Abnormal Agonist-Stimulated Cardiac Parasympathetic Acetylcholine Release in Streptozocin-Induced Diabetes

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We examined the effect of three distinct depolarizing conditions on [^{3}H]ACh release from cardiac postganglionic parasympathetic neurons in age-matched controls and insulin-treated STZ-induced diabetic rats to determine whether alterations in neurotransmitter release were present in the diabetic group. The effect of TTX, which exerts a use- and voltage-dependent block of sodium channels, was examined on the release of ACh stimulated by SRIF14 (preferentially acts at the cell body). We also studied the effect of STZ-induced diabetes on [^{3}H]ACh release by the relatively site-specific depolarizing agent VT (preferentially acts at the axon) and high potassium (non-site-specific). Basal, SRIF14-(10^{-7} M), VT-(10^{-4} M), and K^+ (100 mM)–stimulated [^{3}H]ACh release was similar in control and STZ-induced diabetic animals. However, in STZ-induced diabetic but not control rats, SRIF14-induced [^{3}H]ACh release was resistant to TTX (2×10^{-7} M). In addition, the response to submaximal K^+ (25 mM) stimulation was greater in STZ-induced diabetic compared with control animals. Treatment with insulin corrected these abnormalities. These data indicate that in the acute STZ-induced diabetic rat, SRIF14-, VT-, and high K^+-evoked release of ACH is not impaired, which suggests that the mechanisms associated with ACh storage and release in postganglionic cardiac parasympathetic neurons are not affected in this model. However, the TTX insensitivity and the increase in ACh release in response to submaximal K^+ stimulation in STZ-induced diabetes are consistent with a positive shift in the resting membrane potential in postganglionic cardiac parasympathetic axons similar to that reported in peripheral somatic nerve axons in experimental diabetes. *Diabetes* 42:141–47, 1993

Autonomic neuropathy is a common complication of human and experimental diabetes, and cardiac autonomic neuropathy is thought to contribute significantly to mortality among diabetic patients. STZ-induced diabetic rats display several signs suggesting ANS dysfunction including diarrhea, colonic, and bladder dilatation, infertility in the male, bradycardia, and decreased variability of heartbeat frequencies (1–3). Metabolic abnormalities of cardiac parasympathetic nerves in acutely STZ-induced diabetic rats include down-regulation of cholinergic receptors (4) and an increase in the synthesis and decrease in metabolism of ACh (2) with no change in the rate and extent of the neuronal choline uptake (4). Structural evidence of parasympathetic denervation has been reported in rats only after long-standing diabetes (5). Many studies have explored the effect of diabetes on the extrinsic cardiac vagal and sympathetic ANS (6–9), but biochemical and functional assessment of the intrinsic cardiac ANS is confounded by its weblike spatial distribution within the myocardium. This study used pharmacological techniques to evaluate the functional integrity of the intrinsic postganglionic cardiac atrial parasympathetic ANS at the level of the soma, the axon, and the nerve terminals by measuring the release of [^{3}H]ACh in vitro after peptidergic stimulation of the soma by SRIF14, pharmacological stimulation of axonal Na channels by VT, and direct membrane depolarization by K^+, in the presence of varying concentrations of TTX, the Na channel inhibitor. TTX blocks Na channels in a use- and voltage-dependent manner. The insensitivity of [^{3}H]ACh release to inhibition by TTX and enhanced

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[^{3}H]ACh, [^{3}H]acetylcholine; STZ, streptozocin; TTX, tetrodoxtoxin; SRIF, somatostatin; VT, veratridine; ANS, autonomic nervous system; ANOVA, analysis of variance.

sensitivity to submaximal concentrations of K^+ were consistent with a reduction in the resting membrane Na potential in otherwise intact postganglionic cardiac parasympathetic neurons analogous to that postulated for diabetic peripheral somatic nerve and sympathetic ganglia.

