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Inositol 1,4,5-trisphosphate receptor isoform expression in mouse pancreatic islets: effects of carbachol

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Abstract

The inositol 1,4,5-trisphosphate receptors (IP3Rs) are ligand-gated Ca^{2+} channels that regulate intracellular Ca^{2+} mobilization. Among the IP3R mRNA isoforms I, II, and III, IP3R-I mRNA was expressed in mouse islets and the β-cell line βTC3, and was quantitatively the most abundant isoform as determined by reverse transcriptase–polymerase chain reaction. IP3R-II and -III mRNAs were expressed at similar levels in mouse islets, but neither isoform was detected in βTC3 cells. Culture of mouse islets for 30 min and 2 hr at 20 mM glucose, or for 7 days at 11 mM glucose did not affect IP3R-I mRNA expression compared with islets cultured in 5.5 mM glucose. Culture of islets or βTC3 cells with carbachol (0.5 mM) reduced IP3R-I mRNA expression levels below control. Mouse islet α- and β-cells expressed IP3R-I and -III proteins, but IP3R-II protein was not detected by immunoblot or double-label immunohistochemistry. Culture of islets for up to 6 hr with carbachol reduced IP3R-I and -III protein expression in a time-dependent manner with a half-maximal effect on type I at 1 hr. Glucose (20 mM) stimulation for 2 hr did not affect IP3R-1 levels. The carbachol-induced decrease in IP3R-I and -III protein expression was reversed by carbobenzoxyl-leucinyl-leucinyl-leucinyl-H (MG-132), a proteasome inhibitor. Thus, glucose failed to regulate mouse islet IP3R mRNA expression, whereas carbachol stimulation down-regulated IP3R mRNA and protein. A proteasomal protein degradative pathway appeared to mediate the muscarinic receptor-induced effects on IP3R-I and -III.

Keywords: Inositol 1,4,5-trisphosphate receptor; Insulinoma cell; Islet of Langerhans; Carbamylcholine; Proteasome

1. Introduction

IP3 mediates Ca^{2+} mobilization from intracellular Ca^{2+} stores and plays an important role in insulin secretion from pancreatic β-cells [1]. IP3 exerts its action through specific receptors that are ligand-activated, Ca^{2+}-selective channels [2, 3]. IP3Rs have been localized to the endoplasmic reticulum, nucleus, insulin secretory granules, chromaffin granules, and plasma membranes [4–8]. Molecular cloning and expression studies have revealed that there is a family of IP3Rs with different primary structures and tissue distributions [9]. Full-length sequences for rat IP3R type I [10], type II [11], and type III [12], and partial sequences for putative types IV and V, have been identified [13,14]. Moreover, functional definitions of the IP3R isoforms have been evolving. The isoforms have unique IP3 affinities [11, 15] and calcium sensitivities [16,17], and are regulated by phosphorylation, calmodulin, and ATP [10,11]. When the IP3R-III was activated by calcium in a rat β-cell line, there was a monophasic all-or-none release of intracellular calcium [18]. IP3 also releases calcium from intracellular storage sites in β-cells [19–21]. On the other hand, calcium induces a biphasic activation of IP3R-I, and activation of this receptor isoform results in a localized non-propagating calcium release response [18]. The physiological impact of IP3R activation in the islet may be the participation of these receptors in the calcium oscillatory capacity of β-cells, which has been postulated to modulate insulin secretory responses [22]. IP3Rs are regulated by Ca^{2+} and IP3 ligand binding [18,23]. It is likely that IP3R regulation is related to Ca^{2+} mobilization and insulin secretion in β-cells since it has been reported that fast intracellular Ca^{2+} oscillations in islets depend upon mobilization of Ca^{2+} from intracellular stores [24]. In addition, aberrant Ca^{2+} sequestration and

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Abbreviations: Ara-C, cytosine arabinoside; COX, cytochrome *c* oxidase; DMEM, Dulbecco's modified Eagle's medium; IP3, inositol 1,4,5-trisphosphate; IP3R, inositol 1,4,5-trisphosphate receptor; and RT–PCR, reverse transcriptase–polymerase chain reaction.

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mobilization from the endoplasmic reticulum in db/db mouse islets are responsible for the defective insulin secretion in this mouse model of diabetes [25].

Different patterns of IP3R expression in various tissues suggest that different subtypes confer distinct functions and that mechanisms exist for regulating the differential expression of IP3Rs [12,14]. Several studies have demonstrated that the expression of IP3R isoforms in tissues is responsive to diverse stimuli including glucose/feeding/diabetes, phorbol ester, vitamin D_3, retinoic acid, and cholecystokinin, among others [8,12,26–28]. Previous studies from this laboratory have shown that glucose metabolism and hyperglycemia are related to either the up-regulation or down-regulation of certain isoforms of IP3R mRNA and protein in rat islets and insulinoma cell lines [29–31]. A regulatory influence of glucose on IP3R protein levels has also been suggested for islets from Zucker diabetic fatty (ZDF) rats and RINm5F cells [8]. Although IP3R-I, -II, and -III mRNA isoforms are co-expressed in various tissues [12,13] and the expression of IP3R-I mRNA has been reported to be predominant in mouse pancreatic islets [14], the presence and relative abundance of the IP3R isoforms have not been characterized fully in mouse pancreatic islets. Moreover, IP3R protein levels for mouse islets have not been reported. In the present study, the hypothesis that there is regulated expression of IP3R-I, -II, and -III mRNA and protein in isolated mouse pancreatic islets and the mouse clonal beta cell line (βTC3) was tested using semiquantitative RT–PCR, immunoblot, and double-label immunohistochemistry. The results demonstrated that mouse islets express a unique ratio of IP3R isoforms, and that isoform levels can be regulated by carbachol.

