Ryanodine Receptor Type I and Nicotinic Acid Adenine Dinucleotide Phosphate Receptors Mediate Ca\(^{2+}\) Release from Insulin-containing Vesicles in Living Pancreatic \(\beta\)-Cells (MIN6)*

Kathryn J. Mitchell, F. Anthony Lai§, and Guy A. Rutter§

From the Henry Wellcome Laboratories of Integrated Cell Signaling and Department of Biochemistry, School of Medical Sciences, University Walk, University of Bristol, Bristol BS8 1TD and the Department of Cardiology, Wales Heart Research Institute, University of Wales College of Medicine, Cardiff CF14 4XN, United Kingdom

We have demonstrated recently (Mitchell, K. J., Pinton, P., Varadi, A., Tacchetti, C., Ainscow, E. K., Pozzan, T., Rizzuto, R., and Rutter, G. A. (2001) J. Cell Biol. 155, 41–51) that ryanodine receptors (RyR) are present on insulin-containing secretory vesicles. Here we show that pancreatic islets and derived \(\beta\)-cell lines express type I and II, but not type III, RyRs. Purified by subcellular fractionation and membrane immuno-isolation, dense core secretory vesicles were found to possess a similar level of type I RyR immunoreactivity as Golgi/endoplasmic reticulum (ER) membranes but substantially less RyR II than the latter. Monitored in cells expressing appropriately targeted aequorins, dantrolene, an inhibitor of RyR I channels, elevated free Ca\(^{2+}\) concentrations in the secretory vesicle compartment from 40.1 ± 6.7 to 90.4 ± 14.8 μM (n = 4, p < 0.01), while having no effect on ER Ca\(^{2+}\) concentrations. Furthermore, nicotinic acid adenine dinucleotide phosphate (NAADP), a novel Ca\(^{2+}\)-mobilizing agent, decreased dense core secretory vesicle but not ER free Ca\(^{2+}\) concentrations in permeabilized MIN6 \(\beta\)-cells, and flash photolysis of caged NAADP released Ca\(^{2+}\) from a thapsigargin-insensitive Ca\(^{2+}\) store in single MIN6 cells. Because dantrolene strongly inhibited glucose-stimulated insulin secretion (from 3.07 ± 0.51-fold stimulation to no significant glucose effect; n = 3, p < 0.01), we conclude that RyR I-mediated Ca\(^{2+}\)-induced Ca\(^{2+}\) release from secretory vesicles, possibly potentiated by NAADP, is essential for the activation of insulin secretion.

Increases in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) are important for stimulation of neurosecretion in general (1) and for the activation of insulin secretion from pancreatic islet \(\beta\)-cells (1, 2). In the latter cell type, increases in [Ca\(^{2+}\)]\(_{i}\) usually occur as a result of either nutrient-induced influx of Ca\(^{2+}\) ions through voltage-gated Ca\(^{2+}\) channels on the plasma membrane (3) or via the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores (4). The endoplasmic reticulum (ER) (5, 6) and Golgi apparatus (7) probably represent the major Ca\(^{2+}\) stores in \(\beta\)-cells (8–10) as in other cell types (11). However, we have recently provided evidence that dense core secretory vesicles also play a role in intracellular Ca\(^{2+}\) signaling in \(\beta\)-cells (9, 12). Importantly, secretory vesicles occupy a substantial proportion of the intracellular volume of \(\beta\)-cells (13, 14) and may contain close to half the total cellular Ca\(^{2+}\). As such, these organelles potentially provide a huge store of mobilizable Ca\(^{2+}\) ions (15).

Previous studies involving measurements of intravesicular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{SV}\)) and immunoelectron microscopy (9) indicated that ryanodine, but not inositol 1,4,5-trisphosphate (11), receptors mediate Ca\(^{2+}\) release from secretory vesicles in \(\beta\)-cells (9, 10, 16). cDNAs encoding three RyR isoforms have so far been identified in mammals. The type I isoform (RyR I) is expressed mainly in skeletal muscle (17), whereas RyR II is abundant in the heart (18). RyR III is present in a variety of tissues and cell types, most notably the brain (19). RyR II has been reported previously to be the most abundantly expressed isoform (at the mRNA level) in wild type (20) and ob/ob mouse islets (20, 21), as well as in rat islets (22) and clonal βTC3 cells (22). Moreover, the presence of RyR II protein has also been demonstrated in derived INS-1 \(\beta\)-cells (23). Lower levels of RyR I and RyR III mRNA have also been detected in βTC3 (22) and HIT-T15 cells (24), respectively. However, the physiological role(s) of RyRs in \(\beta\)-cells remains unclear, given that RyR II mRNA levels in ob/ob mouse islets are reportedly ~1000-fold less than in the heart (21), whereas RyR II protein levels in INS-1 \(\beta\)-cells were ~10-fold lower than in brain (23).