RESEARCH DESIGN AND METHODS

TTX, VT, physostigmine, bovine serum albumin, and hemicholinium were purchased from Sigma (St. Louis, MO); S14 from Peninsula Laboratories (Belmont, CA); Bio-Rex 70 cation-exchange resin from Bio-Rad (Richmond, CA); [^{3}H]choline and [^{3}H]ACh from Amersham (Arlington Heights, IL); Protosol from Du Pont-NEN (Boston, MA); and Sephadex G-50 Superfine Resin from Pharmacia LKB (Pleasant Hill, CA).

Subjects were 4-wk-old male Sprague-Dawley rats weighing ~200 g that were randomly divided into three groups: nondiabetic control rats (n = 64), diabetic rats (n = 65), in whom diabetes was induced by i.v. injection of 65 mg/kg body weight STZ (Sigma), and insulin-treated diabetic rats (n = 10) made diabetic with STZ but treated with daily subcutaneous injections of insulin (Ultratard Novo 40 U/kg). Body weight and nonfasting plasma glucose (hexokinase method) were monitored weekly, and GHb was determined by the thiobarbituric acid method (10) at study termination. All the experiments were performed 4 wk after STZ administration, except that the time course of the development of TTX resistance of SRIF14-stimulated [^{3}H]ACh release was studied 1–12 wk after STZ administration.

In vitro cardiac tissue preparation. Rats were anesthetized with intraperitoneal inactin (Byk Gulden, Konstanz, Germany; ~100 mg/kg body weight), after which the heart was surgically removed and immersed in oxygenated (95% O_2–5% CO_2), cold (4°C) buffer (pH 7.38) comprising (final concentration) 125 mM NaCl, 24 mM $NaHCO_3$, 4 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 2.7 mM $CaCl_2$, and 4.44 mM glucose. Slices (1–2 mm) of atrial tissue weighing ~10 mg were transferred to a 10-ml vial containing 2 ml of Krebs buffer containing [^{3}H]choline (0.2 μM, specific activity 80 Ci/mmol) and 50 μM physostigmine at 37°C in an atmosphere of 95% O_2 and 5% CO_2 and incubated for 1 h (11). Unincorporated [^{3}H]choline was removed by 12 5-min washes in 10 ml of label-free buffer.

[^{3}H]ACh release. The washed slices were carried through a 90-min test involving sequential 5-min incubations in buffer containing 50 μM physostigmine and 10 μM hemicholinium-3. After a 30-min baseline collection, the slices were exposed for 5-min periods to sequential pharmacological stimuli (e.g., SRIF14, VT, or K^+) to evaluate release of synthesized [^{3}H]ACh. ^{3}H released into the medium was measured after the addition of 10 ml of scintillation fluid to 1-ml aliquots of buffer from each vial by liquid scintillation spectrometry. In the absence of pharmacological stimulation, basal ^{3}H release remained linear with respect to time for up to 120 min. ^{3}H retained in the tissue slices was measured after solubilization at 55°C for 1 h in 1.0 ml of dihydroxyacetone (Protosol) and neutralization with 50 μl of acetic acid by scintillation spectrometry in 10 ml of scintillation fluid.

Fractional ^{3}H release was expressed as a function of the initial total tissue ^{3}H calculated as the cumulative sum of the released ^{3}H plus that retained in the tissue at the end of the experiment (11). Stimulated ^{3}H release was calculated by subtracting the mean basal rate of ^{3}H release in the 5-min collection periods immediately before and after stimulation from the ^{3}H release after stimulation.

Basal and stimulated ^{3}H release was characterized by column chromatography (11,12). A 3-ml aliquot from each vial was applied to a cation-exchange resin (Bio-Rex 70) column (1 × 120 cm) previously standardized with 0.5 mmol [^{3}H]ACh and [^{3}H]choline. Elution of the column with Na_2HPO_4 buffer (0.1 M, pH 7.0) at a flow rate of 3 ml/h revealed three completely resolved peaks, two with retention times equal to those of ACh and choline and one containing [^{3}H]labeled metabolites (11). Basal ^{3}H release was composed of approximately equal amounts of [^{3}H]ACh and [^{3}H]choline and a slightly smaller amount of other [^{3}H]choline metabolites (data not shown). Stimulation augmented only the [^{3}H]ACh peak, which accounted for >85% of the increase in ^{3}H release over basal.

