The Anx7(+/-) Knockout Mutation Alters Electrical and Secretory Responses to Ca^{2+}-Mobilizing Agents in Pancreatic β-cells

David Mears[1], Charles L. Zimliki[1*], Illani Atwater[2], Eduardo Rojas[2], Mirta Glassman[1], Ximena Leighton[1], Harvey B. Pollard[1] and Meera Srivastava[1]

[1]Department of Anatomy, Physiology & Genetics, Uniformed Services University of the Health Sciences, Bethesda, MD, [2]Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, *Current Address: Center for Devices and Radiological Health, U.S. Food and Drug Administration, Silver Spring, MD

Abstract

Insulin secretion from the pancreatic β-cell is controlled by changes in membrane potential and intracellular Ca^{2+}. The contribution of intracellular Ca^{2+} stores to this process is poorly understood. We have previously shown that β-cells of mice lacking one copy of the Annexin 7 gene (*Anx7(+/-)*) express reduced levels of IP$_3$ receptors and defects in IP$_3$-dependent Ca^{2+} signaling. To further elucidate the effect of the *Anx7(+/-)* mutation on signaling related to intracellular Ca^{2+} stores in the β-cell, we measured the effects of Ca^{2+} mobilizing agents on electrical activity, intracellular Ca^{2+} and insulin secretion in control and mutant β-cells. We found that the muscarinic agonist carbachol and the ryanodine receptor agonists caffeine and 4-chloro-*m*-cresol had more potent depolarizing effects on *Anx7(+/-)* β-cells compared to controls. Accordingly, glucose-induced insulin secretion was augmented to a greater extent by caffeine in mutant islets. Surprisingly, ryanodine receptor-mediated Ca^{2+} mobilization was not affected by the *Anx7(+/-)* mutation, suggesting that the mechanism underlying the observed differences in electrical and secretory responsiveness does not involve intracellular Ca^{2+} stores. Our results provide evidence that both IP$_3$ receptors and ryanodine receptors play important roles in regulating β-cell membrane potential and insulin secretion, and that the *Anx7(+/-)* mutation is associated with alterations in the signaling pathways related to these receptors.

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Introduction

Insulin secretion from the pancreatic β-cell is driven by increases in the intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$). With few exceptions, [Ca^{2+}]$_i$ in the β-cell is determined by the membrane potential, which controls the rate of Ca^{2+} influx through voltage-gated Ca^{2+} channels [1-3]. At low glucose levels, the β-cell membrane is kept hyperpolarized by the activity of adenosine triphosphate-sensitive K$^+$ channels (K$_{ATP}$), and [Ca^{2+}]$_i$ is low. Increases in the cytosolic ATP:ADP ratio following metabolism of glucose cause K$_{ATP}$ channels to close, depolarizing the

membrane and initiating an electrical activity known as bursting. Bursting consists of oscillations between depolarized and hyperpolarized phases [4]. The depolarized phase of bursting is characterized by production of Ca^{2+}-dependent action potentials, and the Ca^{2+} that enters the cell during this phase raises $[Ca^{2+}]_i$ and triggers insulin secretion. Although the mechanism underlying bursting has not yet been deciphered [5, 6], it is clear that many physiological and pharmacological agents that affect glucose-induced insulin secretion act, at least in part, by altering the glucose-induced pattern of electrical activity [7, 8].

The participation of intracellular Ca^{2+} stores in stimulus-secretion coupling is not as well understood. Ca^{2+} is sequestered into numerous organelles within the β-cell, including the endoplasmic reticulum (ER). Inositol-1,4,5-trisphosphate (IP_3)-generating agonists such as acetylcholine stimulate release of Ca^{2+} from the ER by activating IP_3 receptor Ca^{2+} channels on the membrane of the organelle [9-11]. The Ca^{2+} released from the store can contribute to insulin secretion directly, by transiently elevating $[Ca^{2+}]_i$ [12]. Furthermore, depletion of IP_3-sensitive Ca^{2+} stores activates a store-operated non-selective ionic current in the β-cell [13, 14]. This current augments glucose-induced membrane depolarization, thereby producing a sustained enhancement of voltage-gated Ca^{2+} influx [15, 16]. Uptake and release of Ca^{2+} from IP_3 sensitive stores may also play a role in shaping glucose-induced Ca^{2+} oscillations, even in the absence of IP_3-generating agonists [17].