Receptors for nicotinic acid adenine dinucleotide phosphate (NAADP), a novel intracellular Ca\(^{2+}\)-mobilizing agent (25), may represent an alternative pathway for Ca\(^{2+}\) efflux from dense core secretory vesicles (26). Although other studies (27–30) have demonstrated NAADP-induced Ca\(^{2+}\) release in a variety of mammalian cells and cell lines, few data are currently available regarding the role of NAADP in the \(\beta\)-cell. Although functional NAADP-sensitive Ca\(^{2+}\) stores were recently revealed in human \(\beta\)-cells (31), NAADP-induced Ca\(^{2+}\) release was not observed in dispersed \(\beta\)-cells from either normal or ob/ob mouse islets (32). However, in the latter report, neither the RyR agonists caffeine and ryanodine nor cyclic ADP-ribose (cADPr) induced Ca\(^{2+}\) release.

In the present study, we show that islets and MIN6 \(\beta\)-cells express two RyR isoforms, RyR I and RyR II, that display distinct subcellular localizations. Thus, whereas type I RyRs
Ryanodine and NAADP Receptors in Islet β-Cells

are present at approximately equal density in a vesicle/mito-
ochondrial fraction, and in microsomes, RyR II was considerably
more abundant on ER membranes. Surprisingly, dantrolene,
a selective inhibitor of RyR I, increased steady-state free [Ca²⁺]
in secretory vesicles but not in the ER, suggesting the presence
on vesicles of a further activator or channel capable of ampli-
fying the effects of RyRs on Ca²⁺ release. We provide evidence
that receptors for NAADP may serve this role, and we thus
demonstrate that secretory vesicles, but not the ER, are a
NAADP-responsive Ca²⁺ store in β-cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Adenoviral Infection—MIN6 cells, a well differen-
tiated mouse insulinoma β-cell line (33) (passages 20–30), were grown
in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 25 mm
-glucose and 2 mM pyruvate, and supplemented with 15% (v/v) fetal
bovine serum, 200 μg/ml penicillin, 100 μg/ml streptomycin, and 50 μg β-mercaptoethanol, in a humidified atmo-
sphere containing 5% CO₂ (9, 34). Measurements of aequorin bioluminescence were performed using a
purpose-built photomultiplier system 48 h after infection, as described
previously (9, 37).

Detection of mRNA for Ryanodine Receptors in β-Cells and Islets—
Total RNA was extracted from cell line or rat tissue using TRI Rea-
tag™ (Sigma) according to the manufacturer’s instructions and
reverse-transcribed using Moloney murine leukemia virus reverse-transcriptase (Promega). PCR amplification was performed with prim-
ers designed to amplify an isoform-specific region of each of the three
RyR subtypes (35) as follows: RyR I (forward, 5′-GGAGGTTTCGTACAAACGGG-3′; reverse, 5′-TTGCTCTTGATGATCTGGTGCGG-3′); RyR II (forward, 5′-GAATGCACTGAATGATTGGAATG-3′; reverse, 5′-CTGTGTCTTCGTCTCCAAAAGC-3′); and RyR III (forward, 5′-CTTCTGCATACAACTTCTCCTGGC-3′; reverse, 5′-TCTCTCTGGGTGTAATGACCCG-3′). The PCR mix consisted of 5 μl of 10× Buffer 3 (Roche Molecular Biochemicals), 6 μl of 25 mM MgCl₂, 1 μl of 10 mM dNTPs, 0.25 μl of Taq DNA polymerase (Roche Molecular Biochemicals), 5 μl of reverse transcription-PCR cDNA, 0.4 μl forward primer, 0.4 μl reverse primer, and distilled H₂O, at a final volume of 50 μl. Amplifica-
tion conditions are as follows: 94°C for 2 min and then 94°C for 45 s, 55.5°C for 45 s, 72°C for 1 min for 32 cycles and then 72°C for 10 min. Negative controls were performed by omission of the reverse transcription step or fractionation of the template from the PCR. PCR products were
then separated by migration on a 5% (v/v) polyacrylamide gel, transferred onto a mem-
brane, and probed with anti-skeletal muscle RyR antibody or isoform-
specific antibodies, RyR I and RyR III.