Experimental protocols. In preliminary experiments, SRIF14 was tested at concentrations of 10^{-10}–10^{-6} M in tissue from STZ-induced diabetic and control rats before selecting a standard (maximal) stimulating concentration of 10^{-7} M. TTX was used at concentrations of 2×10^{-7}–2×10^{-5} M to inhibit SRIF14-stimulated [^{3}H]ACh release. VT was used at concentrations of 10^{-6} and 10^{-4} M to stimulate [^{3}H]ACh release from tissue derived from STZ-induced diabetic and control rats. K^+ was used at concentrations of 25, 50, and 100 mM to stimulate [^{3}H]ACh release from tissue derived from STZ-induced diabetic and control rats.

Statistics. All results are means ± SE. Each experiment used tissue from one animal, and the number of animals was taken as the sample size. Statistical analysis was performed with the unpaired two-sided Student's t test or ANOVA with repeated measures where appropriate. Statistical significance was defined at the 5% level.

RESULTS

Metabolic parameters. Untreated STZ-induced diabetes blunted normal weight gain, and insulin administration normalized weight gain during the first 4 wk of STZ-induced diabetes (Table 1). Random plasma glucose and GHb were increased three- to fourfold in the untreated STZ-induced diabetic rats and were only partially ameliorated by insulin treatment (Table 1).

Effect of STZ-induced diabetes and insulin-treated STZ-induced diabetes on basal and SRIF14-stimulated ^{3}H release. Basal ^{3}H release from atrial muscle slices prelabeled with [^{3}H]choline did not differ significantly among control, STZ-induced diabetic, and insulin-treated STZ-induced diabetic rats (Fig. 1). Addition of SRIF14 at a concentration of 10^{-7} M to atrial muscle slices from control and STZ-induced diabetic rats prela-

TABLE 1
Metabolic characteristics of control rats, untreated STZ-induced diabetic rats, and insulin-treated STZ-induced diabetic rats

	Rat groups		
	Controls (n = 64)	STZ-induced diabetic (n = 65)	Insulin-treated STZ-induced diabetic (n = 10)
Body weight (g)			
Initial	205 ± 8	206 ± 7	204 ± 6
4 wk	281 ± 10	249 ± 6*	275 ± 12
12 wk	430 ± 14	306 ± 17†	—
Plasma glucose (mM)	6.8 ± 1.4	24.2 ± 2.0†	11.3 ± 2.0†‡
GHb (%)	1.26 ± 0.14	4.08 ± 0.28	2.83 ± 0.2†‡

Values are means ± SE.
*P < 0.01 versus controls.
†P < 0.001 versus controls.
‡P < 0.01 versus STZ-induced diabetic rats.

beled with [^{3}H]choline caused a rapid, transient, and reproducible release of radioactivity (Fig. 2). As previously shown in dogs (11), the stimulatory effect of SRIF14 on [^{3}H]ACh release was dose dependent, reaching maximal at 10^{-7} M (Fig. 3). SRIF14 (10^{-7} M) induced equivalent [^{3}H]ACh release in control, STZ-induced diabetic and insulin-treated STZ-induced diabetic rats (Fig. 4, *left*). SRIF14-induced [^{3}H]ACh release was unaffected by STZ-induced diabetes of up to 12-wk duration (Fig. 5) or by age in the control rats (data not shown).

Effect of TTX on SRIF14-stimulated [^{3}H]ACh release. In control rats, SRIF14-stimulated [^{3}H]ACh release was significantly reduced by 2×10^{-7} M TTX (Fig. 4, *right*) and was virtually eliminated at higher concentrations (Fig. 6). Thus, SRIF14-stimulated [^{3}H]ACh release in STZ-induced diabetic rats, which did not ordinarily differ from that of control rats (Fig. 4, *left*), was significantly greater than that of control rats when TTX (2×10^{-7} M) was present (Fig. 4, *right*) because of insensitivity to TTX inhibition. Sensitivity of [^{3}H]ACh release to inhibition by TTX was restored by insulin administration in the insulin-treated STZ-induced diabetic rats, so that it no longer differed from that of the nondiabetic control rats (Fig. 4), thereby identifying TTX resistance as a consequence of insulin deficiency and/or its attendant hyperglycemia rather than STZ administration. TTX resistance was absent after 1 wk of untreated STZ-induced diabetes, present after 2 wk of untreated STZ-induced diabetes, and fully developed by 4 wk of untreated STZ-induced diabetes, at which time no TTX sensitivity was evident (Fig. 5).