2. Materials and methods

2.1. Materials

Collagenase (type P) was purchased from Roche Molecular Biochemicals. CMRL-1066 and DMEM culture media, random hexamer, TRIzol, PCR buffer, and *Taq* DNA polymerase were from GIBCO/Life Technologies. Fetal bovine serum was from Atlanta Biologicals. Peroxidase-conjugated anti-rabbit IgG secondary antibody was from Bio-Rad Laboratories, Inc. Immobilon-P membrane was from the Millipore Corp. Affinity purified anti-IP3R-I (CT1) and anti-IP3R-II (CT2) antibodies were a gift from Dr. R. Wojcikiewicz (State University of New York at Syracuse). Monoclonal anti-IP3R-III (TL3) antibody to the human IP3R-III was purchased from Transduction Laboratories. Anti-human insulin and anti-glucagon antibodies were from Linco Research. Bio-Rad Protein Assay reagent was from Bio-Rad Laboratories, Inc. Normal goat serum, normal donkey serum, and Texas-red conjugated donkey anti-guinea pig antibody were from the Jackson ImmunoResearch Laboratories. Alexa 488 conjugated goat anti-rabbit, goat anti-mouse antibodies, and Prolong Antifade Reagent were from Molecular Probes, Inc. MG-132 (carbobenzoxyl-leucinyl-leucinyl-leucinyl-H) was purchased from Calbiochem. Carbamylcholine chloride (carbachol) was from the Sigma Chemical Co. Murine pCEP4 βTC3 cells were a gift from Dr. Roland Stein (Vanderbilt University).

2.2. Isolation of mouse pancreatic islets; culture of islets and βTC3 cells

Pancreatic islets from adult C57BL/6 mice were isolated using collagenase digestion. All animal procedures were approved by the Institutional Animal Care and Use Committee. Isolated islets, devoid of any visible adherent acinar tissue, were either used immediately as freshly isolated islets, or they were cultured for 1 day at 5.5 mM glucose, as described previously [30]. Mannitol was included in 1-hr control cultures to maintain osmotic equivalence to glucose-stimulated conditions. In certain experiments, following the prescribed period of time, islets were treated with carbachol as indicated in the text. During islet culture for 7 days, the mitotic inhibitor Ara-C was added to the culture medium. βTC3 cells (0.5×10^6/well) were cultured in 24-well plates at 35^o for 9–10 days in DMEM containing 25 mM glucose, and subsequently cultured for 1 day at 2.8 mM glucose before glucose or carbachol stimulation for 2 hr.

2.3. Insulin secretion

Insulin release was determined by incubating isolated mouse islets (5 islets/sample) in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 16 mM HEPES, 0.01% bovine serum albumin, and 5.5 mM glucose for 60 min, followed by a 60-min incubation in fresh buffer with and without a glucose stimulus. Aliquots of incubation medium were removed at time zero and after the 60-min incubation for assay of insulin by radioimmunoassay. Insulin release (ng) values after 60 min are minus zero-time insulin levels.

2.4. RNA isolation and cDNA synthesis

Total RNA was extracted from mouse pancreatic islets and βTC3 cells using TRIzol as described previously [29]. cDNA was reverse transcribed as described previously [29, 30] from 0.3 to 0.5 μg of total RNA by random hexamer.

2.5. PCR amplification and quantitation of IP3R transcript levels

Polymerization reactions were carried out as described previously [29, 30] with a Hybaid Sprint thermalcycler and using 10.0 μL of a 1:10 dilution of IP3R-I cDNA, and 1:20 dilutions of COX subunit III (COX-III) and β-actin cDNAs as templates in a 25-μL reaction mixture containing 0.2 mM dNTPs, 10 pmol of appropriate oligonucleotide primers, PCR buffer, 2 mM $MgCl_2$, and 1 U *Taq* DNA polymerase.

Amplification primers for IP3R isoform analysis were selected to be distinct for each isoform, and the sequences of primer pairs for each IP3R isoform and for β-actin used in this study were as described previously [29,30,32].The primers recognize conserved sequences between the rat and mouse genes. The COX-III primers were: sense, 5′-GAACATACCAAGGCCACCAC-3′; antisense, 5′-CGTGGAGGCCATGAAATC-3′ (PCR product, 429 bp). Preliminary studies established the linearity of amplification rates under the conditions used for these experiments. The amplification conditions were 35 cycles with denaturation for 1 min at 94°, annealing for 2 min at 55°, and extension for 3 min at 72°, with the final extension for 7 min. The amplimers were separated by electrophoresis in a 1.5% agarose gel in Tris borate buffer. The gel was stained by ethidium bromide and viewed by Gel Doc 1000 (Bio-Rad Laboratories) with density analysis of each PCR fragment by Molecular Analyst software. The image densities of PCR products for IP3R isoforms were compared with the density of co-amplified β-actin or COX-III to determine the ratio of expression. Values are expressed as relative levels of IP3R/β-actin mRNA or IP3R/COX-III mRNA.

