Insulin Promotes the Association of Heat Shock Protein 90 with the Inositol 1,4,5-Trisphosphate Receptor to Dampen Its Ca^{2+} Release Activity

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The inositol 1,4,5-trisphosphate receptor (IP_3R) is a Ca^{2+} release channel that plays a pivotal role in regulating intracellular Ca^{2+} levels in resting cells. Three isoforms of IP_3Rs have been identified, and they all possess a large regulatory domain that covers about 60% of the protein. This regulation is accomplished by interaction with small molecules, posttranslational modifications, and mostly protein-protein interactions. In our search for new binding partners of the IP_3R, we found that 90-kDa heat-shock protein (Hsp90) binds to the IP_3R. This interaction increased on stimulation of HEK293T6.11 cells with insulin but not with G_q protein-coupled receptor (G_qPCR) agonists. Moreover, the Hsp90 inhibitor geldanamycin (GA) disrupted the interaction between Hsp90 and the IP_3R. Pretreatment of HEK293T6.11 cells with GA greatly increased the intracellular Ca^{2+} release induced by a G_qPCR agonist. Insulin alone did not induce any intracellular Ca^{2+} release. However, insulin diminished the intracellular Ca^{2+} release induced by a G_qPCR agonist. Interestingly, GA abolished the inhibitory effect of insulin on G_qPCR-induced intracellular Ca^{2+} release. Furthermore, in our search for a mechanistic explanation to this phenomenon, we found that inhibition of kinases activated downstream of the insulin receptor greatly increased the interaction between Hsp90 and the IP_3R. Of greater interest, we found that the simultaneous inhibition of mammalian target of rapamycin and the Src kinase almost completely disrupted the interaction between Hsp90 and the IP_3R. These results demonstrate that insulin promotes the interaction of Hsp90 with the IP_3R to dampen its Ca^{2+} release activity by a complex mechanism involving mammalian target of rapamycin and the Src kinase. (***Endocrinology*** **150: 2190–2196, 2009)**

The inositol 1,4,5-trisphosphate receptor (IP_3R) is a Ca^{2+} release channel that plays a pivotal role in regulating intracellular Ca^{2+} levels in resting cells (1). In mammals, three different isoforms of IP_3R have been identified, IP_3R-1, IP_3R-2, and IP_3R-3 (2–4). Structurally the IP_3Rs are composed of three domains, an N-terminal domain containing the IP_3 binding site, a large middle domain involved in the coupling with other proteins and in the modulation of the channel activity, and a C-terminal domain forming the pore of the channel (5). Even though they share great sequence homology (70–80%) (6), the three types of IP_3R differ in many aspects, including their tissue distribution (7) and their regulatory mechanisms (1, 8). The regulatory domain shares the least homology between the three isoforms of IP_3R and it is where most regulatory processes take place, such as phosphorylation and interaction with small molecules (like Ca^{2+} or ATP) or other proteins (1). Because protein-protein interactions contribute to an important part of protein regulation, strong efforts have been put on the identification of new binding partners for the IP_3R. Many IP_3R binding partners have already been identified, like calmodulin (9), CaBP1 (10), Homer (11), and IRBIT (12) to name a few. Recent advances in the field of proteomic have offered a new interesting approach using chemical cross-linking combined with mass spectrometry for the identification of transient protein-protein complexes (13–15). Using such a cross-linking approach combined with mass spectrometry, we searched for new binding partners that could be involved

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Abbreviations: CCh, Carbachol; DPDPB, 1,4-di-[3′-(2′-pyridyldithio)-propionamide] GA, geldanamycin; G_qPCR, G_q protein-coupled receptor; HBSS, HEPES-buffered saline solution; Hsp90, heat-shock protein; IP_3R, inositol 1,4,5-trisphosphate receptor; mTOR, mammalian target of rapamycin; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d] pyrimidine.

in the regulation of the IP_3R activity. We found that the 90-kDa heat-shock protein (Hsp90) interacts with the IP_3R in HEK293T6.11 cells. Hsp90 is best known for its role in the proper folding and the maturation of nascent proteins (16). Hsp90 is also well recognized for its buffering function against genetic mutations and environmental stresses (17). Here we demonstrate that insulin promotes the interaction of Hsp90 with the IP_3R to dampen its Ca^{2+} release activity by a complex mechanism involving mammalian target of rapamycin (mTOR) and the Src kinase.

