of the endogenous PTP (Fig. 2). When COS-1 cells were transiently transfected with hIAR and exposed to low or high KCl, no increase in PTP activity was detected in hIAR immunoprecipitates from cells treated with high KCl compared with low KCl (data not shown). This suggests that hIAR activation is specific to cells possessing a regulated secretory pathway and not a general effect of the cell treatment.

Cytoskeleton-associated but Not Membrane-associated hIAR
Is Activated by High KCl—As shown in Fig. 4, ectopically expressed hIAR is present in the membrane and the cytoskeleton fractions of βTC3 cells. To determine whether these populations of hIAR are responsive to high KCl treatment, cytosol, membrane, and cytoskeleton fractions prepared from hIAR-expressing cells after a 20-min treatment with high or low KCl were incubated with anti-hIAR antibody, and the immunoprecipitates were assayed for PTP activity. The PTP activities in the cell fractions prior to immunoprecipitation were essentially as found previously for untransfected βTC-3 cells, with a 44% increase in cytoskeleton PTP activity after high KCl treatment compared with low KCl treatment and virtually no effect of high KCl observed on the PTP activity of the other fractions (Fig. 7A). In the anti-hIAR immunoprecipitates, about 3.5-fold higher PTP activity was present in the cytoskeletal fraction after high KCl treatment (Fig. 7A). In the anti-hIAR immunoprecipitates, about 3.5-fold higher PTP activity was present in the cytoskeletal fraction after high KCl treatment (Fig. 7A). Elevated PTP activity was not present in immunoprecipitates from any other fraction (Fig. 7A), although hIAR was also present, albeit at a lower level, in immunoprecipitates from membrane fractions (Fig. 7B). These results suggest that cytoskeleton-associated but not membrane-associated hIAR is activated upon treatment with high KCl.

**DISCUSSION**

Although the activation of multiple kinases in neuroendocrine cells during the late events leading to hormone secretion has been well documented, the involvement of protein phosphatases in this process has mainly been implied through the use of phosphatase inhibitors. Here we have directly measured the activation of a PTP (or PTPs) in pancreatic β cell lines in response to insulin secretagogues. This PTP is transiently ac-
tivated by exposure of the cells to secretagogues with maximal activation occurring 15–20 min after secretagogue treatment, is associated with the cytoskeleton, and is resistant to SDS. We have identified this PTP (or at least a part of the PTP activity) as IAR, a secretory granule receptor-like PTP.

Treatment of βTC3, HIT-T15, RIN 5AH-12, and A/TC-20 cells with KCl and treatment of HIT-T15 cells with glucose or TPA effects PTP activation. Notably, PTP activation is only observed with concentrations of these compounds that are effective in stimulating hormone secretion, and both events occur in response to high (55 mM) but not low (1 mM) KCl or high (15–20 mM) but not low (1 mM) glucose or with 1 μM TPA. All the above are neuroendocrine cells that possess a regulated secretory pathway, in contrast to most other cells, which can only undergo constitutive secretion. In the latter category, COS-1 and NIH 3T3 cells did not exhibit any alteration in PTP activity in response to high KCl. We have identified the IAR-PTP as a component of the secretagogue-responsive PTP activity (see next paragraph), and IAR mRNA expression is not detectable in NIH 3T3 cells (31). Furthermore, expression of hIAR in COS-1 cells was not sufficient to permit its activation upon exposure of the cells to KCl. This indicates that these cells lack the necessary KCl-specific response to effect activation and/or that the PTP is absent or, even if present, is unresponsive. Our findings suggest that the observed PTP activation is connected with regulated secretion and restricted to certain specialized cells.