Effect of STZ-D on VT- and K^+-stimulated [^{3}H]ACh release. VT in usual pharmacological concentrations (12–14) produced a similar dose-dependent increase in [^{3}H]ACh release in control and STZ-D rats (Fig. 7). Fully depolarizing concentrations of K^+ (100, 50 mM) induced similar [^{3}H]ACh release in control and STZ-induced dia-

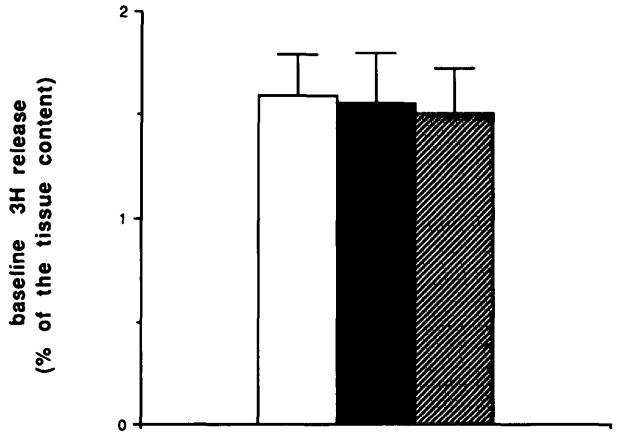

FIG. 1. Baseline ^{3}H release from atrial slices during in vitro incubation expressed as a percentage of tissue ^{3}H content. Baseline ^{3}H release during 5-min incubation in the absence of agonists was low compared with total tissue ^{3}H content and did not differ between atrial slices from control rats (□), STZ-induced diabetic rats (■), or insulin-treated STZ-induced diabetic rats (▨). n = 6 for each group.

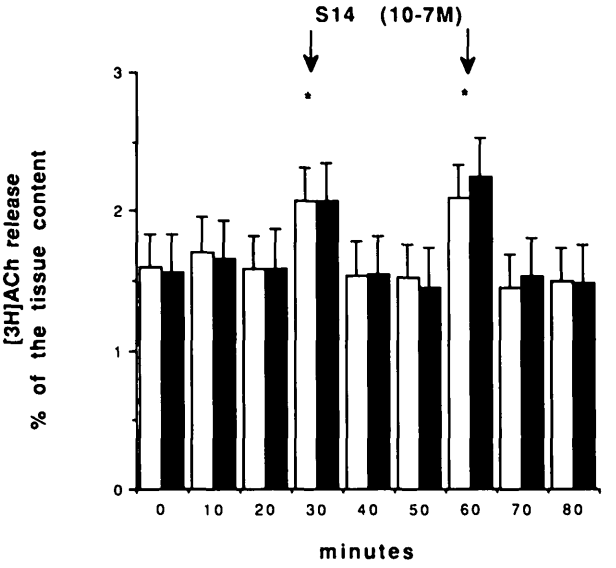

FIG. 2. SRIF14-stimulated [^{3}H]ACh release from atrial slices from control and STZ-induced diabetic rats. After preincubation with [^{3}H]choline, addition of SRIF14 to the medium containing atrial tissue induced a rapid and reproducible release of [^{3}H]ACh that did not differ between control (□) and STZ-induced diabetic (■) rats. n = 6 for each group, *P < 0.01 from the basal value.

