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European Journal of Pharmacology 298 (1996) 299–306

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Altered intracellular Ca^{2+} regulation in pancreatic acinar cells from acute streptozotocin-induced diabetic rats

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Received 13 July 1995; revised 14 November 1995; accepted 17 November 1995

Abstract

We investigated intracellular Ca^{2+} regulation in pancreatic acinar cells from rats with diabetes induced by a single injection of streptozotocin (80 mg/kg). Experiments were performed 2 days and 7 days after the injection of streptozotocin. The density of muscarinic receptors, measured by [^{3}H]*N*-methyl scopolamine binding, was unchanged in 2-day-diabetic rats, but was significantly increased in 7-day-diabetic rats. The percentage of high affinity receptors (R_H) and low affinity receptors (R_L) determined from the competitive curves with [^{3}H]*N*-methyl scopolamine and carbachol was not change in 2-day-diabetic rats compared to controls, whereas 7-day-diabetic rats showed a decrease in $\%R_H$ and an increase in $\%R_L$. The carbachol-evoked initial peak of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was increased in 2-day-diabetic rats and decreased in 7-day-diabetic rats, compared to controls. In the carbachol-induced sustained phase in $[Ca^{2+}]_i$, the response in 7-day-diabetic rats was significantly decreased; however, there was no difference between controls and 2-day-diabetic rats. Carbachol (100 μM)-induced [^{3}H]inositol 1,3,4-trisphosphate generation was significantly lower in diabetic rats than in the controls. The addition of inositol 1,4,5-trisphosphate (1,4,5-IP_3) significantly increased $^{45}Ca^{2+}$ release from saponin-permeabilized cells in 2-day-diabetic rats, but did not do so in 7-day-diabetic rats. Ca^{2+} refilling into the intracellular stores, determined by second cholecystokinin-8 (10 nM) stimulation after 10 μM carbachol stimulation, was increased in 2-day-diabetic rats and decreased in 7-day-diabetic rats. These observations indicate that the alterations in intracellular Ca^{2+} regulation accompanied by changes in transmembrane signaling occur in the earlier stage of the diabetic state. The findings also suggest that the increase in the carbachol-evoked $[Ca^{2+}]_i$ peak in 2-day-diabetic rats is related predominantly to the higher sensitivity of 1,4,5-IP_3-sensitive Ca^{2+} stores and the increase in the capacity of Ca^{2+} refilling in these animals, whereas the reduction in the $[Ca^{2+}]_i$ peak in 7-day-diabetic rats appears to be related to the essential decrease in receptor-mediated 1,4,5-IP_3 generation and the decrease in Ca^{2+} refilling capacity.

Keywords: Diabetic pancreas; Ca^{2+} mobilization; 1,4,5-IP_3-sensitive Ca^{2+} store; Ca^{2+} refilling; Muscarinic receptor

1. Introduction

Diabetes induces disorders of exocrine pancreatic function (Vacca et al., 1964; Domschke et al., 1975; Adler and Kern, 1975). Some reports (Studer and Ganas, 1989; Levy et al., 1990; Ohara et al., 1991; Taira et al., 1991) have shown that experimental diabetes induces abnormalities in cell Ca^{2+} homeostasis, although the nature of the alteration is often tissue specific; however, the precise mechanism involved in these findings is not known. It therefore seems that abnormalities in cell Ca^{2+} homeostasis are closely related to the disorder of pancreatic function.

Experimental diabetes induces alterations of signal transduction pathways activated by external stimuli such as hormones, neurotransmitters, and agonists that interact specifically with plasma membrane receptors in several tissues (Bushfield et al., 1990; Shima et al., 1992; Izawa et al., 1993; Inoguchi et al., 1994; Yu et al., 1994). However, there have been few systematic studies of changes in the signal transduction pathway in the diabetic pancreas (Korc and Schoni, 1988; Chandrasekar and Korc, 1991). Pancreatic secretory response to agonists such as acetylcholine and cholecystokinin is substantially mediated through intracellular Ca^{2+} mobilization. Accordingly, the disorder of exocrine pancreatic function induced by diabetes may be related to the impairment of intracellular Ca^{2+} regulation. To our knowledge, control of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in diabetic acinar cells in response

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SSDI 0014-2999(95)00796-2

to muscarinic stimulation has not been studied in the earlier stage of the diabetic state. Therefore, to elucidate the process leading to the abnormalities in cell Ca^{2+} homeostasis and the disorder of pancreatic function, it would be of interest to investigate muscarinic receptor-mediated $[Ca^{2+}]_i$ responses in diabetic acinar cells. In this study, we examined alterations in intracellular Ca^{2+} regulation in the earlier stage of the diabetic state in pancreatic acinar cells obtained from rats with diabetes induced by a single injection of streptozotocin. Our findings indicate that alterations in intracellular Ca^{2+} regulation occur in the earlier stage of the diabetic state and are accompanied by changes in transmembrane signaling.

