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Effects of the Nitric Oxide Synthase Inhibitor N^{ω} Nitro-L-arginine Methyl Ester on Electrical Activity and Ion Channels of Mouse Pancreatic B Cells

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The effects of the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) were studied on electrical activity, K^+_{ATP} currents and voltage-dependent K^+ and Ca^{2+} channel currents in mouse pancreatic B cells. In the presence of 15 mM glucose, L-NAME (>5 mM) depolarized the B cell membrane and electrical activity became continuous. The depolarization caused by L-NAME was not reversed by the addition of a membrane permeant cyclic GMP analogue. L-NAME inhibited the whole-cell K^+_{ATP} current reversibly by reducing the single channel current conductance and open probability. The results are consistent with the assumption that L-NAME depolarizes the B cell membrane by a direct action on the K^+_{ATP} channel. L-NAME also influenced the whole-cell current through voltage-dependent K^+ and Ca^{2+} channels.

Recently, it has been shown that nitric oxide (NO) markedly influenced electrical activity and ion channels in pancreatic B cells [1, 2]. Besides its cytotoxic, cell deleterious effects, NO is known to be involved in the regulation of cell function like regulation of vascular tone, synaptic transmission in the brain and platelet aggregation [3-6]. Since it is still debatable whether NO produced by a constitutive NO synthase plays a role in the regulation of insulin secretion under physiological conditions [7-9] we have recently tested whether the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) alters glucose-induced electrical activity in B cells [10]. L-NAME did not influence glucose induced electrical activity at a concentration of 100 μM, at which the NO synthase is completely inhibited in other tissues [11]. This observation speaks against an important contribution of NO to the regulation of B cell electrical activity. However, at higher concentrations (5-20 mM) L-NAME reversibly depolarized the membrane potential as it has been shown for its mother compound L-arginine [12, 13]. It has been reported by others that L-NAME, similar to L-arginine, stimulates insulin secretion in this concentration range [14-16] and it has been concluded from these data that NO is involved in the regulation of insulin secretion. We now provide evidence that the depolarization caused by L-NAME is independent from the NO synthase activity. Moreover, despite the similarity in structure and effects of L-arginine and L-NAME, the mechanism of depolarization seems to be different for the two molecules. L-Arginine has been shown to depolarize B cells because it enters the cells as a positively charged molecule [13, 17]. In the present paper we demonstrate that L-NAME directly influences the activity of ion channels in B cells. Concerning the probable use of L-NAME as a drug, it is certainly of relevance to understand its pharmacological and toxic actions. Several diseases are considered to be connected to the overproduction of NO, e.g. diabetes or septic shock [18-20]. It is under investigation whether NO synthase

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inhibitors are beneficial in the treatment or protection against different pathological states in animals [21-25].

MATERIALS AND METHODS

The experiments were performed on islets of fed female NMRI mice (25-30 g), killed by cervical dislocation. Membrane potential measurements were done using high resistance microelectrodes [26] with an extracellular fluid composed of (in mM): 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, 15 glucose, gassed with 95% O_2 and 5% CO_2 to maintain a pH of 7.4 at 37° C. Patch-clamp experiments were performed with islet cells isolated by collagenase digestion, dispersed in Ca^{2+} free medium, and cultured for up to 4 days in RPMI 1640 medium [27]. Whole-cell K^+_{ATP} currents were measured at a holding potential of −70 mV and during 300 ms pulses to −80 mV and −60 mV at 15 s intervals. Voltage-dependent whole-cell K^+ and Ca^{2+} currents were elicited by applying depolarizing pulses from the holding potential of −70 mV to 0 mV. Experiments were performed at 32° C. Single K^+_{ATP} channel currents were recorded in inside-out patches at membrane potentials of −30 or −50 mV. Open probabilities (P_o) were calculated as the sum of time at which the current of each single channel was greater than half the amplitude of the single channel, devided by the number of available channels in the patch and the total recording time (1 min). P_o is expressed as percentage of control values. Single channel measurements were done at 25° C. Whole-cell K^+_{ATP} and voltage-dependent K^+ current recordings were performed with a pipette solution containing (in mM): 130 KCl, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 0.65 Na_2ATP, 20 HEPES, pH 7.15 adjusted with KOH. Bath solution was composed of (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 0.5 glucose, 10 HEPES, pH 7.4 adjusted with NaOH. For the measurement of voltage-dependent K^+ currents 100 μM tolbutamide was added to the bath solution to inhibit the K^+_{ATP} current [28]. B Cell whole-cell Ca^{2+} currents were recorded in a solution containing (in mM): 115 NaCl, 20 TEACl, 10 $CaCl_2$, 1.2 $MgCl_2$, 0.1 tolbutamide, 15 glucose, 10 HEPES, pH 7.4 adjusted with NaOH. The pipette solution was composed of (in mM): 50 CsCl, 70 N-methyl-D-glucamine, 58 HCl, 4 $MgCl_2$, 3 Na_2ATP, 10 EGTA, 2 $CaCl_2$, 10 HEPES, pH 7.15 adjusted with CsOH. To record currents through single K^+_{ATP} channels, seals were obtained using a pipette solution composed of (in mM): 130 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 HEPES, pH 7.4 adjusted with KOH and the same bath solution as for the measurement of whole-cell K^+_{ATP} currents. Before patch excision, the bath solution was replaced by a Mg^{2+} free solution with the following composition (in mM): 130 KCl, 4.6 $CaCl_2$, 10 EDTA, 20 HEPES, pH 7.20 adjusted with KOH. The lack of Mg^{2+} and ATP in this solution prevents phosphorylation and probably reduced K^+_{ATP} channel rundown by this mechanism [29, 30].