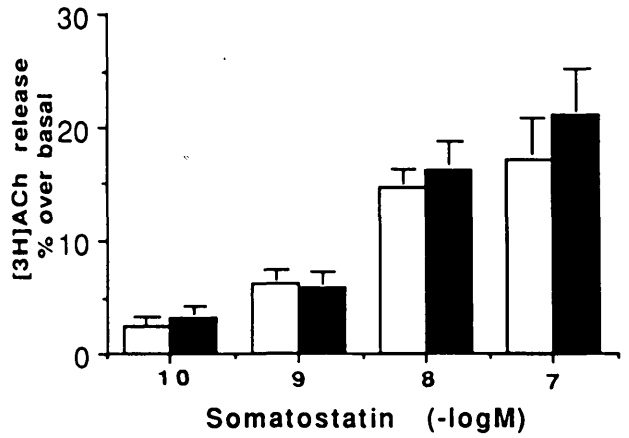

FIG. 3. Dose-dependent increase in [^{3}H]ACh release by SRIF14 from atrial slices from control and STZ-induced diabetic rats. Stimulated release was maximal at 10^{-7} M SRIF14 in both control (□) and STZ-induced diabetic (■) rats (17.2 ± 2.5 and 21 ± 2.8% over basal levels, respectively, $n = 6$ for both groups, $P < 0.001$), because higher doses (not shown) did not stimulate additional increments in [^{3}H]ACh release. ED_{50} stimulatory concentrations of SRIF14 of 5×10^{-9} M in STZ-induced diabetic and 2×10^{-9} M in control rats produced respective increases of 8.4 ± 1.1 and 17.2 ± 0.8% in [^{3}H]ACh release.

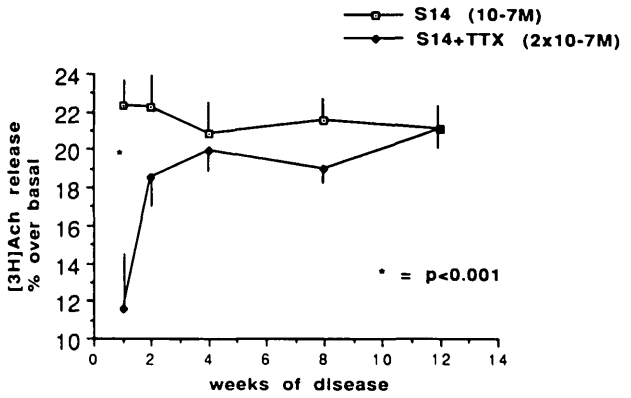

FIG. 5. Time course of the development of TTX resistance of atrial SRIF14-stimulated [^{3}H]ACh release in untreated STZ-induced diabetic rats. The effect of TTX (compare ⊡ and ■ at each time point) was diminished by 2 wk and absent by 4 wk after induction of STZ-diabetes. *$P < 0.001$.

betic rats (Fig. 8), but a lower, near-threshold concentration of K^+ (25 mM) induced a smaller submaximal release of [^{3}H]ACh in control rats; this response to 25 mM K^+ in STZ-induced diabetic rats was undiminished compared with 50 or 100 mM K^+ (Fig. 8).

DISCUSSION

This study was undertaken to explore the functional status of intrinsic cardiac neurons in STZ-induced diabetic rats. The diffuse anatomical localization of these nerve fibers within the cardiac atrial wall greatly complicates direct electrophysiological and biochemical evaluation (15). Therefore, indirect pharmacological procedures were used to assess the effect of diabetes and insulin treatment on [^{3}H]ACh release with four different excitatory or inhibitory substances acting at three different levels within the neuron: SRIF14 is a peptidergic neuropeptide whose action is thought to be mediated by cell surface receptors on the cell soma and/or dendrites (11); VT and TTX bind to and stimulate or inhibit, respectively, voltage-dependent Na channels concentrated along the axon (12–14); and depolarizing concentrations of K^+ are thought to generally depolarize all excitable plasma membrane components, including those of the nerve terminal. Because SRIF14 reacts specifically with cell bodies in this system (11), it targets only intrinsic postganglionic neurons of the cardiac autonomic nervous system. In contrast, VT and K^+ might in addition act on preganglionic cholinergic axons and terminals within the heart wall but derived from extrinsic neurons such as from the vagus nerve. However, because preganglionic axons and terminals constitute only a minor proportion of the cardiac cholinergic system (15), we may presume that our study focused mainly on the pathophysiology of the postganglionic parasympathetic neurons.