2.6. Western immunoblot

Mouse pancreatic islets were sonicated, and the protein concentration was determined by Bio-Rad protein assay using bovine serum albumin as standard. The sonicates were precipitated with acetone and pelleted by centrifugation at 14,000 *g* for 30 min at 4°. The pellet was resuspended in sample buffer, and for each experiment equal amounts of sample protein (10–15 μg) were loaded per lane for separation by 5% SDS/PAGE, followed by transfer to an Immobilon-P membrane. The membrane was then blocked with 10% nonfat dried milk, and incubated with antisera against the IP3R isoforms as described previously [30]. Antisera were generated against synthetic peptides corresponding to the C-termini of rat IP3R-I, -II, and III, and affinity purified to yield antisera specific to their cognate proteins [26]. After washing, the membrane was incubated with peroxidase-conjugated anti-rabbit IgG secondary antibody. The bound antibody was localized by chemiluminescence, and the density of each band was determined by densitometric scanning using Molecular Analyst software.

2.7. Immunofluorescence and confocal microscopy

The 5- to 10-μm thick frozen sections of mouse pancreas were fixed for 10 min with 2% paraformaldehyde in PBS (pH 7.4), followed by a 10-min permeabilization with 0.1% Triton X-100, as described previously [33]. The slides were washed in PBS and incubated sequentially with primary antibodies [anti-IP3R-I antibody (CT1) and anti-IP3R-II antibody (CT2); monoclonal anti-IP3R-III antibody (TL3); anti-human insulin antibody; anti-glucagon antibody], followed by Alexa 488 conjugated goat anti-rabbit or goat anti-mouse antibodies or Texas-red conjugated donkey anti-guinea pig antibody. After the sections were washed with PBS containing Triton X-100 and covered with Prolong Antifade Reagent, they were viewed using confocal microscopy as described previously [32].

Table 1
Relative expression of IP3R mRNA isoforms in mouse pancreatic islets and βTC3 cells

	IP3R mRNA/β-actin mRNA	
	Mouse islets	BTC3 cells
IP3R-I	0.38 ± 0.03 (95 ± 3%)	0.25 ± 0.001 (100%)
IP3R-II	0.01 ± 0.01 (2 ± 1%)	ND
IP3R-III	0.01 ± 0.01 (2 ± 1%)	ND

Islets were freshly isolated and cultured for 1 hr in CMRL-1066 medium containing 5.5 mM glucose. βTC3 cells (0.5×10^6/well) were cultured for 1 day at 2.8 mM glucose. IP3R-I, -II, and -III mRNA expression levels were determined after normalization to β-actin mRNA following RT-PCR. Values are means ± SEM for 3 independent determinations for mouse pancreatic islets and for 8 independent determinations for βTC3 cells. Numbers in parentheses are percent of total IP3R isoforms expressed. ND = not detectable.

2.8. Statistical analysis

Values are means ± SEM. Significant differences between treatment groups were determined by Student's *t*-test (paired) or one-way ANOVA with post-hoc analysis using the Student–Newman–Keuls multiple-comparison test. Values of $P \leq 0.05$ were accepted as significant. Values expressed as percent of control were determined using mRNA expression ratio values.

3. Results

3.1. Expression of IP3R isoform mRNA in mouse pancreatic islets and mouse insulinoma cells

RT-PCR was used to determine the expression level of IP3R isoform mRNA in isolated mouse pancreatic islets and mouse βTC3 insulinoma cells. IP3R-I was the most abundant subtype expressed in mouse islets, relative to β-actin mRNA, and constituted approximately 95% of IP3R mRNA (Table 1). In contrast, IP3R-II and -III mRNA isoforms were expressed at similar levels of only 2% of total (Table 1). In βTC3 cells, the IP3R-I mRNA isoform was also most abundant, whereas IP3R-II and -III mRNAs were not detected (Table 1).

3.2. IP3R isoform protein expression in mouse islets

Immunofluorescence labeling and confocal microscopy were used to identify the localization of IP3R-I, -II, and -III

