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Effects of Pyridine Nucleotides on the Gating of ATP-Sensitive Potassium Channels in Insulin-Secreting Cells

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Summary. The single-channel current recording technique has been used to study the influences that the pyridine nucleotides NAD, NADH, NADP and NADPH have on the gating of ATP-sensitive K^+ channels in an insulin-secreting cell line (RINm5F). The effects of the nucleotides were studied at the intracellular surface using either excised inside-out membrane patches or permeabilized cells. All four pyridine nucleotides were found to evoke similar effects. At low concentrations, 100 μM and less, each promoted channel opening whereas high concentrations, 500 μM and above, evoked channel closure. The degree of K^+ channel activation by pyridine nucleotides (low conc.) was found to be similar to that evoked by the same concentrations of ADP or GTP, whereas the degree of K^+ channel inhibition (high conc.) was less marked than that evoked by the same concentrations of ATP, and never resulted in refreshment of K^+ channels following removal. The effects of NAD, NADH, NADP and NADPH seemed to interact with those of ATP and ADP. In the presence of 1 mM ADP and 4 mM ATP, 10 to 100 μM concentrations of the pyridine nucleotides could not evoke channel opening, whereas concentrations of 500 μM and above were found to evoke channel closure. In the presence of 2 mM ATP and 0.5 mM ADP, however, 10 to 100 μM concentrations of the pyridine nucleotides were able to activate K^+ channels.

Key Words K^+ channel · ATP · NAD(P) · NAD(P)H · RINm5F cell

Introduction

It is now widely considered that the metabolism of glucose by pancreatic B-cells is the physiological signal for the release of insulin (Malaisse, et al., 1979*b*; Ashcroft, 1980; Hedeskov, 1980; Meglasson & Matschinsky, 1986; Wollheim & Biden, 1987). Carbohydrate-induced insulin release is associated with a depolarization of the plasma membrane, mediated via a decrease in the permeability to K^+ (Dean & Matthews, 1970; Sehlin & Täljedal, 1975; Meissner, 1976; Henquin & Meissner, 1984), a prerequisite for the opening of voltage-gated calcium channels (Matthews & Sakamoto, 1975). Recent studies carried out using the single-channel current-recording technique have shown that the initial depolarization of insulin-secreting cells is brought about by the closure of a specific class of K^+-selective channel (Ashcroft, Harrison & Ashcroft, 1984; Rorsman & Trube, 1985; Dunne et al., 1986; Misler et al., 1986; Petersen & Findlay, 1987). The gating of these channels can be influenced by a number of nucleotides including ATP (Cook & Hales, 1984; Findlay, Dunne & Petersen, 1985; Findlay & Dunne, 1986; Dunne, Illot & Petersen, 1987; Ribalet & Ciani, 1987), ADP (Dunne & Petersen, 1986*a*; Kakei et al., 1986) and GTP and GDP (Dunne & Petersen, 1986*b*).

The physiological link(s) that couple the metabolism of glucose and other carbohydrates to the release of insulin, via the closure of the nucleotide-sensitive K^+ channel, is unknown. One hypothesis is that the K^+ channel closure is mediated through the pyridine nucleotides: reduced and nonreduced nicotinamide-adenine dinucleotide (NADH and NAD, respectively) and reduced and nonreduced nicotinamide-adenine dinucleotide phosphate (NADPH and NADP, respectively), and in particular via changes in the redox ratio [NAD(P)H]/NAD(P)] (Panten et al., 1973). Indeed there is evidence to suggest that changes in the concentration ratios NADH/NAD and NADPH/NADP do take place when insulin-secreting cells are exposed to glucose or other insulin-secretagogues (Ashcroft & Christie, 1979; Malaisse et al., 1979*a,b*; Malaisse et al., 1982; Sener et al., 1984; Matschinsky et al., 1986; Hedeskov, Capito & Thams, 1987).

In this study we have employed the single-channel current recording technique to study the influence of NAD, NADP, NADH and NADPH on the gating of nucleotide-sensitive K^+ channels in the clonal insulin-secreting cell line RINm5F.

Materials and Methods

Cell Isolation and Maintenance

All experiments were carried out on the insulin-secreting cell line RINm5F (Halban, Praz & Wollheim, 1983; Praz et al., 1983) maintained as previously described (Dunne et al., 1986).

Media

Patch-clamp recording pipettes (Type 101-PB, Ceebee Glass, Denmark) coated with Sylgard® (Dow Corning Corp., USA), had a final resistance of around 5 MΩ when filled with a Na^+-rich solution of the following composition (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 2.5 glucose, 10 HEPES and 1 EGTA. No calcium was added and the pH was set at 7.2 (NaOH). Initially, cells were maintained in the Na^+-rich solution containing 2 mM $CaCl_2$ and no added EGTA. Prior to cell permeabilization or patch excision this solution was switched to a K^+-rich fluid of the following composition (mM): 140 KCl, 1.13 $MgCl_2$, 2.5 glucose, 10 HEPES and 1 EGTA. No calcium was added and the pH was set at 7.2 (KOH). The osmolality of all solutions was 290 ± 5 mosm/kg.

Recording and Analysis

Single-channel current records were obtained, using the principles described by Hamill et al. (1981), from both permeabilized or open cells and excised inside-out membrane patches, as previously described in detail from our laboratory (Findlay et al., 1985*a*; Dunne et al., 1986, 1987). Gigaohm seal formation was achieved in the continuous flow of the K^+-rich bathing solution from one of a series of eight outlet pipes, the other pipes containing various test solutions. The internal diameter of each outlet was around 150 μm. Exchange of solution from control to test was achieved manually under visual control.

The open-cell recording configuration was obtained by exposing the cell-attached membrane patch very briefly to a concentration of saponin less than 0.05% (wt/vol) through the use of a blunted patch-clamp pipette, placed at right angles to the outlet pipes (Dunne et al., 1987). Air exposure of membrane patches from open cells invariably led to the formation of an excised inside-out membrane patch.

Analysis of data to determine channel open-state probability was performed on stretches of continuous current records (filtered at 1 kHz, low pass) lasting between 10 and 20 sec. Open-state probability determinations were restricted to records, where at the time of analysis, no more than 6 coincident K^+ channel events were recorded. All data was digitized at 8 kHz (CED, 1401, Cambridge, U.K.) and analyzed using a Tandon microcomputer in conjunction with a software package supplied by CED Cambridge, U.K. Idealized current records were obtained from computerized threshold analysis using a preselected threshold current level. The open-state probability values presented have been determined based on the maximal number of coincident K^+ channel events recorded initially, seconds after the permeabilization of the cell or patch excision, i.e. before channel run-down had taken place.

In the record presented in Fig. 7, the number of active K^+ channels exceeded the limitation of the analysis system and hence the results are expressed as maximal patch-current values, i.e. the peak current recorded in each particular situation.

In all patch-clamp current records illustrated, upward deflections represent outward-current flow (i.e. from the inside to the outside of the patch membrane), the pipette voltage in all cases was held at 0 mV throughout and all current records photographed directly from the oscilloscope screen, have been filtered at 400 Hz (low pass).