Ryanodine receptors (RyRs) are another important class of intracellular Ca^{2+} channels. RyRs mediate Ca^{2+}-induced Ca^{2+} release (CICR) in many cell types, but the role of these channels in the β-cell has been particularly challenging to decipher. Over the past decade, a low level of RyR expression has been detected in β-cells and immortalized β-cell lines from different species by various molecular techniques [18-21]. In accord with these reports, numerous studies using pharmacological agents have shown that RyRs mediate Ca^{2+} release and influence insulin secretion under certain conditions [22-25]. These reports have led to the suggestion that RyR-mediated CICR contributes to glucose-induced insulin secretion and/ or its enhancement by agents such as caffeine, GLP-1 and growth hormone. Other studies point to a role for basal RyR activity in enhancing β-cell survival [18, 26]. On the other hand, several studies have found no evidence for a functional role for RyRs in the β-cell [27-29]. Despite this controversy, the emerging picture is that intracellular Ca^{2+} stores are important regulators of membrane

potential, Ca^{2+} signaling and insulin secretion in β-cells.

The quantitative study of Ca^{2+} sequestering organelles and Ca^{2+} release channels in the β-cell is impeded by their small size and broad intracellular localization. An alternative approach to gain insight into the functional roles of β-cell Ca^{2+} stores is to study β-cell function in animal models with altered Ca^{2+} store signaling. One such model is the annexin 7 [$Anx7(+/-)$] knockout mouse. Annexin7 (also called synexin) is a Ca^{2+}/GTP-dependent fusion protein that is a tumor suppressor, has Ca^{2+} channel activity, releases Ca^{2+} from the ER in brain tissue, and is involved in regulating vesicle fusion and exocytosis in β-cells and other neuroendocrine cells [30-32]. We have previously shown that the phenotype of the $Anx7(+/-)$ mutation includes islet hyperplasia, β-cell hypertrophy, aberrant regulation of islet gene expression by the fed/fasted state, and an alteration in the Ca^{2+}-dependence of glucose-induced insulin secretion [33, 34]. Most relevant to this work, we also noted that mutant islets displayed markedly reduced IP_3 receptor expression, accompanied by attenuations in the magnitude and rise time of β-cell $[Ca^{2+}]_i$ changes induced by both IP_3-generating agonists and inhibitors of the sarco-endoplasmic reticulum Ca^{2+}-ATPase responsible for filling IP_3-sensitive Ca^{2+} stores [33, 35]. Given the important role of Ca^{2+} stores in both β-cell electrical activity and insulin secretion, we hypothesized that electrical and secretory responses, particularly to Ca^{2+}-mobilizing agents, would be altered in β-cells of the mutant animals. We report here that mutant islets exhibit exaggerated depolarizing electrical responses to both IP_3 and ryanodine receptor agonists, providing further evidence that a major consequence of the $Anx7(+/-)$ mutation is dysregulation of intracellular Ca^{2+} store function in pancreatic β-cells. We suggest that this dysregulation may underlie alterations in secretory behavior observed in mutant β-cells in this and previous studies.

Materials and Methods

Derivation of Anx7(+/-) knockout mice

The $Anx7(+/-)$ mutant mice were generated by targeted gene disruption as described by Srivastava and colleagues [33]. Briefly, genomic $anx7$ DNA clones were isolated from an isogenic strain of mice (129/sv) and were used to construct a gene-targeting vector in which $anx7$ sequences encompassing exon 6 were replaced with the neo gene. The homozygous $Anx7(-/-)$ mutation is lethal by E10. Heterozygous mice were identified by PCR analysis of genomic DNA taken from tail samples of the offspring. The experiments reported here were carried out on adult $Anx7(+/-)$ animals and $Anx7(+/+)$ littermate

controls in the age range of 2-6 months.