Materials and Methods

Materials

Cell culture media, serum, geneticin (G418), penicillin/streptomycin, L-glutamine, HEPES, and trypsin were purchased from Invitrogen (Burlington, Ontario, Canada). Wortmannin, PD98059, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d] pyrimidine (PP2), rapamycin, 17-allylamino-17-demethoxygeldanamycin, and fura-2/AM were from Calbiochem (La Jolla, CA). Recombinant human insulin was from Sigma (St. Louis, MO). Geldanamycin was from HMH Therafect Inc. (Sherbrooke, Québec, Canada). 1,4-di-[3′-(2′-pyridyldithio)-propionamide] (DPDPB) was from Pierce (Rockford, IL). Mouse monoclonal anti-IP_3R type 3 antibody was from BD Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-Hsp90 antibody was from Stressgen (Victoria, British Columbia, Canada). Horseradish peroxidase-conjugated sheep anti-mouse and peroxidase-conjugated donkey antirabbit antibodies were from Amersham Biosciences (Piscataway, NJ). All other reagents and chemicals were from Laboratoire MAT Inc. (Beauport, Québec, Canada).

Cell culture

HEK293T6.11 cells stably expressing the Ca^{2+}-permeable channel TRPC6 were cultured in DMEM supplemented with 10% fetal bovine serum in the presence of 400 μg/ml G418, 5 mM glutamine, 50 μg/ml streptomycin, and 50 U/ml penicillin at 37 C under a humidified 5% CO_2 environment.

Cross-linking, cell lysis, and immunoprecipitation assays

For cross-linking experiments, 3.5 million cells were washed twice with cold PBS and incubated with 725 μM DPDPB for 20 min at 4 C. DPDPB is a cell-permeable homobifunctional agent reactive toward sulfhydryl groups and cleavable under reducing conditions. The cross-linking reaction was stopped by washing the cells with cold PBS. The cells were then lysed in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM EDTA, 0.1% sodium dodecyl sulfate, 1 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride for 30 min at 4 C followed by 20 passages through a 20-gauge needle and five passages through a 25-gauge needle. The cell lysate was centrifuged at 15,000 $\times$ *g* for 30 min to remove insoluble materials and the supernatant was incubated for 16 h at 4 C with the anti-IP_3R-3 antibody immobilized on protein A-Sepharose beads (Amersham Biosciences). Beads were then washed three times by centrifugation at 10,000 $\times$ *g* for 1 min.

Mass spectrometry and protein identification

For mass spectrometry analysis, 8.5 million cells were lysed. Immunoprecipitated proteins were separated by SDS-PAGE (under reducing conditions) and stained with Colloidal Blue. Proteins of interest were cut from the gel, washed in 100 mM ammonium bicarbonate for 15 min at room temperature, and dehydrated in acetonitrile. Reduction was performed with 5 μg/μl dithiothreitol at 37 C, and alkylation was carried out using 25 μg/μl iodoacetamide. The gel pieces were then washed in 100 mM ammonium bicarbonate and dehydrated in acetonitrile before drying in a SpeedVac. The dried gel pieces were rehydrated in 100 mM ammonium bicarbonate containing 40% dimethylformamide and 100 ng/μl trypsin for 15 min at room temperature. After removal of excess trypsin solution and addition of 50 μl of 50 mM ammonium bicarbonate, the proteolytic digestion was carried out overnight at 37 C. Peptides from in-gel digestion were analyzed by nano-liquid chromatography, electrospray ionization, time of flight, and tandem mass spectrometry system consisting of a capillary LC system with autosampler (Waters, Milford, MA) and a Magic C18 AQ reversed-phase column (200A, 5 μm; Michrom BioResources, Auburn, CA) coupled on-line to a QTOF 2E mass spectrometer (Micromass, Manchester, UK). After tandem mass spectrometry, data files were created separately with the MassLynx 3.5 software, spectra were searched with the MASCOT algorithm (www.matrixscience.com).

Western blotting

For Western blot analysis, the beads were boiled in 2$\times$ Laemmli buffer containing 40 mg/ml dithiothreitol to denature proteins. Note that DPDPB is a sulfhydryl-reactive homobifunctional cross-linking agent that is cleaved under these denaturing conditions. Proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA) in a buffer containing 25 mM Tris, 200 mM glycine, and 20% methanol. After staining with Ponceau S, blots were washed with TBS-T [20 mM Tris-HCI (pH 7.4), 140 mM NaCl, 0.3% Tween-20] and incubated for 16 h at 4 C in 5% nonfat milk. For detection, blots were incubated with indicated antibodies for 3 h at room temperature, followed by incubation with horseradish peroxidase-coupled anti-antibodies for 90 min at room temperature, and revealed with enhanced chemiluminescence technique.