Results

Effects of NAD, NADP, NADH, and NADPH Alone

The massive increase in patch current observed when either an excised inside-out or outside-out membrane patch is formed (Findlay et al., 1985*b*) or when an open-cell is generated (Findlay & Dunne, 1986; Dunne et al., 1986), is mediated by openings of the nucleotide-sensitive K^+ channels. The influence of NAD, NADP, NADH and NADPH on the gating of these channels is illustrated in Figs. 1 and 2.

Figure 1 shows how channels are influenced by 100, 500 and 1000 μM NAD. The addition of 500 and 1000 μM NAD to the membrane inside was found to evoke dose-dependent closure of K^+ channels whereas 100 μM NAD applied to the same patch of membrane resulted in opening of the channels. In the record shown, 500 μM NAD reduced the open-state probability (P) of K^+ channels from 0.061 to 0.024, i.e. a decrease to 40% of the control level of activity (100%). 1000 μM NAD evoked a further reduction to about 18% of the control, by lowering P from 0.053 to 0.0087. When 100 μM NAD was added P was increased from 0.013 to 0.018, 138% of the prestimulus level of activity. The effects of all three concentrations of NAD were reversible; removal of NAD and returning the membrane to the control situation resulted in P values of 87, 68 and 75% of the prestimulus level of activity, respectively.

Similar results were found for NADH, NADP and NADPH as shown in Fig. 2. NADH (500 μM) reduced P from 0.13 to 0.069, to 53% of the control level, whereas 50 μM NADH increased P from 0.066 to 0.094, to 143% of the control value. 1000 μM NADP lowers P to 21% of the control value whereas 50 μM NADP increases P to 155% of the precontrol value (P was changed from 0.098 to 0.021 and 0.029 to 0.045, respectively). Similarly, 1000 μM NADPH, reduced P from 0.47 to 0.077, 16% of the control value, whereas 10 μM NADPH increased P from 0.17 to 0.28, 164% of the control value. The effects of each concentration of NADH, NADP and NADPH were found to be reversible upon return to the control situation.

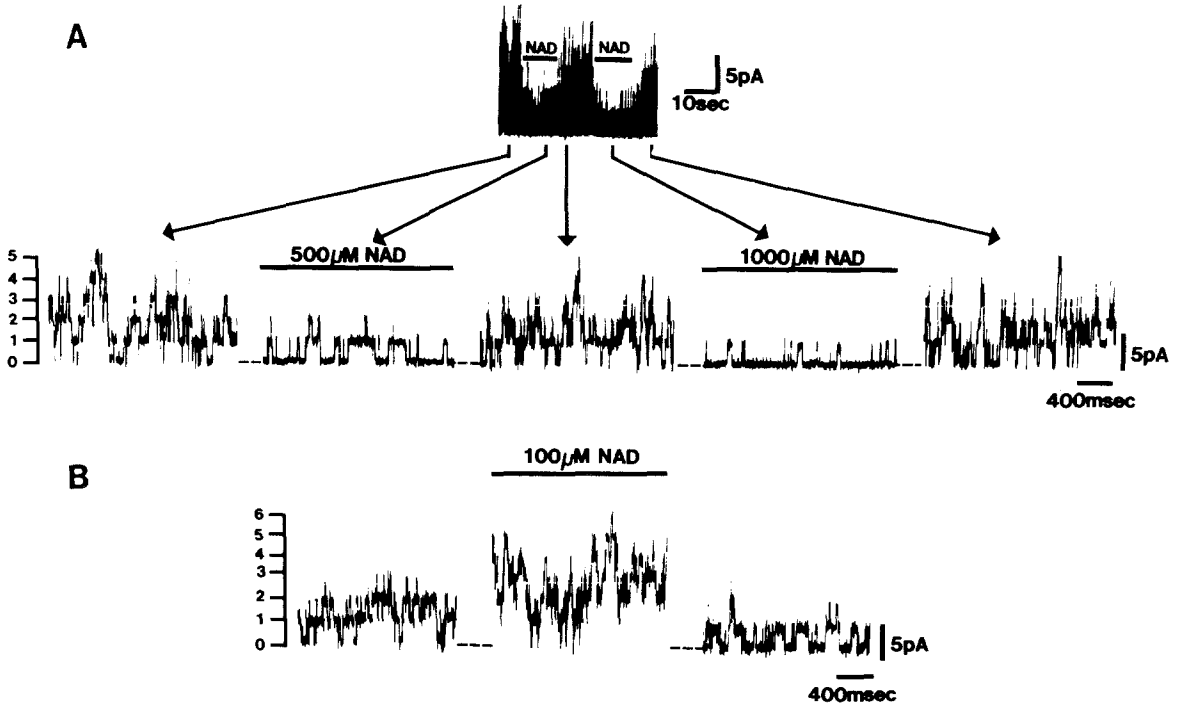

Fig. 1. Dual effects of NAD on ATP-sensitive K^+ channels in insulin-secreting cell line. All single-channel current recordings presented are from the same cell-attached membrane patch after permeabilization of the plasma membrane, outside of the isolated patch area, has taken place. Panel (*A*) shows that NAD is able to evoke a dose-dependent inhibition of channels at concentrations of 500 and 1000 μM. Panel (*B*) shows the stimulatory effect of 100 μM NAD. The continuous open-cell current record shown in (*A*) begins 320 sec after permeabilization of the cell with 0.05% saponin, which yielded an initial patch current of 50 pA. The interval between (*A*) and (*B*) is 200 sec. The scale bar on the left-hand side of each current trace corresponds to the number of coincident K^+ channel openings