Membrane potential recordings

Membrane potential was recorded from β-cells within intact islets of Langerhans using intracellular microelectrodes [36, 37]. Briefly, individual islets of Langerhans were dissected from the pancreas by hand under a microscope and pinned to the bottom of a perfusion chamber though the surrounding acinar tissue. The islets were continuously perifused with a bicarbonate-buffered Krebs solution composed of: (in mM) 120 NaCl, 5 KCl, 1 $MgCl_2$, 2.6 $CaCl_2$, 25 $NaHCO_3$, equilibrated with 95% O_2:5% CO_2 to maintain a pH of 7.4 at 37 °C. Glucose and other agents were added to this solution as indicated in the figures, without adjusting for osmolarity. Microelectrodes were formed from thick-walled borosilicate capillary glass (2.0 mm O.D., 0.7 mm I.D., FHC, Brunswick, ME) using a vertical puller, and had electrical resistances of 180-220 mΩ when filled with a 1:1 mixture of 1M KCl: 1M K-citrate. Electrodes were inserted into individual cells within the islet, and membrane potential was monitored using a current-clamp amplifier designed by E. Rojas. Beta cells were identified based on the presence of "bursting" electrical activity in the presence of 11 mM glucose [38]. Voltage records were digitized at 250 Hz and stored on a computer for later analysis. The results shown are representative of 3 to 6 experiments under the given experimental conditions.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} was measured in clusters of islet cells using microfluorimetry, with indo-1 as the fluorescent Ca^{2+} indicator. Islets of Langerhans were isolated by collagenase digestion of the pancreas, and were dispersed into single cells and cell clusters by shaking in a Ca^{2+}-free medium containing dispase (Boehringer Mannheim, Indianapolis, IN), as described previously [13]. The cells were plated onto glass coverslips and allowed to attach for 1-2 days in culture. The culture medium was RPMI 1640 supplemented with 10% fetal bovine serum. For experiments, cells were loaded with Indo-1 for 40 min at 37 °C in bicarbonate-buffered Krebs solution containing 0.5 μM Indo-1-AM (Molecular Probes/Invitrogen, Eugene, OR). The coverslips were used to form the bottom of a sample chamber that was placed on the stage of an inverted microscope and continuously perfused with Krebs solution (as used for intracellular recordings). The dye in the cells was continuously excited with 340 nm UV light, and intensity of the emitted fluorescent light was measured at 405 nm and 485 nm using two photomultiplier tubes. The output of each photomultiplier was sampled at 3 Hz and the ratio of emitted fluorescence (F_{405}/F_{485}) was used as an estimate of the relative intracellular Ca^{2+} concentration.

Insulin Secretion

Islets of Langerhans were microdissected from the pancreas of *Anx7*(+/-) mice and *Anx7*(+/+) littermate controls as described above. For each experiment, one mutant and one control animal were used, and 10 islets were removed from each pancreas and placed into perfusion chambers (5 islets/chamber, 2 chambers/mouse). The chambers were continuously
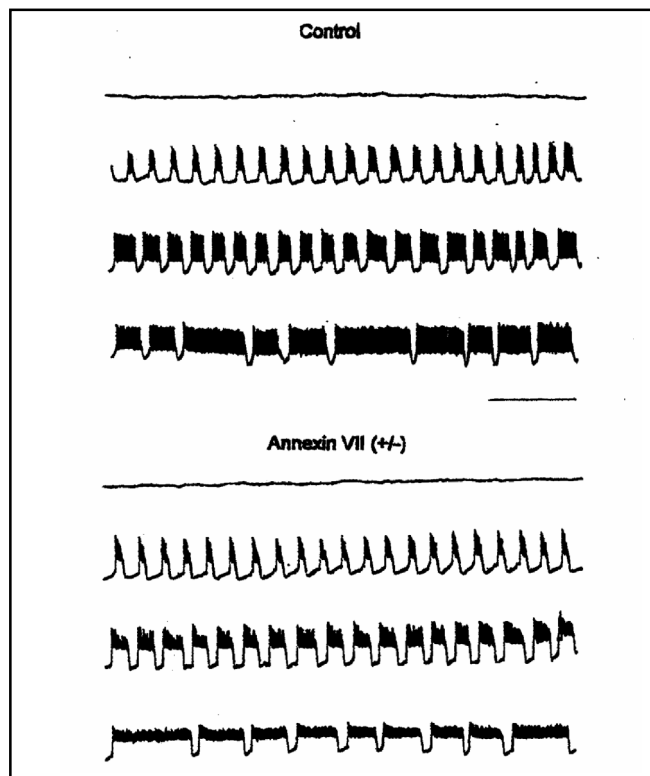