Ryanodine Receptor Type I Antiserum Production and Purification—
Rabbits (New Zealand White) were immunized with the keyhole limpet hemocyanin-conju-
gated peptide as described previously (41), and antibody specificity was
confirmed by enzyme-linked immunosorbent assay and immunoblot
analysis with brain, skeletal, and cardiac muscle microsomes, prepared
as described previously (41). For immunoblot analysis, microsomes
were separated on a 5% (v/v) polyacrylamide gel (30 μg of protein/lane), and proteins were electrophoretically transferred to polyvinylidene difluoride membrane before probing with antibody at a dilution of 1:1000, Affinity-purified antibody (anti-RyR I; number 2142) was prepared by
acid elution following incubation of the crude RyR I antiserum either with
skeletal muscle RyR protein immobilized on polyvinylidene difluoride
membrane strips or on protein A-agarose columns (Sigma).

Immunoblotting—Protein samples were resolved by SDS-PAGE on
5% (v/v) polyacrylamide gels and transferred onto Immobilon-P transfer
membrane (Millipore) following a standard protocol. Membranes were
probed with anti-skeletal muscle RyR antibody (1:2500; Upstate Bio-
technology, Inc.), anti-RyR I (1:500; number 2142), anti-RyR II (1:200; Affinity BioReagents), and anti-RyR III antibodies (1:500; number 110E) (42). Immunostaining was revealed with horseradish peroxidase-conju-
gated anti-rabbit IgG (Sigma; 1:100 000), anti-mouse IgG (Sigma; 1:10,000), or alkaline phosphatase-labeled anti-rabbit or anti-mouse IgG (1:1000) using an enhanced chemilu-
minescence (ECL) detection system (Roche Diagnostics).

Measurements of Free Ca²⁺ Concentration with Recombinant Tar-
ged Aequorins—Cells were depleted of Ca²⁺ by incubation with
ionomycin (10 μM), monensin (10 μM), and cyclopiazonic acid (10 μM) in
modified Krebs-Ringer bicarbonate buffer (KRB: 140 mM NaCl, 3.5 mM
KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 5 mM glucose, 10 mM Hepes, 2
mM NaHCO₃, pH 7.4) supplemented with 1 mM EGTA, for 10 min at
4°C (9). Aequorin was reconstituted with 5 μM coelenterazine n (43) for 1–2 h at 4°C in KRB supplemented with 1 mM EGTA.

Intact cells were perfused with KRB plus additions as stated at 2
mM/min in a thermostatted chamber (37°C) in close proximity to a
photomultiplier tube (ThornEMI) (44). Where indicated, cells were per-
meabilized with 20 μM digitonin for 1 min at 37°C and subsequently
perfused in intracellular buffer (IB: 140 mM KCl, 10 mM NaCl, 1 mM
KH₂PO₄, 5.5 mM glucose, 2 mM MgSO₄, 1 mM ATP, 2 mM sodium
succinate, 20 mM Hepes, pH 7.05). Additions to this buffer were as