N^{ω}-Nitro-L-arginine methyl ester (L-NAME) and 8-bromoguanosine 3′:5′-cyclic monophosphate (8-Br-cGMP) were purchased from Sigma, Deisenhofen, FRG. All other chemicals were purchased from Merck, Darmstadt, FRG in the purest form available. Where applicable, experimental data are expressed as arithmetic means ± SEM. Statistical significance was accepted at $P \leqslant 0.05$.

RESULTS

Effects of L-NAME on Electrical Activity of Mouse Pancreatic B Cells

In the presence of 15 mM glucose B cells exhibited typical oscillations of membrane potential with Ca^{2+} action potentials superimposed on the plateau potential (figure 1). Under control conditions the fraction of plateau phase (percentage of time with spike activity, calculated for the last 4 min before change of solution) was 47 ± 5 % (n=5) in this series of experiments. Addition of 15 mM L-NAME to the perifusion medium led to depolarization of the cells and the spike activity became continuous in all experiments. After wash-out of L-NAME, oscillations reappeared and the fraction of plateau phase was 54 ± 9 %. The addition of 100 μM of the membrane permeant cGMP derivative 8-Br-cGMP in the presence of L-NAME did not alter electrical activity (n=5; figure 1). In another series of experiments a lower concentration of L-NAME (5 mM) was tested (not shown). The percentage of plateau phase increased from 46 ± 3 % under control conditions to 73 ± 6 % after addition of 5 mM L-NAME (n=5; $P<0.01$). Again, the application of 100 μM 8-Br-cGMP in the presence of L-NAME was without effect on the electrical activity. The addition of 8-Br-cGMP alone did not change electrical activity (not shown). The fraction of plateau phase under control conditions was 59 ± 8 % and 63 ± 5 % after a 10 min treatment of the cells with 100 μM 8-Br-cGMP (n=5).

Effects of L-NAME on K^+_{ATP} Channels

L-NAME (5 and 20 mM) decreased the whole-cell K^+_{ATP} current in a concentration dependent manner (figure 2A). The amplitude of the current, measured during the 10 mV depolarizing