2. Materials and methods

2.1. Materials

Myo-[2-^{3}H]inositol (10–20 Ci/mmol), [^{3}H]*N*-methyl scopolamine, and $^{45}CaCl_2$ (1000 Ci/mmol) were purchased from Amersham Co. Fura-2/AM was obtained from Dojindo Lab. (Japan); collagenase A (0.255 U/mg) from Boehringer Mannheim; cholecystokinin-8 from Peptide Institute (Japan); and carbachol, atropine, trypsin inhibitor (type I-S), D-myo-inositol 1,4,5-trisphosphate (1,4,5-IP_3), 5'-guanylylimidodiphosphate (Gpp(NH)p), and streptozotocin were from Sigma Chemical Co. All other reagents were of analytic grade.

2.2. Animals

Male Wistar rats, originally weighing 160–180 g, were used in this study, and diabetes was induced by a single intravenous injection of streptozotocin (80 mg/kg) in 0.1 M sodium citrate, pH 4.5. Control rats were age-matched and injected with the vehicle. All animals were maintained under a 12-h light-dark cycle, and allowed free access to food and water. Only animals demonstrating nonfasting blood glucose levels > 400 mg/dl were considered diabetic for the purpose of this study. Experiments were performed 2 days and 7 days after the injection.

2.3. Preparation of pancreatic acinar cells

Pancreatic acinar cells, prepared by collagenase digestion, as described previously (Komabayashi et al., 1990), were incubated in Krebs-Henseleit medium containing (mM): NaCl 98; KCl 5; KH_2PO_4 1.2; $CaCl_2$ 1.3; $MgSO_4$ 1.2; $NaHCO_3$ 2.4; Hepes-Na 10; dextrose 11; and 0.1% (w/v) bovine serum albumin. The medium also contained essential amino acids and was maintained at pH 7.4 under an atmosphere of 95% oxygen and 5% carbon dioxide. The number of cells was determined microscopically in a hemocytometer.

2.4. Specific [^{3}H]*N*-methyl scopolamine binding experiments

Specific binding of [^{3}H]*N*-methyl scopolamine was determined according to the method of Vinayek et al. (1990). Pancreatic acinar cells (10^6 cells/ml) were suspended in the medium and incubated with [^{3}H]*N*-methyl scopolamine (200 ~ 3200 pM) for 30 min at 37°C. After incubation, the reaction was terminated by adding an ice-cold wash solution (mM, pH 7.4): NaCl 154; KH_2PO_4 1; and Na_2HPO_4 3.4. Competitive experiments with carbachol and [^{3}H]*N*-methyl scopolamine were carried out with crude membranes, prepared by the method of Watson and Jacobson (1986). Samples (2 mg protein/tube) were suspended in buffer (mM): $MgCl_2$ 20 and Tris-HCl 10, pH 7.4. The concentration of [^{3}H]*N*-methyl scopolamine was similar (control, 100; 2-day-diabetic rats, 130; and 7-day-diabetic rats, 170 pM) to dissociation constant (K_d) value determined from [^{3}H]*N*-methyl scopolamine binding experiments. Samples were incubated with [^{3}H]*N*-methyl scopolamine and carbachol (0.01 ~ 1000 μM) for 30 min in the presence and absence of 100 μM Gpp(NH)p, and the reaction was terminated by adding an ice-cold wash solution.

Each sample was filtered rapidly through Whatman GF/F glass filters and then washed three times with the wash solution. The filters were placed in vials containing scintillation cocktail, and the radioactivity was determined by liquid scintillation spectrometry. Nonspecific binding was determined as the amount of [^{3}H]*N*-methyl scopolamine bound in the presence of 1 μM atropine. Specific binding was defined as the total minus the nonspecific binding.

Binding densities (B_{max}) and K_d for [^{3}H]*N*-methyl scopolamine were evaluated from linear Scatchard plots. Based on competitive curves with [^{3}H]*N*-methyl scopolamine and carbachol, the percentage of high affinity receptors (R_H) and low affinity receptors (R_L) was determined by computer analysis, according to the method of Hulme et al. (1978) and U'Prichard et al. (1980).