Fig. 1. The traces are segments of continuous recordings of intracellular membrane potential from an *Anx7*(+/+) islet (top) and an *Anx7*(+/-) islet (bottom). Each trace shows steady-state electrical activity observed in the presence of the glucose concentration indicated at the right. Traces were obtained during the last 5 minutes of 10-minute exposures to each glucose concentration.

perfused (1 ml/min) with the Krebs solution described above, supplemented with 0.5% bovine serum albumin and 5.6 mM glucose. After a 60-minute pre-perfusion, samples were collected at 2 or 5 minute intervals for analysis of insulin content by radioimmunoassay [39]. During the test perfusion, islets were exposed to basal (5.6 mM) or stimulatory (22 mM) glucose, with or without other agents as indicated in the figures. The stimulatory concentration of 22 mM was chosen because it induces a submaximal secretory response, which can then be modulated upward or downward by agents under study [8]. The total amount of insulin secreted during one hour of exposure to the test conditions was calculated and divided by the exposure time. Data was analyzed for statistical significance using Student's t-test.

Results

Glucose-induced electrical activity in Anx7(+/+) and Anx7(+/-) β-cells.

Figure 1 shows representative examples of glucose-induced electrical activity recorded from *Anx7*(+/+) islets
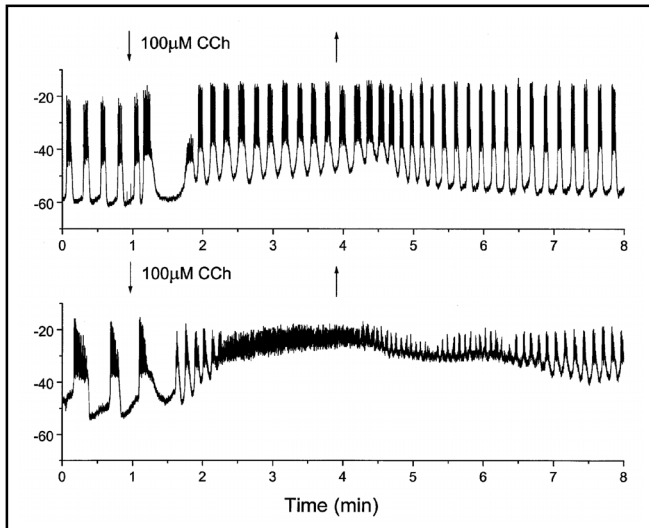
Fig. 2. Electrical activity was recorded from an *Anx7*(+/+) islet (top) and an *Anx7*(+/-) islet (bottom) in the presence of 11 mM glucose. Carbachol (CCh) was added and removed as indicated by the arrows above each trace.

Fig. 3. Electrical activity was recorded from an *Anx7*(+/+) islet (top) and an *Anx7*(+/-) islet (bottom) in the presence of 11 mM glucose. Caffeine was added and removed as indicated by the arrows above each trace.

(top panel) and *Anx7*(+/-) islets (bottom panel) using the classic intracellular microelectrode recording technique. Both control and mutant islets displayed typical glucose-sensitive bursting electrical activity, with plateau fraction (duty cycle of the oscillation) increasing with concentration of the sugar. No differences were observed between the two genotypes in burst frequency, plateau fraction or action potential characteristics at the glucose concentrations used. These results indicate that the cellular mechanisms for producing glucose-induced membrane depolarization and electrical oscillations are intact in β-cells of *Anx7*(+/-) mice.