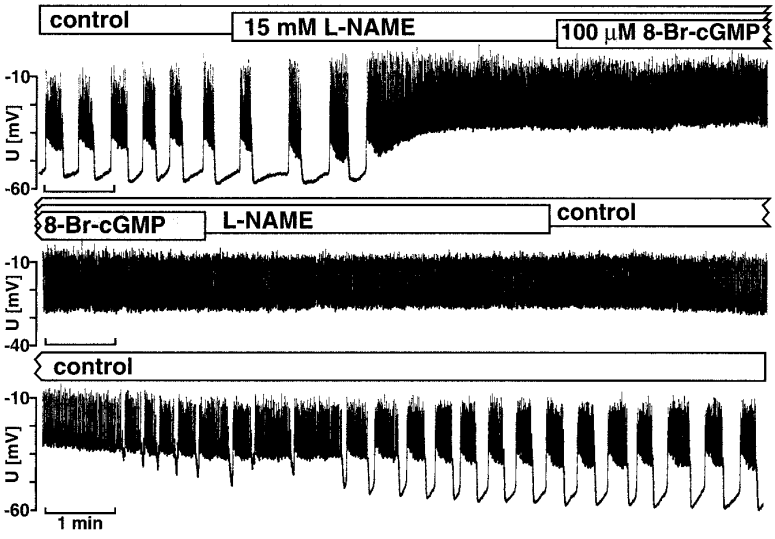

FIG. 1. Effects of 15 mM L-NAME and 100 μM 8-Br-cGMP in the presence of L-NAME on the membrane potential of single mouse pancreatic B cells perifused with a medium containing 15 mM glucose. The drugs were added for the periods indicated by the bars. The three panels show one experiment without interruption. This record is representative of results obtained in five similar experiments.

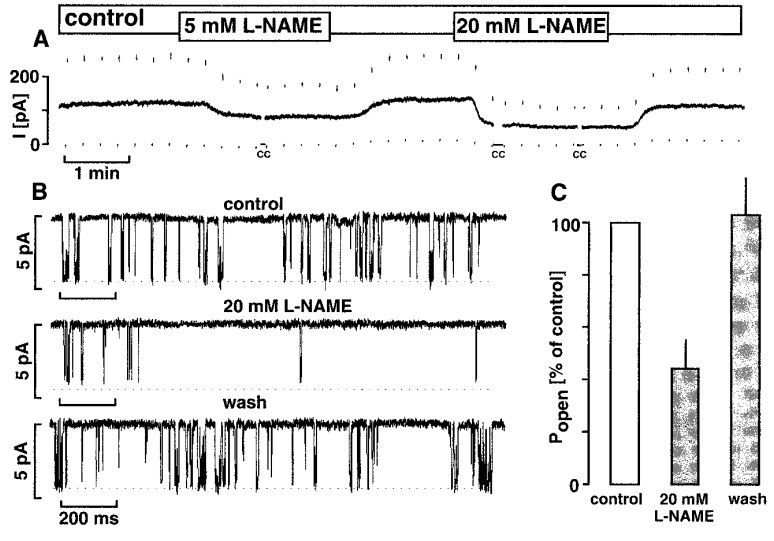

FIG. 2. (A) Effect of 5 mM and 20 mM L-NAME on ATP-sensitive whole-cell K^+ currents in a single cultured mouse pancreatic B cell. The currents are shown at the holding potential of -70 mV (solid line) and during 300 ms steps to -80 and -60 mV (lower dashed trace and upper dashed trace, respectively). L-NAME was added at the periods indicated by the bars. At the time points marked with cc, membrane potential was measured in the current-clamp mode. The record is representative of eight experiments with similar results. (B) Effect of 20 mM L-NAME on single K^+_{ATP} channel currents recorded in the inside-out patch configuration at a membrane potential of -50 mV under control conditions (upper panel), after addition of L-NAME (middle panel) and after removal of L-NAME (lower panel). Data were filtered at 1 kHz for display. For comparison, the dashed lines in each panel indicate the channel amplitude under control conditions. The record is representative of six experiments with similar results. (C) Channel open probability calculated for these six experiments as a percentage of control.