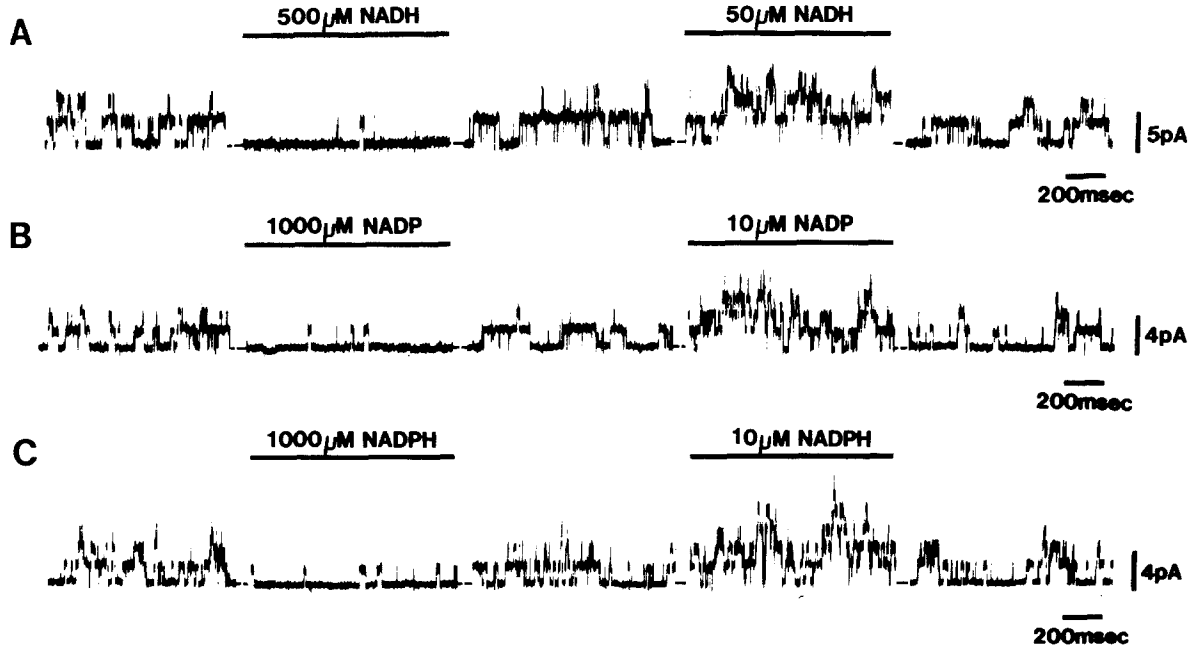

Fig. 2. Concentration-dependent inhibition and stimulation of K^+ channels by NADH, NADP and NADPH. Panel (*A*) shows that 500 μM NADH inhibits whereas 50 μM NADH stimulates K^+ channels in the same open-cell recording. The effects of each concentration of NADH are fully reversible upon removal of the nucleotide. Panels (*B*) and (*C*) show that NADP and NADPH, respectively, elicit effects similar to those of NADH at concentrations of 1000 and 10 μM. Records (*A*), (*B*) and (*C*) come from three separate membrane patches

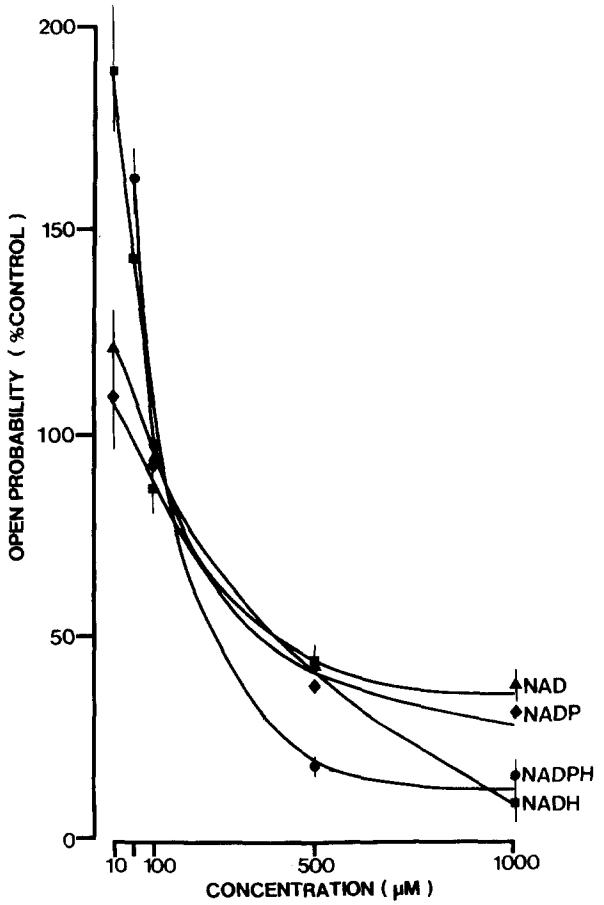

Fig. 3. Relationship between the open-state probability of K^+ channels and the concentration of NAD, NADP, NADH and NADPH. The open probability has been expressed as a percentage of the prestimulus control level of activity (100%). Solid squares represent data points for NADH, solid circles NADPH, solid diamonds NADP and solid triangles NAD. Standard error bars have been added wherever the error is greater than the symbol used. All lines have been fitted by eye

The data presented in Figs. 1 and 2 were found to be typical of 85 and 61 separate membrane-patch recordings, respectively, obtained from either open cells or excised inside-out membrane patches. 99% of all applications of 10 or 50 μM NAD (added 79 times) and 80% of all applications of 100 μM NAD (added 25 times) resulted in channel activation. High concentrations of NAD (500, 1000 or 5000 μM) consistently evoked channel inhibition in every one of the 84 applications. The effects of NADP, NADH and NADPH were similar to those described for NAD since 500, 1000 and 5000 μM concentrations of each consistently inhibited K^+ channels in everyone of the 30 (NADH), 53 (NADP) and 31 (NADPH) applications. NADH and NADPH in concentrations ranging between 10 and 50 μM, evoked activation of K^+ channels in 89% of all applications (added 27 and 28 times, respectively), whereas NADP and NAD in the same concentrations, activated channels in 95% (n = 19) and 99% (n = 79) of the applications, respectively.

Figure 3 shows graphically the relationship between channel open-state probability, expressed as a percentage of the prestimulus level of activity and the concentration of each of the nucleotides. The data presented are based upon analysis of 23 separate open-cell records, where a number of concentrations of each of the nucleotides were used. On average, 1 mM concentrations of NAD, NADP, NADH and NADPH reduced P to 39 ± 5% (SE) (n = 7 patches), 32 ± 5% (n = 8), 9 ± 4% (n = 5) and 16 ± 5% (n = 5) of the initial control value, respectively. Similar results were found with 0.5 mM concentrations of NAD, NADP, NADH and NADPH which reduced P values to 42 ± 6% (n = 3), 38 ± 6% (n = 3), 44 ± 6% (n = 3) and 19 ± 8% (n = 3) of the control, respectively. Upon removal of the nucleotides P increased to 69 ± 18% (n = 3), 75 ± 12% (n = 3), 86 ± 14% (n = 3) and 98 ± 14% (n = 3), respectively.

Data analysis of the records obtained using 0.1 mM concentrations of NAD, NADP, NADH and NADPH may appear to suggest that these concentrations had little effect, as the average value of P was 98 ± 3% (n = 4), 94 ± 4% (n = 3), 86 ± 6% (n = 4) and 95 ± 5% (n = 3) of the prestimulatory level of activity, respectively. Although clear inhibitory effects of 0.1 mM concentrations of the pyridine nucleotides were obtained in a number of individual experiments, the majority of experiments showed clear activating effects of NAD, NADP, NADH and NADPH. This discrepancy between the overall data analysis and the inspection of individual records results from the fact that in the patches of membrane analyzed the stimulatory effects of the nucleotides are masked by the inherent run-down of K^+ channels. This explanation is supported by the fact that on return to the control situation the open-state probability was found to be, on average, only 65 ± 12% (n = 4), 72 ± 9% (n = 3), 71 ± 11% (n = 4) and 76 ± 10% (n = 3) of the initial control value for NAD, NADP, NADH and NADPH, respectively.