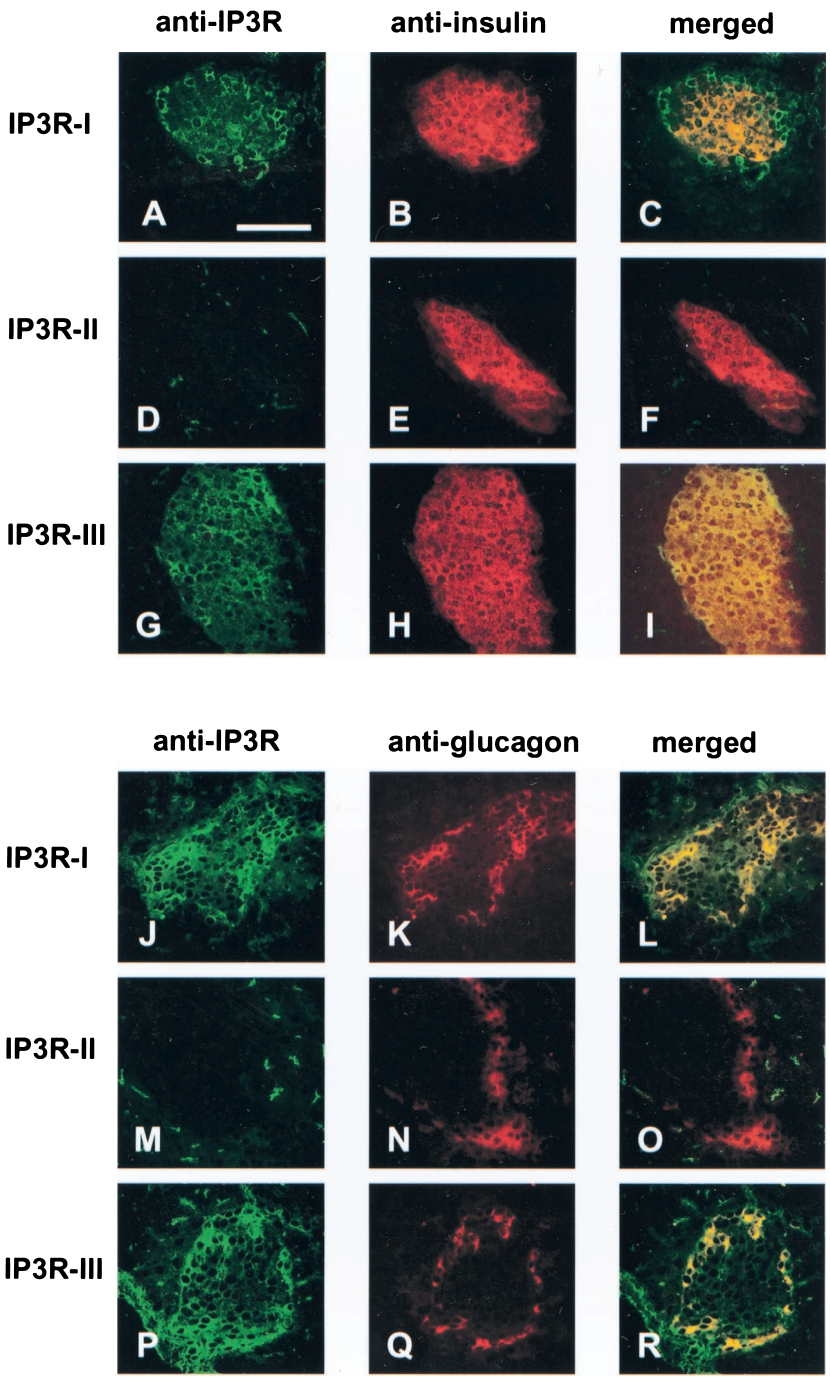

Fig. 1. Colocalization of IP3Rs and insulin or glucagon in mouse pancreatic islets. Confocal images showing cells fixed, permeabilized, and stained with anti-IP3R-I (A–C; J–L), -IP3R-II (D–F; M–O), and -IP3R-III antibodies (G–I; P–R), anti-human insulin antibody (B, C, E, F, H, I), or anti-glucagon antibody (K, L, N, O, Q, R), followed by Alexa 488-conjugated secondary antibodies (for IP3Rs) and Texas-red conjugated secondary antibody (for anti-insulin and anti-glucagon antibodies). Bar, 60 μm.

isoforms in mouse pancreatic α- and β-cells. As shown in Fig. 1, islet cells were double-labeled with anti-IP3R antibodies and anti-insulin or anti-glucagon antibodies. Anti-IP3R-I (Fig. 1A) and -III (Fig. 1G) antibodies were colocalized with the anti-insulin antibody (Fig. 1, B and H, respectively) as indicated by the orange color resulting from overlaying the Alexa 488 signal (green) from the anti-IP3R antibodies with the Texas-red signal (red) from the anti-insulin antibody (Fig. 1, C and I). However, the anti-IP3R-II antibody did not localize in islet cells and was not colocalized with the anti-insulin antibody (Fig. 1, D–F). Thus, IP3R-I and -III are present in mouse islet β-cells. Control sections in which primary antibody was omitted and only secondary antibody was allowed to react with the tissue

Table 2
IP3R-I mRNA in mouse pancreatic islets and βTC3 cells stimulated with glucose

	Mouse islets IP3R-I mRNA/β-actin mRNA	
	Control	Glucose-treated
30 min	0.40 ± 0.02	0.36 ± 0.05 (n = 3) (90 ± 7%)
2 hr	0.48 ± 0.02	0.45 ± 0.01 (n = 3) (95 ± 5%)
7 days	0.42 ± 0.03	0.39 ± 0.03 (n = 5) (95 ± 10%)
	βTC3 cell IP3R-I mRNA/β-actin mRNA	
2 hr	0.20 ± 0.02	0.20 ± 0.03 (n = 3–5) (103 ± 7%)

Isolated islets were cultured for 30 min or 2 hr in the presence of 5.5 mM glucose (control) or 20 mM glucose. Islets were also cultured for 7 days at 5.5 mM glucose control) or 11 mM glucose. βTC3 cells (0.5×10^6 well) were cultured at 2.8 mM glucose (control) and then stimulated for 2 hr with 25 mM glucose. IP3R-I mRNA expression was determined after normalization to β-actin mRNA following RT-PCR. Numbers in parentheses are percent of control. Values are means ± SEM for the number of independent determinations indicated (N).