2.5. Determination of [^{3}H]phosphatidylinositol level

Isolated cells were incubated with myo-[2-^{3}H]inositol (15 μCi/ml) at 37°C for 90 min. The cells were washed and centrifuged twice with fresh medium. Lipids were extracted with chloroform/methanol/12 N HCl (1 : 2 : 0.1, v/v). The chloroform phase was dried under a stream of nitrogen. Lipids were dissolved in a chloroform/methanol mixture (95 : 5, v/v) and separated by thin layer chromatography using a solvent system of chloroform/acetone/methanol/acetic acid/water (40 : 15 : 13 : 12 : 8). Because the spots of [^{3}H]phosphatidylinositol 4-monophosphate and [^{3}H]phosphatidylinositol 4,5-bisphosphate were not clearly detected under these conditions,

only the spot corresponding to [^{3}H]phosphatidylinositol was scraped, and the radioactivity was determined by liquid scintillation spectrometry.

2.6. Assay of [^{3}H]inositol 1,3,4-trisphosphate ([^{3}H]IP$_3$)

Cells were prelabeled with myo-[2-^{3}H]inositol (30 μCi/ml) at 37°C for 90 min. Labeled cells (10^6 cells/ml) were incubated with an agonist in the presence of 10 mM LiCl for 30 min. [^{3}H]Inositol phosphates were extracted with 4.5% perchlorate on ice for 20 min. After centrifugation at $1000 \times g$ for 5 min, the supernatant was removed, and the pH was adjusted to 8–9 by the addition of 0.5 M KOH/9 mM sodium tetraborate/1.9 mM EDTA. The [^{3}H]IP$_3$ fraction was separated by anion-exchange chromatography and analyzed by the method of Berridge et al. (1983). For the determination of [^{3}H]IP$_3$, total lipids were extracted. The results were expressed as the percentage of total [^{3}H]inositol lipids.

2.7. Measurement of $[Ca^{2+}]_i$

Isolated cells were loaded with 2 μM fura-2/AM for 30 min at 37°C. The loaded cells ($4 \sim 8 \times 10^6$ cells/ml) were washed twice with Krebs-Henseleit medium, and the cells were then suspended in the same medium. Experiments were performed on a calcium analyzer (CAF-100, Nihon Bunkou, Japan), thermostatically maintained at 37°C. Drugs were added directly to the cuvette under conditions of continuous stirring. $[Ca^{2+}]_i$ was determined by the method of Grynkiewicz et al. (1985).

2.8. Determination of $^{45}Ca^{2+}$ release in permeabilized acinar cells

Determination of $^{45}Ca^{2+}$ release was based on the method of Fujinami et al. (1993). The cells were washed twice with K^+ medium (mM): KCl 120; NaCl 1; NaH_2PO_4 0.96; $MgSO_4$ 5; EGTA 1; Hepes-Tris, pH 7.2, and then incubated with the same medium containing saponin (50 μg/ml) at 4°C for 10 min. After incubation, the cells were washed and centrifuged twice with the high K^+ medium containing 0.2% bovine serum albumin and suspended in the same medium containing 0.3 mM $CaCl_2$ and 0.2% bovine serum albumin. More than 95% of the cells were stained with trypan blue under these conditions. Cells (10^6 cells/ml) were incubated in the presence of $^{45}Ca^{2+}$ (10 μCi/ml) at 37°C for 5 min and Mg-ATP (final concentration of 1.5 mM) was then added. An equilibrium state of $^{45}Ca^{2+}$ uptake was attained at 10 min, and this remained steady for up to 30 min after the addition of Mg-ATP. Mitochondrial inhibitors were not added to the incubation medium, since some investigators (Fleming et al., 1989; Willems et al., 1989) have reported that $^{45}Ca^{2+}$ accumulation in mitochondria is negligible in rat submandibular gland and rabbit pancreas. Based on these results, 1,4,5-IP$_3$ was added 10 min after the addition of Mg-ATP. Because reuptake of $^{45}Ca^{2+}$ was not observed for at least 90 s following the addition of 1,4,5-IP$_3$, experiments for $^{45}Ca^{2+}$ release from Ca^{2+} stores were performed for 1 min. The incubation was terminated with 4 ml of ice-cold washing medium (mM): KCl 150; Hepes-Tris 2, pH 7, and the suspension was rapidly filtered through Whatman GF/B filters. The filters were washed several times with ice-cold wash medium, dissolved in scintillation cocktail, and the radioactivity was determined. $^{45}Ca^{2+}$ release was expressed as a percentage of $^{45}Ca^{2+}$ retained after the filtering of unstimulated samples.