The activating effects of 10 μM concentrations of NAD, NADP, NADH and NADPH are also shown. P increased to 120 ± 10% (n = 4), 110 ± 12% (n = 5), 189 ± 15% (n = 3) and 159 ± 4% (n = 4) of the control, respectively, whereas P values decreased to 95 ± 6% (n = 4), 96 ± 12% (n = 5) 102 ± 15% (n = 3) and 90 ± 14% (n = 4), respectively, upon return to the control solution.

The effects of 50-μM concentration of NADH and NADPH were also analyzed in six separate patches. NADH increased P to 143 ± 10% and NADPH to 165 ± 9% of the prestimulatory control values.

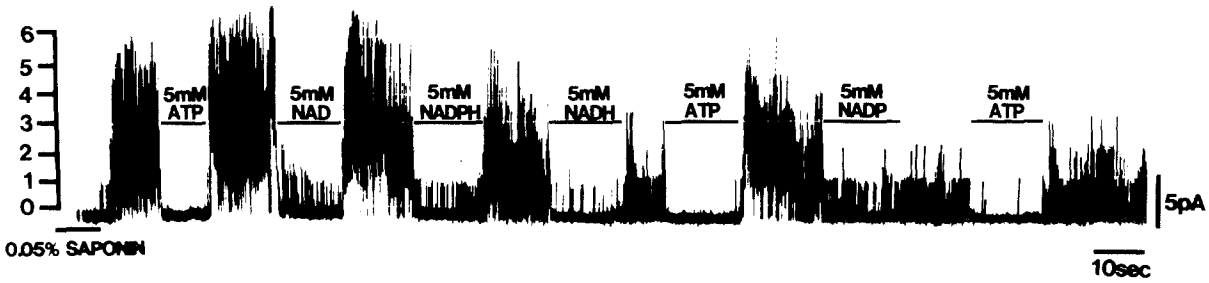

Fig. 4. Comparison of the inhibitory effects of pyridine nucleotides with ATP. The continuous trace shown begins 60 sec after the formation of a gigaohm seal. Permeabilization of the cell yielded an open cell with an initial patch current of 15 pA. Adding 5 mM concentrations of ATP, NAD, NADP, NADH and NADPH reversibly reduced single-channel currents to varying degrees, with 5 mM ATP having the most powerful inhibitory influence. The scale bar on the left-hand side of the current trace corresponds to the number of coincident K^+ channel openings

Figure 4 shows the result of a typical experiment in which the inhibitory effects of 5 mM concentrations of NAD, NADP, NADPH and NADH, respectively, are compared to the inhibitory effect of 5 mM ATP. Briefly exposing the intact cell to 0.05% saponin resulted in the activation of a large K^+ current consisting of coincident openings from several channels ($P = 0.13$). This current is completely abolished when 5 mM ATP is added to the bath ($P = 0$). Removal of ATP generates refreshment of channel activity as previously described (Findlay & Dunne, 1986) and P increased to 0.14. The subsequent application of NAD, NADPH, NADH and NADP consistently evoked inhibition of K^+ channels, reducing P from 0.14 to 0.012 (9% of the control value), from 0.12 to 0.015 (13% of the control) from 0.12 to 0.017 (14% of the control) and from 0.14 to 0.015 (11% of the control), respectively. K^+ channel closure evoked by each of the pyridine nucleotides was less marked than the degree of inhibition evoked by the same concentration of ATP, which reduced P to on average 0.026 ± 0.01% ($n = 3$) of the initial control values. The pyridine nucleotides also failed to evoke refreshment of K^+ channels following their removal, so typical of the effects of ATP, which increased P to 107, 378 and 131% of the control value upon its removal, whereas following removal of NAD, NADPH, NADH and NADP P values returned to 91, 84, 98 and 89% of the control, respectively.

The data shown in Fig. 4 are representative of nine separate membrane-patch recordings. On average, based on data obtained from four separate membrane patches, NADH reduced P to 6 ± 3% of the prestimulus level of activity, NADPH to 7 ± 2%, NADP to 11 ± 3% and NAD to 28 ± 8%. ATP proved to be the most powerful inhibitor of the channels, reducing on average P to 0.03 ± 0.001% of the control level of activity.

Figure 5 shows the result of an experiment were the stimulatory effects of NAD were compared to those evoked by ATP, ADP and GTP. The data illustrated is typical of 13 separate open-cell recordings where 50-μM concentrations of each of the nucleotides were applied 75 times and consistently evoked channel activation. In the example illustrated, GTP increased the maximal patch current to 285% of the prestimulus level, ADP to 242%, ATP to 342% and NAD to 218%. All effects were reversible after removal of the nucleotides. Quantitative analysis of a further five separate membrane patches revealed that on average 50 μM NAD increased the patch current to 167 ± 24% of the prestimulus control level of activity (100%), 50 μM ADP increased it to 172 ± 16% and 50 μM GTP to 126 ± 25%. Again all effects were reversible, as upon return to the control environment removing NAD lowered the current to 110 ± 30% of the initial control and ADP and GTP to 76 ± 20% and 60 ± 10%, respectively.

Inhibition of K^+ Channels by Pyridine Nucleotides using Different Lactate/Pyruvate and Malate/Pyruvate Ratios

One interpretation of the findings presented in Figs. 1 to 4, that essentially each of the pyridine nucleotides acts in the same manner, is that in solution only one of the forms of each of the two classes is stable, i.e. either NAD and NADP are quickly transformed into NADH and NADPH or vice versa. Malic enzyme and lactate dehydrogenase may be present in the open-cell preparation and could, therefore, catalyze such conversions. On the assumption that these two enzymes are active in the permeabilized cells, we have carried out a series of experiments in which the attempt has been made to clamp each of the nucleotides in their two forms,

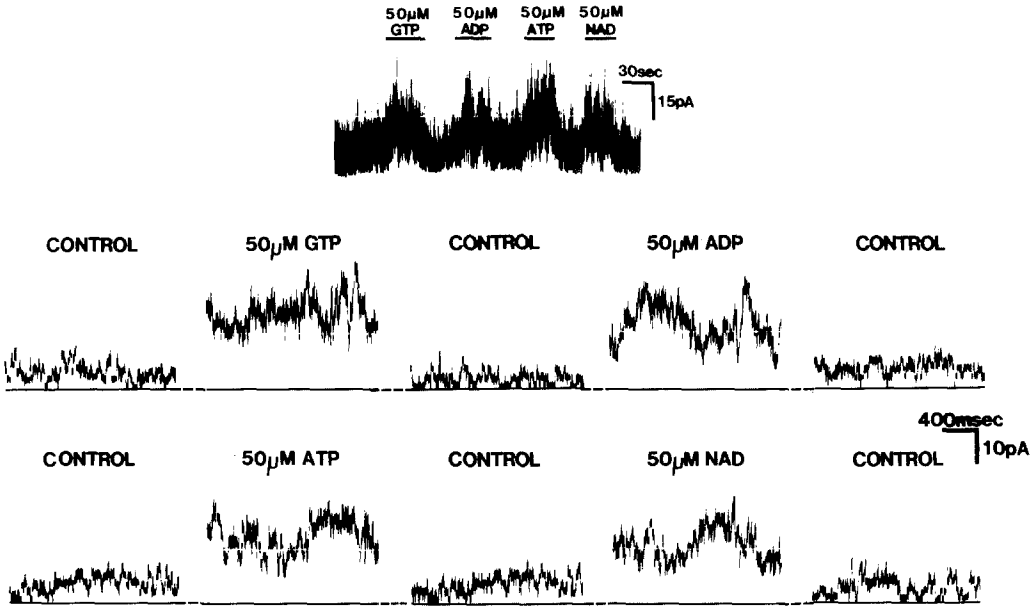

Fig. 5. Comparison of the stimulatory effects of 50 μM concentrations of GTP, ATP, ADP and NAD. The continuous trace shown begins 450 sec after formation of an open cell which evoked an initial patch current of 140 pA. The insets showing traces at higher time resolution come from the periods indicated.