FIG. 1. Immunoblot of RyRs in MIN6 β-cells. A, subcellular frac-
tions of MIN6 cells obtained by differential centrifugation were mi-
gated on a 5% (v/v) polyacrylamide gel, transferred onto an Immob-
ilon-P transfer membrane, and stained with anti-skeletal muscle RyR
(type I) or anti-RyR II antibody. The latter antibody is known to also
weakly react with RyR I. Lanes were loaded as follows: lane 1, whole
cell lysate; lane 2, cytosolic fraction; lane 3, nuclear fraction; lane 4,
microsodium and secretory vesicles; lane 5, ER and Golgi. B, whole cell
lysate from MIN6 cells (lane 1), skeletal muscle protein (lane 2), and
MIN6 cell vesicle protein (see “Experimental Procedures”; lane 3) were
separated on a 5% (v/v) polyacrylamide gel, transferred onto a mem-
brane, and probed with anti-skeletal muscle RyR antibody or isoform-
specific antibodies, RyR I and RyR III.
Fig. 2. Effect of ryanodine and dantrolene on steady-state Ca\textsuperscript{2+} concentrations in the secretory vesicle and ER. After Ca\textsuperscript{2+} depletion and aequorin reconstitution, MIN6 cells infected with VAMP.Aq (A and C), ER.Aq (B and D), or Cyt.Aq (E) encoding adenoviruses were perfused in KRB containing 1 mM EGTA. Where indicated, EGTA was replaced with 1.5 mM CaCl\textsubscript{2} in the absence (open symbols) or presence (closed symbols) of ryanodine (Ryan.) (10 μM; A and B) or dantrolene (Dan.) (10 μM; C–E). Data are the means of four separate experiments.

**RESULTS**

Detection of Ryanodine Receptor mRNAs in β-Cell Lines and Primary Islets—Using isofrom-specific primers, PCR products corresponding to RyR I and RyR II cDNA were readily amplified from MIN6-, INS1-, or rat islet-derived cDNAs (data not shown). By contrast, RyR III cDNA was not amplified from these sources using the chosen primer pair (see “Experimental Procedures”), although RyR III from brain cDNA was amplified, as expected. The identity of each of the generated PCR products was confirmed by both restriction analysis and by automatic sequencing, which revealed 100% identity with the corresponding mouse (GenBank\textsuperscript{TM} accession numbers X83932 and AF295105 for RyR I and RyR II, respectively) and rat (GenBank\textsuperscript{TM} accession numbers AF130879 and U95157) cDNAs. Semi-quantitative PCR revealed 5-fold lower RyR I mRNA levels in rat islet-derived than in skeletal muscle-derived cDNA, and RyR II mRNA levels were 8-fold lower in islet than in heart cDNA (data not shown).

Expression of Ryanodine Receptor Protein in MIN6 Cells—In order to confirm the presence, and identify the intracellular
localization, of RyRs in MIN6 β-cells, subcellular fractionation and immunoblotting (Western) was performed. Probing of crude MIN6 cell fractions with a subtype-specific RyR antibody, which recognizes type I (and III) isoforms, indicated similar levels of RyR immunoreactivity in both mitochondrial/dense core secretory vesicle and microsomal fractions (Fig. 1 A, upper panel). By contrast, RyR II immunoreactivity was much more abundant on the latter fraction, with only weak staining for RyR II in the crude vesicle/mitochondria fraction (Fig. 1 A, lower panel). To demonstrate that RyR I immunoreactivity was present on secretory vesicles in the crude secretory vesicle/mitochondrial fraction examined above, immunoblotting was also performed with immunopurified dense core secretory vesicles (40). Immunoreactivity toward both anti-RyR I/III, and to a selective anti-RyR I antibody (see “Experimental Procedures”), was clearly evident in these membranes (Fig. 1 B, upper panels), whereas reactivity to RyR III (Fig. 1 B, lower panel) was undetectable.

Effect of Ryanodine Receptor Inhibition on Secretory Vesicle and ER Ca\(^{2+}\) Concentrations—The above fractionation studies indicated that the relative abundance of type I/type II RyRs was higher in secretory vesicles than the ER but provided no information on the relative abundance of the two isoforms on either membrane, considered alone. To determine the relative importance of RyR I and RyR II on each organelle, we therefore used a functional approach in living MIN6 cells. Recombinant aequorins, targeted specifically to either organelle by the addition of appropriate presequences (9), were utilized to monitor free Ca\(^{2+}\) concentrations in each compartment. Concentrations of ryanodine (10 \(\mu M\)) expected to lead to closure of all RyR isoforms (51) substantially raised the steady-state concentrations of free Ca\(^{2+}\) in both the secretory vesicle matrix ([Ca\(^{2+}\)]\(_{SV}\)) and the ER lumen ([Ca\(^{2+}\)]\(_{ER}\)) (Fig. 2, A and B). In contrast, the skeletal muscle relaxant dantrolene (52) significantly increased [Ca\(^{2+}\)]\(_{SV}\) (from 40.1 ± 6.7 to 90.4 ± 14.8 \(\mu M\); \(n = 4, p < 0.01\); Fig. 2 C), whereas neither [Ca\(^{2+}\)]\(_{ER}\) (Fig. 2 D) nor [Ca\(^{2+}\)]\(_c\) (Fig. 2 E) was affected by this agent. These findings are in agreement with previous studies where dantrolene was shown to bind directly to and inhibit pig and rabbit skeletal muscle (types I and III), but not cardiac (type II), RyRs (52–55), suggesting that dantrolene inhibits the type I, but not the type II, RyR in MIN6 β-cells.