Measurement of intracellular Ca^{2+}

We used the method described by Zhu *et al.* (18) to measure intracellular Ca^{2+}. Briefly, cells attached to poly-L-lysine-treated coverslips were washed twice with HEPES-buffered saline solution (HBSS) and loaded with fura-2/AM (0.2 μM in HBSS) for 20 min at room temperature in the dark. After a deesterification step (washing and incubating in fresh HBSS for 20 min at room temperature), the coverslips were then transferred to a measuring chamber in which the cells were continuously superfused with HBSS. The chamber was then placed on the stage of a Zeiss Axovert microscope fitted with an Attofluor digital imaging and photometry system (Attofluor Inc., Rockville, MD). About 70–80 isolated fura-2-loaded cells were selected. Measurements of intracellular Ca^{2+} in single cells were obtained by using alternate excitation wavelengths of 334 and 380 nm and monitoring emitted fluorescence at 520 nm. Free Ca^{2+} concentration was calculated from 334:380 fluorescence ratios following the method of Grynkiewicz *et al.* (19). All reagents were diluted to their final concentrations in HBSS and applied to the cells by surface perfusion.

Statistical analysis

Means $\pm$ SD were calculated for at least three independent experiments. Data were analyzed statistically using Student's *t* test. Results were considered statistically significant (*) when $P < 0.05$.

Results

Hsp90 interacts with IP_3R-3 in HEK293T6.11 cells

To find new IP_3R binding partners, a cross-linking approach followed by coimmunoprecipitation was used (see *Materials and Methods*). Due to the abundant expression of IP_3R-3 in HEK293T6.11 cells, an antibody against this specific isoform was used for the immunoprecipitation step. The immunoprecipitate contained a protein migrating with a molecular weight of 90 kDa on SDS-PAGE that was identified as Hsp90 by mass