respectively, through the use of different ratios of their substrates (Hedeskov et al., 1987). The results of two of these experiments are shown in Fig. 6.

Figure 6(*A*) shows that K^+ channels inhibited to 46% of their prestimulus level of activity by 1 mM NADPH in the presence of a $1:10^{-3}$ ratio of malate/pyruvate, are unaffected when this ratio is switched to $10^{-3}:1$ (P = 40% of control) and back again to $1:10^{-3}$ (P = 29% of precontrol). P was also unaffected (36% of control) by the removal of the malate/pyruvate solutions from the inside of the plasma membrane in the continued presence of 1 mM NADPH. Finally returning the membrane to the prestimulus control solution evoked a recovery of channel activity to 65% of the initial control level of activity. The data shown in Fig. 6(*A*) is typical of four separate membrane-patch recordings where this experiment was carried out six times.

Figure 6(*B*) shows a similar experiment in which a lactate/pyruvate solution was used either to attempt to prevent NADH formation from NAD or conversely to encourage this transformation. The gating of NAD-inhibited K^+ channels was not affected by (i) the introduction of a $1:10^{-5}$ ratio of lactate/pyruvate, in the continued presence of 1 mM NAD (P = 50% of the initial control value), (ii) by changes in the ratio from $1:10^{-5}$ (P = 49%), to $10^{-5}:1$ (P = 55%) and back to $1:10^{-5}$ (P = 50%), and finally (iii) channel gating was unaffected by the removal of the lactate/pyruvate solution in the continued presence of NAD (P = 41%). The effects were reversible since the channel activity was 100% of the prestimulus level of activity after returning to the control solution. These results are typical of seven separate open-cell recordings where this experiment was carried out a total of 17 times.

In a control series of experiments channel activity was not influenced by any of the malate/pyruvate or lactate/pyruvate solutions, at concentrations ranging from 1×10^{-8} to 1×10^{-2} M, in the absence of pyridine nucleotides (14 times in 12 separate open-cell recordings).

Interactions Between ATP and NAD, NADP, NADH and NADPH

Figure 7 shows the result of a typical experiment in which interactions between a pyridine nucleotide and ATP were studied.

In Fig. 7(*A*) 5 mM NAD is seen to close K^+ channels. Incomplete block typically resulted (*see also* Fig. 4), with P being reduced by NAD from 5.1×10^{-2} to 3.9×10^{-3}, or some 8% of the control level of activity. 5 mM ATP added to the bath in the presence of NAD further suppressed P to 0.04% of the control level (2.1×10^{-5}). After ATP removal the level of channel activity was higher than that observed prior to addition, increasing P to 24% of the control level (1.2×10^{-2}), indicating that even in the presence of an inhibitory dose of NAD, ATP is still able to evoke refreshment of K^+ channels. Fi-

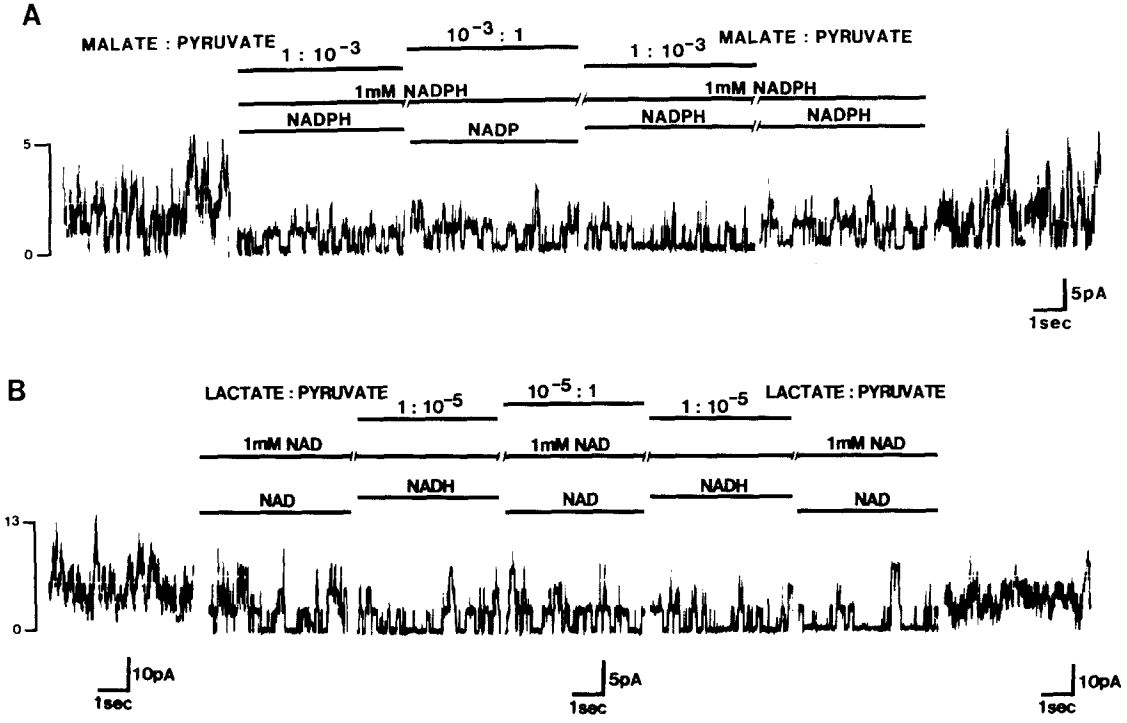

Fig. 6. K^+ channel inhibition by pyridine nucleotides at different lactate/pyruvate and malate/pyruvate concentration ratios. Panel (*A*) shows how the gating of K^+ channels in an open cell is inhibited by 1 mM NADPH and a $1 : 10^{-3}$ ratio of malate/pyruvate (1 and 10^{-3} mM concentrations, respectively) but is unaffected when the ratio is changed from $1 : 10^{-3}$ to $10^{-3} : 1$ and back to $1 : 10^{-3}$. Panel (*B*) shows a similar experiment in which 1 mM NAD-inhibited K^+ channels are exposed to a $1 : 10^{-5}$ and a $10^{-5} : 1$ ratio of lactate/pyruvate (1 and 10^{-5} mM concentrations, respectively). The current traces shown in panels (*A*) and (*B*) come from two separate membrane patches and begin 65 and 55 sec, respectively, after permeabilization of the cells. The maximum number of coincident channel openings observed in each record was 6 and 15 for (*A*) and (*B*), respectively. The scale bars on the left-hand side of each current trace correspond to the number of coincident K^+ channel openings. Note in panel (*B*) that the vertical scale has been expanded during the test period