2.9. Statistical analysis

Comparison of all groups was done initially by one-way analysis of variance (F-test). If the F-test was significant at $P < 0.05$, subsequent comparisons between groups were done by Student's t-test. All values are expressed as means ± S.E. In each figure, where S.E. bars are not shown, the values lie within the symbol.

3. Results

3.1. General characteristics of diabetic rats

Control rats gained weight, whereas streptozotocin-treated rats lost weight. Two days after the streptozotocin injection, the diabetic rats showed a transient initial weight loss (2%) and their body weight then gradually increased. Blood glucose levels in diabetic rats increased > 400 mg/dl 24 h after streptozotocin injection compared with control rats (< 200 mg/dl), and the levels were maintained for 7 days. In contrast, plasma insulin levels decreased from about 30 μU/ml to < 5 μU/ml 24 h after the injection, and the levels remained at a similar degree thereafter.

3.2. Alterations in muscarinic receptors induced by diabetes

The receptor characteristics are summarized in Table 1. The density of pancreatic muscarinic receptors in 2-day-di-

Table 1
Characteristics of pancreatic muscarinic receptors in diabetic rats

	Control	Diabetic	
		2 days	7 days
B_{max} (fmol/10^6 cells)	10.8 ± 0.3	11.3 ± 0.4	17.0 ± 1.5 [a]
K_d (pM)	97.6 ± 1.3	126.6 ± 24.2	174.9 ± 15.7 [a]
%R_H	66.5 ± 6.4	60.5 ± 4.5	38.2 ± 5.5 [b]
%R_L	23.9 ± 6.5	34.2 ± 4.7	51.9 ± 5.5 [a]

Each value represents the mean ± S.E. of quadruplicate determinations from 5 separate experiments. B_{max}, density of specific [^{3}H]N-methyl scopolamine binding sites; K_d, dissociation constant of specific [^{3}H]N-methyl scopolamine binding sites; R_H, high affinity receptors; R_L, low affinity receptors. [a] $P < 0.05$; [b] $P < 0.01$ vs. control.

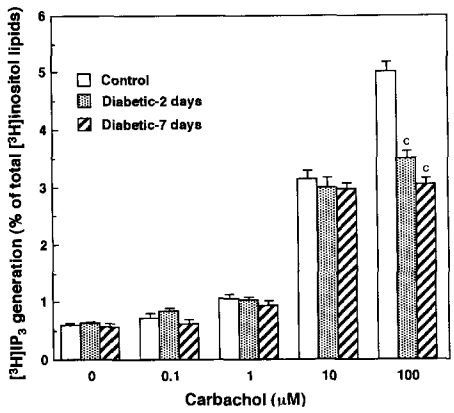

Fig. 1. Effect of carbachol on [^{3}H]IP_3 generation in control and diabetic rats. Each column represents the mean ± S.E. of quadruplicate determinations from 4 separate experiments. ^{c} $P < 0.001$ vs. control.

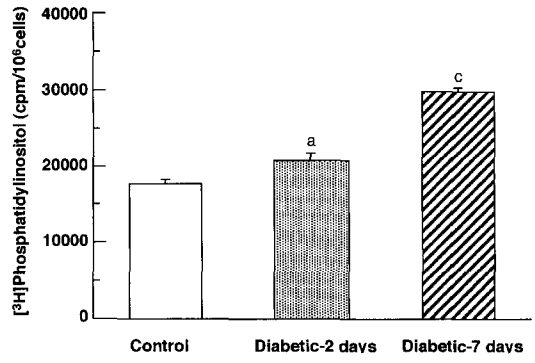

Fig. 2. Effect of diabetes on [^{3}H]phosphatidylinositol level. Each column represents the mean ± S.E. of quintuplicate determinations from 3 separate experiments. ^{a} $P < 0.05$; ^{c} $P < 0.001$ vs. control.

abetic rats was unchanged, but that in 7-day-diabetic rats was significantly increased. The affinity of the receptors for [^{3}H]*N*-methyl scopolamine was not changed in 2-day-diabetic rats, but was significantly increased in 7-day-diabetic rats. We further examined carbachol competition curves in several conditioned rats. In the presence of 100 μM Gpp(NH)p in the reaction medium, the competition curves for control, 2-day-diabetic, and 7-day-diabetic rats were identical. In the absence of Gpp(NH)p, the competition curves for control and 2-day-diabetic rats were identical. The percentage of receptors in the high affinity state (%R_H) for control and 2-day-diabetic rats was 61–67%. However, the competition curve for 7-day-diabetic rats was steeper than for the control rats; %R_H was approximately 40%. Thus, receptors in the high affinity state were significantly reduced in 7-day-diabetic rats.

