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The Effects of Pyridine Nucleotides on the Activity of a Calcium-activated Nonselective Cation Channel in the Rat Insulinoma Cell Line, CRI-G1

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Abstract. The activity of a calcium-activated nonselective (Ca-NS$^+$) channel in a rat insulinoma cell line (CRI-G1) is inhibited by pyridine nucleotides in excised patches. The effects of all four pyridine nucleotides tested, β-NAD$^+$, β-NADH, β-NADP$^+$ and β-NADPH were very similar when tested at 0.1 mM, and at 1 mM the phosphorylated forms, β-NADP$^+$ and β-NADPH, appeared to be slightly more potent than β-NAD$^+$ and β-NADH. All the pyridine nucleotides tested reduced both the open state probability of the channel and the number of functional channels observed in a single patch.

The application of β-NAD$^+$, but not of the other nucleotides tested, to the cytoplasmic surface of isolated inside-out patches from CRI-G1 cells opened a novel nonselective cation channel (the β-NAD$^+$-NS$^+$ channel). The activity of this new channel is calcium sensitive and may also be inhibited by AMP.

Key words: Calcium-activated nonselective channel — Rat insulinoma cell line, CRI-G1 — Pyridine nucleotides β-NAD$^+$-NS$^+$ channel — Nucleotide regulation — AMP

Introduction

The nature of the factor coupling the metabolism of glucose and other carbohydrates to the depolarization of the pancreatic β-cell has been a matter of great debate (Ashcroft, 1980; Ashcroft & Rorsman, 1991). However, it has now become well established that membrane depolarization arises due to the closure of the ATP-K$^+$ channel and that the intracellular ratio of ATP/ADP constitutes the primary determinant of ATP-K$^+$ channel activity in the intact β-cell (Ashcroft, Harrison & Ashcroft, 1984; Cook et al., 1988; Ashcroft & Rorsman, 1991).

Other mechanisms that have been suggested to regulate the membrane potential of the β-cell include protein kinase C, which may alter the activity of the ATP-K$^+$ channel by phosphorylation (Wollheim et al., 1988), and changes in the redox ratio of nicotinamide-adenine dinucleotides, [NAD(P)H]/[NAD(P)$^+$] (Panten et al., 1973; Ashcroft & Christie, 1979; Matschinsky et al., 1986; Hedeskov, Capito & Thams, 1987). However, in the latter case this ratio may not be the triggering factor coupling metabolism to insulin secretion, since all four pyridine nucleotides are capable of producing a similar concentration-dependent effect on the activity of the ATP-K$^+$ channel in excised patches from an insulin-secreting cell line (RINm5F) (Dunne, Findlay & Petersen, 1988). In this case low concentrations (100 μM and below) of the nucleotides increase channel activity and higher concentrations (500 μM and above) decrease channel activity. Thus, since there is a complex concentration-dependent interaction between the effects of adenine nucleotides and the pyridine nucleotides on the activity of the ATP-K$^+$ channel, the latter nucleotides may well contribute to the tonic inhibition of the ATP-K$^+$ channel under physiological conditions.

A complex regulatory mechanism is also emerging for another ion channel that may well be involved in the regulation of the release of insulin from the pancreatic β-cell, namely the calcium-activated nonselective cation (Ca-NS$^+$) channel. This channel is present in the plasma membrane of the insulin-secreting cell line CRI-G1 (Carrington et al., 1986) and may also be present in intact β-cells since a channel of similar conductance and kinetics has been reported in cell-attached patches from adult (Ashcroft, Ashcroft & Harrison, 1987, 1988) and fetal

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Table 1. Solutions (mM)

Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	EGTA	HEPES	pH adjusted to 7.2 with	Final $[Ca^{2+}]$
A		140	0.9	1	1	10	KOH	0.001
B		140	1	1	1	10	KOH	0.01
C		140	0.2	1		10	KOH	0.20
D		140	0.5	1		10	KOH	0.50
E	135	5	0.5	1		10	NaOH	0.50
F	135	5	1	1		10	NaOH	1
G	140		0.5	1		10	NaOH	0.50
H		92.4	0.5	1		10	KOH	0.50

(Rorsman et al., 1989) β-cells. At present, the physiological function of this channel is not clear. However, in the CRI-G1 cells the Ca-NS^+ channel activity is regulated by both adenine nucleotides (Sturgess, Hales & Ashford, 1986, 1987; Reale, Hales & Ashford; 1994) and by cyclic nucleotides (Reale, Hales & Ashford, 1992; Reale et al., 1994). The activity of the channel is reduced by both AMP and ADP (1–100 μM) in a concentration-dependent manner with AMP being more potent than ADP. However, at lower concentrations (0.1–5 μM) both ADP and AMP activate the channel in some patches. Cyclic AMP also exhibits a dual effect, predominantly increasing channel activity at low concentrations (0.1–10 μM) and reducing it at higher concentrations (100 μM and 1 mM). In view of the complex effects of pyridine nucleotides on the activity of the ATP-K^+ channel, the present investigation has examined the effects of pyridine nucleotides on the activity of the Ca-NS^+ channel in excised patches from CRI-G1 cells. The results are discussed in terms of the regulatory properties of nucleotide derivatives on Ca-NS^+ channels and the possible physiological significance of such modulation.

Materials and Methods

Cell Culture

Cells of the rat pancreatic islet cell line, CRI-G1, were used in all experiments. These were grown in Dulbecco's modified Eagle's medium at 37°C in an humidified atmosphere of 95% air and 5% CO_2 and passaged at weekly intervals (Carrington et al., 1986). For the patch clamp studies, cells 2–6 days old were used. After this time they become unsuitable for experimentation because of their high density.

Recording and Analysis

In all the experiments shown in this paper, the inside-out configuration of isolated membrane patches (Hamill et al., 1981) was used. The recording pipettes used had resistances of 8–10 MΩ. Single channel currents were recorded using a Dagan 8900 patch clamp amplifier, coupled to an 8930 probe (Dagan, Minneapolis, MN). Seal formation was monitored using a digital oscilloscope (Gould 1421) and information stored on video cassettes (VHS video cassette recorder, SLV-201, Sony; Digital pulse code modulation, PCM-701ES). Recorded data from the experiments were played back into a Gould 3000 chart recorder that filtered the signals at 0.14 kHz. Outward currents (defined as the current flowing from the intra- to extracellular side of the membrane) are indicated as upward deflections of the trace.