Effect of Anx7(+/-) mutation on β-cell electrical responses to Ca²⁺-mobilizing agents.

Because we have previously observed that Ca^{2+} mobilization by IP$_3$-generating agonists is attenuated in *Anx7*(+/-) β-cells [33], we next measured the electrical activity of control and mutant islets exposed to the muscarinic agonist carbachol (CCh). In control islets, 100 µM CCh had a triphasic effect on glucose-induced bursting, consisting of an initial prolonged active phase, a transient hyperpolarized phase, and finally the return of a burst pattern having higher frequency and more depolarized silent phases that that produced by glucose alone (Fig. 2A). This triphasic electrical activity has been reported previously, and appears to be the typical response of normal β-cells to muscarinic stimulation [15, 40, 41]. As shown in Figure 2B, we found that muscarinic stimulation had a more dramatic depolarizing effect on
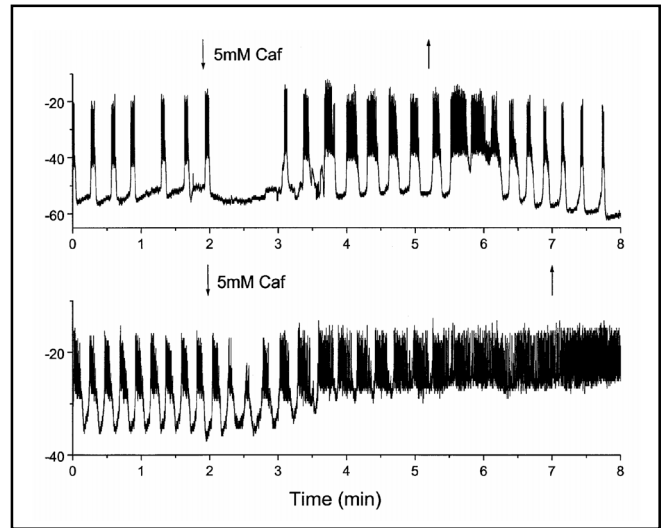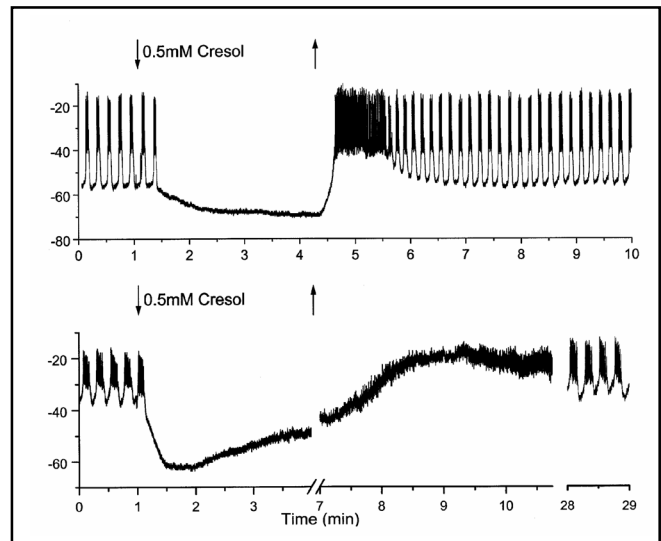

Fig. 4. Electrical activity was recorded from an *Anx7*(+/+) islet (top) and an *Anx7*(+/-) islet (bottom) in the presence of 11 mM glucose. The ryanodine receptor agonist 4-chloro-*m*-cresol was added and removed as indicated by the arrows above each trace.

Anx7(+/-) islets than controls. Firstly, the transient hyperpolarization was attenuated and shortened in the mutant islets. Secondly, the steady-state electrical activity in the presence of CCh consisted of continuous action potential generation from a depolarized plateau potential rather than the fast spiking pattern observed in control islets.