3.3. Effects of diabetes on carbachol-induced [^{3}H]IP_3 generation and [^{3}H]phosphatidylinositol level

Since the initial transient increase in $[Ca^{2+}]_i$ is mediated through 1,4,5-IP_3, we next investigated the effect of diabetes on phosphoinositide breakdown. As shown in Fig. 1, carbachol evoked [^{3}H]IP_3 generation in a dose-dependent manner. Carbachol-induced [^{3}H]IP_3 generation in diabetic rats reached maximal level at 10 μM, whereas that in control rats did not. At 100 μM of carbachol, [^{3}H]IP_3 generation was significantly lower in diabetic rats than in control rats. This reduction may have resulted from differences in ligand incorporation into cells. To clarify this point, we further investigated the incorporation of myo-[2-^{3}H]inositol into cellular phosphatidylinositol. As shown in Fig. 2, [^{3}H]phosphatidylinositol level in diabetic rats increased relative to that in control rats. These results indicate that the decrease in diabetes-induced [^{3}H]IP_3 generation is not a result of decreased [^{3}H]inositol incorporation.

3.4. Effect of diabetes on carbachol-induced $[Ca^{2+}]_i$

Carbachol elevates $[Ca^{2+}]_i$ in a wide variety of cell types. This elevation of $[Ca^{2+}]_i$ consists of an initial Ca^{2+} release from intracellular stores and a more delayed Ca^{2+} influx across the plasma membrane. As shown in Fig. 3A, carbachol dose-dependently evoked a rapid increase in $[Ca^{2+}]_i$ in all groups. These responses were increased in 2-day-diabetic rats and decreased in 7-day-diabetic rats, compared to control rats. On the other hand, carbachol (1 and 10 μM) also increased the sustained phase in $[Ca^{2+}]_i$, but increasing the concentration to 100 μM did not cause any further increase in $[Ca^{2+}]_i$ (Fig. 3B). These responses in 7-day-diabetic rats were less than those in control rats at all concentrations used, but there were no differences between control and 2-day-diabetic rats. Baseline $[Ca^{2+}]_i$ in 7-day-diabetic rats was significantly decreased compared with that in control rats, but that in 2-day-diabetic rats was similar to the results in control rats (control, 160 ± 11; 2-day-diabetic rats, 165 ± 15; 7-day-diabetic rats, 135 ± 12 nM, $P < 0.05$ vs. control).

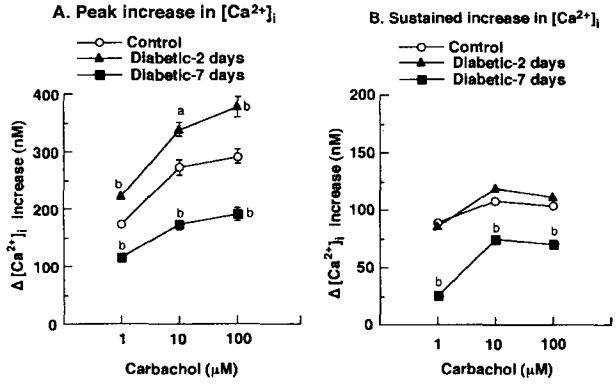

Fig. 3. Dose-dependence curves of $[Ca^{2+}]_i$ for carbachol in control and diabetic rats. Each point represents the mean ± S.E. of duplicate determinations from 5 separate experiments. ^{a} $P < 0.05$; ^{b} $P < 0.01$ vs. control.

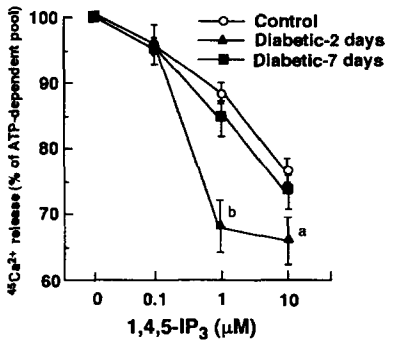

Fig. 4. Effect of diabetes on 1,4,5-IP_3-evoked $^{45}Ca^{2+}$ release from saponin-permeabilized pancreatic acinar cells. Each point represents the mean ± S.E. of duplicate determinations from 5 separate experiments. [a] $P < 0.05$; [b] $P < 0.01$ vs. control.