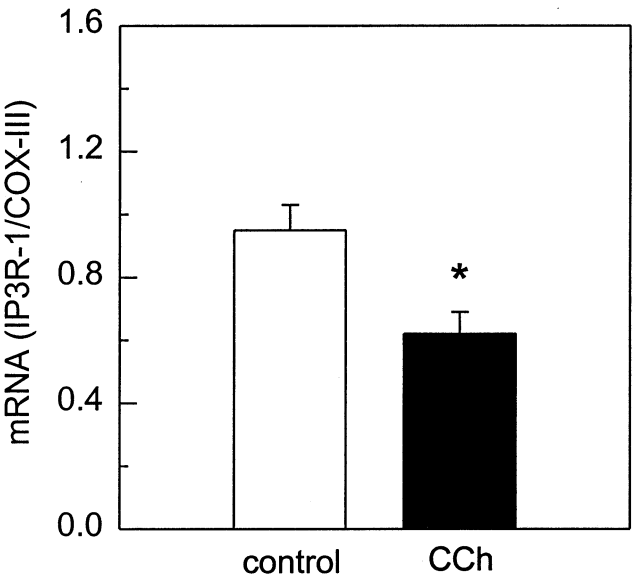

Fig. 2. Effect of carbachol (CCh) on IP3R-I/COX-III mRNA ratio in mouse islets. Isolated islets were cultured for 1 hr with 5.5 mM glucose in the absence (control) or presence of CCh (0.5 mM). Values are the means ± SEM for 6 independent determinations. Significant differences between groups were determined by Student's *t*-test (paired). Key: (*) $P < 0.05$ vs control.

sections did not show any binding fluorescence (data not shown).

Anti-glucagon antibody clearly defined cells in the perimeter of islets (Fig. 1, K, N, and Q), and anti-IP3R-I (Fig. 1J) and anti-IP3R-III (Fig. 1P) antibodies colocalized with the anti-glucagon antibody, as indicated by the yellow color (Fig. 1, L and R, respectively). However, the anti-IP3R-II antibody showed no binding in islets (Fig. 1M) and was not colocalized with the anti-glucagon antibody (Fig. 1O). Thus, IP3R-I and -III are present in mouse islet α-cells. IP3R-I and -III were also expressed in pancreatic acinar tissue surrounding islets (Fig. 1, J and P), and IP3R-II, although not detectable in islets, was expressed in acinar tissue (Fig. 1M). Control sections in which primary antibody was omitted and only secondary antibody was allowed to react with the tissue sections did not show any binding fluorescence (data not shown).

3.3. Regulation of IP3R mRNA and protein expression in mouse pancreatic islets

Acute regulation of expression of IP3R-I mRNA was determined in islets cultured at 5.5 mM glucose (control) or 20 mM glucose (glucose-treated). Osmotic equivalence between the treatment groups was maintained by the addition of mannitol to control islets. After 30 min or 2 hr of culture with a maximally secretagogic concentration of glucose (20 mM), the expression of islet IP3R-I mRNA was unchanged at 90 and 95%, respectively, of control values (Table 2). In βTC3 cells, glucose (25 mM) stimulation for 2 hr also did not affect IP3R-I mRNA expression significantly (Table 2).

Islets were also cultured for 7 days to characterize the long-term effects of a physiological, but hyperglycemic concentration of glucose (11 mM) on IP3R-I mRNA expression. Even with chronic glucose stimulation, islet IP3R-I mRNA remained unchanged relative to control (Table 2).

The effect of muscarinic receptor stimulation on IP3R-I mRNA expression was characterized in islets treated with carbachol. COX-III mRNA was chosen as a stable mitochondrial gene with which to compare changes of IP3R-I mRNA, since COX-III mRNA levels were not changed by carbachol or glucose stimulation (data not shown). In contrast, β- actin mRNA levels increased in response to carbachol stimulation in comparison with cyclophilin mRNA levels in rat islets (data not shown) and were not used to normalize data in these experiments. In islets treated for 1 hr at 0.5 mM carbachol, IP3R-I mRNA was reduced 35 ± 4% below control levels (Fig. 2). A similar down-regulation of IP3R-I mRNA isoform was also demonstrated in βTC3 cells. After 2 hr of culture with carbachol (0.5 mM), the expression of βTC3 cell IP3R-I mRNA was reduced 31 ± 8% ($P < 0.01$) below control levels.

Mouse islets expressed IP3R-I and -III proteins, but not IP3R-II, as determined by immunoblot analysis (Fig. 3A). Islets stimulated with glucose (20 mM) for 2 hr had IP3R-I protein levels that were 94 ± 10% of control ($P > 0.05$). However, the 20 mM glucose stimulus increased insulin release more than 18-fold (8.0 ± 2.2 ng insulin) over basal levels (0.43 ± 0.09 ng insulin). In contrast, stimulation with carbachol induced a time-dependent change in IP3R-I levels in mouse islets. Carbachol stimulation reduced islet IP3R-I protein after 1 hr to 40% below control and after 2 hr to 86% below control levels (Fig. 3, B and C). After 6 hr of carba-