The open state probability, single channel current amplitudes, and mean open and closed times of the channels were quantified off-line using an analysis system consisting of an Elonex PC286C-100 microcomputer and an analysis program that incorporated a 50% threshold crossing parameter to detect events (Dempster, 1988). Data segments between 1–4 min were replayed to the computer at the recorded speed, filtered at 600 Hz using an 8-pole Bessel filter, and digitized at a frequency of 3.3 kHz using a Data Translation 2801A interface. The open state probability was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time recorded; the number of channels observed in the patch was taken into consideration. All data in the text and figures are presented as means ± SEM.

Solutions

The compositions of the solutions used in this study are listed in Table 1.

The cell culture medium was washed from the experimental dish with four applications of Solution A. The free Ca^{2+} and Mg^{2+} concentrations of this solution, calculated using the computer programme "METLIG" (P. England, and R. Denton, University of Bristol), were 1 μM and 1 mM, respectively. This is the same extracellular medium in which the inside-out patch was first obtained. The recording pipette contained Solution F. On the formation of an inside-out patch the bathing solution facing the intracellular face was replaced in most experiments by Solution D. This solution with a high calcium concentration is needed to produce a significant activation of the Ca-NS^+ channel (Sturgess et al., 1987).

For the studies of the ion selectivity of the β-NAD^+-NS^+ channel, four different sets of ionic solutions were used in the pipette and in the bath: (i) Symmetrical KCl solutions: Solution D was used both inside the pipette and in the bath. (ii) Symmetrical NaCl solutions: Solution G was used both inside the pipette and in the bath. (iii) Asymmetrical distribution: Solution E was used in the recording pipette; and a Solution D was used in the bath. (iv) Diluted asymmetrical distribution: the recording pipette contained Solution E; and Solution H was used in the bath. A series of Solutions (A, B, C and D) with differing levels of free calcium (0.001, 0.01, 0.2 and 0.5 [mM], respectively) were used in the bath to test the calcium sensitivity of the β-NAD^+-NS^+ channel.

All the pyridine nucleotides used were applied directly by super-

fusion to the cytoplasmic face of the membrane, diluted in the appropriate saline solution, and removed by suction. They were applied at a rate of approximately 0.5 ml/sec, which allowed a complete solution exchange within 45 sec. All experiments were performed at room temperature, 22–25°C. All reagents used were obtained from Sigma Chemical (Poole, Dorset, UK).

Results

To investigate whether or not the Ca-NS$^+$ channel from the CRI-G1 cell line is sensitive to pyridine nucleotides, we tested the effects of a range of these nucleotides (β-NAD$^+$, β-NADH, β-NADP$^+$ and β-NADPH) on single channel activity in inside-out patches. The β-forms of the pyridine nucleotides are the common forms of the nucleotides that are used as coenzymes. The structurally related α-forms are not used as coenzymes, and were not tested in the present investigation.

The sensitivity of the Ca-NS$^+$ channel activity to two concentrations of β-NAD$^+$ is shown in Fig. 1 in patches held at −45 mV in the presence of 0.5 mM Ca^{2+}. Figure 1*A* shows an example of a patch in which channel activity was decreased by exposure to 0.1 mM β-NAD$^+$ to 15.7% of the control level. These effects were reversible on washing the patch in nucleotide-free solution ($n = 45$). The effects of β-NAD$^+$ were concentration dependent, since exposure of another patch to 1 mM β-NAD$^+$ (Fig. 1*B*) further reduced the activity of the channel to 4.7% of the control level and produced a reduction of the number of functional channels observed, from five under control conditions, to two in the presence of the dinucleotide. These effects were also fully reversible upon washing in nucleotide-free solution ($n = 18$).

To establish if the decrease in the activity of the Ca-NS$^+$ channel induced by β-NAD$^+$ was a result of a reduction in P_{open} (P_o) and/or a reduction in the number of functional channels observed (N_f), we carried out a binomial analysis of the single channel activity as described previously (Kozlowski, Hales & Ashford, 1989). This analysis (*results not shown*) established that β-NAD$^+$ also appears to change both parameters, at least over the time periods used for the analysis of the data. Thus, to present the single channel data quantitatively, since no change in channel amplitude (i) was observed, $N_f \cdot P_o$ was used ($I = N_f \cdot P_o \cdot i$). The threshold for a significant reduction in Ca-NS$^+$ channel activity occurred between 1 and 10 μM β-NAD$^+$ (10 μM β-NAD$^+$ reduced channel activity to 27.5 and 85.8% of control levels in two different patches, while 1 μM produced no significant change from control levels; $n = 3$). No increase in channel activity was observed in the concentration range 1 μM to 1 mM β-NAD$^+$ in any of the patches tested.

The ability of β-NAD$^+$ to alter the kinetic properties of the Ca-NS$^+$ channel was also investigated. The mean open and closed times of the Ca-NS$^+$ channel were measured from records in which only one channel was apparently active, that is, from patches where no overlaps of the unitary current were observed throughout the recording period. Table 2*A* compares the mean open and closed times under control conditions, with those obtained in the presence of 1 mM β-NAD$^+$. It can be seen that in the presence of β-NAD$^+$, the mean open time of the channel is decreased, while the mean closed time of