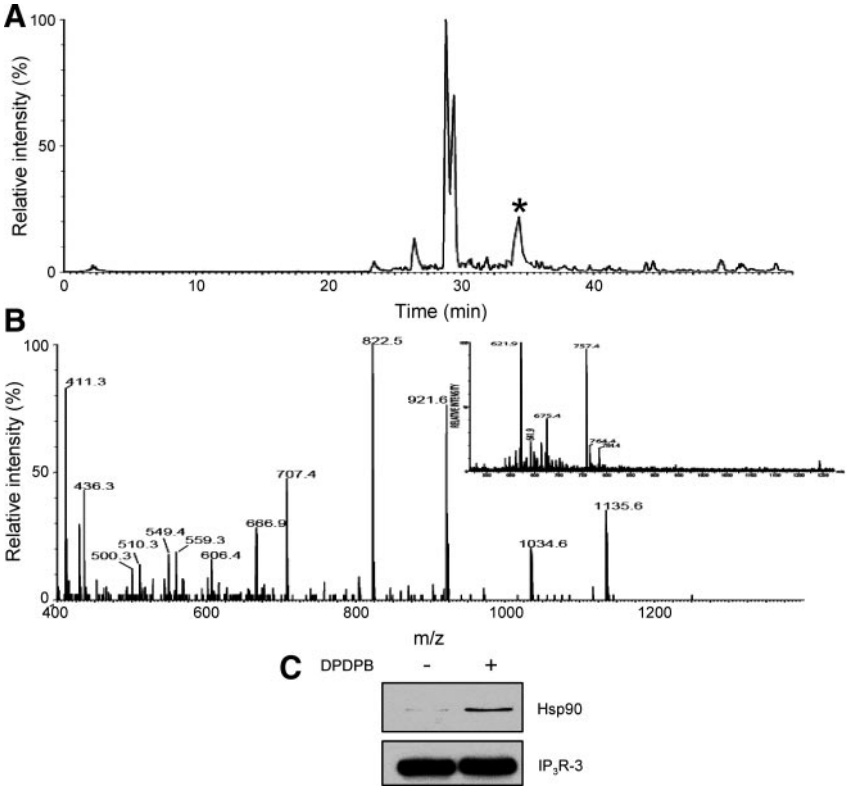

FIG. 1. Hsp90 interacts with IP_3R-3 in HEK293T6.11 cells. Cells were cross-linked for 20 min with 725 μM DPDPB. After cell lysis, proteins were immunoprecipitated with anti-IP_3R-3 antibody and separated by SDS-PAGE. A, Nano-liquid chromatography, electrospray ionization, time of flight, and tandem mass spectrometry analysis of spot 90. Base peak intensity chromatogram of survey scans 400–1400 mass to charge ratio (m/z). The fraction with one of the ions selected for tandem mass spectrometry is indicated by a *star*. B, Tandem mass spectrometry analysis of the doubly charged ion m/z 675.4 selected from the survey scan shown in the *insert*. C, Western blot analysis using anti-Hsp90 or anti-IP_3R-3 antibodies. This typical experiment is representative of three independent experiments.

spectrometry analysis (spectra shown in Fig. 1, A and B, and analyzed in Table 1). Hsp90 is a chaperone protein known to play an essential role in protein folding and maturation (16). To further confirm the interaction between Hsp90 and IP_3R-3, the immunoprecipitated material was analyzed by Western blot. Figure 1B shows that Hsp90 coimmunoprecipitated with IP_3R-3. These results are consistent with a physical interaction of Hsp90 with IP_3R-3 in HEK293T6.11 cells.

Insulin promotes the interaction between IP_3R-3 and Hsp90

The activation of G_q protein-coupled receptors (G_qPCR) and tyrosine kinase receptors are two major pathways involved in the production of IP_3, hence leading to the activation of IP_3R. We verified whether the interaction between IP_3R and Hsp90 could be increased under conditions that activate the IP_3R. Figure 2A shows that preincubation of HEK293T6.11 cells with carbachol (CCh) or ATP, two G_qPCR agonists, did not increase the coimmunoprecipitation of Hsp90 with IP_3R-3. Interestingly, the preincubation of HEK293T6.11 cells with insulin, a tyrosine kinase receptor agonist, significantly increased the coimmunoprecipitation of Hsp90 with IP_3R-3, even under conditions where no cross-linking agent was used. The interaction of Hsp90 with client proteins is dependent on its ability to bind and hydrolyze ATP, and this interaction is efficiently disrupted by ATP-mimetic drugs such as geldanamycin (GA) (20). After pretreating HEK293T6.11 cells with GA, insulin could no more increase the coimmunoprecipitation of Hsp90 with IP_3R-3 (Fig. 2B). These results suggest that IP_3R-3 is a new client protein of Hsp90 and that their interaction is promoted by insulin.

GA potentiates CCh-induced intracellular Ca^{2+} release

To determine the effect of Hsp90 on IP_3R activity, we treated HEK293T6.11 cells with GA and measured CCh-induced in-

TABLE 1. Sequence assignment by MASCOT of peptide ions selected for tandem mass spectrometry and used to identify Hsp90-1β (*Homo sapiens*)

Observed mass[a]	Calculated mass[a]	Expected mass[a]	δ-Mass	Score	Peptide sequence
576.3029	1150.5506	1150.5912	0.0407	23	YIDQEELNK
580.8173	1159.5761	1159.6200	0.0440	33	SIYYITGESK
597.8510	1193.6404	1193.6890	0.0486	17	IDIIPNPQER
621.8765	1241.6979	1241.7384	0.0405	45	ADLINNLGTIAK
625.3354	1248.6098	1248.6562	0.0464	27	EQVANSAFVER
638.3464	1274.6354	1274.6782	0.0429	23	ELISNASDALDK
675.3928	1348.7272	1348.7710	0.0438	70	TLTLVDTGIGMTK
683.3947	1364.7221	1364.7748	0.0527	39	TLTLVDTGIGMTK + ox(M)
757.4204	1512.7784	1512.8262	0.0479	86	GVVDSEDLPLNISR
764.4036	1526.7365	1526.7926	0.0561	49	SLTNDWEDHLAVK
772.9508	1543.8205	1543.8870	0.0665	43	ELISNASDALDKIR
924.4381	1846.7897	1846.8616	0.0719	27	NPDDITQEEYGEFYK

Individual ion scores greater than 42 indicate identity or extensive homology ($P < 0.05$). Parameters used to search the human protein database: fixed modifications, carbamidomethylation; variable modifications, oxidation; peptide mass tolerance, 0.5 Da; fragment mass tolerance, 0.25 Da.