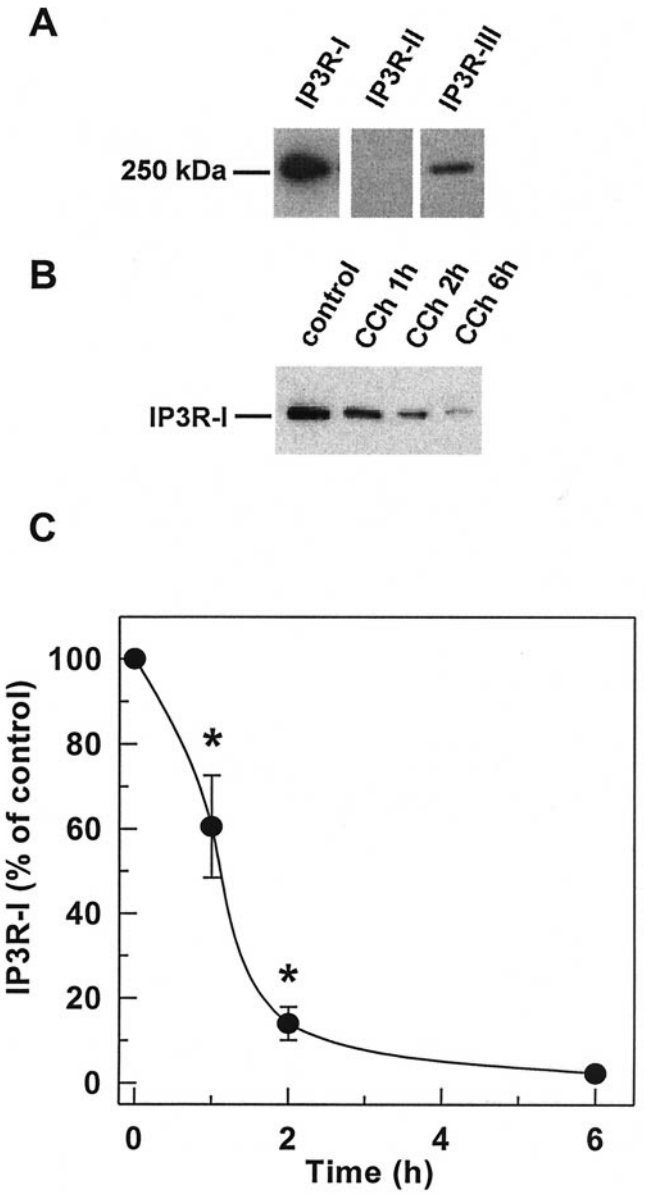

Fig. 3. IP3R regulation in mouse islets. (A) Western blot densitometric scan of IP3R isoforms in mouse islets. Islet homogenates were analyzed by SDS–PAGE and chemiluminescent immunoblot for IP3R isoforms, I, II, or III, as indicated. Equal amounts of protein (12 μg) were loaded per lane. (B and C) Time course of IP3R-I down-regulation in isolated islets. Islets were incubated in the absence (control) or presence of carbachol (CCh) (0.5 mM) for 1, 2, and 6 hr, and immunoblots were probed with an anti-IP3R-I antibody. (C) Values are expressed as percent of control, and are the means ± SEM for 3 or 4 independent determinations, except for the 6-hr value which is for one determination. Significant differences between the fraction of IP3R-I remaining at 1 and 2 hr were determined by one-way ANOVA and the Student–Newman–Keuls multiple-comparison test. Key: (*) $P < 0.05$ vs control.

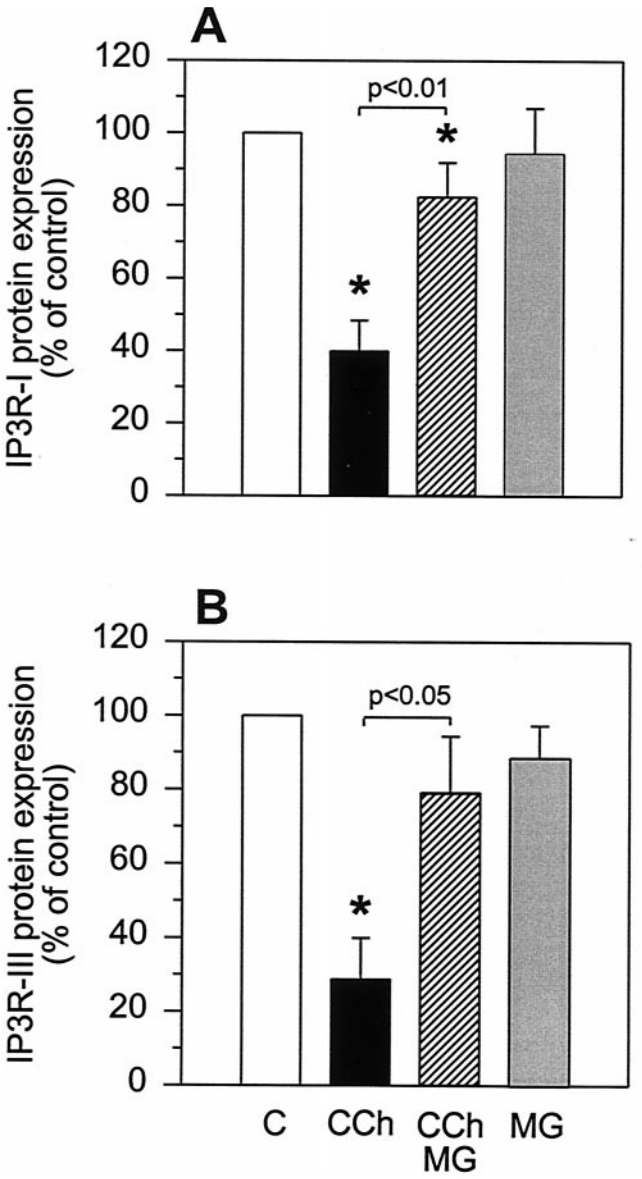

Fig. 4. Effect of MG-132 on carbachol-induced changes in IP3R protein expression. Mouse islets cultured at 5.5 mM glucose were incubated in the absence (C) or presence of MG-132 (MG) (50 μM) and carbachol (CCh) (0.5 mM). Values are the means ± SEM for 4–6 independent determinations. The relative density units for control IP3R-I and -III were 2.66 ± 0.02 and 4.32 ± 0.43, respectively. Significant differences between groups were determined by one-way ANOVA and the Student–Newman–Keuls multiple-comparison test. Key: (*) $P < 0.05$ vs control.

chol stimulation, islet IP3R-I levels were barely detectable. Carbachol stimulation for 2 hr down-regulated islet IP3R-I and -III levels to a similar extent (Fig. 4, A and B). In contrast to carbachol, after 2 hr of phorbol 12,13-dibutyrate (1 μM) treatment, islet IP3R-I levels were similar to control (101 ± 14% of control) ($P > 0.05$).