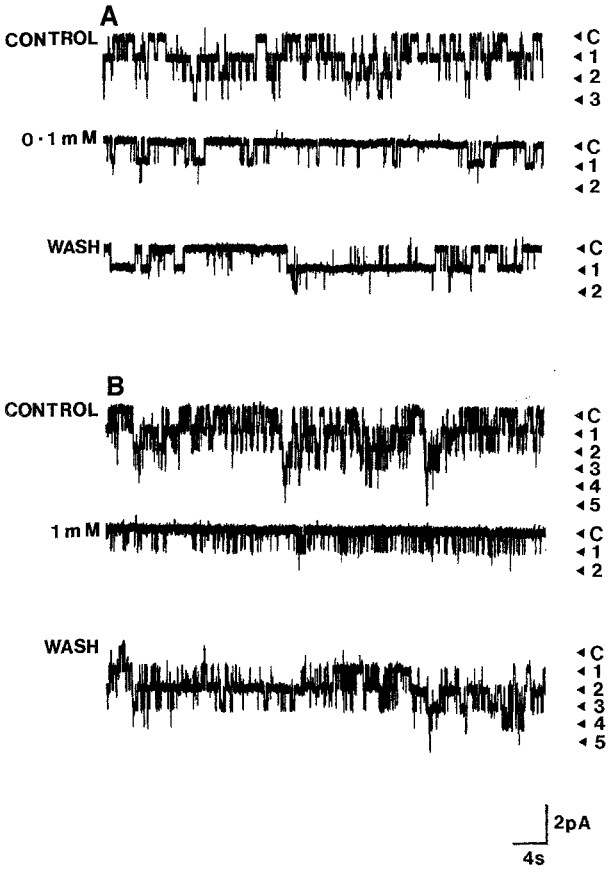

Fig. 1. Single channel current records showing the inhibitory effects of different concentrations of β-NAD$^+$, 0.1 mM (*A*) and 1 mM (*B*), in two separate patches which did not contain the β-NAD$^+$-NS$^+$ channel. Ca-NS$^+$ channel currents were recorded from excised inside-out patches in the presence of 0.5 mM Ca^{2+} on the inside. Single channel openings are denoted by downward deflections (inward currents) and the membrane potential was maintained at −45 mV. The values for $N_f \cdot P_o$ are as follows: (*A*) *Control,* 1.06; *0.1 mM* β-NAD$^+$, 0.17; *Wash,* 0.44. (*B*) *Control,* 1.12; *1 mM* β-NAD$^+$, 0.05; *Wash,* 1.38. In *A* the traces start 170 sec after patch formation. The gap between the top and middle traces was 162 sec, of which the patch spent 44 sec in the presence of 0.1 mM β-NAD$^+$. The gap between the middle and bottom traces was 256 sec, of which the patch spent 76 sec in the wash. In *B* the traces start 118 sec after patch formation. The gap between the top and middle traces was 180 sec of which the patch spent 100 sec in the presence of 1 mM β-NAD$^+$. The gap between the middle and bottom traces was 219 sec of which the patch spent 99 sec in the wash. In this figure, and all subsequent figures, the current levels are indicated on the right (c = closed; *1* = one channel open; *2* = two channels simultaneously open, etc).

Table 2. Kinetic parameters of Ca-NS$^+$ channel in the presence and absence of pyridine nucleotides

	Mean open times (msec)	Mean closed times (msec)	(Intervals)
A			
Control	130.0	13.9	(768)
1 mM β-NAD$^+$	40.1	26.1	(1,765)
B			
Control	30.5	17.3	(1,208)
0.1 mM β-NADPH	6.0	93.6	(572)

Table 3. The specificity of Ca-NS$^+$ channel inhibition by pyridine-containing nucleotides

Concentration	$N_f \cdot P_o$(test)/$N_f \cdot P_o$ (control)		
	0.1 mM (*n*)	1 mM	(*n*)
β-NAD$^+$	0.14 ± 0.08 (8)	0.09 ± 0.05	(10)
β-NADH	0.23 ± 0.13 (3)	0.13 ± 0.10	(4)
β-NADP$^+$	0.17 ± 0.05 (6)	0	(5)
β-NADPH	0.12 ± 0.05 (6)	0	(3)

The results are expressed as the mean relative change in $N_f \cdot P_o$ ± SEM. The number of patches (*n*) used are shown in parentheses.

the channel is increased. This analysis was performed on the data obtained from three separate patches and similar qualitative changes were obtained in the mean open and closed times of the channel in all cases.

The ATP-K$^+$ channel showed very little selectivity between the effects of the different nicotinamide-adenine dinucleotides tested (Dunne et al., 1988). To see if the Ca-NS$^+$ channel binding site for the pyridine-dinucleotides exhibited a similar lack of specificity, we compared the effects of β-NAD$^+$ to those observed with its reduced form, β-NADH, and with their corresponding phosphorylated derivatives, β-NADP$^+$ and β-NADPH. The Ca-NS$^+$ channel was inhibited by all the pyridine nucleotides tested. Table 2*B* compares the mean open and closed times under control conditions and in the presence of 0.1 mM β-NADPH. It can be seen that just as for β-NAD$^+$, β-NADPH also decreased the mean open time of the channel, while increasing the closed time of the channel.

Binomial analyses of the effects of β-NADH, β-NADP$^+$ and β-NADPH indicated that the actions of all four of the pyridine nucleotides on the Ca-NS$^+$ channel activity are best expressed by the term $N_f \cdot P_o$, since both parameters are altered in the presence of each of the pyridine nucleotides tested. A quantitative comparison of the relative inhibitory effects of the four different nicotinamide-adenine dinucleotides tested on the activity of the Ca-NS$^+$ channel at two different concentrations (0.1 and 1 mM) is shown in Table 3. It can be seen that when tested at a concentration of 0.1 mM, there was little difference in the inhibitory effects of the dinucleotides tested. At this concentration, the mean effect of all the pyridine nucleotides tested, [NAD(P)(H)], was a reduction in channel activity to 15.3 ± 3.4% (mean ± SE) (n = 23) of control values with a recovery to 85.8 ± 9.6% (mean ± SE) (n = 23) of initial control values during the subsequent wash periods. However, when tested at a concentration of 1 mM, the phosphorylated dinucleotides, β-NADP$^+$ and β-NADPH, were slightly more effective than both β-NAD$^+$ and β-NADH in inhibiting the activity of the Ca-NS$^+$ channel.