[a] Monoisotopic masses were used.

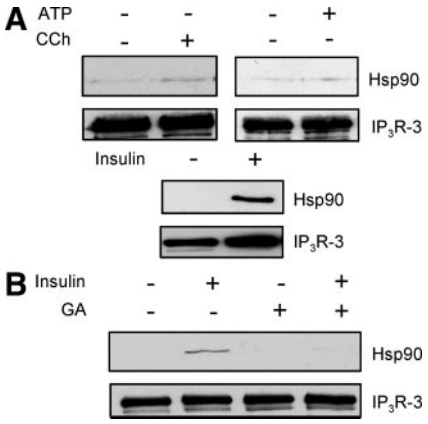

FIG. 2. Insulin increases the interaction of IP_3R-3 with Hsp90 in HEK293T6.11 cells. A, Cells were stimulated for 15 min with CCh (50 μM), ATP (100 μM), or insulin (1 μM). B, Cells were pretreated for 1 h with GA (5 μM) before stimulation for 15 min with insulin (1 μM). After cell lysis, proteins were immunoprecipitated with the anti-IP_3R-3 antibody, separated by SDS-PAGE, and identified by Western blotting with anti-Hsp90 or anti-IP_3R-3 antibodies. Note that these experiments were done without using any cross-linking agent. These typical experiments are representative of at least three independent experiments.

tracellular Ca^{2+} releases in cells bathing in a Ca^{2+}-free extracellular medium. In the absence of extracellular Ca^{2+}, any intracellular Ca^{2+} increase is due exclusively to the release of Ca^{2+} from the intracellular store. Under control conditions, 2 μM CCh raised the intracellular Ca^{2+} concentration from a basal level of 89 nM to a high level of 136 nM (Fig. 3A). After a pretreatment with GA, 2 μM CCh raised the intracellular Ca^{2+} concentration from a basal level of 89 nM to a high level of 193 nM. Figure 3B shows that under control conditions, 2 μM CCh increased the intracellular Ca^{2+} concentration by 46.8 ± 7.9 nM (mean ± SD of three independent experiments performed each with 70–80 individual cells), whereas after a pretreatment with GA, 2 μM CCh increased the intracellular Ca^{2+} concentration by 104.1 ± 5.8 nM (mean ± SD of three independent experiments performed each with 70–80 individual cells). We verified that the CCh-induced production of inositol phosphates was not modified after treatment with GA (data not shown). These results demonstrate that the inhibitor of Hsp90 potentiates agonist-induced intracellular Ca^{2+} release by a mechanism that does not increase the production of IP_3.

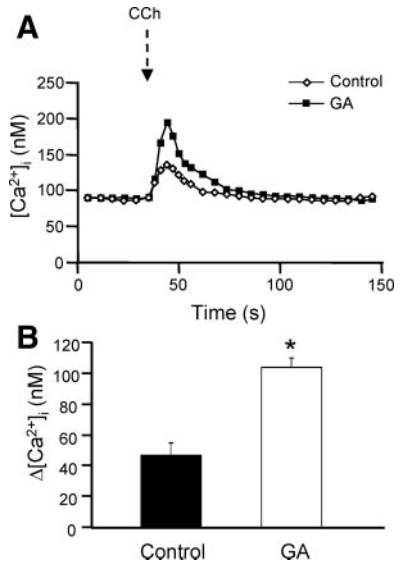

FIG. 3. GA potentiates intracellular Ca^{2+} release. A, HEK293T6.11 cells were pretreated for 1 h with GA (2 μM), and their intracellular Ca^{2+} concentration was measured after loading for 40 min with fura-2/AM, in a Ca^{2+}-free extracellular medium. A, Typical traces depicting the intracellular Ca^{2+} released by 2 μM CCh in cells (average response of 70–80 cells) pretreated with GA or vehicle (control). B, Peak value Ca^{2+} responses (mean ± SD of three independent experiments performed each with 70–80 individual cells. *, $P < 0.05$) obtained under both conditions.

