Effects of Insulin on Potassium Currents of Rat Ventricular Myocytes in Streptozotocin Diabetes

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Abstract. Membrane currents of ventricular cardiomyocytes isolated from control, diabetic and insulin-treated diabetic Wistar rats have been measured using the whole cell configuration of the patch-clamp technique. Insulin restored the density of the 4-aminopyridine-sensitive early transient component of the calcium-independent outward potassium currents which decreased in diabetes. The inactivation rate of the transients increased in diabetes and was normalised by insulin. The late 4-aminopyridine-insensitive component of the outward currents showed the same diabetes- and insulin-related changes. This current could reflect the activation of the delayed rectifier channels although pharmacological identification of this component could not be achieved.

Key words: Diabetes — Insulin — Outward potassium currents — 4-Aminopyridine

Introduction

The streptozotocin (STZ)-treated rat is a well-established model of Type I diabetes mellitus (IDDM). Heart preparations of these animals exhibit altered contractility characterised by reduced force and slower relaxation (Fein et al. 1980). The changes may indicate the development of diabetic cardiomyopathy (Regan et al. 1977) and can be a result of modified intracellular calcium homeostasis (Penpargkul et al. 1981; Heyliger et al. 1987; Noda et al. 1992) and/or of an increased duration of the surface action potential (Nobe et al. 1990; Magyar et al. 1992; Jourdon and Feuvray 1993; Shimoni et al. 1994). Most of the contractile disorders can be normalised by insulin treatment (Fein et al. 1981; Schaible et al. 1983) proving further their diabetes-related origin.

Previous results rendered probable that decreased density and faster inacti-

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vation of the transient outward potassium current might be responsible for the prolongation of the ventricular action potentials (Magyar et al. 1992; Jourdon and Feuvray 1993; Shimoni et al. 1994). Normalisation of the action potential duration following insulin treatment of diabetic rats was also observed (Magyar et al. 1992). Experiments reported herein bring evidence for insulin being able to normalise the diabetes-related alterations of calcium-independent transient potassium currents in STZ-diabetic rats.

Materials and Methods

General procedures, isolation of ventricular myocytes

The experiments were carried out on 12 weeks old Wistar rats weighing 150 200 g. Diabetes was induced in 23 animals by a single intravenous dose of STZ (65 mg/kg body weight; Sigma) while the controls (11 rats) were treated with the solvent of the STZ (50 mmol/l citrate buffer; Magyar et al. 1992). Blood glucose levels of the animals were checked twice a week, their value ranged from 17.6 to 27.7 mmol/l in the diabetic rats and from 4.2 to 6.1 mmol/l in the controls. Starting four weeks after the STZ injection, insulin was administered for two weeks to 9 animals of the diabetic group (2–5 IU/day/animal; Ultralente, Novo Industri AS, Copenhagen). These doses of insulin kept the animals in normoglycemic state (blood glucose concentrations 3.9 6.2 mmol/l). The rats received the last insulin dose 24 hours before being sacrificed.

The animals were killed with a blow on the neck. Single ventricular myocytes were isolated with collagenase digestion (Sigma, Type I; Mitra and Morad 1985; Magyar et al. 1992). The excised heart was perfused through the aorta, first with Tyrode's solution (in mmol/l: NaCl, 150.0; KCl, 5.4; $MgCl_2$, 0.5; HEPES, 5.0; glucose, 10.0; pH adjusted to 7.2 by NaOH) containing 1 mmol/l Ca^{2+}, then with Ca^{2+}-free Tyrode's solution for 5 min, and finally with the enzyme-containing Tyrode's solution for 20–30 min (180 μmol/l Ca^{2+} added). The isolated cells were suspended in a solution similar to that described by Hescheler et al. (1986), in mmol/l: KCl, 85.0; KH_2PO_4, 30.0; $MgCl_2$, 3.0; glucose, 10.0; K-glutamate, 16.0; EGTA, 0.5; $KHCO_3$, 3.0; pyruvic acid, 5.0; HEPES, 10.0; taurine, 20.0; pH set to 7.4 by KOH. The myocytes were stored in a refrigerator at 5 °C for 2–6 hours before use.