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We next studied the effects of ryanodine receptor agonists on β-cell electrical activity. Figure 3A shows the response of a control islet of Langerhans to caffeine. The agent induced a prolonged silent phase followed by recovery of a burst pattern having similar frequency but higher plateau fraction than observed before addition of the agonist. As with CCh, caffeine had a more dramatic depolarizing effect on mutant β-cells than controls. In the mutant islets, no initial hyperpolarized phase was induced by caffeine, and the steady-state response consisted of continuous spiking rather than oscillatory activity (Fig. 3B). To continue this line of investigation, we tested electrical responses to another ryanodine receptor agonist, 4-chloro-*m*-cresol (cresol). In control islets (Fig. 4A), cresol hyperpolarized the membrane for the duration of the exposure (4 min). Upon removal of the drug, a transient phase of continuous spiking was observed, followed by the recovery of the original burst pattern. Cresol also caused an initial hyperpolarization in the mutant islets, but in contrast to the control situation, the cell then began to depolarize (Fig. 4B). Also, following removal of the drug, the mutant β-cells remained depolarized for nearly 20 minutes before the burst pattern recovered. Together, Figures 3 through 5 demonstrate that, regardless of the Ca^{2+} store targeted, *Anx7(+/-)* β-cells are more sensitive to depolarization by Ca^{2+}-mobilizing agonists than controls.

Ca^{2+} release from ryanodine sensitive Ca^{2+} stores in control and mutant β-cells.

In order to determine if the altered electrical responses to Ca^{2+} mobilizing agents in *Anx7(+/-)* β-cells result from differences in the Ca^{2+} release pattern induced by such agents, we made ratiometric intracellular Ca^{2+} $[Ca^{2+}]_i$ measurements from clusters of cultured islet cells. Figure 5 shows representative results. In the presence of 11 mM glucose, both control and *Anx7(+/-)* β-cells exhibited slow $[Ca^{2+}]_i$ oscillations. This pattern, which is slower than the electrical bursting pattern observed in freshly microdissected whole islets, is the typical response of cultured β- cells. In order to distinguish Ca^{2+} release from Ca^{2+} influx through voltage-gated Ca^{2+} channels, diazoxide was added to the perfusate to hyperpolarize the cells. As expected, the K_{ATP} channel agonist inhibited the glucose-induced oscillations. Addition of cresol under these conditions caused a reversible increase in $[Ca^{2+}]_i$, demonstrating that the agent does indeed release stored Ca^{2+}. We observed cresol-induced Ca^{2+} release in 6 of 6 *Anx7(+/+)* β-cells and 4 of 4 *Anx7(+/-)* β-cells. The magnitude and kinetics of the $[Ca^{2+}]_i$ rise induced by cresol
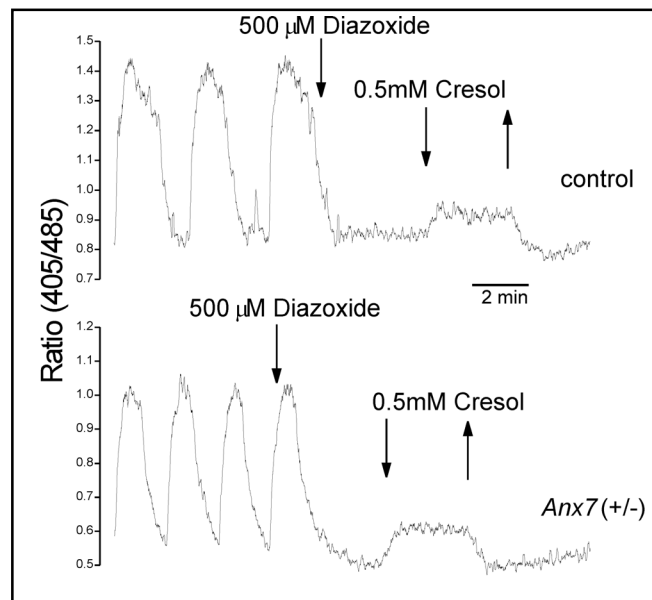
Fig. 5. Intracellular Ca^{2+} was measured from clusters of islet cells (top- *Anx7(+/+)*; bottom- *Anx7(+/-)*) using the ratiometric indicator Indo-1. Glucose (11 mM) was present throughout the recordings. The K_{ATP} agonist diazoxide and the ryanodine receptor agonist 4-chloro-*m*-cresol (cresol) were added as indicated by the arrows above each trace. Data points represent the averages of 10 consecutive points acquired at 3 Hz.
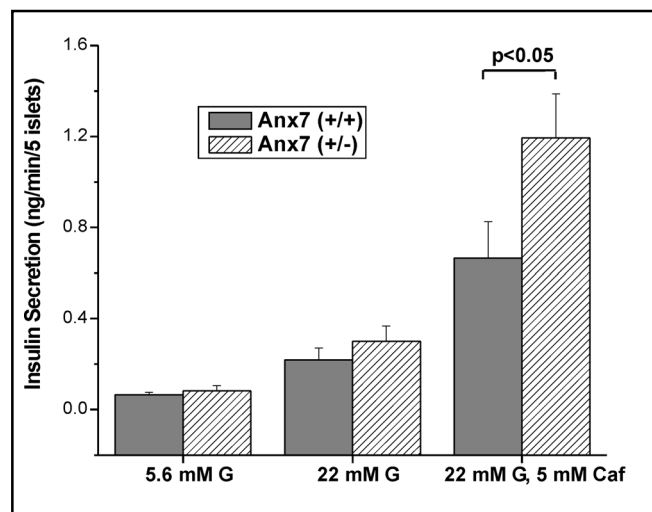
Fig. 6. Batches of *Anx7(+/+)* islets (filled bars) and *Anx7(+/-)* islets (hatched bars) (5 islets per chamber) were exposed to the indicated concentrations of glucose (G) and caffeine (Caf) during dynamic perfusion. The amount of insulin secreted per minute under each condition is shown (presented as mean +/- s.e.m.).