Effect of NAADP on Secretory Vesicle and ER Ca\(^{2+}\) Concentrations—The above results suggest that although ryanodine-sensitive Ca\(^{2+}\) efflux from secretory vesicles is likely to be mediated principally via type I RyRs, this channel subtype apparently plays a minor role, if any, in mediating Ca\(^{2+}\) release from the ER in MIN6 β-cells. This result was unexpected given that subcellular fractions enriched with ER membranes apparently contained the same amount or more immunoreactivity to

![Fig. 3. Effect of NAADP on secretory vesicle and ER Ca\(^{2+}\) concentrations in permeabilized MIN6 cells.](image-url)
Ryanodine and NAADP Receptors in Islet β-Cells

RyR I as a crude secretory vesicle/mitochondria fraction (Fig. 1A, upper panel). One simple explanation of this observation is that the absolute number of type II RyRs on the ER is very much greater than type I receptors, a difference that may not be apparent given the different antibodies and dilutions used to quantify each of these isoforms (Fig. 1).

However, as an alternative explanation, we next explored the possibility that another Ca\[^{2+}\] release channel may be functional on secretory vesicles, whose presence may stimulate the activity of neighboring RyR I channels. The effects on [Ca\[^{2+}\]]\(_{SV}\) of the recently identified Ca\[^{2+}\]-mobilizing molecule NAADP (25, 56) were therefore examined in permeabilized cells at a concentration of this compound previously shown to be optimal in human β-cells (31) and other mammalian cell types (26). 100 nM NAADP caused a small but highly significant decrease in [Ca\[^{2+}\]]\(_{SV}\) (Fig. 3A) but was completely without effect on [Ca\[^{2+}\]]\(_{ER}\) (Fig. 3B).

To determine whether the effects of NAADP may be mediated by a receptor identical or similar to that described previously (26) in mammalian and other cell systems, we next explored the pharmacology of the observed NAADP-induced changes in secretory vesicle [Ca\[^{2+}\]]. Concentrations of ryanodine sufficient to inhibit all RyR isoforms (51), but known to have no effect on NAADP receptor activity (57), failed to alter NAADP-induced [Ca\[^{2+}\]]\(_{SV}\) changes (Fig. 3C). Similarly, NAADP-induced release of secretory vesicle Ca\[^{2+}\] was unaffected by dantrolene (Fig. 3D). By contrast, nimodipine, an inhibitor of L-type Ca\[^{2+}\] channels shown previously (26) to block NAADP receptors, completely blocked NAADP-induced changes in [Ca\[^{2+}\]]\(_{SV}\) (Fig. 3E).

Effect of Photorelease of Caged NAADP on [Ca\[^{2+}\]], in Intact MIN6 Cells—To determine whether (i) NAADP may mediate Ca\[^{2+}\] release selectively from dense core vesicles in living cells, and (ii) to explore the impact of this release of cytosolic Ca\[^{2+}\] concentrations, we next micro-injected an inactive precursor of NAADP (“caged NAADP”) (48) into single MIN6 β-cells, and monitored the impact of its rapid uncaging by flash photolysis (Fig. 4). Photo-released NAADP provoked an increase in the fluorescence of the co-microinjected Ca\[^{2+}\] reporter, Oregon Green, of 4.3 ± 2.1% with respect to basal fluorescence (n = 7 cells; Fig. 4A), consistent with the mobilization of intracellular Ca\[^{2+}\]. The magnitude of this increase was not significantly affected by depletion of ER/Golgi Ca\[^{2+}\] stores with the sarco-(endo)plasmic reticulum Ca\[^{2+}\]-ATPase inhibitor, thapsigargin (5.6 ± 1.4%; n = 8 cells; Fig. 4B), nor by incubation with ryanodine (3.7 ± 0.9%; n = 7 cells; Fig. 4C).