Neither STZ-induced diabetes nor insulin-treated STZ-induced diabetes affected basal ^{3}H release or agonist-stimulated [^{3}H]ACh release, suggesting that the postganglionic parasympathetic fibers remain generally intact during the first 3 mo of experimental diabetes.

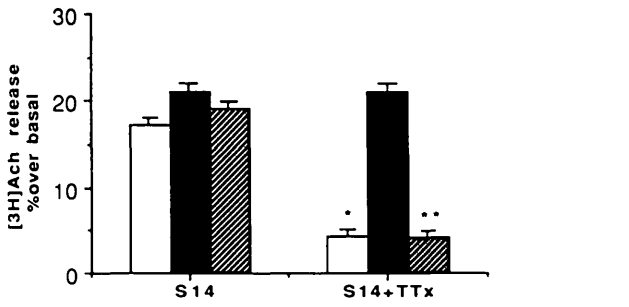

FIG. 4. Inhibition by TTX (*right*) of SRIF14-stimulated atrial [^{3}H]ACh release (*left*) in control (□), and insulin-treated STZ-induced diabetic (▨) but not untreated STZ-induced diabetic (■) rats. *$P < 0.01$, **$P < 0.001$, $n = 5$ for each group.

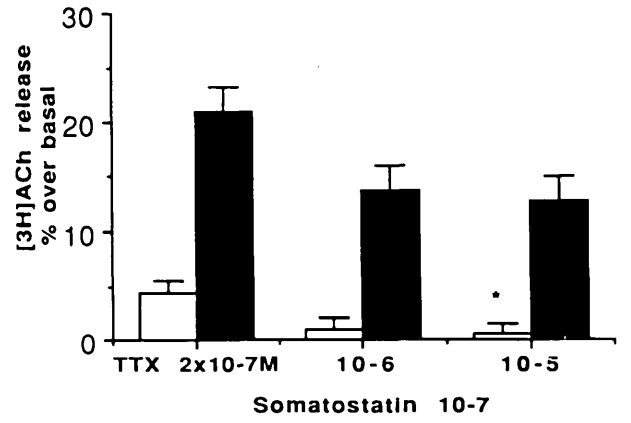

FIG. 6. The effect of increasing concentrations of TTX on SRIF14-stimulated [^{3}H]ACh release in atria from control (□) and untreated STZ-induced diabetic (■) rats. [^{3}H]ACh release was significantly ($P < 0.001$) greater in STZ-induced diabetic versus control rats at each TTX concentration ($n = 5$). [^{3}H]ACh release was significantly lower in control rats at 2×10^{-5} versus 2×10^{-7} M TTX (*$P < 0.05$).

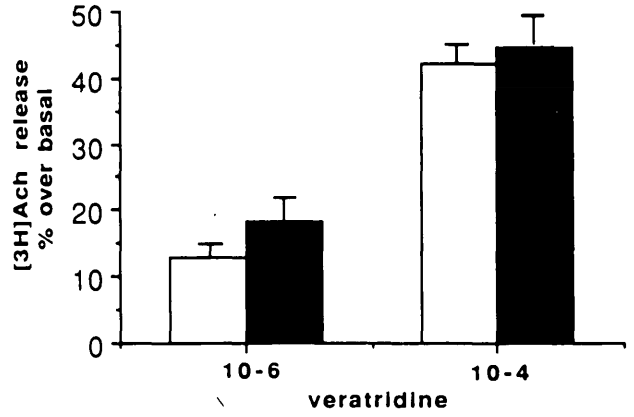

FIG. 7. Concentration-dependent stimulation of [^{3}H]ACh release by VT in atria from control (□) and untreated STZ-induced diabetic (■) rats. VT-stimulated [^{3}H]ACh release was not significantly different in control and STZ-induced diabetic rats at either concentration of VT. n = 5 in each group.