3.5. 1,4,5-IP_3-evoked $^{45}Ca^{2+}$ release from saponin-permeabilized cells of diabetic rats

Agonist-evoked initial elevation of $[Ca^{2+}]_i$ is attributed to the release of Ca^{2+} from 1,4,5-IP_3-sensitive Ca^{2+} stores. We therefore considered the possibility that diabetes affects 1,4,5-IP_3-sensitive Ca^{2+} stores. To explore this possibility, we measured $^{45}Ca^{2+}$ release from the intracellular Ca^{2+} stores. Under our conditions, 1,4,5-IP_3 caused $^{45}Ca^{2+}$ release from intracellular Ca^{2+} stores in a dose-dependent manner. As shown in Fig. 4, the $^{45}Ca^{2+}$ release in 7-day-diabetic rats was unchanged compared to controls, but that in 2-day-diabetic rats was significantly increased at concentrations of 1 and 10 μM.

3.6. Capacity of Ca^{2+} refilling in pancreatic acinar cells from diabetic rats

Cholecystokinin-8 and carbachol are thought to share a similar, if not identical, intracellular pathway (Williams and Blevins, 1993). Although the biological responses of the pancreas to cholecystokinin-8 depend upon the concentration, the $[Ca^{2+}]_i$ peak evoked by cholecystokinin-8 in the three groups was not significantly different at supramaximal concentrations of 10 nM (750 ± 30 in control, 780 ± 52 in 2-day-diabetic rats, and 740 ± 45 nM in 7-day-diabetic rats, respectively). Based on these results, we investigated the effect of diabetes on the capacity of Ca^{2+} refilling, using the method shown in Fig. 5A. In these experiments, the action of carbachol was terminated by atropine, and refilling of the intracellular agonist-sensitive Ca^{2+} stores was determined by the subsequent addition of 10 nM cholecystokinin-8. As shown in Fig. 5B, the $[Ca^{2+}]_i$ peak evoked by cholecystokinin-8 was increased in 2-day-diabetic rats and decreased in 7-day-diabetic rats, similar to the results obtained during carbachol stimulation (Fig. 1). The effect of diabetes was not observed in a nominally Ca^{2+}-free medium containing 1 mM EGTA (Fig. 5C).

4. Discussion

Some studies have shown that abnormal intracellular Ca^{2+} metabolism involving one or more regulatory mechanisms is a common defect in experimentally induced diabetes (Studer and Ganas, 1989; Levy et al., 1990; Ohara et al., 1991; Taira et al., 1991). The present study provides evidence that the changes in intracellular Ca^{2+} mobilization occur in the earlier stage of the streptozotocin-induced diabetic state. These changes might depend on the insulin deficiency or hyperglycemia, although direct effect(s) of streptozotocin cannot be ruled out.

Williams et al. (1983) reported that diabetes, 8 weeks after the injection of streptozotocin (50 mg/kg), had no effect on the density of myocardial muscarinic receptors. In this study, we found that the density of muscarinic receptors on the acinar cell surface was not changed in 2-day-diabetic rats, but was significantly increased in 7-day-diabetic rats (Table 1). The results we obtained with 7-day-diabetic rats differed from the results of Williams et al. (1983), but were similar to those of Latifpour et al. (1989), who showed the increase in muscarinic receptor

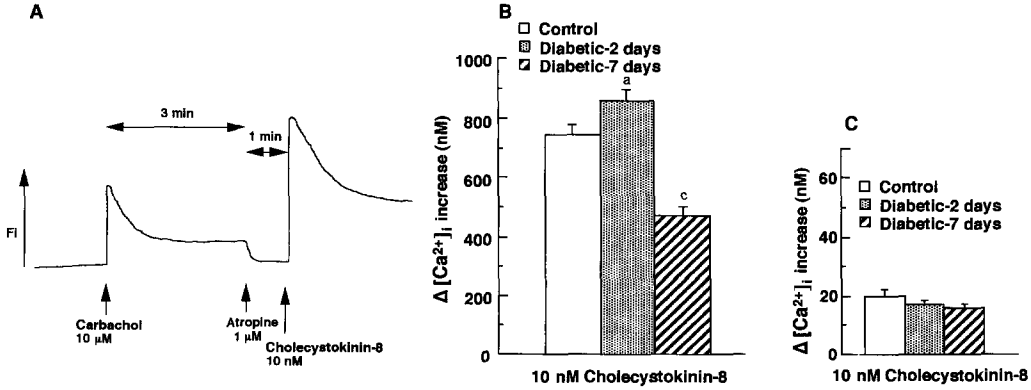

Fig. 5. Effect of diabetes on the refilling of the agonist-sensitive Ca^{2+} store in pancreatic acinar cells. Each column represents the mean ± S.E. of triplicate determinations from 3 separate experiments. Panel A, experimental protocol; panel B, 10 nM cholecystokinin-8-induced $[Ca^{2+}]_i$ peak in the normal medium; panel C, 10 nM cholecystokinin-8-induced $[Ca^{2+}]_i$ peak in nominally Ca^{2+}-free medium containing 1 mM EGTA. [a] $P < 0.05$; [c] $P < 0.001$ vs. control.