To determine the mechanism whereby carbachol induced changes in IP3R levels, an inhibitor of the proteasome complex (26S) pathway, MG-132, was included in the islet cultures. MG-132 at concentrations similar to those used in this study has been demonstrated previously to inhibit proteasome-associated activities in isolated islets [34]. In the presence of MG-132, the carbachol-induced down-regulation of islet IP3R-I and -III was largely or completely prevented (Fig. 4, A and B). MG-132 alone had no effect on IP3R-I or -III levels.

4. Discussion

The results of this study indicate that IP3R-I, -II, and -III mRNA are expressed in mouse pancreatic islets, with IP3R-I mRNA the predominant isoform. βTC3 cells, generated from β-cell tumors [35,36], confirmed subtype expression in a homogeneous population of β-cells, compared with islets that are composed of endocrine and non-endocrine cells. Since only IP3R-I mRNA was detectable in βTC3 cells, and IP3R-II was not detected in islet sections but was detected in pancreatic acinar tissue in immunohistochemical studies, the results prompt speculation that a small amount of acinar tissue in islet preparations may account for the IP3R-II mRNA detected in mouse islets. IP3R-I mRNA was reported previously to be most abundant in mouse islets [14], although a recent study failed to detect IP3R-I mRNA in them [37]. However, the latter study appears not to have used efficient primers since in RT-PCR they also did not detect abundant IP3R-I mRNA in brain where it has been well characterized [12,14,27]. Previously, the mouse pancreatic β-cell line βHC9 was characterized as having predominantly IP3R-II and -III mRNA isoforms, using RT–PCR and primers identical to those used in the present study [14,29]. Since βHC9 cells are derived from pancreatic islets with β-cell hyperplasia [37], they may not be identical to βTC3 cells in terms of IP3R gene expression. However, the most marked difference in relative IP3R mRNA levels is found upon comparison between species—in rat islets, IP3R-III mRNA is the major isoform and IP3R-I mRNA is least abundant [29], whereas in mouse islets IP3R-I mRNA is the most abundant isoform.

This study is the first to provide immunohistochemical evidence that IP3R-I and -III proteins are present in mouse pancreatic α- and β-cells, where they colocalized with glucagon and insulin antibody, respectively. Localization of IP3Rs to α- and β-cells suggests that these calcium-mobilizing receptors play a role in insulin and glucagon secretion. Localization of mouse IP3R to α-cells differs from results reported for the rat islet where IP3R-III was not present in α-cells but only in β- and δ-cells [8]. The data demonstrate that low levels of IP3R-III mRNA in mouse islets are translated into detectable levels of protein, in contrast with IP3R-II. Mammalian IP3R-I, -II, and -III cDNA have between 60 and 70% identity to each other [11, 12,38–40]. Putative IP3R-IV and -V cDNAs have been partially sequenced and have a high degree of identity to IP3R-II cDNA [13,14]. It is possible that IP3R-II mRNA in the present study includes IP3R-IV and/or -V, since a significant level of IP3R-V is present in mouse islets [14]. Alternatively, IP3R-II mRNA may not be translated at detectable levels or the protein is broken down rapidly.

The IP3R profile for mouse islets, where IP3R-I and -III were clearly evident but IP3R-II was not, represents a dramatic difference compared with rat islets possessing IP3R-I, -II, and -III [12,26,30,41]. The absence of IP3R-II in mouse islets does not appear to be due to lack of antibody recognition of the mouse protein since antibody binding was demonstrated by immunofluorescence in pancreatic acinar tissue in this study, and by western blot of mouse pancreas [42]. The data suggest that IP3R-I and IP3R-III are responsible for mediating a stimulatory effect of IP3 on insulin and glucagon secretion in mouse pancreatic β-cells, whereas IP3R-I, -II, and -III may play an important role in rat β-cells, and IP3R-II may have a unique role in rat α-cells [8]. Interaction between the types I and III isoforms as a heteroligomer [43] may provide an additional layer of regulation in both rat and mouse islets. The differential expression of IP3R isoforms in mouse and rat islets likely contributes to unique Ca^{2+} mobilizing actions of IP3 given that the receptors have differences in structure, function, regulation, and cellular distribution [8,18,44,45].