were similar in both wild-type and mutant β-cells. This observation suggests that the difference in electrical responsiveness of the two genotypes to cresol is not

related to differences in the pattern of Ca^{2+} release induced by the agent.

Effect of ryanodine receptor agonists on insulin-secretion from Anx7(+/+) and Anx7(+/-) islets of Langerhans.

We next tested whether the differences in electrical responsiveness of control and mutant islets to Ca^{2+}-mobilizing agents are accompanied by corresponding differences in secretory responsiveness. Insulin secretion was measured from control and *Anx7(+/-)* mutant islets in exposed to glucose and caffeine, with an extracellular Ca^{2+} concentration of 2.5 mM. As observed in Figure 6, no differences in basal insulin secretion or glucose-stimulated insulin secretion were observed between control and mutant islets. However, consistent with the observed differences in electrical responsiveness to caffeine, the ryanodine receptor agonist enhanced glucose-stimulated insulin secretion more potently in *Anx7(+/-)* islets than controls.

Discussion

In recent years it has become apparent that intracellular Ca^{2+} sequestration and release play an important role in controlling β-cell membrane potential, $[Ca^{2+}]_i$ and insulin secretion. However, the molecular mechanisms that regulate β-cell Ca^{2+} stores remain poorly understood. The *Anx7(+/-)* knockout mouse is a unique tool for studying the properties and functional roles of β-cell Ca^{2+} stores, since one consequence of the mutation is a reduction of IP_3 receptor expression in pancreatic β-cells, resulting in decreases in the magnitude and rate of IP_3-induced $[Ca^{2+}]_i$ rises [33, 34]. In this study, we have taken advantage of this model to further explore the relationship between Ca^{2+} stores and β-cell function, by measuring electrical and secretory responses to Ca^{2+}-mobilizing agents in control and mutant islets.