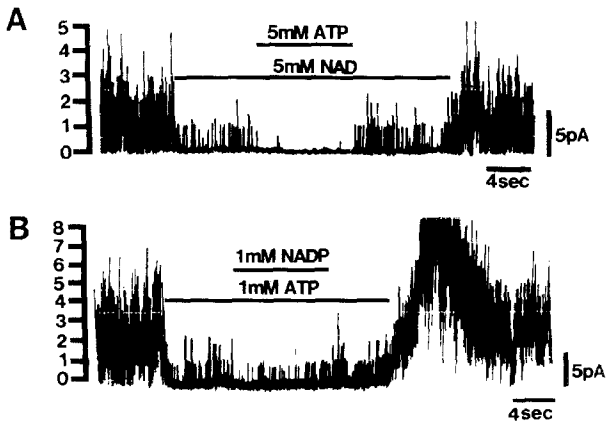

Fig. 7. Interactions between ATP, NAD and NADP. Panel (*A*) shows that NAD-inhibited channels can be further suppressed by ATP. Panel (*B*) shows the converse experiment, NADP suppression of ATP-inhibited channels. The two continuous traces shown come from separate open-cell recordings. Record (*A*) begins 220 sec after permeabilization and record (*B*) 400 sec after. The initial patch current observed in each case was 28 and 45 pA, respectively

nally, returning to the control situation increased *P* to 107% of the prestimulus level of activity. This finding is interesting (and observed in 10 out of 13 such experiments), as in 106 ATP-free experiments removal of inhibitory concentrations of the pyridine nucleotides never once evoked refreshment of channels (Fig. 4). Results similar to those presented in Fig. 7(*A*) were observed in six separate membrane patches in which interactions between NAD or NADP (at concentrations ranging from 500 μM to 5 mM) and ATP (1 to 5 mM) were studied 13 times.

Interactions between NAD, NADP and ATP were quantified in three additional patches. ATP was found to further suppress *P* of NAD-inhibited K^+ channels to 4 and 11% of the value during NAD exposure alone, and reduce *P* of NADP-inhibited K^+ channels to 15% of the value during NADP exposure. Upon the removal of ATP a refreshment of K^+ channels in the presence of NAD or NADP was always observed as the level of activity was increased to 180, 140 and 166%, respectively.

The converse experiment, Fig. 7(*B*), shows that

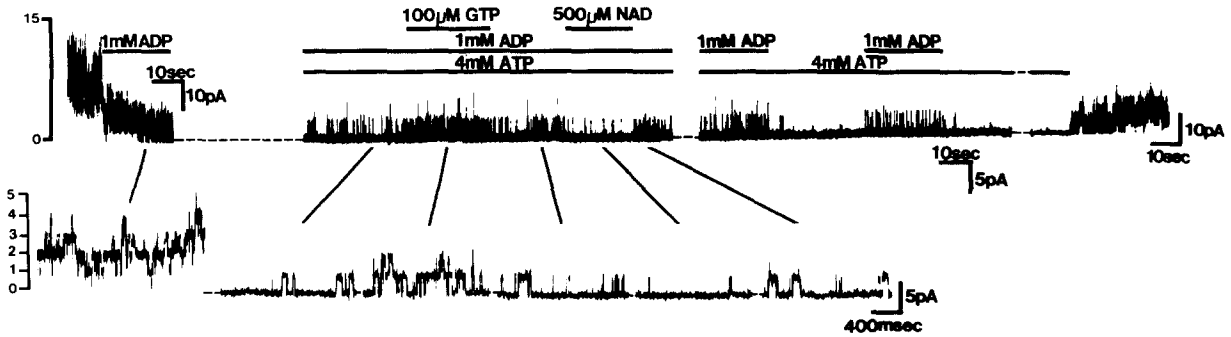

Fig. 8. NAD inhibition and GTP stimulation of K^+ channels in the presence of ATP and ADP. The continuous trace shown is taken from an inside-out membrane patch and begins 40 sec after excision of the patch from the intact cell. Channels inhibited by 1 mM ADP are further suppressed by the addition of 4 mM ATP. In the continuous presence of ATP and ADP the basal level of channel activity is enhanced by adding 100 μM GTP and further suppressed by the addition of 500 μM NAD. Removal of ADP in the presence of ATP completely abolishes channel activity. Channel reactivation occurs by the subsequent readmission of ADP. During the three periods indicated by the dotted lines the contents of the bath were drained and because of the artifacts generated these parts of the record have been omitted. Note that the vertical scale has been expanded during the test period

channel inhibition induced by 1 mM ATP to 14% (P = 0.007) of the prestimulus level of activity (P = 0.05), can be further suppressed by adding 1 mM NADP in the continued presence of ATP, reducing P to 10% (0.005). Removing NADP increased P to 12% (0.0062), which is further enhanced to 110% when ATP is finally removed.

NAD and NADP in 1 and 5 mM concentrations consistently evoked further closure of ATP-inhibited K^+ channels, seen seven times in four open cells; but at concentrations of 100 μM and less, neither NAD or NADP had any effect on channel gating in the presence of ATP, at 1 mM or greater concentrations studied in three separate membrane-patch recordings. In one typical experiment 5 mM ATP closed K^+ channels to 0.2% of the control level of activity, a value that remained constant upon the addition of NAD (10 μM) and its subsequent removal (0.21% and 0.25%, respectively).

Interactions Between ATP/ADP and NAD, NADP, NADH and NADPH

Figure 8 shows that in the continued presence of ATP and ADP, 500 μM NAD evokes inhibition of K^+ channels. When 1 mM ADP is added to the solution bathing the intracellular surface of an inside-out membrane patch the number of single-channel open events is significantly reduced. Further inhibition was evoked when ATP (4 mM) was added in the continued presence of ADP, resulting in an average P of $3.1 \pm 0.8 \times 10^{-3}$ (n = 3). In the presence of both ATP and ADP, 100 μM GTP was able to evoke channel activation, increasing P to an average of $9.9 \pm 1.3 \times 10^{-3}$ (n = 3) (whereas 500 μM NAD evoked channel inhibition, reducing P to on average $1.03 \pm 0.11 \times 10^{-3}$ (n = 3). The effects of both GTP and NAD were reversible. Removal of ADP in the presence of ATP inhibited K^+ channels and reduced P from $3.3 \pm 0.75 \times 10^{-3}$ to $0.09 \pm 0.01 \times 10^{-3}$ (n = 3), and reactivated K^+ channels upon its readmission, increasing P to an average of $1.75 \pm 0.20 \times 10^{-3}$ (n = 3).