During the measurements, the cells were bathed in Tyrode's solution containing 0.02 mmol/l tetrodotoxin (TTX) and 3 mmol/l $CoCl_2$ to block sodium and calcium channels, respectively. In some experiments 4-aminopyridine (4-AP, Sigma) or $BaCl_2$ (Ba^{2+}, Reanal) was added to the Tyrode's solution. The measurements were carried out at room temperature ($T = 20$ 23 °C).

Data acquisition and analysis

The whole-cell configuration of the patch-clamp technique (Hamill et al. 1981) was used for recording membrane currents. The microelectrodes (2–5 MOhms) were filled with the following solution (in mmol/l): K-aspartate, 130.0; KCl, 20.0; KH_2PO_4, 1.0; $MgCl_2$, 1.0; EGTA, 5.0; Na_2-ATP, 3.0; HEPES, 5.0; pH adjusted to 7.3 by KOH.

The analogue current traces were filtered using a four-pole low-pass Bessel filter (5 kHz). The sampling rate of a 12 bit A/D converter (Labmaster TM40, Scientific Solutions, Foster City, CA) varied between 2.8 and 33 kHz. The pCLAMP 5.5.1 software (Axon Instruments, Foster City, CA) was used for data acquisition and analysis.

Membrane capacitance of the myocytes was determined at a holding potential of −85 mV by means of depolarising and hyperpolarising pulses of 5 mV. The leakage current was determined by hyperpolarising pulses of 5 mV, and its linearly extrapolated value was subtracted from each current trace before further analysis. No correction was made for the linear leakage if the input resistance was higher than 1 GOhm. 4-AP- and Ba^{2+}-sensitive current components were derived by subtracting current families recorded in the presence of 4-AP or $BaCl_2$ from the current families obtained on the same myocyte prior to the addition of the blocker. Fitted parameter values were determined by the Levenberg-Marquardt algorithm.

Statistics

Data were analysed statistically using Student's unpaired t-test. Scatter of the means is given as standard deviation (± S.D.). The letter "n" always refers to the number of the individual cells.

When evaluating data obtained by fitting Eqs. 3 and 4, the parameter values of the individual fits were grouped into a histogram with a uniform bin width and chosen to make the histogram similar to Gaussian distribution in shape. Each bar height was equal to the number of the points found in the given interval divided by the total number of the points and by the bin width. The histogram was fitted by the probability density function of the Gaussian distribution

$$f(x) = ((2\pi\sigma^2)^{-0.5}) \exp(-(x - m)^2/(2\sigma^2)) \quad (1)$$

which gave the best fit value of the mean (m) and of the standard deviation (σ), respectively.

Results

Effects of insulin on the potassium current densities in diabetic rats

Fig. 1*A* shows characteristic current families accompanying test pulses of 500 ms duration from a holding potential of −85 mV to voltages between −50 and +50 mV. In order to avoid complications arising from frequency-dependent phenomena (Shimoni et al. 1994), the pulses were delivered at a constant rate of 0.2 Hz throughout the experiments. A prepulse of 7 ms to −20 mV preceded each test pulse to inactivate TTX-insensitive sodium currents (Apkon and Nerbonne 1991). The prepulse did not appreciably alter the early part of the outward current but without it a fast inward current was always observable. Due to the composition of the solutions used in the experiments, the traces can be regarded as calcium-independent potassium currents. As reported earlier, both the early peak and the sustained level of these currents decreased in diabetes (Magyar et al. 1992; Jourdon and Feuvray 1993; Shimoni et al. 1994). Two weeks of insulin treatment, however, considerably normalised the current densities.