Despite the fact that IP_3-mediated Ca^{2+} release is diminished in *Anx7(+/-)* β-cells, we found that carbachol, an IP_3-generating muscarinic receptor agonist, enhanced glucose-induced electrical activity more strongly in mutant islets than controls. This genotype-specific difference in cholinergic electrical responsiveness can be interpreted in terms of previous electrophysiological studies suggesting that muscarinic agonists activate both depolarizing and hyperpolarizing ionic currents in the β-cell, which act in concert to produce the fast burst pattern that is observed when these agents are applied at stimulatory glucose

levels. The depolarizing effects of muscarinic agonists are now attributed to two distinct non-selective currents, both of which have been measured directly in whole-cell patch-clamp recordings. The first is a store-operated current (SOC) that activates following IP_3-dependent release of Ca^{2+} from the ER [13, 14]. The other non-selective current activates independently of G-proteins and intracellular Ca^{2+} release. [13, 42]. The latter current may correspond molecularly to a putative ion channel originally cloned by Lee and co-workers (1999), which was recently shown to form complexes with M_3 muscarinic receptors in β-cells [43, 44]. Both of these non-selective currents could contribute to the enhancement of glucose-induced β-cell electrical activity by muscarinic agonists. The resulting increase in voltage-dependent Ca^{2+} influx, together with IP_3-dependent leak of Ca^{2+} from the ER, raises $[Ca^{2+}]_i$ sufficiently to activate charybdotoxin-sensitive, Ca^{2+}-activated K^+ channels (K_{Ca}), which produce the cyclic hyperpolarizations observed during the muscarinic agonist-induced fast bursting pattern [40].

Since IP_3-induced Ca^{2+} release is deficient in Anx7(+/-) β-cells, we expect that carbachol would activate the store-operated non-selective current minimally if at all. In the case of Fig. 2B, the muscarinic agonist-induced depolarization must be attributed only to the store-independent non-selective current. This is feasible given that the store-independent current has a more positive reversal potential than the store-operated current, giving it a greater influence on the membrane potential [13]. The observation that fast oscillations do not occur in mutant β-cells stimulated with muscarinic agonists is also in accord with the model presented above, since KCa are not expected to activate in the absence of IP_3-induced Ca^{2+} release.

Our results indicate that ryanodine receptor agonists release stored Ca^{2+} in β-cells, in accord with reports from numerous laboratories[20, 45-47]. Here we have shown that release of Ca^{2+} from ryanodine-sensitive stores also affects β-cell membrane potential and insulin secretion. Similar to results with CCh, *Anx7(+/-)* β-cells were more strongly depolarized by ryanodine receptor agonists than controls. In the case of caffeine, this enhanced depolarizing effect was associated with a more potent augmentation of glucose-induced insulin secretion. However, the differences in responsiveness do not appear to be related to differences in agonist-induced Ca^{2+} release, since the *Anx7(+/-)* mutation did not affect the magnitude or dynamics of Ca^{2+} release from ryanodine-sensitive stores (Fig. 6). A recent report demonstrated

the presence of functional ryanodine receptors in insulin-secreting RINm5F cells [48]. This pool of ryanodine receptors may be responsible for the membrane depolarization induced by ryanodine receptor agonists. It would appear that this pool of receptors is enhanced in the Anx7(+/-) β-cells, most likely as a compensation for the relative inability of IP_3 to activate depolarizing currents. An alternative explanation would be that release of Ca^{2+} from ryanodine sensitive stores is more effective in activating store-operated currents in the mutant cells than in controls, which could also be a compensation for the reduced sensitivity to IP_3. Regardless of the mechanism underlying the altered responsiveness in the Anx7(+/-) islets, our data contribute to the mounting evidence that ryanodine sensitive Ca^{2+} stores play an important role in β-cell signaling.

In summary, our results demonstrate that the Anx7(+/-) mutation results in alterations in the electrical and secretory responsiveness of pancreatic β-cells to mobilization of both IP_3- and ryanodine-sensitive Ca^{2+} stores. The changes in responsiveness to IP_3-generating agonists most likely result from a reduction of IP_3 receptor expression and the associated attenuation of IP_3-mediated Ca^{2+} release. Conversely, the change in sensitivity to ryanodine receptor-mediated Ca^{2+} release seems to result from alterations in cell signaling elements that are independent of Ca^{2+} stores. The Anx7(+/-) knockout mouse therefore promises to be a useful tool for studying the function and regulation of cellular processes related to intracellular Ca^{2+} sequestration in the β-cell.

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