An unsuspected and intriguing finding from the present investigation was the ability of β-NAD$^+$ to open another channel when applied to the cytoplasmic surface of isolated inside-out patches from CRI-G1 cells. When applied to an isolated patch at concentrations of either 0.1 or 1 mM (but not at lower concentrations) in the presence of 0.5 mM Ca^{2+}, β-NAD$^+$ induced the opening of a channel passing a larger amount of current than the Ca-NS$^+$ channel, in addition to closing the Ca-NS$^+$ channel. Figure 2*A* shows an example of the effect of applying 1 mM β-NAD$^+$ to an inside-out patch held at a membrane potential of −45 mV in the presence of 0.5 mM Ca^{2+}, with symmetrical ionic concentrations on both faces of the membrane patch (135 mM NaCl and 5 mM KCl). It causes almost a complete closure of the four Ca-NS$^+$ channels apparently active in this patch, plus the opening of a channel carrying a larger current. The channel activity disappears immediately after washing the membrane patch with nucleotide-free solution (*not shown*). Figure 2*B* shows an example of the effect of applying 0.1 mM β-NAD$^+$ to an inside-out patch held under the same conditions as in Fig. 1. It causes a reduction in the Ca-NS$^+$ channel activity, plus a number of brief openings of the larger channel. In this patch, both channels can be observed at the same time in the presence of 0.1 mM β-NAD$^+$. The activity of the large channel again disappears immediately after washing the membrane patch with nucleotide-free solution and the activity of the Ca-NS$^+$ channel also begins to recover. The larger channel has a mean single channel current of 3.94 ± 0.08 pA (mean ± SE, n = 16 separate experiments) at a membrane potential of −45 mV, using the solutions described in Materials and Methods. This channel was not opened by β-NADH (n = 9), β-NADP$^+$ (n = 14) or β-NADPH (n = 9) at concentrations up to 1 mM under the conditions used. The channel was observed in 40 out of 59 inside-out patches exposed to high concentrations of β-NAD$^+$ and at an average frequency of three to four channels per patch.

To determine the ion selectivity of this β-NAD$^+$-activated channel, plots of single channel current amplitude against membrane voltage (*I-V* plots) were obtained under a variety of ionic conditions across the membrane patch. With symmetrical 140 mM KCl solutions on both sides of the membrane patch and with 0.5 mM Ca^{2+} and

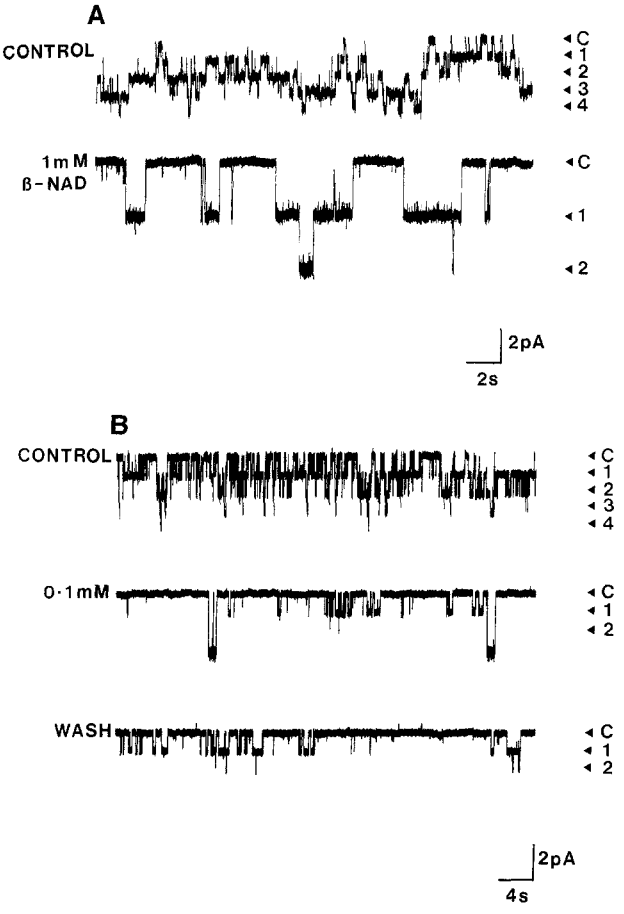

Fig. 2. Single channel current records illustrating the opening of a β-NAD^+-NS^+ cation channel and the inhibition of Ca-NS^+ channel activity by 1 mM (*A*) and 0.1 mM (*B*) β-NAD^+. Channel currents were recorded from excised inside-out patches held at a membrane potential of −45 mV in the presence of 0.5 mM Ca^{2+}. The values for $N_f \cdot P_o$ are: (*A*) *Control,* Ca-NS^+ channel, 1.23; *1 mM* β-*NAD^+* on Ca-NS^+ channel, 0.00 and on β-NAD^+-NS^+ channel, 0.6. (*B*) For Ca-NS^+ channel, *Control,* 1.06; *0.1 mM* β-*NAD^+*, 0.11, *Wash,* 0.40. The traces start 384 sec (*A*) and 118 sec (*B*) after patch formation. In *A* the gap between the two traces was 154 sec, of which 48 sec was spent in the presence of 1 mM β-NAD^+. In *B* the gap between the first two traces was 270 sec, of which 106 sec was spent in the presence of 0.1 mM β-NAD^+ and the gap between the second and third traces was 197 sec, of which 77 sec was spent in the wash.

0.1 mM β-NAD^+ in the bath (Fig. 3*A*, open circles), the *I-V* curve was approximately linear for the potentials studied (−45 to +45 mV), and exhibited a reversal potential of 0 mV and a single channel conductance of 87.3 ± 3.4 pS ($n = 3$). A similar *I-V* curve was obtained with symmetrical 140 mM NaCl across the membrane patch in the presence of 0.5 mM Ca^{2+} and 0.1 mM β-NAD^+ in the bath (Fig. 3*A*, filled circles) and this resulted in a reversal potential of 0 mV with a single channel conductance of 81.1 ± 3.4 pS ($n = 6$). Under these conditions, the *I-V* relationship was also approximately linear for the potentials studied (−45 to +45 mV). Single channel currents obtained under symmetrical conditions (140 mM NaCl in both the pipette and the bath) are illustrated in Fig. 4*A*.