To identify pharmacologically the outward potassium currents, 4-AP, a widely used blocker of the transient outward current (I_{to}) was applied at a concentration

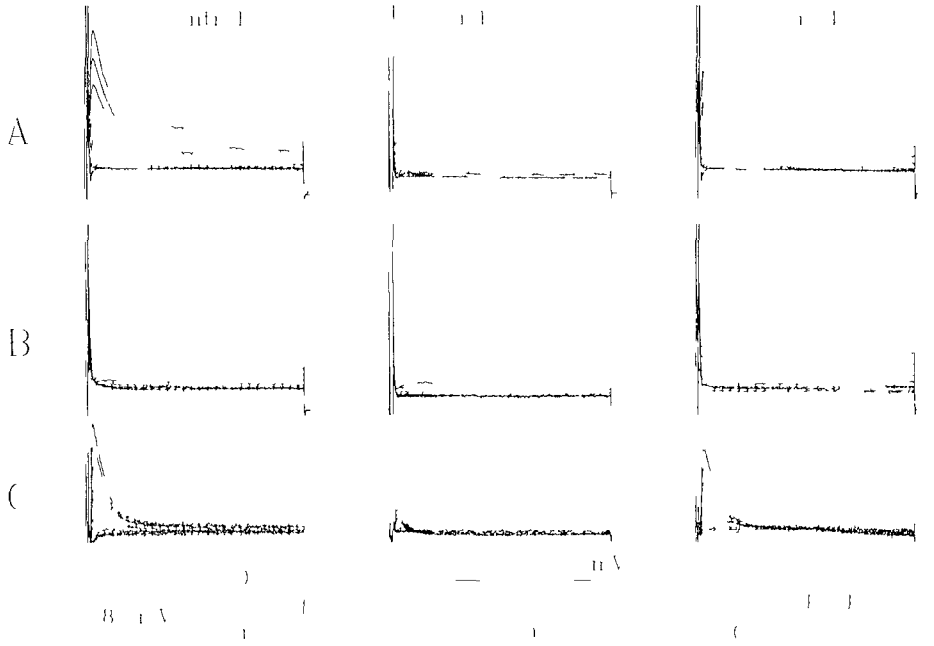

Figure 1. Isolation of the 4-AP-sensitive current (I_t). Currents were elicited by the pulse protocol indicated at the bottom of the Figure. Analogical traces were recorded in Tyrode's solution (*A*) and 5–10 minutes after the addition of 4-AP (*B*) at a concentration of either 3 mmol/l (myocytes from a control and an insulin-treated diabetic rat) or 6 mmol/l (cell from a diabetic rat). The 4-AP-sensitive currents (*C*) were derived by subtracting the curves in *B* from the corresponding traces in *A*. The broken line indicates the zero current level in each case.

of 3–6 mmol/l (Kenyon and Gibbons 1979, Benndorf and Nilius 1988, Apkon and Nerbonne 1991, Jourdon and Feuvray 1993). Current traces shown in Fig. 1*B* were evoked in the presence of 4-AP, which eliminated the early transient component in all groups of animals leaving the sustained level unaltered.

The difference traces in Fig. 1*C* show the 4-AP sensitive I_{to} components. The peak amplitude of the transients decreased in diabetic tissue and recovered nearly completely following insulin treatment. The sustained level of the 4-AP-sensitive traces was negligible in most instances.

The current–voltage relationships of the 4-AP sensitive early peaks are displayed in Fig. 2. The amplitudes were determined by subtracting the end-pulse level of the difference traces from the peak values. The current density was significantly smaller in the diabetic animals than that in the control rats while after

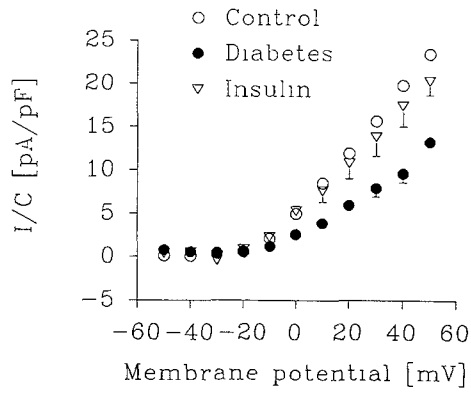

Figure 2. Current-voltage relationships of the inactivating 4-AP-sensitive currents. The points represent averages of 3-6 individual measurements each.