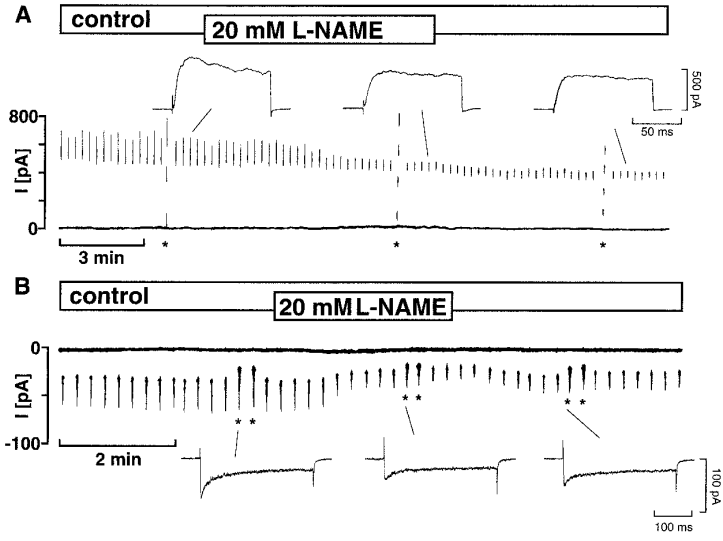

FIG. 3. (A) Effects of 20 mM L-NAME on currents through voltage dependent K^+ channels of mouse B cells. Currents were monitored every 15 s during 100 ms voltage steps from the holding potential of −70 mV to 0 mV. L-NAME was added at the period indicated by the bar. At the points marked by an asterisk I-V curves were recorded. Currents before, during, and after application of L-NAME are shown above the continuous current trace on an extended time scale. This record is representative of five experiments with similar results. (B) Effect of 20 mM L-NAME on voltage-dependent Ca^{2+} currents. Currents were elicited every 15 s by 50 ms voltage steps from the holding potential of −70 mV to 0 mV. To evaluate effects on channel inactivation 2 pulses of 300 ms duration (indicated by *) were applied under each experimental condition before solution exchange. Below the continuous trace, currents are shown before, during, and after addition of L-NAME on an extended time scale. This record is representative of nine experiments with similar results.

step, was reduced by 48 % from 118 ± 8 pA to 61 ± 7 pA (n=8; $P<0.001$) by 5 mM L-NAME. The effect seemed not to be fully reversible, probably due to channel rundown. Removal of L-NAME resulted in a current amplitude of 79 ± 8 pA (n=8). The addition of 20 mM L-NAME caused a reduction of the current amplitude by 62 % from 91 ± 12 pA to 35 ± 5 pA (n=8; $P<0.01$). After wash-out the current was 64 ± 13 pA (n=8). Simultaneous measurements in the current-clamp mode showed that the reduction in K^+_{ATP} current depolarized the membrane potential from −81.3 ± 0.4 mV to −78.5 ± 1.0 mV (n=6; $P<0.02$) and −81.1 ± 0.3 mV to −77.1 ± 1.1 mV (n=7; $P<0.01$) after application of 5 and 20 mM L-NAME, respectively.

The effect of L-NAME was also tested on single K^+_{ATP} channels in the excised inside-out patch configuration. As shown in figure 2B, L-NAME inhibited K^+_{ATP} channels by reducing the single channel conductance and the open probability (P_o). The single channel conductance decreased from 77 ± 1 pS under control conditions to 61 ± 2 pS after application of 20 mM L-NAME to the perifusion medium (n=6; $P<0.001$). After removal of L-NAME the single channel conductance increased again to 76 ± 1 pS (n=6; $P<0.001$). The open probability was quite variable in the patches tested (range from 0.012 to 0.274). Thus it was expressed as percentage of the open probability under control conditions. P_o decreased to 44 ±11 % (n=6, $p<0.005$) during the application of 20 mM L-NAME and increased again after removal of L-NAME to 103 ± 14 % (n=6, $p<0.005$) (figure 2C).

Effects of L-NAME on Voltage-Dependent K^+ and Ca^{2+} Channels

As demonstrated in figure 3A, 20 mM L-NAME inhibited the current through voltage-dependent K^+ channels. The amplitude of the peak current was reduced from 762 ± 143 pA

to 542 ± 78 pA (n=5; P<0.05). The L-NAME effect on this channel type was not reversible. The mean amplitude was estimated to be 592 ± 120 pA after removal of L-NAME (n=3). The current inactivation was partly blocked. As a measure for the degree of inactivation, the current recorded at the end of a 100 ms pulse was divided by the peak current resulting in a mean value of 0.73 ± 0.05 under control conditions, 0.81 ± 0.02 after application of L-NAME (n=5, p<0.05) and 0.84 ± 0.03 after wash-out of L-NAME (n=3).

Figure 3B shows that 20 mM L-NAME also reduced the current through voltage-dependent Ca^{2+} channels. The amplitude decreased from −90 ± 12 pA to −44 ± 6 pA (n=9; P<0.01). The effect was only partly reversible. After removal of L-NAME the amplitude reached a value of −66 ± 14 pA (n=5). L-NAME also slowed Ca^{2+} current inactivation. The quotient calculated by dividing the steady state current recorded after 300 ms by the peak current was 0.47 ± 0.03 under control conditions and 0.59 ± 0.04 after treatment of the cells with L-NAME (n=9; P<0.05). This effect was hardly reversible after removal of L-NAME.