This study shows for the first time that the expression of IP3R-I mRNA and protein are down-regulated by muscarinic receptor activation in mouse islets. Carbachol also down-regulated IP3R-III protein levels in mouse islets. The results with carbachol contrast with those of glucose stimulation since glucose did not affect IP3R mRNA or protein levels when compared with control, even though islet glucose-responsive insulin release was maintained. However, it has been reported previously [28] that the duration of IP3 generation is proportional to the ability of an agonist to down-regulate IP3R levels. Both glucose [46,47] and carbachol [46,48] rapidly stimulate IP3 generation in islets. The effect of carbachol (0.5 mM) on IP3 generation is reversed by the muscarinic receptor antagonist atropine [49]. The duration of action of glucose and of carbachol may be one reason for the differences noted in IP3R down-regulation. Carbachol (0.5 mM) has been reported to increase islet inositol phosphate production during a 5-min exposure to the receptor agonist [48], whereas glucose effects appear to be maximal after 1 min [47,50]. The absence of glucose-stimulatory effects on IP3R-I mRNA and protein levels in rat pancreatic islets, RINm5F cells, and βHC9 cells has also been reported [29,30]. Conversely, glucose stimulation induces an increase in IP3R-III mRNA expression in rat islets, βHC9 cells, and RINm5F cells [8,29], whereas this response is lacking in mouse islets. The lack of responsivity of IP3R-III mRNA to glucose stimulation in mouse islets suggests that regulation of this isoform is specific for different species, and tends to suggest a minor if any regulatory role for type III receptor in mouse islets.

Both mRNA and protein levels for IP3R-I declined soon after carbachol stimulation; protein levels for IP3R-III were similarly reduced. It is possible that carbachol stimulation induced a significant decrease in IP3R-I mRNA in islet α- and/or β-cells. Since carbachol stimulation induced a decrease in IP3R-I mRNA in βTC3 cells, it is likely that at least islet β- cells respond to carbachol in this manner. In addition, glucose did not induce any change in IP3R-I mRNA in βTC3 cells, suggesting that IP3R-I mRNA expression is not induced by glucose stimulation but is changed by muscarinic receptor activation in pancreatic

β-cells. The mechanism for the reduction in IP3R-I mRNA is not known but may be related to slowed transcriptional activity of the gene, or to destabilization of the message.

The mechanism by which carbachol reduces IP3R protein is probably not accounted for entirely by reduced mRNA levels. IP3R-I protein has a $T_{1/2}$ of $>$ 8 hr, and receptor degradation is likely to be the mechanism accounting for the rapid carbachol effects [51]. Precedent for this hypothesis is provided by observations of other investigators that activation of phosphoinositidase-C-linked receptors (as occurs in islets in response to muscarinic receptor activation) leads to the down-regulation of IP3R-I, -II, and -III protein expression in several cell types, including the rat insulinoma cell line INS-1 [28,52,53]. The down-regulation is induced by an acceleration of the proteolysis of IP3R-I, -II, and -III through the ubiquitin/proteasome pathway, since all three isoforms of IP3R are ubiquitinated [28]. In islets, it is likely that the carbachol-stimulated down-regulation of IP3R-I and -III proteins is mediated by ubiquitination and degradation by proteases, since the response is rapid with a half-maximal effect at 1 hr and was largely or totally prevented by the proteasome complex (26S) inhibitor MG-132. The small component of IP3R-1 protein that was lost even in the presence of MG-132 may be reflective of the loss of mRNA. MG-132 was reported previously to inhibit proteasome-associated cytokine-induced nuclear transcription factor-κB activation and nitric oxide synthase expression in islets [34]. The lack of effect of phorbol 12,13-dibutyrate, a protein kinase C activating agent, on mouse islet IP3R-1 levels suggests that carbachol effects are not mediated by protein kinase C activation subsequent to phospholipase C stimulation and diacylglycerol production. No nonspecific effects of MG-132 on phospholipase C or protein kinase C activities have been reported, although it cannot be ruled out that nonspecific effects of this agent contribute to the responses observed. It is likely that carbachol induces its effects through IP3 formation and binding to its receptor in islets as suggested for other cell types [28].

Functional characterization of IP3R isoforms is a work-in-progress. IP3R-I is phosphorylated in response to hormones that activate cyclic AMP, whereas IP3R-II and -III lack the relevant consensus sequences [34,54]. Significant differences between IP3R-I and -II are found in the region of the Ca^{2+} channel, suggesting that the gating properties of the receptor/Ca^{2+} channels may be different [11]. IP3R-I, -II, and -III have different binding affinities for IP3: the relative order of affinities are type II $>$ I $>$ III [15]. In addition, Ca^{2+} can inhibit IP3 binding to IP3R-I but stimulate (at intermediate Ca^{2+} levels) or inhibit (at high Ca^{2+} levels) IP3 binding to IP3R-III [16,17]. Calmodulin can also inhibit IP3 binding to IP3R-I but not IP3R-III [55]. In addition, IP3Rs may have distinct functions related to their subcellular localization in the endoplasmic reticulum, nucleus, secretory granules, and plasma membrane [4–6,8]. Thus, co-expressed IP3Rs are regulated independently and may have different IP3 binding characteristics and subcellular distribution, which could account for the selective regulation of receptor activity in tissues.

In summary, mouse islet and/or insulinoma cells express IP3R-I, -II, and -III mRNA; however, IP3R-II protein is conspicuously absent. Moreover, IP3R-I appears to be transcriptionally and post-transcriptionally regulated by muscarinic receptor activation of β-cells. Post-transcriptional regulation of IP3R-I and -III is rapid and suggests that IP3R levels may mediate changes in β-cell responsivity to certain secretagogues. It remains to be determined if the down-regulation of IP3R contributes to desensitization of glucose-stimulated insulin secretion, or to changes in glucagon secretion, in islets pretreated with carbachol [56].

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