density in rat bladder smooth muscle, 2, 4 and 8 weeks after the injection of streptozotocin (65 mg/kg). Although the exact mechanism(s) for the increase in receptor density remain(s) unclear, it seems that the effect of diabetes on muscarinic receptor density is tissue specific and is influenced by the severity of diabetes. We also showed that the $\%R_H$, which implies a high binding affinity of agonist to the receptors, was unchanged in 2-day-diabetic rats and decreased in 7-day-diabetic rats, respectively (Table 1). On the other hand, a significant increase in the $\%R_L$ was observed in 7-day-diabetic rats. When the amount of high- and low-affinity receptors is given in a percentage, the sum of these two receptors was 90–95%. At present, we are not certain of the reason for the remaining 5-10%. Generally, since the capacity for receptor coupling to GTP-binding protein is larger in R_H than in R_L, the R_H is thought to be functional receptors. It therefore seems likely that the increased receptors in 7-day-diabetic rats are non-functional receptors that have low affinity for the agonist and low coupling capacity to GTP-binding protein. Thus, diabetes apparently had different effects in 2-day-diabetic and 7-day-diabetic rats. As shown in Fig. 1, carbachol (100 μM)-induced $[^3H]IP_3$ generation was significantly reduced by diabetes. This reduction in $[^3H]IP_3$ generation in 2-day-diabetic rats could be related to changes in post-receptor events, presumably due to changes in phospholipase C activity, since there was no change in receptor density or $\%R_H$. In addition, the reduction in $[^3H]IP_3$ generation may result in the high sensitivity of Ca^{2+} stores (see below) as a compensatory response. On the other hand, the reduction in $[^3H]IP_3$ generation in 7-day-diabetic rats could be related predominantly to functional alterations in muscarinic receptor properties, although changes in post-receptor events cannot be ruled out.

Two phases of carbachol-induced Ca^{2+} mobilization in exocrine cells can be distinguished, a rapid Ca^{2+} release from intracellular stores and a delayed Ca^{2+} entry from the extracellular space (Streb et al., 1983; Mertz et al., 1990b). As shown in Fig. 3A, the carbachol-induced initial rapid phase of $[Ca^{2+}]_i$ increased in 2-day-diabetic rats and decreased in 7-day-diabetic rats. On the other hand, the carbachol-induced sustained phase of $[Ca^{2+}]_i$ was decreased in 7-day-diabetic rats, but was not decreased in 2-day-diabetic rats (Fig. 3B). These results suggest that $[Ca^{2+}]_i$ regulation in 2-day-diabetic rats occurs at the Ca^{2+} store site prior to the alteration in the Ca^{2+} entry pathway.

The initial rapid release of Ca^{2+} from the intracellular stores is modified by at least the following two factors: (a) 1,4,5-IP_3, a phosphoinositide breakdown product that releases Ca^{2+} from endoplasmic reticulum stores, and (b) the sensitivity of 1,4,5-IP_3-sensitive Ca^{2+} stores. To explore these points, we assessed the effect of diabetes on phosphoinositide breakdown. Carbachol-induced $[^3H]IP_3$ generation was not clearly affected by diabetes at concentrations of 0.1–10 μM, but was significantly reduced at 100 μM (Fig. 1). We further showed that 1,4,5-IP_3-induced $^{45}Ca^{2+}$ release from Ca^{2+} stores in permeabilized cells was unchanged in 7-day-diabetic rats, but was significantly increased in 2-day-diabetic rats compared to controls (Fig. 4). These results indicate that, even if $[^3H]IP_3$ generation was decreased, the increase in the initial peak of $[Ca^{2+}]_i$ in 2-day-diabetic rats can be explained by the higher sensitivity of 1,4,5-IP_3-sensitive Ca^{2+} stores. On the other hand, although 1,4,5-IP_3-induced $^{45}Ca^{2+}$ release was not distinctly affected in 7-day-diabetic rats, the carbachol-evoked $[Ca^{2+}]_i$ peak was significantly decreased. It is possible that this decrease could be closely related to the reduction in receptor-mediated 1,4,5-IP_3 generation. In these experiments with 7-day-diabetic rats, the carbachol-evoked $[Ca^{2+}]_i$ peak significantly decreased at 1 and 10 μM, but the decrease in $[^3H]IP_3$ generation was slight. This difference may result from the determination of $[^3H]IP_3$ (1,4,5-IP_3 isomer). It is generally thought that 1,4,5-IP_3, which induces the Ca^{2+} release, is metabolized to both inositol 1,4-bisphosphate and inositol 1,3,4,5-tetrakisphosphate, and the latter is then dephosphorylated to 1,3,4-IP_3. Therefore, this metabolism may result in a gap between the changes in $[^3H]IP_3$ generation and those in $[Ca^{2+}]_i$.