DISCUSSION

The present paper demonstrates that the NO synthase inhibitor L-NAME has profound effects on electrical activity and ion channel currents in mouse pancreatic B cells. Several studies have shown that L-NAME enhances insulin secretion in the concentration range used in our experiments [14-16]. This effect is not unique for L-NAME but was also observed with N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NNA) in cultured rat islets [31]. On the contrary, two other reports show that L-NMMA inhibits insulin secretion [7, 8]. The reason for this discrepancy is unknown.

A stimulation of insulin secretion is in good agreement with the membrane depolarization induced by L-NAME observed in our experiments [10 and this paper]. It has been speculated [14, 31] that the increase in insulin secretion is due to the fact that NO synthase inhibitors are transported into cells as positively charged molecules as it has been demonstrated for L-arginine [13, 17]. It has been shown with aortic endothelial cells and a murine macrophage cell line that L-NMMA and N^G-iminoethyl-L-ornithine are transported via the cationic transport system y^+ but not L-NNA nor its methyl ester L-NAME, which should be neutral at physiological pH [32, 33]. Thus, it appears that the mechanism by which L-NAME depolarizes B cells is different from that of L-arginine.

We show here that L-NAME reduces the whole-cell K^+_{ATP} current and that it reversibly decreases single K^+_{ATP} channel activity by diminishing single channel conductance and open probability. This action is different from that of L-arginine which does not influence K^+_{ATP} current [34], again suggesting that the two molecules depolarize B cells by different mechanisms. Concerning L-NAME, the observed inhibition of K^+_{ATP} channel activity and subsequent membrane depolarization is assumed to be sufficient to stimulate insulin secretion. The experiments performed with excised patches confirm that L-NAME directly acts on the channels and not via intracellular messenger molecules.

Action potential generation in pancreatic B cells has been attributed to Ca^{2+} inward and K^+ outward currents through voltage-dependent ion channels [35]. In mouse B cells spike repolarization mainly results from the opening of the delayed rectifying K^+ channel. The Ca^{2+} dependent K^+ channel may participate in action potential repolarization but seems not to play a major role [36]. L-NAME partly inhibited voltage-dependent Ca^{2+} and K^+ currents and simultaneously slowed inactivation of both currents. These alterations in the amplitude and kinetics of the channels are assumed to explain the changes in the spike pattern obtained after application of L-NAME [10].

A role of NO formed by a constitutive NO synthase in the modulation of glucose-induced insulin secretion in B cells has been deduced from the observations that NO synthase inhibitors alter insulin secretion [7, 8, 15], that glucose and arginine enhance the cGMP concentration

in islets [7] and that insulin and arginine release NO from a clonal pancreatic B cell line [8]. However, the effects of NO synthase inhibitors on insulin secretion are controversial and the effects of glucose and arginine on NO release were not confirmed with islets [9]. We have previously shown that low concentrations of L-NAME (100 μM) which have been shown to almost completely inhibit NO synthase activity in other tissues [11] did not influence glucose-induced electrical activity in B cells [10]. In this context, it is interesting to mention that Jones *et al.* [9] did not find any effect of L-NAME or L-NMMA on basal, glucose, and glucose plus arginine stimulated insulin secretion measured with isolated rat islets in the micromolar concentration range. In another study 100 μM L-NAME was without effect on insulin secretion elicited in response to an elevation of the glucose concentration from 3.9 to 7.2 mM in the perfused rat pancreas [37]. The results presented in this paper confirm our conclusions that NO does not substantially participate, at least in the regulation of glucose-induced electrical activity. First, addition of 100 μM 8-Br-cGMP did not alter electrical activity induced by 15 mM glucose. Secondly, if the depolarization resulted from a decrease in the cGMP concentration, which could be a consequence of the inhibition of the NO synthase, the addition of a membrane permeant cGMP analogue should repolarize the cells. However, cGMP did not reverse the effect of L-NAME on B cell electrical activity.

In conclusion, the NO synthase inhibitor L-NAME augments electrical activity of pancreatic B cells by depolarizing the cells via direct closure of K^+_{ATP} channels. L-NAME also partly inhibited voltage-dependent Ca^{2+} and K^+ channels and alters the kinetic of these channels. The profound effects of L-NAME on B cell function seem to have no connection with the suppression of NO synthase activity.

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