insulin treatment, a nearly complete recovery can be seen. At +30 mV, for example, the current density was 16 ± 4 pA/pF ($n = 6$) in controls, 8 ± 1 pA/pF ($n = 6$) in diabetic animals and 14 ± 2 pA/pF ($n = 3$) in insulin-treated diabetic animals. The diabetes-related decrease was significant at $p < 0.05$ between 0 and +50 mV. There was no significant difference between the values for control and insulin-treated animals.

Fig. 1*B* clearly demonstrates the presence, as well as the diabetes- and insulin-related changes, of a 4-AP-insensitive, virtually non-inactivating outward current. Experiments were done to test whether the activation of the inward rectifier potassium channels (I_{K1}) was responsible for this current component. Neither diabetes nor insulin treatment modified significantly the density of the I_{K1} identified as a Ba^{2+}-sensitive current (Kilborn and Fedida 1990; data not shown).

According to recent reports (Apkon and Nerbonne 1991; Jourdon and Feuvray 1993; Shimoni et al. 1994), the non-inactivating component might be a manifestation of the delayed outward current (I_K). Pharmacological (TEA) identification of such a component failed.

Insulin and the activation-inactivation properties of the transient outward current

The inactivation kinetics of the transient outward current component was described by fitting the declining phase of the 4-AP-sensitive traces with the single exponential function

$$I(t) = A_1 \exp(-t/\tau_1) + A_2 \tag{2}$$

where τ_1 is the inactivation time constant, A_1 is the y axis intercept at $t = 0$. The sustained component (A_2) was usually negligible. The traces recorded at membrane potentials between 0 and +50 mV were always suitable for fitting while at −10 mV the transients were too small to be used regularly.

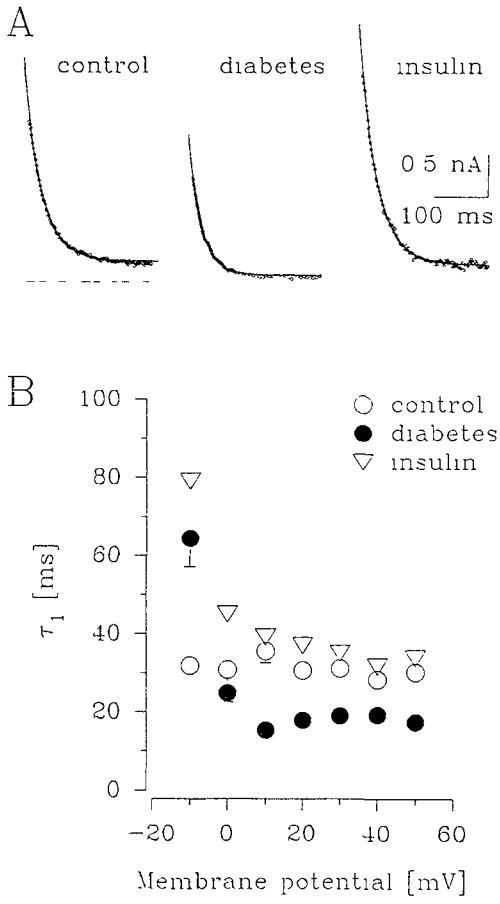

Figure 3. Voltage dependence of inactivation time constant (τ_1) of the 4-AP-sensitive current. *A*: Current transients similar to those seen in Fig. 1*C* (membrane potential +50 mV, circles). The continuous lines through the circles were calculated using Eq. 2 and the parameter values obtained from the fits. The τ_1 values are 31.0, 23.8 and 33.4 ms for cells isolated from control, diabetic and insulin-treated diabetic animals, respectively. The dotted lines represent the zero current level. *B:* Inactivation time constants as a function of the membrane potential. Data points represent averages of 3–6 individual measurements each.