As shown by others (McMillian et al., 1988; Ambudkar et al., 1990; Mertz et al., 1990a; Pandol and Schoeffield-Payne, 1990), the agonist-sensitive intracellular Ca^{2+} store refills with extracellular Ca^{2+} following blockage of a stimulatory signal by an antagonist. However, although it has been reported that cyclic GMP and small GTP-binding protein are involved in the mechanism connecting the storing and transmembrane influx of Ca^{2+} (Mertz et al., 1990b; Pandol and Schoeffield-Payne, 1990; Fasolato et al., 1993), the precise mechanism underlying this process is still unresolved. Thus, Ca^{2+} entering the cytosol increases $[Ca^{2+}]_i$ and is available for refilling the agonist-sensitive Ca^{2+} stores. In this study, we used cholecystokinin-8 as a tool of second stimulation after carbachol stimulation. The $[Ca^{2+}]_i$ peak induced by cholecystokinin-8 alone was not affected by diabetes. This result was distinct from the case of carbachol stimulation. In the experiment illustrated in Fig. 5, we examined whether diabetes had an effect on agonist-sensitive Ca^{2+} store refilling. The addition of cholecystokinin-8 (10 nM) evoked a higher $[Ca^{2+}]_i$ peak in 2-day-diabetic rats than in the controls, whereas the response in 7-day-diabetic rats was significantly decreased (Fig. 5B). These changes in $[Ca^{2+}]_i$ were similar to the results obtained during stimulation with carbachol alone (Fig. 3A). Although an additional intracellular regulatory mechanism is required for the changes in Ca^{2+} refilling, this difference may be explained by changes in endoplasmic reticular Ca^{2+}-ATPase activity, in intracellular redistribution of Ca^{2+} between compartments, and/or in transmembrane influx of Ca^{2+}. These results suggest that diabetes has an effect on Ca^{2+} refilling into agonist-sensitive Ca^{2+} stores. In addition, when cholecys-

tokinin-8 was added to a nominally Ca^{2+}-free medium containing 1 mM EGTA, the effect of diabetes was not observed (Fig. 5C). Since EGTA might have reduced the size of Ca^{2+} pools, the influence of the chelator may be explained by this effect rather than inhibiting the uptake of extracellular Ca^{2+}. Moreover, the reduction in baseline $[Ca^{2+}]_i$ level observed in 7-day-diabetic rats may be associated with this impairment of Ca^{2+} regulation.

We have demonstrated, in this report, that the alterations in intracellular Ca^{2+} regulation accompanied by changes in transmembrane signaling occur in the earlier stage of the diabetic state. In addition, diabetes may result in an abnormality in cell Ca^{2+} homeostasis through this impairment of Ca^{2+} regulation and, in turn, lead to the disorder of pancreas function. The present studies also show that agonist-induced changes in $[Ca^{2+}]_i$ in 2-day-diabetic and 7-day-diabetic rats are regulated by some different steps. The increase in the carbachol-induced $[Ca^{2+}]_i$ peak in 2-day-diabetic rats appears to be related predominantly to the higher sensitivity of 1,4,5-IP_3-sensitive Ca^{2+} stores and the increase in the capacity of Ca^{2+} refilling in these animals. On the other hand, the reduction in carbachol-induced $[Ca^{2+}]_i$ peak in 7-day-diabetic rats appears to be related to the essential decrease in 1,4,5-IP_3 generation in these animals, which is led by functional alteration in receptor properties, and to the decrease in the capacity of Ca^{2+} refilling. Additionally, the decrease in the sustained phase of carbachol-induced $[Ca^{2+}]_i$ may also be related to the change in the Ca^{2+} entry process (Fig. 3B). Although we have shown that the alterations in intracellular Ca^{2+} regulation occur in the development of diabetic state, physiological significance that diabetes results in these alterations remains to be established.

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