Importance of Secretory Vesicle Ca\[^{2+}\] Release for Glucose-stimulated Insulin Secretion—The above studies indicated that release of Ca\[^{2+}\] from secretory vesicles, mediated by either type I RyR or NAADP receptors, may be important for the triggering of insulin secretion by nutrients. To determine whether Ca\[^{2+}\]-induced Ca\[^{2+}\] release via type I RyRs or Ca\[^{2+}\] release through NAADP receptors was qualitatively the more important pathway for Ca\[^{2+}\] efflux in living cells, we blocked the former with dantrolene (see above). In accordance with previous findings in islets (58) and INS1E cells (59), the stimulation of insulin release from MIN6 β-cells by 30 mM (versus 3 mM) glucose (3.07 ± 0.51-fold; n = 3, p < 0.01) was completely inhibited in the presence of the drug (Fig. 5A). Similarly, glucose-induced insulin secretion was abolished (from 2.02 ± 0.11-fold stimulation to no significant effect; n = 4, p < 0.01) in the presence of 100 μM ryanodine (Fig. 5B). This latter finding is in contrast to previous reports where ryanodine had little or no effect on insulin secretion in mouse β-cells (60) nor in INS1E cells (59), a result attributed to the poor permeation of ryanodine across the cell membrane in these systems. By contrast, both the present (Fig. 2, A and B) and previous studies (61) indicate that intact MIN6 cells may be more permeable to this drug.

DISCUSSION

Ryanodine Receptor mRNA Expression in Pancreatic β-Cells—In this study, we found that mRNAs encoding both RyR I and RyR II are expressed in β-cell lines as well as in rat islets at approximately equal levels. These findings are in apparent contrast with previous reports (20–22) indicating that RyR II mRNA was by far the most abundant RyR isoform, at least in mouse β-cells. Thus, in these earlier studies, only faint reverse transcriptase-PCR products were generated with RyR I primers in mouse-derived βTC3 cells, whereas the same primers did not detect any RyR I mRNA in rat islets (22). Similarly, ob/ob mouse islets, which are enriched in β-cells, were shown to contain ~1000-fold less RyR II mRNA than the heart (21). A number of factors may underlie these apparently discrepant results. First, the primers used to detect RyR I in previous reports may have been less efficient in amplifying the β-cell RyR I than those used in the present study. Consistent with this view, the primers used by Takasawa et al. (20) scarcely detected RyR I mRNA in the brain, a tissue in which this isoform is abundant (19, 62). Second, the presence and
MIN6 cells were cultured for 16 h with 3 mM glucose and then incubated with KRB supplemented with 3 mM glucose for 30 min. Cells were then incubated for a further 30 min with 3 or 30 mM glucose, with additions as stated. Released insulin was measured by radioimmunoassay (see “Experimental Procedures”). Data are the means of at least three separate experiments.

density of RyRs in different β-cell lines and rodent strains may well differ. Thus, RyR II protein was hardly detectable in RINm5F cells, whereas INS1 cells were shown to express significant amounts (23). Similarly, one group (21) described low levels of RyR II mRNA in ob/ob mice islets, whereas another (20) failed to detect this message in islets from distinct colonies of ob/ob mice. An intriguing possibility is that these differences in RyR II expression in the islet may contribute to the differing severities of diabetes in the different mouse strains.

Ryanodine Receptor Protein Expression and Subcellular Localization in MIN6 β-Cells—RyR subtypes are expressed in various combinations in specific tissues and cell types. Thus, RyR II and III are expressed in the heart, RyR I and RyR III in skeletal muscle, and all three subtypes in smooth muscle and brain (62). Although the relevance of multiple isoform expression is not clearly understood, one possibility is that co-expression serves to amplify Ca2+ signals. Indeed, such a mechanism may explain why the presence of both RyR I and RyR II is required for the activation of Ca2+-induced Ca2+ release upon Ca2+ influx in vascular myocytes (63).