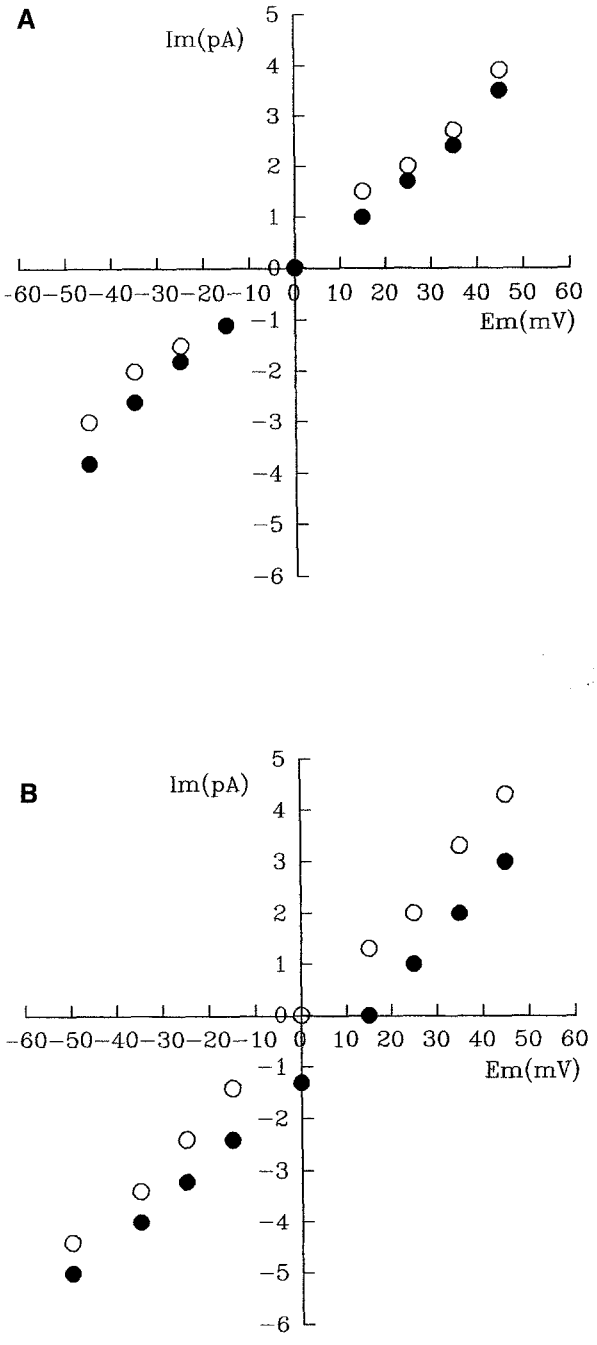

Fig. 3. Current-voltage relationships for the β-NAD^+-NS^+ channel induced by 0.1 mM β-NAD^+ in the presence of 0.5 mM Ca^{2+}. The relationships shown in *A* were obtained from two separate inside-out patches under symmetrical 140 mM KCl conditions (open circles) and under symmetrical 140 mM NaCl conditions (filled circles). Those in *B* were obtained from two separate inside-out patches with 135 mM NaCl and 5 mM KCl in the recording pipette and 140 mM KCl in the bath (open circles), and with 135 mM NaCl and 5 mM KCl in the recording pipette and 92.4 mM KCl in the bath (filled circles).

In experiments where an asymmetrical distribution of cations across the membrane (mM: 140 KCl inside, and 135 NaCl plus 5 KCl outside) was present, the *I-V* curve for the large channel induced by 0.1 mM β-NAD^+

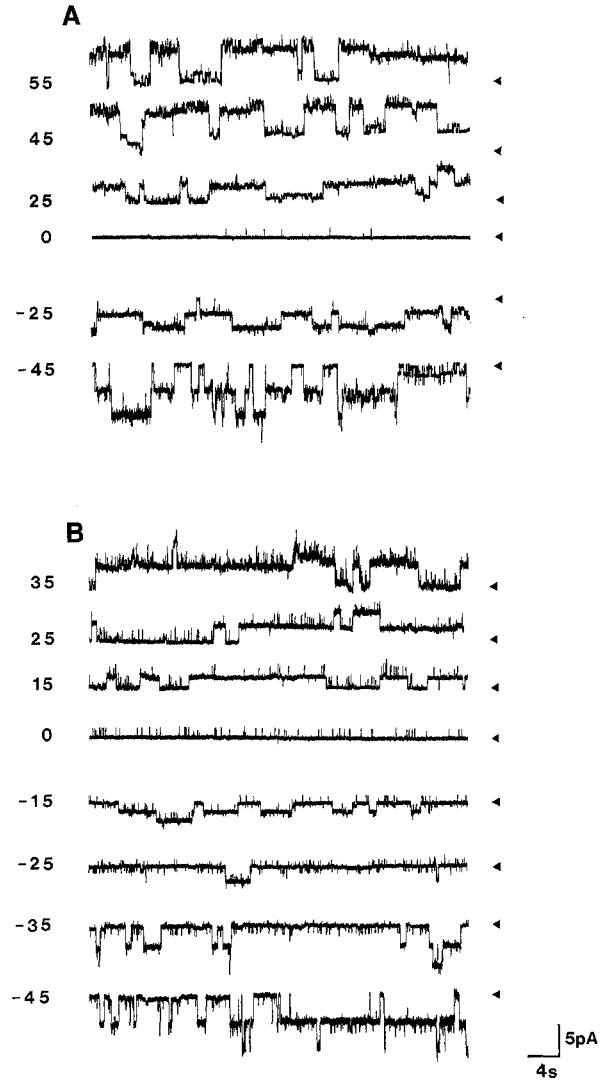

Fig. 4. (*A*) Single channel current recordings of the β-NAD^+-NS^+ channel obtained under symmetrical ionic conditions from a single inside-out patch held at a range of different patch membrane potentials from +55 to −45 mV. The solution in the recording pipette contained 140 mM NaCl, and that in the bath contained 140 mM NaCl plus and 0.1 mM β-NAD^+. The −45 mV trace starts 618 sec after patch formation and 258 sec after exposure to 0.1 mM β-NAD^+. The gaps between the traces are as follows: 147, 48, 28, 180 and 28 sec. (*B*) Single channel current recordings of the β-NAD^+-NS^+ channel obtained under asymmetrical ionic conditions from a single inside-out patch held at a range of different patch membrane potentials from +35 to −45 mV. The solution in the recording pipette contained 135 mM NaCl and 5 mM KCl, and that in the bath contained 140 mM KCl plus and 0.1 mM β-NAD^+. The −45 mV trace starts 120 sec after patch formation and 20 sec after exposure to 0.1 mM β-NAD^+. The gaps between the traces are as follows: 48, 24, 16, 22, 16, 10, and 64 sec. In both *A* and *B* the numbers to the left of the traces refer to the patch membrane potentials (mV) and the arrows to the right of the traces indicate the closed levels of the channels. Inward currents are shown as downward deflections at negative membrane potentials. The openings superimposed on that of the large conductance big channel are due to the Ca-NS^+ and ATP-K^+ channels that are not completely closed in some patches by this concentration of β-NAD^+.