Fig. 3*A* illustrates the reliability of the fitting. There is acceptable overlap of the circles representing the original records and of the traces generated using the parameters obtained from the fits. Fig. 3*B* shows a faster inactivation of the 4-AP-sensitive current in the diabetic cells and an obvious normalisation following insulin treatment ($p < 0.05$ between 0 and +50 mV when comparing myocytes of diabetic animals to either controls or insulin-reverted cells). The τ_1 seems voltage-independent in controls while in other cases the data suggest a membrane-potential dependence although the differences seen along the voltage axis did not reach significance.

The voltage dependence of the steady-state inactivation of the transient outward current was determined by means of a double pulse protocol. The membrane potential during the prepulse (700 ms) varied between −100 and −20 mV while being +50 mV in each case during the test pulse (700 ms). The amplitude of the

transient component was determined as the difference between the early peak and the sustained level at the end of the pulse. The individual values were normalised to that obtained with a prepulse of −100 mV in the same myocyte. The normalised data of a given cell (I_{norm}) were fitted by the equation

$$I_{norm} = 1/(1 + \exp((E_{pp} - E_{0.5})/s)) \quad (3)$$

where E_{pp} is the prepulse potential, $E_{0.5}$ is the half inactivation voltage, and s is the slope factor. Table 1 indicates that neither the diabetic state nor the insulin treatment modified the steady-state inactivation of the transient outward current.

Table 1. Parameter values characterising the voltage dependence of the activation and the steady-state inactivation of I_{to} as well as its recovery from inactivation

	Steady-state inactivation			Recovery from inactivation			Activation			
	$E_{0.5}$ [mV]	s [mV]	n	I_{max}	τ [ms]	n	g_{max} [pS/pF]	$E_{0.5}$ [mV]	s [mV]	n
Control	−49 ± 5	5 ± 2	25	1.0 ± 0.1	26 ± 3	9	84 ± 17	−1 ± 6	−12 ± 4	29
Diabetes	−48 ± 1	6 ± 2	29	1.0 ± 0.1	26 ± 3	9	36 ± 10*	−1 ± 8	−12 ± 6	66
Insulin	−49 ± 1	5 ± 2	23	1.0 ± 0.1	40 ± 8*	9	85 ± 18	−1 ± 5	−13 ± 4	60

The mean and standard deviation values were determined from the Gaussian distribution of the parameters. * significantly different from the corresponding value of control and diabetic values ($p < 0.001$). None of the other differences are statistically significant.

The recovery from inactivation was also determined by a double pulse protocol. In this case the parameters of the two pulses were the same and constant (depolarisation from −85 to +50 mV for 700 ms) while the interpulse interval was changed between 2 and 500 ms. The amplitude of the transient component during the second pulse (I_{II}, determined as above) was divided by the corresponding value belonging to the first pulse (I_I). The ratios obtained in the individual cells were fitted by the equation

$$I_{II}/I_I = I_{max}(1 - \exp(-t/\tau)) \quad (4)$$

where t is the interpulse interval, I_{max} is the maximum ratio in the given myocyte, and τ is the time constant of the recovery from inactivation, which was the same in control and diabetic rats (Table 1). Insulin, however, significantly decreased the recovery rate.

The voltage dependence of the activation of the outward current was derived from the current-voltage relationships of the early peak of the total I_{to} currents (in this case the peak amplitudes were measured as deviation from the zero level). The individual current-voltage curves were fitted by the equation

$$I = (E_p - E_e) \cdot g_{max}/(1 + \exp((E_p - E_{0.5})/s)) \quad (5)$$

where the E_p is the membrane potential during the pulses, E_e is the equilibrium potential (taken as -85 mV), $E_{0\,5}$ is the half activation voltage, g_{max} represents the maximum conductance value, and s is the slope factor.