SRIF14, which stimulates ACh release via a TTX-sensitive pathway (11), is thought to stimulate ACh release via axonal conduction of an evoked action potential. The preservation of normal SRIF14-stimulated [^{3}H]ACh release in STZ-induced diabetes suggests that peptide binding, transmembrane signal transduction, and action potential generation and axonal conduction to nerve terminals remain qualitatively unaffected by 3 mo of STZ-induced diabetes.

VT depolarizes nerve fibers by interfering with the Na inactivation of the axonal Na^+ conductance (13,14) and thus maximally increasing Na channel–mediated Na^+ permeability. The ionic fluxes elicited by VT are therefore considered more physiological than those elicited by high extracellular K^+ concentration, which are independent of Na channel function and are Ca^{2+} independent (13,16). The similar patterns of [^{3}H]ACh release elicited by VT in STZ-induced diabetic and control rats attests to the overall functional integrity of the Na^+ channels.

Elevation of extracellular K^+ above 30 mM is routinely used in vitro to mimic the depolarizing effect of an action

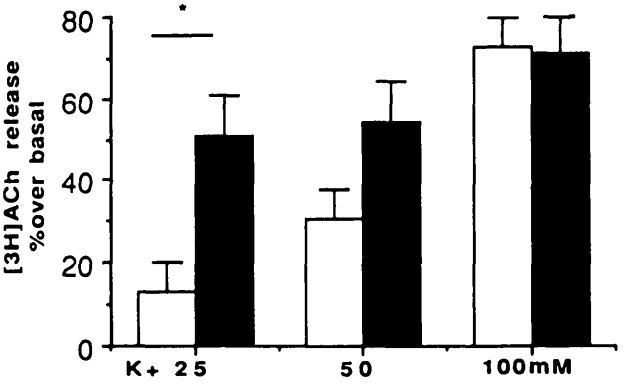

FIG. 8. Concentration-dependent stimulation of [^{3}H]ACh release by K^+ in atria from control (□) and untreated STZ-D rats. Atria from control but not STZ-induced diabetic (■) rats exhibited dose-dependent K^+-stimulated [^{3}H]ACh release. Atrial [^{3}H]ACh release was significantly greater in STZ-induced diabetic versus control rats at 25 mM K^+ (*$P < 0.01$).

potential at nerve endings (17). This causes the release of putative neurotransmitters independent of axonal conduction, requires an intact synaptic vesicle storage and release mechanism, but does not require the presence of an intact neuron (12,18). Depolarizing concentrations of K^+ (50–100 mM) induced equivalent [^{3}H]ACh release in control and diabetic rats, suggesting the presence of intact ACh storage and release mechanism. However, the increased sensitivity of STZ-induced diabetic rats to low, near-threshold K^+ concentrations could reflect a reduction in the resulting membrane potential of diabetic fibers.

Voltage-sensitive Na^+ channels are responsible for the generation and conduction of action potentials along neuronal membranes. TTX binds to voltage-dependent Na channels, thereby reversibly inhibiting, sequentially, the fast inward Na^+ current, the progression of the action potential, and the release of the neurotransmitters (19,20). SRIF14-induced [^{3}H]ACh release from postganglionic intrinsic cardiac parasympathetic fibers is highly sensitive to TTX inhibition (11) in control rats, but STZ-induced diabetic rats are highly resistant to the effects of TTX on SRIF14-induced [^{3}H]ACh release. Yet, VT-induced [^{3}H]ACh release was normal in STZ-induced diabetic rats, even though both TTX and VT have been reported to bind directly to Na channels (21), although their binding sites may differ somewhat.