was again linear and the reversal potential remained at 0 mV with a single channel slope conductance of 89.8 ± 3.0 pS (n = 6) (Fig. 3*B*, open circles). Single channel currents obtained under asymmetrical conditions (mM: 140 KCl outside, and 135 NaCl and 5 KCl inside) are illustrated in Fig. 4*B*. In these recordings (and in Fig. 4*A* above) the openings superimposed on that of the large conductance channel are the Ca-NS^+ and ATP-K^+ channels that are not completely closed in some patches. At 0 mV the few upward deflections observed are due to the ATP-K^+ channel. The fact that the reversal potential did not change under any of the conditions used, suggests that the large channel is either relatively nonselective to cations or is anion selective. It is possible to differentiate between these two possibilities by simply diluting the intracellular solution to two-thirds of the control (i.e., to 92.4 mM KCl) to alter the salt gradient across the membrane. Under these conditions (Fig. 3*B*, filled circles), the *I-V* curve was still linear with a slope conductance of 82.6 ± 2.8 pS (n = 3), but displaced in the positive direction. There was a concomitant shift in the reversal potential to +11, +12 and +9.5 mV for three different patches. These values are close to that predicted from the Nernst equation (+10.5 mV) for a purely cation-selective channel. Thus, the large channel induced by β-NAD^+ is another nonselective cation channel that we will refer to as the β-NAD^+-NS^+ channel.

In view of the calcium dependence of the Ca-NS^+ channel from the CRI-G1 cells, we investigated the sensitivity of the β-NAD^+ –NS^+ channel to intracellular calcium levels. Figure 5 illustrates an experiment where the β-NAD^+-NS^+ channel was induced by application of 0.1 mM β-NAD^+ to an inside-out patch held at −45 mV and subsequently exposed to calcium concentrations of 1, 10, 200 and 500 μM Ca^{2+}. At a calcium concentration of 500 μM, the channel spends a large proportion of time in the open state (P_{open} = 0.55) and this value was much reduced in the presence of 200 μM Ca^{2+} (P_{open} = 0.05) and 10 μM Ca^{2+} (P_{open} = 0.01). No channel openings were observed at a concentration of 1 μM Ca^{2+} (n = 3).

The Ca-NS^+ channel from the CRI-G1 cells is also sensitive to adenine nucleotides which inhibit channel activity (Sturgess et al., 1986, 1987; Reale et al., 1994). Thus, to see if the β-NAD^+-NS^+ channel was similarly sensitive to such nucleotides it was activated in inside-out patches by the application of 0.1 mM β-NAD^+, and the effects of ATP and AMP on its activity were assessed. In three out of the four patches tested, 0.1 mM ATP had no significant effect, only increasing the activity of the β-NAD^+ –NS^+ channel to 119.1 ± 15.0% of its control level before the addition of ATP. On the other hand, 0.1 mM AMP produced a variable degree of inhibition of the β-NAD^+ –NS^+ channel activity in the four patches tested. In two patches, it reduced the activity of the channel (to 42.8 and 47.2% of control levels) and in the other two patches it completely abolished the activity

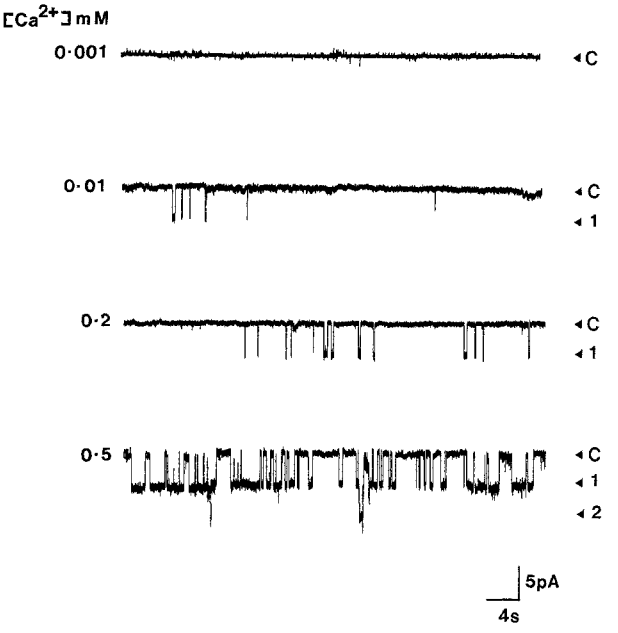

Fig. 5. Single channel currents recorded from a single inside-out patch showing the calcium sensitivity of the β-NAD^+-NS^+ channel in the presence of 0.1 mM β-NAD^+. The membrane potential of the patch was held at −45 mV. The traces show the activity of the channel at four different Ca^{2+} concentrations: 0.001, 0.01, 0.2 and 0.5 mM. The values of P_o for the four traces are 0.000, 0.005, 0.051 and 0.549, respectively. The traces start 34 sec after patch formation and 30 sec after exposure to 0.1 mM β-NAD^+. The gaps between the traces are 60, 310 and 18 sec, respectively.

of the channel. Further, more detailed experiments are required to confirm this apparent sensitivity of the β-NAD^+ –NS^+ channel to adenine nucleotides.

Discussion

It is clear from the present study that the activity of the Ca-NS^+ channel in the insulin-secreting cell line, CRI-G1, can be reduced by pyridine nucleotides. The effects of all four of the pyridine nucleotides tested, β-NAD^+, β-NADH, β-$NADP^+$ and β-NADPH, were very similar when tested at 0.1 mM, and at 1 mM the phosphorylated forms, β-$NADP^+$ and β-NADPH, appeared to be slightly more potent than β-NAD^+ and β-NADH.