Insulin dampens CCh-induced Ca^{2+} response

Because insulin promotes the interaction of Hsp90 with the IP_3R, we expected that it would also modify the cellular Ca^{2+} response. Figure 4A shows a typical Ca^{2+} response of an individual HEK293T6.11 cell stimulated with 1 μM CCh in the presence of extracellular Ca^{2+}. Upon addition of CCh, the intracellular Ca^{2+} concentration rapidly raised from a basal level of 45 nM Ca^{2+} to a high level of 227 nM Ca^{2+}, to form an initial Ca^{2+} spike that rapidly declined to an intermediate level around 150 nM Ca^{2+}. This intermediate level slowly declined toward the basal level but remained above the basal level of Ca^{2+} for several minutes (to constitute a plateau phase). This typical spike and plateau Ca^{2+} profile was observed in the majority of HEK293T6.11 cells in response to 1 μM CCh. Upon addition of insulin, no change in the intracellular Ca^{2+} concentration was observed (data not shown). When HEK293T6.11 cells were simultaneously stimulated with 1 μM CCh and 1 μM insulin, they responded with repetitive Ca^{2+} spikes more commonly identified as intracellular Ca^{2+} oscillations (Fig. 4B). It is generally recognized that an oscillatory response is obtained after a weak stimulation (low concentration of agonist), whereas a spike and plateau response is obtained after a strong stimulation (high concentration of agonist) (21, 22). Therefore, by switching the spike and plateau response into an oscillatory response we interpret the effect of insulin as a dampening of the CCh-induced Ca^{2+} response. Interestingly, after a pretreatment with 17-AAG (an analog of geldanamycin), HEK293T6.11 cells responded to a simultaneous stimulation with CCh and insulin by producing a spike and plateau profile, restoring the response obtained with 1 μM CCh (Fig. 4C). Figure 4D shows that as few as 5.1 ± 7.2% of cells stimulated with CCh only (mean ± SD of values obtained with 119 individual cells) responded with an oscillatory pattern, whereas as much as 51.8 ± 5.5% of cells stimulated simultaneously with CCh and insulin (mean ± SD of values obtained with 134 individual cells) responded with an oscillatory profile. In the presence of 17-AAG, the proportion of cells that produced Ca^{2+} oscillations in response to CCh and insulin simultaneously was 6.6 ± 9.3% (mean ± SD of values obtained with 136 individual cells). The regulatory effect of insulin on CCh-induced Ca^{2+} response was further illustrated by adding insulin 5 min after stimulating the cells with CCh. Under these conditions, the spike and plateau profile induced by CCh was modified for an oscillatory profile upon addition of insulin (Fig. 4E). Thus, these results suggest that by promoting the association of