The present study revealed the presence of both RyR I and RyR II in MIN6 β-cells and islets at both the mRNA and protein levels (Fig. 1). Moreover, using subcellular fractionation as well as vesicle immunopurification, we demonstrate a distinct subcellular localization for each RyR isoform in this cell type. Thus, type I RyRs were present on both the ER and secretory vesicles, whereas RyR II immunoreactivity was more abundant in the former. Although we also detected a small amount of RyR II immunoreactivity in the crude mitochondrial/secretory vesicle fraction (Fig. L4), it should be noted that at least part of this reactivity may result from contamination of this fraction with ER/Golgi fragments and/or cross-reactivity with RyR I, because the anti-type II RyR antibody used also weakly cross-reacts with RyR I.

Supporting the view that Ca2+-induced Ca2+ release from secretory vesicles is mediated principally via RyR 1 channels, and from the ER via RyR II, blockade of RyR I receptors in whole cells with dantrolene (55) affected steady-state Ca2+ concentrations only in the former compartment (Fig. 2, C versus D). Together, these data therefore provide both structural and functional evidence that RyR I and RyR II are located on distinct organelles in MIN6 β-cells.

Interestingly, inhibition of type I RyRs, shown here (Fig. 5) and in earlier studies (58) to block glucose-induced insulin secretion, now seems likely to involve largely a blockade of Ca2+ release from secretory vesicles, rather than from the ER (Fig. 2). This result, which accords well with the reported effects of depleting vesicle Ca2+ on insulin release (64), reinforces the view that the release of vesicle Ca2+ plays an important role in triggering or facilitating the exocytotic release of insulin (Fig. 6).

Role of NAADP Receptors in β-Cells—An unexpected finding of the present study was that dantrolene, which blocks RyR I channels, affected Ca2+ concentrations in secretory vesicles but not in the ER despite the presence of RyR I receptors on both organelles. One possible explanation for this result is our demonstration that MIN6 β-cells possess an NAADP-sensitive intracellular Ca2+ store that appears to coincide, at least in part, with secretory vesicles (Figs. 3 and 4). Importantly, these data are consistent with previous findings that have suggested NAADP releases Ca2+ from a non-ER Ca2+ pool at the frog neuromuscular junction (65) and that the NAADP-sensitive Ca2+ pool in sea urchin eggs is thapsigargin-insensitive (66).

We show here that in pancreatic β-cells, NAADP-induced Ca2+ release is insensitive to ryodanline and dantrolene, confirming previous reports (26) that NAADP acts on a channel distinct from the RyR (Figs. 3, C and D, and 4C). Although the receptor for NAADP is as yet unidentified in molecular terms,
in agreement with previous findings in sea urchin eggs (67),
brain (28), smooth muscle (68), and heart (57), L-type Ca^2+-
channel inhibitors were found here to block NAADP-induced Ca^2+
release from β-cell vesicles (Fig. 3E), suggesting that a com-
mon or similar receptor is involved in each of these cellular
systems. Interestingly, NAADP-mediated Ca^2+ release in liv-
ing β-cells was not significantly affected by blockade of RyR8s
(Fig. 4, A and B), a result also consistent with an action of this
mesenger via a non-RyR channel.

Two Converging Pathways Are Involved in Ca^2+ Release
from Secretory Vesicles—cADPr, the proposed endogenous li-
gand for RyRs (69, 70), and NAADP are likely to be synthesized
in β-cells by the same enzyme, an ADP ribosyl cyclase termed
NAADP. Thus, the Ca^2+ signal is exposed to the extracellular
space (72). However, internalized catalytically active CD38 is
found in non-clathrin-
coated vesicles (73). Moreover, endocytotic vesicles have been
shown previously to accumulate the precursor for cADPr,
NaADP, via an unidentified dinucleotide transport system (74).

As well as catalyzing the conversion of β-NAD to cADPr, CD38
itself is thought to be involved in pumping the cyclic nucleotide
into the cytosol (75), thus providing a potential mechanism for
the accumulation of intracellular cADPr and potentially
NAADP.