To date, very few studies have examined the modulatory effects of pyridine nucleotides on ion channel activity using single channel recording techniques. One exception is the study of the actions of pyridine nucleotides on the activity of the ATP-K^+ channel in another insulin-secreting cell line, RINm5F (Dunne et al., 1988). This study also found very little qualitative or quantitative differences between the effects of the four different nicotinamide-adenine dinucleotides tested, except that at low concentrations (10 and 100 μM) each promoted channel opening, whereas high concentrations (500 μM and above) evoked channel closure. In each case the reduced forms of the dinucleotides were slightly more effective than the nonreduced forms. Unlike the ATP-K^+ channel (Dunne et al., 1988) there was no activation of the Ca-NS^+ channel at low concentrations of pyridine nucleotides (1–10 μM). The levels of endogenous pyridine nucleotides in the unstimulated cell are within the micromolar range (β-NADH, 20–70 μM; β-NAD^+, 200–350 μM; β-NADPH, 30–70 μM; and β-$NADP^+$, 30–100 μM; Dunne et al., 1988), while the levels of ATP and ADP are 5 and 2 mM, respectively (Malaisse et al., 1979; Kakei, Kelly & Ashcroft; 1986). Thus, since the activating effects of the pyridine nucleotides seem to be blocked by 4 mM ATP plus 1 mM ADP, Dunne et al. (1988) concluded that the only effects that would be present in the cell would be the inhibitory effect of β-NAD^+. However, they point out that this conclusion might be altered by the results of Malaisse and Sener (1987) who reported that the levels of ATP and ADP in the cytosol may be less than 0.5 mM and less than 0.2 mM, respectively. Under these conditions where the concentrations of ATP and ADP would not inhibit the stimulatory effect of the pyridine nucleotides, both the stimulatory effects of β-$NADP^+$, β-NADH and β-NADPH would also be expressed, as would the overall inhibitory effects of β-NAD^+. One possible physiological role for β-NAD^+ would be that in the intact cell it may contribute to the tonic inhibition of the ATP-K^+ channel. Since the Ca-NS^+ channel is inhibited by the same concentration range of pyridine nucleotides as the ATP-K^+ channel, it is likely that it too will be tonically modulated by them in the intact cell. However, the lack of specificity of the pyridine nucleotides on the Ca-NS^+ channel would argue against any glucose-mediated changes in the [NAD(P)H/[$NAD(P)^+$] ratio being able to alter the activity of this channel. Nevertheless, the functional significance of the modulation of the Ca-NS^+ channel by pyridine nucleotides can only be speculated on at present until their effects have been assessed in the presence of physiologically relevant concentrations of ATP (and ADP).

Since both the adenine nucleotides and the pyridine nucleotides are capable of reducing the activity of the Ca-NS^+ channel and since the nicotinamide-adenine dinucleotides, β-NAD^+ and β-NADH, contain the structure of AMP, this raises the question of whether they are all acting at the same site or on different sites on the channel. Low concentrations of adenine nucleotides have been shown to activate the Ca-NS^+ channel (Reale et al., 1994), while a parallel effect was not observed for the nicotinamide-adenine dinucleotides. Consequently, it seems likely that the activation site for adenine nucleotides is separate from the pyridine nucleotide site, unless the pyridine nucleotides can bind to, but not activate, this site. In the absence of specific agents to block the inhibition of Ca-NS^+ channel activity by either adenine nucleotides or pyridine nucleotides, it is impossible to

distinguish if they are acting on separate sites on the Ca-NS^+ channel.

It is also possible that some of the effects of β-NAD^+ described in the present paper could result from its enzymatic breakdown to active metabolites. There is currently much interest in the role of cyclic ADP ribose, a metabolite of β-NAD^+, in modulating calcium release from ryanodine-sensitive stores (*see* Berridge, 1994). Although this mechanism seems unlikely to function in isolated membrane patches, further experiments are required to rule out the possibility that enzymatic breakdown of β-NAD^+ is not involved in the regulation of Ca-NS^+ channel activity.

An unexpected finding of the present study was that β-NAD^+, in contrast to the other pyridine nucleotides studied, induced the opening of a new channel with a single channel current amplitude of 3.9 pA and a conductance of 80–90 pS (n = 9). The β-NAD^+-induced channel has some similarities in its properties with the Ca-NS^+ channel since they are both nonselective cation channels that are activated by Ca^{2+} and sensitive to AMP. The exact physiological role of the β-NAD^+-NS^+ channel is not known at present and it has not been established whether it is present in β-cells. The possibility that the β-NAD^+-NS^+ channel is really a different form of the Ca-NS^+ channel whose conductance has been changed in the presence of β-NAD^+ seems unlikely since both channels can be shown to be present at the same time in many patches. In addition, preliminary experiments (*data not shown*) suggest that 0.1 mM AMP can completely block Ca-NS^+ channel activity in patches where the β-NAD^+-NS^+ channel is still partially active. One can speculate that this β-NAD^+-NS^+ channel contributes to the cell depolarization during insulin secretion evoked by metabolizable substrates. However, if the ideas of Matschinsky *et al.* (1986) are correct and increases in the glucose concentrations around β-cells cause an increase in the [NADH]/[NAD^+] ratio, then this would reduce the effective concentration of β-NAD^+ and make the β-NAD^+-NS^+ channel less likely to open. Under such circumstances the β-NAD^+-NS^+ channel would be unlikely to be involved in insulin release, since its contribution to the general depolarizing background current would be decreased.

The pyridine nucleotides, such as β-NAD^+ and β-$NADP^+$ are ubiquitous coenzymes for a wide variety of enzymes involved in metabolism. β-NAD^+ undergoes a reduction to β-NADH during oxidative degradation reactions and it is the subsequent oxidation of NADH by the cytochromes of the electron transport chain that constitutes the principal source of ATP in aerobic organisms. Conversely, β-$NADP^+$ is confined, with very few exceptions, to the reactions of reductive biosynthesis, such as the synthesis of fatty acids and steroids, and the synthesis of sugars during photosynthesis (Scrutton et al., 1990). It is interesting that both coenzymes are now emerging as potential direct modulators of ion channels, extending their role to encompass that of coupling factors between metabolism and membrane potential-dependent processes. To understand the physiological significance of the newly discovered β-NAD^+-NS^+ channel, it will be important to study its nucleotide sensitivity in detail, including possible modulation by cyclic nucleotides since the Ca-NS^+ channel is sensitive to cyclic nucleotides (Reale et al., 1992, 1994) and to look for its presence and frequency of occurrence in β-cells. At present, the physiological significance to the cell of the inactivation of one type of nonselective cation channel and the activation of another by β-NAD^+ is not clear. In the future it will also be important to determine if either the β-NAD^+-NS^+ or the Ca-NS^+ channels is involved in the control of the release of insulin.