The secretagogue-activated PTP is localized in the Triton X-100 insoluble fraction of the cell, often termed the cytoskeleton-associated fraction. The similar localization of ectopically expressed hIAR, a transmembrane PTP of secretory granules, together with the known cytoskeletal association of secretory granules, prompted us to examine whether IAR was in fact the endogenous secretagogue-activated PTP. Indeed, ectopically expressed hIAR is activated by high KCl or high glucose treatment in βTC3 or HIT-T15 cells in a time-dependent manner identical to the endogenous PTP, the hIAR activity is SDS-resistant, and the activated hIAR is found associated with cytoskeleton, confirming its identity with the endogenous PTP. Whether IA-2, a transmembrane PTP of secretory granules that is closely related to IAR (27, 28, 35), comprises another portion of the secretagogue-stimulated PTP activity of neuroendocrine cells remains to be determined. Ectopically expressed hIAR is present in both the Triton X-100-soluble membrane fraction and the Triton X-100-insoluble cytoskeletal fraction of βTC3 cells. Nevertheless, activation of the membrane-associated IAR cannot be detected following secretagogue treatment. This IAR could represent unsorted and recycled IAR in the endoplasmic reticulum or trans-Golgi network and/or IAR in secretory granules that are not cytoskeleton-associated. The absence of activated ectopic or endogenous IAR in the membrane fraction also suggests that activation does not involve the IAR that is transiently present on the plasma membrane following granule fusion with the plasma membrane and exocytosis of granule contents. We hypothesize that IAR must be in the budded secretory granule (possibly even in the mature secretory granule) and in association with the cytoskeleton to be able to respond to the secretagogue signal. The specific nature of the signal(s) resulting in IAR activation is unknown and requires further investigation. Our results indicate that the IAR activator lies downstream of K+ channel closure and likely of the ensuing Ca2+ influx, which is the central requirement for hormone secretion. At present we only know that IAR activation does not appear to involve phosphorylation of IAR by a tyrosine kinase, because although IAR contains tyrosine phosphate, the levels of this are unaltered by exposure to high versus low concentrations of secretagogues.2 In view of the serine/threonine protein kinases activated by secretagogues, modification and activation of IAR by serine/threonine phosphorylation are possibilities that we are investigating. At least one such secretagogue-activated kinase, Ca2+/calmodulin-dependent protein kinase II, is primarily found in the cytoskeletal fraction of insulin secreting cells and co-purifies with secretory granules (41).

Although the recombinant, cytosolic portion of IAR has PTP activity in vitro (32), this activity is extremely low. Our assays of this recombinant IAR toward the substrate used in the present study, RR-src peptide, found a barely detectable phosphatase activity even with very high (0.6–0.7 mg/ml) concentrations of the enzyme. In contrast, immunoprecipitated hIAR from transfected and high KCl-treated β cells was a much more effective RR-src phosphatase, even though far lower enzyme concentrations (as estimated from Western blotting) were used. This indicates that the transmembrane form of IAR and/or conditions in the cell may be critical for phosphatase activity. The assays of immunoprecipitated hIAR probably still underestimate IAR-phosphatase activity. The activity of the endogenous secretagogue-responsive PTP and of ectopic hIAR in SDS is unusual, because we found that addition of the same concentration of SDS (0.5%) to cytosol or Triton X-100 extracted membrane fractions virtually abolished all PTP activity. We do not know whether IAR activity is completely or only partially preserved in SDS. If the latter is true, the cellular activity could be much higher. Also, we are measuring phosphatase activity toward an in vitro, artificial substrate. The activated IAR may be a much more potent PTP with its specific physiological substrate.

Several observations indicate that secretagogue-induced PTP activation is unlikely to be directly linked to hormone secretion. Firstly, maximal PTP activation occurred 15–20 min after initial exposure of the cells to secretagogue, whereas insulin secretion could be detected much more rapidly. Thus, the two events did not exhibit a temporal correlation, and PTP activation is not required for secretion. Secondly, the extent of PTP activation did not reflect the potency of the secretagogue in hormone release, for example in HIT-T15 cells, a 30-min treatment with high KCl resulted in about a 15-fold increase in secreted insulin over treatment with low KCl, and 30-min treatments with high glucose or with TPA effected a less than 3-fold increase in secreted insulin over appropriate control treatments, yet the extent of PTP activation was similar with all three secretagogues. Thus PTP (or IAR) activation may represent a cellular event or process secondary to hormone secretion. This is reminiscent of the finding that although MAP kinase is activated in neuroendocrine cells by secretagogues, this is not required for insulin secretion per se (23). The precise timing of PTP (IAR) activation that we have characterized here may be important in identifying an IAR substrate, which is perhaps transiently modified by tyrosine phosphorylation. This will be key in elucidating the cellular pathway in which IAR plays a role, evidently one that diverges from immediate secretory events.

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