Interestingly, transgenic mice overexpressing CD38 specifi-
cally in β-cells show enhanced glucose-induced insulin secre-
tion (76), whereas deletion of both alleles of CD38 gives rise to
glucose intolerance (77). Because CD38 activity is regulated
allosterically by ATP (76), glucose-induced increases in the
intracellular free concentration of this nucleotide (47, 78) may
lead to increases in the intracellular concentrations of either
cADPr, NAADP, or both. Although glucose-dependent increases
in cADPr have been reported previously (69), key fu-
g future goals will be to determine whether glucose is able to
increase NAADP levels in β-cells and whether such increases
are altered in models of type 2 diabetes mellitus.

Acknowledgments—We thank Dr. Mark Jesop and Alan Leard
(Bristol MRC Imaging Facility) for help with confocal imaging and
Dr. Aniko Varadi for help with flash photolysis. We also thank
Dr. Judith A. Airey (University of Nevada) for providing the anti-RyR
III antibodies (University of Naples) for caged
NAADP, and Dr. Frederique Diraison (University of Bristol) for
the preparation of rat islets.

REFERENCES
1. Rutter, G. A. (2001) Mol. Aspects Med. 22, 247–284
2. Wolfle, C. B., Blundel, B., Trueheart, P. A., Renold, A. E., and Sharp, G. W.
(1975) J. Biol. Chem. 250, 1354–1361
3. Sadaayi, H., Haase, H., Kramer, U., Bihlmayer, A., Reisenfeldt, M., Ammon,
H. P., Froschmayr, M., Cassidy, T. N., Morano, I., Ahilian, M. K., and
Streissgr.ig, J. (1997) Mol. Endocrinol. 11, 619–629
4. Wolthuis, C. B., and Bider, T. J. (1986) J. Biol. Chem. 261, 8314–8319
5. Streb, H., Baderoffer, E., Haase, W., Irvine, R. F., and Schulz, I. (1984) J.
Membr. Biol. 81, 241–253
6. Rizzuto, R., Murgia, M., and Pozzan, T. (1993) Science 262, 744–747
7. Pinton, P., Pozzan, T., and Rizzuto, R. (1998) EMBO J. 17, 5296–5308
8. Kennedy, E. D., Rizzuto, R., Theler, J. M., and Hécaen, H. (1999) J. Biol.
Chem. 274, 13810–13816
9. Brailoiu, E., Miyamoto, M. D., and Dun, N. J. (2001) J. Mol. Biol. 312, 328–329
10. Janjic, D., Wollheim, C. B., and Sharp, G. W. (1982) J. Biol. Chem. 257,
138–148
11. Baderoffer, H., Hirth, I., and Rutter, G. A. (1991) Biochem. J. 273, 1169–1173
12. Brailoiu, E., Miyamoto, M. D., and Dun, N. J. (2001) Biochem. J. 369,
287–299
13. Brailoiu, E., Miyamoto, M. D., and Dun, N. J. (2001) Biochem. J. 369,
287–299
14. Brailoiu, E., Miyamoto, M. D., and Dun, N. J. (2001) Biochem. J. 369,
287–299
Ryanodine and NAADP Receptors in Islet β-Cells

71. Lee, H. C. (1999) *Biol. Chem.* **380**, 785–793
72. Jackson, D. G., and Bell, J. I. (1999) *J. Immunol.* **144**, 2811–2815
73. Zocchi, E., Franco, L., Guida, L., Piccini, D., Tacchetti, C., and De Flora, A. (1996) *PEBS Lett.* **386**, 327–332
74. Zocchi, E., Usai, C., Guida, L., Franco, L., Bruzzzone, S., Passalacqua, M., and De Flora, A. (1999) *FASEB J.* **13**, 273–283
75. Franco, L., Guida, L., Bruzzzone, S., Zocchi, E., Usai, C., and De Flora, A. (1998) *FASEB J.* **12**, 1507–1520
76. Kato, I., Takasawa, S., Akahane, A., Tanaka, O., Abe, H., Takamura, T., Suzuki, Y., Nata, K., Yonekura, H., Yoshimoto, T., and Okamoto, H. (1995) *J. Biol. Chem.* **270**, 30045–30050
77. Kato, I., Yamamoto, Y., Fujimura, M., Noguchi, N., Takasawa, S., and Okamoto, H. (1999) *J. Biol. Chem.* **274**, 1869–1872
78. Ainscow, E. K., and Rutter, G. A. (2001) *Biochem. J.* **353**, 175–180