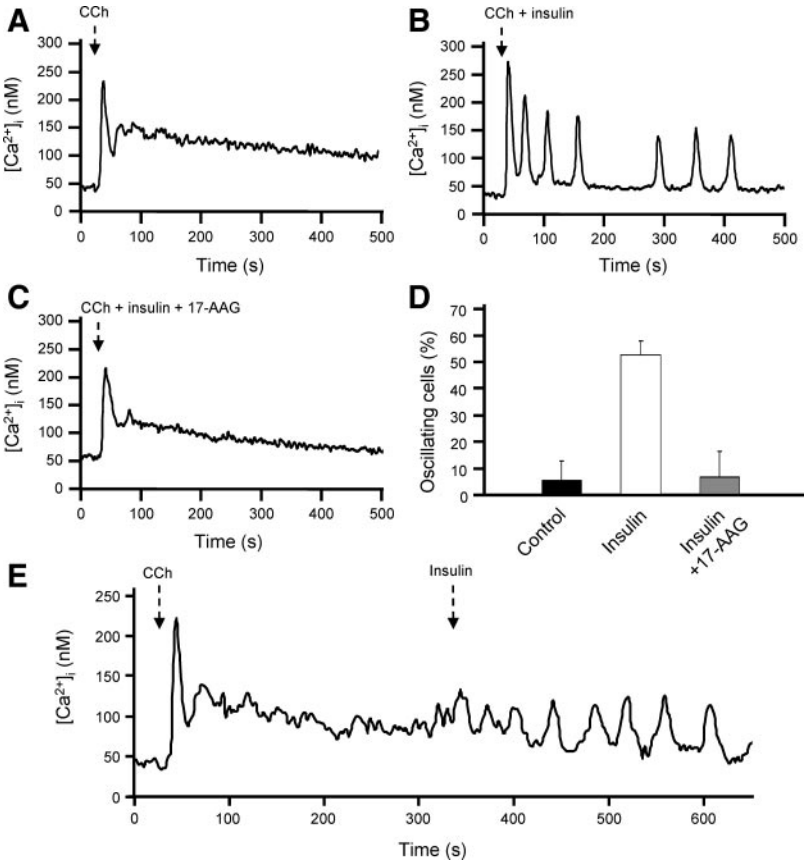

FIG. 4. Insulin affects IP_3R activity by promoting an oscillatory pattern for Ca^{2+} response. Representative response of a single HEK293T6.11 cell stimulated with 1 μM CCh (A) or simultaneously with 1 μM CCh and 1 μM insulin (B). Representative response of a single HEK293T6.11 cell pretreated for 1 h with 2.5 μM 17-AAG and then stimulated simultaneously with 1 μM CCh and 1 μM insulin (C). D, Percentage of cells that, under each condition, responded by producing Ca^{2+} oscillations. The results are the mean ± SD of values obtained with 100–150 individual cells in three independent experiments. E, Representative response of a single HEK293T6.11 cell that was stimulated for the first 5 min with 1 μM CCh after which 1 μM insulin was added for the remainder of the experiment.

Hsp90 with the IP_3R, insulin dampens the CCh-induced Ca^{2+} response of HEK293T6.11 cells.

The insulin-triggered association between Hsp90 and IP_3R depends on the simultaneous activity of mTOR and Src kinase

To explore the pathway responsible for the insulin-induced interaction between Hsp90 and the IP_3R, we treated HEK293T6.11 cells with known inhibitors of different kinases activated downstream of the insulin receptor. Paradoxically, wortmannin (phosphatidylinositol 3-kinase inhibitor), PD98059 (MAPK inhibitor), PP2 (Src kinase inhibitor), and rapamycin (mTOR inhibitor) all increased the insulin-induced coimmunoprecipitation of Hsp90 with IP_3R-3 (Fig. 5, A and B). The only condition that decreased the effect of insulin was when cells were simultaneously treated with rapamycin and PP2 (Fig. 5B). Under this condition, the effect of insulin was almost completely abolished. These results suggest that the downstream effectors mTOR and Src are involved in the effect of insulin by a mechanism that needs their simultaneous actions. Further studies are needed to clarify this complex mechanism.

Discussion

In our search for new IP_3R binding partners, we found out that Hsp90 coimmunoprecipitates with the IP_3R in HEK293T6.11 cell extracts. To our knowledge, we are the first to report a functional association between Hsp90 and the IP_3R. To date, more than 25 proteins have been reported to interact with the IP_3R, influencing its function in a variety of ways (1). Among them, many scaffolding proteins such as Homer (11), and the protein 4.1N (23, 24) are involved in maintaining a stable localization of the IP_3R within the cell. Hsp90 does not appear to play a mere scaffolding function for the IP_3R because we found out that its association with the IP_3R is regulated by insulin. Interestingly, we showed that G_qPCR agonists (CCh and ATP) do not influence the association of Hsp90 with the IP_3R. It therefore appears that the association of Hsp90 with the IP_3R is not a common event in the typical G_qPCR-induced Ca^{2+} signaling cascade but rather that it occurs under some circumstances, in the response of cells to insulin.

More than 100 proteins are known to associate with Hsp90, including kinases, receptors, transcription factors, and a variety of other client proteins (25, 26). Hsp90 exerts many different effects such as a chaperone activity for the maturation of clients, such as the cardiac potassium channel human Ether-à-gogo related (27). Hsp90 is also well known for its role in maintaining some clients in an inactive conformation, such as the glucocorticoid receptor (28) or other steroid receptors (29), that are maintained by Hsp90 in a state capable of binding hormone. Hsp90 was recently shown to associate with and inhibit the inositol hexakisphosphate kinase-2, an enzyme involved in cell apoptosis (30). This negative regulatory effect of Hsp90 was interpreted as a protective effect against environmental stresses and apoptosis.