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References

Ashcroft, S.J.H. 1980. Glucoreceptor mechanisms and the control of insulin release and biosynthesis. *Diabetologia* **18:**5–15

Ashcroft, F.M., Ashcroft, S.J.H., Harrison, D.E. 1987. Effects of 2-ketoisocaproic acid on insulin secretion and single potassium channel activity in dispersed rat pancreatic β-cells. *J. Physiol.* **385:**517–529

Ashcroft, F.M., Ashcroft, S.J.H., Harrison, D.E. 1988. Properties of single potassium channels modulated by glucose in rat pancreatic β-cells. *J. Physiol.* **400:**501–527

Ashcroft, F.M., Harrison, D.E., Ashcroft, S.J.H. 1984. Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. *Nature* **312:**446–448

Ashcroft, F.M., Christie, M.R. 1979. Effects of glucose on the cytosolic ratio of reduced/oxidised nicotinamide-adenine dinucleotide phosphate in rat islets of Langerhans. *Biochem. J.* **184:**697–700

Ashcroft, F.M., Rorsman, P. 1991. Electrophysiology of the pancreatic β-cell. *Prog. Biophys. Molec. Biol.* **54:**87–143

Berridge, M.J. 1993. A tale of two messengers. *Nature* **365:**388–389

Carrington, C.A., Rubery, E.D., Pearson, E.C., Hales, C.N. 1986. Five new insulin-producing cell lines with differing secretory properties. *J. Endocrinol.* **109:**193–200

Cook, D.L., Satin, L.S., Ashford, M.L.J., Hales, C.N. 1988. ATP-sensitive K^+ channels in pancreatic β-cells. Spare-channel hypothesis. *Diabetes* **37:**495–498

Dempster, J. 1988. Computer analysis of electrophysiological signals. *In:* Microcomputers in Physiology: A Practical Approach. P.J. Fraser, editor. pp. 51–93. IRL, Oxford

Dunne, M.J., Findlay, I., Petersen, O.H. 1988. The effects of pyridine nucleotides on gating of ATP-sensitive K^+-channels in insulin-secreting cells. *J. Membrane Biol.* **102:**205–216

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high resolution recording from cells and cell-free membrane patches. *Pfluegers Arch.*, **391:**85–100

Hedeskov, C.J., Capito, K., Thams, P. 1987. Cytosolic ratios of free [NADPH]/[$NADP^+$] and [NADH]/[NAD^+] in mouse pancreatic islets, and nutrient-induced insulin secretion. *Biochem. J.* **241:**161–167

Kakei, M., Kelly, R.P., Ashcroft, F.M. 1986. The ATP-sensitivity of K^+ channels in rat pancreatic β-cells is modulated by ADP. *FEBS Lett.* **208:**63–66

Kozlowski, R.Z., Hales, C.N., Ashford, M.L.J. 1989. Dual effects of diazoxide on ATP-K^+-currents recorded from an insulin-secreting cell line. *Br. J. Pharmacol.* **97:**1039–1050

Malaisse, W.J., Hutton, J.C., Kawazu, S., Herchuelz, A., Valaerde, I., Sener, A. 1979. The stimulus secretion coupling of glucose induced insulin release: the links between metabolism and cationic events. *Diabetologia* **16:**331–341

Malaisse, W.J., Sener, A. 1987. Glucose induced changes in cytosolic ATP content in pancreatic islets. *Biochim. Biophys. Acta* **927:**190–195

Matschinsky, F.M., Gosh, A.K., Meglasson, M.D., Prentki, M., June, V., von Allman, D. 1986. Metabolic concomitants in pure pancreatic beta cells during glucose-stimulated insulin secretion. *J. Biol. Chem.* **261:**14057–14061

Panten, U., Christians, J., von Kriegstein, E., Poser, W., Hasselblatt, A. 1973. Effect of carbohydrates upon fluorescence of reduced pyridine nucleotides from perifused isolated pancreatic islets. *Diabetologia* **9:**477–482

Reale, V., Hales, C.N., Ashford, M.L.J. 1992. Cyclic AMP regulates a calcium-activated non-selective cation channel in a rat insulinoma cell line. *J. Physiol.* **446:**312*P*

Reale, V., Hales, C.N., Ashford, M.L.J. 1994. Nucleotide regulation of a calcium-activated cation channel in a rat insulinoma cell line, CRI-G1. *J. Membrane Biol.* **141:**101–112

Rorsman, P., Arkhammar, P., Bokvist, K., Hellerström, C., Nilsson, T., Welsh, M., Welsh, N., Berggren, P.-O. 1989. Failure of glucose to elicit a normal secretory response in fetal pancreatic beta cells results from glucose insensitivity of the ATP-regulated K^+ channels. *Proc. Natl. Acad. Sci. USA* **86:**4505–4509

Scrutton, N.S., Berry, A., Penham, R.N. 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. *Nature* **343:**38–43

Sturgess, N.C., Hales, C.N., Ashford, M.L.J. 1986. Inhibition of a calcium-activated non-selective cation channel, in a rat insulinoma cell line, by adenine derivatives. *FEBS Lett.* **208:**397–400

Sturgess, N.C., Hales, C.N., Ashford, M.L.J. 1987. Calcium and ATP regulate the activity of a non-selective cation channel in a rat insulinoma cell line. *Pfluegers Arch.* **409:**607–615

Wollheim, C.B., Dunne, M.J., Peter-Riesch, B., Bruzzone, R., Pozzan, T., Petersen, O.H. 1988. Activators of protein kinase C depolarize insulin-secreting cells by closing K^+ channels. *EMBO J.* **7:**2443–2449