The results of this fitting are listed in Table 1. The diabetes-induced decrease of the g_{max} ($p < 0.001$ compared either to the values of control or the insulin-treated animals) might indicate that i) the functional channel density became smaller, or ii) the mean open time of the single channels decreased, or iii) the single channel conductance decreased. Following insulin treatment, a complete recovery of g_{max} was observed. Neither diabetes nor insulin administration modified significantly the half activation voltage and the slope factor.

Discussion

Effects of insulin on the potassium current densities

Earlier studies have shown that STZ-induced diabetes in rats is associated with action potential prolongation (Fein et al. 1983; Horackova and Murphy 1988) and with alterations in the outward potassium currents of isolated ventricular cardiomyocytes (Magyar et al. 1992; Jourdon and Feuvray 1993; Shimoni et al. 1994). The present experiments confirm the previous findings and show that two weeks of insulin administration recovered the current densities.

The outward potassium currents of various species have components with different kinetic features and pharmacological sensitivities (Kenyon and Gibbons 1979; Siegelbaum and Tsien 1980; Josephson et al. 1984). On the basis of its 4-AP sensitivity, the early peak current of the present experiments seems identical with the transient outward current (I_{to}) characterised earlier (Josephson et al. 1984; Apkon and Nerbonne 1991; Jourdon and Feuvray 1993).

The sustained part of the outward current proved 4-AP-insensitive but showed diabetes- and insulin-related changes. Earlier works (Jourdon and Feuvray 1993; Shimoni et al. 1994) excluded the role of inward calcium currents in maintaining this component therefore the activation of the delayed outward current seemed to be a possible explanation (Apkon and Nerbonne 1991; Shimoni et al. 1994). Identification of a TEA-sensitive component, however, was not achieved in the present experiments. In a few cases the activation of the 4-AP-insensitive component could be fitted by a single exponential function resulting in time constants between 5 and

15 ms. The activation rates calculated from these values are close to that of I_K given by Apkon and Nerbonne (1991).

Effects of insulin on the inactivation characteristics of the transient outward current

Development of diabetic state did not induce any change in the rate of recovery from inactivation in the present experiments. In contrast, a slowing of this process was reported earlier (Shimoni et al. 1994). A possible explanation of this discrepancy may be that in the cited work longer reactivation intervals were applied and the sum of two exponentials was used to fit the data. Under these conditions the diabetes influenced more markedly the slow component which was probably overlooked in our experiments.

Following insulin treatment, the recovery of the transient component from inactivation became significantly slower. This finding raises the question whether beyond the restoration of diabetes-evoked modifications, insulin had direct influence on the outward potassium currents. To address this question, insulin was added to the bathing solution of isolated cardiomyocytes ($n = 3$) but no effect on any parameter of the outward currents was found. However, differences between the acute and chronic insulin effects have already been reported (Fein et al. 1981; Schaible et al. 1983).

When interpreting the slower recovery from inactivation seen after insulin treatment, the significance of aging has to be considered, too. Namely, the insulin-treated diabetic rats were two weeks older than the controls or the diabetic animals at the time of the measurements. However, the role of aging can be excluded by the observation that rats in which diabetes persisted for six weeks, showed the same activation-inactivation features as the diabetic animals used in these experiments.

Besides decreasing the density of I_{to}, diabetes increased the inactivation rate of this component. The same conclusion was reached earlier when analysing the falling phase of the total outward current (Magyar et al. 1992). In another report, however, no change in the inactivation rate of either the I_{to} or the total current was found (Jourdon and Feuvray 1993). The latter paper described a voltage-independent inactivation time constant of approximately 70 ms for the 4-AP-sensitive component. In our case, considerably smaller time constants were found, and a membrane potential dependent character could be neither proved nor excluded. Although the origin of the above differences is not clear, the validity of the diabetes-induced kinetic change is supported by the recovery seen after insulin treatment.

The experiments presented here failed to identify pharmacologically the sustained component of the outward current but the role of the I_K seems most likely (Apkon and Nerbonne 1991).

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