Several hypothesis can be invoked to interpret TTX resistance in postganglionic parasympathetic cardiac nerve fibers in STZ-induced diabetic rats, such as direct interference by STZ-induced diabetes with the binding of TTX to, or the number or functional distribution of, the Na channels of the postganglionic neuron. Specific neuronal binding of radiolabeled TTX cannot be measured directly in this experimental model because of the high TTX binding capacity of cardiac membrane preparations (22). However, the disappearance of voltage-sensitive Na channels from the cell surface is marked by a parallel reduction in TTX binding and VT-stimulated Na^+ uptake (23). By this convention, the preservation of normal VT-stimulated [^{3}H]ACh release in STZ-induced diabetic rats would argue against the hypothesis that TTX resistance in STZ-induced diabetes is attributable to a loss of Na channels. Similarly, a reduction in Na channel number would not explain the simultaneously enhanced sensitivity of [^{3}H]ACh release to stimulation by near-threshold concentrations of K^+ in STZ-induced diabetes. Moreover, restoration of normal TTX sensitivity by insulin administration to STZ-induced diabetic rats precludes a direct toxic effect of STZ on Na channel TTX binding or function.

Pharmacological and physiological studies in various excitable cells and at different stages of differentiation suggest the presence of at least four classes of Na channels: *1*) Na channels with high TTX affinity that cannot be activated by electrical stimulation or channel-activating toxins such as sea anemone toxin II or VT; *2*) Na channels with high TTX affinity that cannot be activated electrically but only chemically by sea anemone toxin II, VT, or analogous toxins; *3*) Na channels with high affinity for TTX that are activated by both membrane

depolarization and neurotoxins; and *4*) Na channels with low affinity for TTX that are activated by membrane depolarization and lipid-soluble or polypeptide toxins (24). Furthermore, Na channels may shift between two alternate states with differing TTX sensitivities: TTX-sensitive Na channels inhibited by 10^{-8}–10^{-7} M TTX, and TTX-resistant Na channels inhibited by 10^{-6}–10^{-5} M TTX (24–29). Although a shift to the TTX-resistant form in STZ-induced diabetes might reduce TTX sensitivity in the 10^{-7}–10^{-6} M concentration range, the persistence of TTX resistance at 10^{-5} M TTX is not readily explained by this construct.

An alternative hypothesis could account for TTX resistance at high TTX concentrations, enhanced sensitivity to near-threshold concentrations of K^+, and preservation of normal VT-stimulated ACh release. By this formulation, STZ-induced diabetes would reduce the resting membrane potential of postganglionic cardiac parasympathetic neurons in a fashion analogous to that reported in peripheral somatic myelinated axons. The resting membrane potential would be reduced to the point that threshold could be attained through residual voltage-sensitive Na channel activity in the presence of TTX (2×10^{-5} M). At the same time, a reduced resting membrane potential would be expected to enhance the sensitivity of the membrane to depolarization by near-threshold concentrations of K^+. The unaltered sensitivity to VT would be explained by the ability of this drug to decouple Na channel function from Na inactivation (14) so that VT-stimulated depolarization would be unaffected by raised intracellular Na^+. The effect of a putative reduction in resting membrane potential alone would not be expected to substantially affect TTX binding directly, based on recent studies by Lonnendonker in frog myelinated nerve fibers (30). He concluded that "binding of TTX exhibits a weak voltage dependence with toxin affinities decreasing at more negative holding potentials (30)." However, complex effects of secondary alterations in dynamic membrane fluxes associated with alterations in membrane potential on TTX binding affinity cannot be excluded (31).

In summary, pharmacological assessment of cardiac postganglionic parasympathetic function in acute STZ-induced diabetes associates short-term insulin deficiency and/or hyperglycemia with reduced TTX sensitivity of agonist-stimulated ACh release without diminished responsiveness to direct Na channel agonists or K^+ depolarization. This pattern plus the accompanying heightened sensitivity of ACh release to submaximal K^+ concentrations are not inconsistent with the presence of reduced Na-K-ATPase activity, increased intra-axonal Na^+, and reduced resting membrane potential analogous to that described in somatic and autonomic nerve fibers in STZ-induced diabetes (32–34). This speculation will require more extensive experimental evaluation, such as confirmation of TTX resistance in postganglionic parasympathetic fibers in other animal models and other nerve fiber populations known to exhibit reduced Na-K-ATPase activity, and its responsiveness to aldose reductase inhibitors (33) and *myo*-inositol supplementation (35) that correct Na-K-ATPase activity.

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