The data presented in Fig. 8 show that in the absence of ATP, 1 mM ADP consistently inhibits channels whereas in the presence of ATP, ADP consistently stimulates K^+ channels. The data shown is typical of nine separate membrane-patch recordings where ADP-induced stimulation of ATP-inhibited channels was seen 24 times. In the presence of 4 mM ATP and 1 mM ADP, GTP (100 μM) stimulation was seen nine times in four patches and NAD inhibition of K^+ channels seen eight times in five separate patches.

Concentrations of NAD lower than 500 μM had no effect on channel activity in the presence of 4 mM ATP/1 mM ADP. NAD stimulation of K^+ channels was, however, seen when the concentrations of both ATP and ADP were lowered to 2 and 0.5 mM, respectively (Fig. 9). Initial permeabilization of the cell evoked coincident openings from at least six K^+ channels and the activity of these channels was completely suppressed by 2 mM ATP. Channel recovery by removal of ATP [P = 0.022 (100%)] was followed once again by ATP-evoked inhibition of K^+ channels, lowering P to 0.013% of the control level. Adding 0.5 mM ADP in the presence of ATP reactivated the ATP-inhibited channels and increased P to 2.7% of the initial control. P was further increased, up to 17.5%, when 50 μM NAD was added. Removal of NAD lowered P to 0.9%, which was decreased further, to 0.07%, upon the complete removal of ADP in the continued presence of ATP.

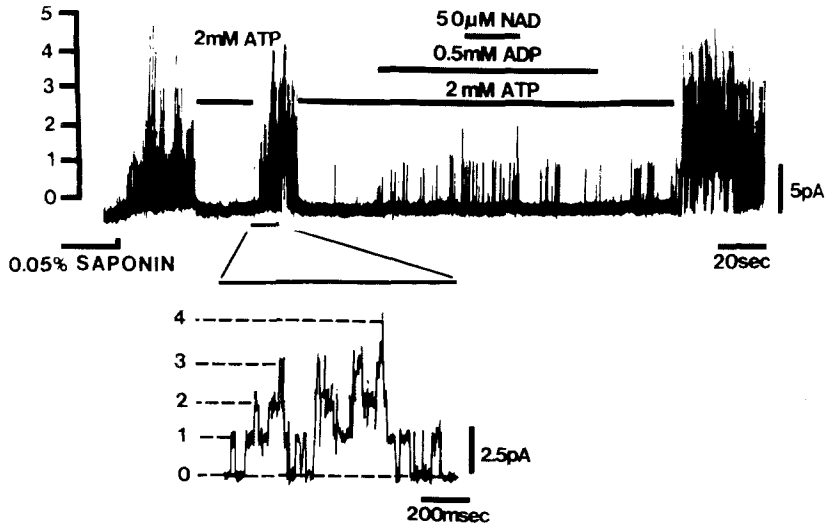

Fig. 9. Activation of K^+ channels by 50 μM NAD in the presence of a 4:1 ratio of ATP/ADP. In the open-cell current record shown, K^+ channel inhibition by 2 mM ATP is followed in the continual presence of ATP by channel stimulation after addition of 0.5 mM ADP. 50 μM NAD in the presence of ATP and ADP brings about further channel stimulation. The record begins 30 sec after the formation of a gigaohm seal between cell and the patch-clamp pipette. The initial patch current was 16 pA

Finally, returning to the control situation evoked a profound stimulation of K^+ channels, activating them to around 300% of the initial control level of activity. In a similar experiment ATP closed channels to 0.04% of the control level of activity, ADP increased P in the continued presence of ATP to 7%, which was further enhanced when 50 μM NAD was added, increasing P to 13% of the initial control. The effects were reversible; when NAD was removed P dropped to 7%, and when ADP was removed in the presence of ATP it was lowered further to 0.013%. Once again a rebound of channel activity was observed when all nucleotides were removed from the membrane inside, as P increased to 290% of the initial control value.

Figure 9 is typical of eight separate membrane patches. ADP (0.5 mM) stimulated K^+ channels in the presence of 2 mM ATP on all 22 occasions tested. 50 μM NAD was found to evoke channel activation on 17 out of 19 occasions tested in the presence of ATP and ADP, two applications having no effect.

Discussion

In this study we have investigated the influences that the pyridine nucleotides NAD, NADP, NADH and NADPH have on the gating of the ATP-sensitive postassium-selective channel in insulin-secreting cells.

Both qualitatively and quantitatively the effects of the four pyridine nucleotides appear to be similar with 10 to 100 μM concentrations of each evoking channel activation (Figs. 1, 2 and 3). This channel activation is as marked as that evoked by the same concentrations of ADP, GTP and ATP (Fig. 5). At concentrations ranging between 500 μM and 5 mM consistent inhibition of K^+ channels was found (Figs. 1, 2 and 3), but this inhibition was smaller than that evoked by the same concentrations of ATP (Fig. 4). The dose-response relationships (Fig. 3) show that even though all the nucleotides exert similar effects on channel gating, the reduced forms appear to have a more powerful influence than the nonreduced forms. The magnitude of channel inhibition evoked by NADH and NADPH is more marked than that induced by the same concentrations of NAD and NADP. Similarly, NADH and NADPH activate K^+ channels more powerfully than NAD and NADP at the same concentration.

It is interesting to note that the overall effects of the pyridine nucleotides are qualitatively similar to those evoked by ATP and ADP. ADP is both able to stimulate and inhibit K^+ channels, a response that is dependent both on the concentration of ADP used (*compare* Figs. 5 and 8) and on the degree of channel run-down (Dunne & Petersen, 1986*a*).

ATP also has a dual controlling influence. The inhibitory effects of ATP have been fully documented in a number of studies (Cook & Hales, 1984; Rorsman & Trube, 1985; Findlay et al., 1985*a*). ATP does, however, also have a clear stimulatory influence since channel closure evoked by ATP is invariably followed, after its removal, by a refreshment or rebound activation of K^+ channels (Findlay & Dunne, 1986; Misler et al., 1986; Ohno-Shosaku, Zünkler & Trube, 1987) (*see* Figs. 4, 7 and 9), the magnitude of which depends on both the dose of ATP and the time of exposure (Findlay & Dunne, 1986). A further stimulatory effect of ATP is seen when the nucleotide is added to the inner membrane for extended periods of time, since the initial channel inhibition is followed, in the continued presence

of ATP, by channel reactivation (Dunne et al., 1986). In this current series of experiments (*see* Fig. 5) concentrations of ATP between 10 and 50 μM corresponding to free ATP (ATP^{4-}) concentrations of about 1 and 5 μM, respectively, were consistently found to evoke a degree of channel stimulation no less powerful than that evoked by either GTP, ADP or the pyridine nucleotides. The latter activating effects of ATP, would seem to be in disagreement with the findings of Cook and Hales (1984) and Kakei et al. (1986), where only inhibition of K^+ channels was seen in experiments carried out on excised inside-out membrane patches from pancreatic islet cells. This discrepancy may in part be explained by the fact that in the open cell relatively higher concentrations of ATP are required to evoke the same degree of channel closure seen in excised inside-out patches from the same cell (Kakei, Noma & Shibasaki, 1985; Dunne et al., 1986).