We demonstrated that the Hsp90 inhibitor GA potentiates agonist-induced intracellular Ca^{2+} release. In a study designed to investigate the signaling pathway through which GA induces the expression of certain heat shock proteins, Lai and colleagues (31, 32) were the first to observe that GA causes a transient increase of intracellular Ca^{2+} level in human nonsmall cell lung cancer H460 cells. The authors did not identify the underlying mechanism responsible for this effect of GA. Our results suggest that Hsp90 associates with and diminishes the Ca^{2+} release activity of the IP_3R. Three pieces of evidence support this interpretation: first, we have shown that Hsp90 coimmunoprecipitates with the IP_3R and this association is disrupted by GA; second, we have shown that GA increases the intracellular Ca^{2+} release induced by G_qPCR agonists in HEK293T6.11 cells incubated in a Ca^{2+}-free medium, a condition under which the Ca^{2+} response is exclusively due to the activity of the IP_3R; third, we showed that

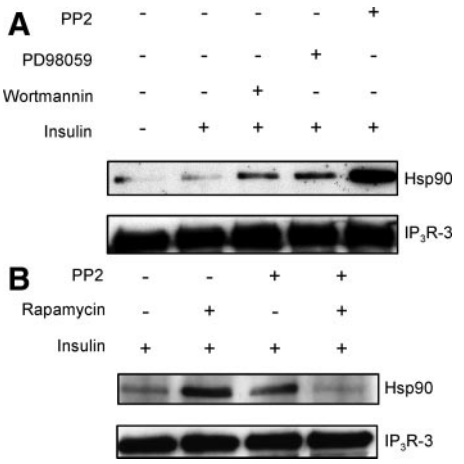

FIG. 5. The insulin-triggered association between Hsp90 and IP_3R depends on the simultaneous activity of mTOR and Src kinase. A, HEK293T6.11 cells were pretreated for 30 min with PP2 (5 μM), PD98059 (10 μM), or wortmannin (100 nM) before stimulation for 15 min with insulin (1 μM). B, Cells were pretreated for 30 min with PP2 (5 μM) and rapamycin (10 μM) before stimulation for 15 min with insulin (1 μM). After cell lysis, proteins were immunoprecipitated with the anti-IP_3R-3 antibody, separated by SDS-PAGE, and identified by Western blotting with anti-Hsp90 or anti-IP_3R-3 antibodies. These typical experiments are representative of at least three independent experiments.

insulin promotes the association of Hsp90 with the IP_3R, and also that insulin dampens the CCh-induced Ca^{2+} response of HEK293T6.11 cells and that this effect is abolished by GA.

Kisfalvi *et al.* (33) recently reported that insulin potentiates Ca^{2+} signaling through an Akt/mTOR-dependent pathway in human pancreatic cancer cell lines. Akt is a kinase that activates mTOR and that plays a central role in the insulin signaling (34). These results of Kisfalvi *et al.* are in apparent contradiction with our results showing a negative regulatory effect of insulin on CCh-induced Ca^{2+} responses in HEK293T6.11 cells. Interestingly, Meares *et al.* (17) previously showed that inhibition of Hsp90 with GA amplified Akt phosphorylation induced by insulin, indicating that Hsp90 normally buffers these signals. Furthermore, Koga et *al.* (35) showed that the Hsp90 inhibitor GA stimulates Akt via transient activation of Src kinase and that Src kinase activation is likely caused by its dissociation from Hsp90. Altogether these results suggest that by activating the Akt/mTOR pathway, insulin is potentiating the Ca^{2+} signaling, but under some circumstances Hsp90 dampens this regulatory effect of insulin. Our results show that the simultaneous inhibition of Src kinase and mTOR decreases the association of Hsp90 with IP_3R; thus, these kinases downstream of the insulin receptor appear to mediate a negative regulatory effect of insulin on Ca^{2+} signaling. Interestingly, in the recent study of Kisfalvi *et al.* (33), it was observed that the addition of insulin after G_qPCR agonists did not produce any additional increase in the intracellular Ca^{2+} level. The authors suggested the existence of a feedback mechanism that would prevent excessive G_qPCR-induced increases of intracellular Ca^{2+} levels in cells receiving a concomitant stimulation with insulin. In light of our results, this feedback mechanism could be achieved through Hsp90. Thus, it is likely that insulin exerts a positive regulatory effect on Ca^{2+} signaling by a mechanism involving Akt/mTOR and a negative regulatory effect by a mechanism involving the association of Hsp90 with the IP_3R. This association of Hsp90 with the IP_3R appears to necessitate the simultaneous actions of mTOR and Src kinase.

In conclusion, our results demonstrate that insulin promotes the association of Hsp90 with the IP_3R to dampen its Ca^{2+} release activity. Further work is needed to evaluate the physiological significance of the insulin-triggered interaction between Hsp90 and IP_3R in the native insulin-target tissues such as muscle, fat, and liver.

Acknowledgments

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