One interpretation of our finding that each of the pyridine nucleotides exhibits similar effects is that under our test conditions only one form of each nucleotide is stable, i.e. either the reduced or the oxidized form. It is possible that enzymes promoting the conversion of NADH to NAD or NADPH to NADP and vice versa remain active in the preparation after permeabilization of the cell, and lead to the predominance of one form of the nucleotide over another. In the experiments presented in Fig. 6(*A*) and (*B*) we have attempted to clamp each of the nucleotides in their different forms (Hedeskov et al., 1987). In Fig. 6(*A*) a high malate/pyruvate ratio in the presence of added NADPH has been used to prevent conversion of NADPH to NADP. When the malate/pyruvate ratio is reversed the system would tend to generate NADP. In a similar series of experiments presented in Fig. 6(*B*) lactate/pyruvate in the presence of added NAD was used to either prevent or promote generation of NADH. Under these experimental conditions it was found that drastic changes in the ratios of either malate/pyruvate or lactate/pyruvate had no significant effect on the gating of the NADPH- or NAD-inhibited K^+ channels. These findings further support the data presented in Figs. 1 to 5 indicating that all four of the pyridine nucleotides influence the ATP-sensitive K^+ channels in a similar fashion.

Interactions between ATP, ADP and the pyridine nucleotides for control of channel gating have been found. NAD and NADP in 5 and 1 mM concentrations further suppress the activity of 5- or 1-mM ATP-inhibited channels (Fig. 7). At concentrations of 100 μM or less the pyridine nucleotides are unable to influence channel openings in the presence of ATP. Similar effects were seen when channel activity was studied in the presence of ATP and ADP. Under conditions where 4 mM ATP and 1 mM ADP were added to the inside of the membrane, 500 μM NAD was found to further suppress the basal level of channel activity (Fig. 8). In the presence of 4 mM ATP and 1 mM ADP, stimulatory concentrations of the pyridine nucleotides (i.e. <100 μM) were unable to evoke activation of K^+ channels. Only when the concentrations of both ATP and ADP were lowered, could the stimulatory effects of 50 and 100 μm concentrations of the pyridine nucleotides be observed (Fig. 9).

The data shown in Figs. 8 and 9 may have important implications. Interactions between ATP and ADP have been proposed to be involved in stimulus-secretion coupling in insulin-secreting cells (Dunne & Petersen, 1986*a*; Kakei et al., 1986). It is therefore of interest to note that in the absence of ATP, 1 mM ADP clearly closes K^+ channels whereas in the presence of ATP, ADP promotes K^+-channel activation.

The physiological concentrations of the pyridine nucleotides, expressed as pmol/islet, have been evaluated by a number of workers (Hutton, Sener & Malaisse, 1979; Malaisse et al., 1979*a*; Hutton et al., 1980; Malaisse-Lagae, et al., 1982; Malaisse et al., 1982; Hoenig & Matschinsky, 1987). To estimate the approximate intracellular concentrations in millimoles/liter these values must be divided by 2 nl, the approximate islet cell volume (Malaisse & Sener, 1987). The range of intracellular concentrations for each of the nucleotides in the unstimulated cell are therefore: 20 to 70 μM for NADH, 200 to 350 μM for NAD, 30 to 100 μM for NADP and 30 to 70 μM for NADPH. The overall effects of the nucleotides will therefore be, based on the data presented in Figs. 1–3, stimulatory for NADH, NADP and NADPH and inhibitory for NAD. In the intact cell the intracellular concentrations of ATP and ADP are thought to be around 5 and 2 mM, respectively (Malaisse et al., 1979*a*, 1982; Kakei et al., 1986). In the face of such concentrations of ATP and ADP and based on the data represented by Figs. 7–9 the stimulatory effects of NADH, NADP and NADPH would not be expressed in the intact cell. NAD, however, present in the cell at inhibitory concentrations, would be able to exert its inhibitory influence on K^+ channels (Fig. 8). These conclusions, however, may be erroneous. In a recent study by Malaisse and Sener (1987) the concentrations of ATP and ADP in the cytosol have been reevaluated and thought to be less than 0.5 mM and less than 0.2 mM, respectively. Under these conditions both the stimulatory effects of NADP, NADH and NADPH would be expressed in the intact cell (based on data presented in Fig. 9) as would the overall inhibitory effects of NAD.

One possible physiological role of NAD would be that in the intact cell it may contribute to the tonic inhibition of nucleotide-sensitive K^+ channels which is mainly the result of the high intracellular ATP concentration (Dunne et al., 1986; Dunne & Petersen, 1986*a*).

The physiological significance of changes in the redox potential [NAD(P)H]/[NAD(P)] for stimulus-secretion coupling in insulin-secreting cells remains controversial. Two recent studies have presented conflicting findings. Hedeskov et al. (1987) working on mouse pancreatic islet cells conclude that the cytosolic ratio [NADPH]/[NADP] increases in response to elevation of the extracellular glucose concentration whereas glucose had no effect on the cytosolic free ratio [NADH]/[NAD]. These findings confirmed earlier studies by Ashcroft and Christie (1979) and Sener et al. (1984) who also found a glucose-dependent increase in the ratio [NADPH]/[NADP]. Matschinsky et al. (1986) present a different story. In their experiments carried out on a pure rat pancreatic B-cell preparation, quickly frozen upon exposure of the cells to glucose, the ratio [NADPH]/[NADP] was not significantly elevated. Increases in the glucose concentration were, however, found to increase the ratio [NADH]/[NAD]. The concentration of NADH was increased, but only after the onset of insulin release, and no change in the concentration of NADPH was found earlier than one minute after stimulation. Matschinsky et al. (1986), therefore, conclude that changes in the ratio [NAD(P)H]/[NAD(P)] do not coincide with the onset of insulin release, and that this ratio is not the triggering factor coupling glucose metabolism to insulin secretion, a coupling event that is mediated through the closure of the nucleotide-sensitive K^+ channel. Our findings showing that all four pyridine nucleotides essentially influence the K^+ channels in a similar manner adds further support to this argument.

In conclusion, our results show that the pyridine-nucleotides NAD, NADP, NADH and NADPH like the adenosine- and guanosine-based nucleotides are able to modulate the gating of K^+ channels in insulin-secreting cells. All three classes of nucleotides evoke inhibitory as well as stimulatory effects on K^+ channels depending on the concentration of the nucleotide used. Physiological concentrations of NAD are able to inhibit K^+ channels in the presence of quasi-physiological concentrations of ATP and ADP, and it is therefore proposed that in the intact cell NAD may contribute to the tonic inhibition of these channels.

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