Nitric Oxide Opens ATP-sensitive K+ Channels through Suppression of Phosphofructokinase Activity and Inhibits Glucose-induced Insulin Release in Pancreatic β Cells

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ABSTRACT Nitric oxide (NO) is known to be a potent messenger in the intracellular signal transduction system in many tissues. In pancreatic β cells, NO has been reported to be formed from L-arginine through NO synthase. To elucidate the effect of NO on insulin secretion and to investigate the intracellular mechanism of its effect, we have used sodium nitroprusside (SNP) as a NO donor. SNP inhibited glucose-induced insulin secretion in a dose-dependent manner, and its effect was reversed by hemoglobin, a known NO scavenger. However, glyceraldehyde-induced insulin secretion was not affected by SNP. Since the closure of ATP-sensitive K+ channels (K_{ATP} channel) has been established as a key step in glucose-induced insulin secretion, we have directly assessed the effect of SNP on K_{ATP} channel activity using the patch clamp technique. The K_{ATP} channel activity reduced by glucose was found to be reversibly activated by the addition of SNP, and this activation was able to be similarly reproduced by applying S-Nitroso-N-acetyl-DL-penicillamine (SNAP), another NO generator. Furthermore, these activating effects were completely eliminated by hemoglobin, in accordance with the reversibility in inhibition of glucose-induced insulin release. However, SNP could not affect the K_{ATP} channel suppression by ATP applied to the inside of the plasma membrane. The activation of the K_{ATP} channel by NO, therefore, seems to be due to the decreased ATP production attributable to impairment of glucose metabolism in β cells. Since SNP exhibited no effect on glyceraldehyde-induced K_{ATP} channel inhibition, NO may disturb a glycolytic step before glyceraldehyde-3-phosphate. The K_{ATP} channel activation by 2-deoxyglucose through presumable ATP consumption due to its phosphorylation by glucokinase was, however, not affected even in the presence of SNP. But in the permeabilized β cells made by exposure to a low concentration

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(0.02 U/ml) of streptolysin O (open cell-attached configuration), SNP reopens K_{ATP} channels which have been eliminated by fructose-6-phosphate, while this effect was not observed in the K_{ATP} channels inhibited by fructose-1,6-bisphosphate. On the other hand, in rat ventricular myocyte K_{ATP} channels were not activated by SNP even under a low concentration of glucose. From these observations, the inhibition of phosphofructokinase activity is probably the site responsible for the impairment of glucose metabolism induced by NO in pancreatic β cells. NO, therefore, seems to be a factor in the deterioration of glucose-induced insulin secretion from pancreatic β cells through a unique intracellular mechanism.

INTRODUCTION

Glucose-induced insulin secretion has been shown to be regulated by intracellular metabolic and ionic events (Leahy, Bonner-Weir, and Weir, 1992). In this process, various intracellular messengers modulate the insulin secretory mechanism. Nitric oxide (NO), a noxious, stable, free radical gas, has been recognized as a potent mediator in intracellular signaling (Lowenstein and Sydner, 1992). Insulin-producing pancreatic β cells reportedly express both a constitutive (Schmidt, Warner, Ishii, Sheng, and Murad, 1992) and an inducible (Eizirik, Cagliero, Bjorklund, and Welsh, 1992) NO synthase, the enzyme responsible for NO formation, and endogenous NO has been shown to be derived from L-arginine (Schmidt et al., 1992). Thus, NO might be expected to play a physiological role in β cell function.

Sodium nitroprusside (SNP), a potent stimulator of cGMP formation in various tissues including rat pancreatic islets (Laychock, Modica, and Cavanaugh, 1991), has been known to be a NO donor (Southam, and Garthwaite, 1991; Knowles, Palacios, Palmer, and Moncada, 1990). By binding to iron in the heme at the active site of guanylyl cyclase, NO is believed to generate cGMP through the enzyme activation (Lowenstein et al., 1992). However, the effect of SNP and cGMP on pancreatic β cell function is still controversial (Schmidt et al., 1992; Boqueist, 1989; Laychoch, 1987; Jones, Persaud, Bjaaland, Pearson, and Howell, 1992) and the physiological significance of NO as a possible modulator of glucose-induced insulin secretion has not yet been clarified.

It has been well established that the closure of the ATP-sensitive K^+ channel (K_{ATP} channel) is a key step in glucose-induced insulin secretion, which is mediated through intracellular ATP production by glucose metabolism in pancreatic β cells (Leahy et al., 1992). In the present study, accordingly, we have investigated the effect of SNP on K_{ATP} channels in β cells by means of a direct assessment of channel activity using the patch clamp technique. In addition, the specificity of the SNP effect on β cell K_{ATP} channel activity also was assessed by comparison with K_{ATP} channels of ventricular myocytes. We found that SNP inhibits glucose-induced insulin release and activates K_{ATP} channel activity exclusively in β cells, probably through impairment of intracellular glucose metabolism, and these effects were eliminated by hemoglobin, a known NO scavenger (Southam et al., 1991; Izumi, Clifford, and Zorumski, 1992). As a further step, in order to determine the target site of NO in the glucose metabolic pathway of β cells, we investigated the effects of SNP on K_{ATP} channels inhibited by nonpermeable intermediates in glycolytic pathway, including fructose-6-phosphate, using β cells permeabilized by a smaller quantity of streptolysin O, referred to as

open cell-attached configuration (Dunne, Findlay, Petersen, and Wollheim, 1986; Ashcroft and Rorsman, 1989).

MATERIALS AND METHODS

Chemicals

Sodium nitroprusside dihydrate (SNP), 2,4-dinitrophenol (DNP), 2-deoxy-D-glucose (2-DG), fructose-1,6-bisphosphate trisodium salt, and D-glyceraldehyde were purchased from Nacalai Tesque (Kyoto, Japan). Adenosine-5'-triphosphate potassium salt (ATP), bovine hemoglobin, D-fructose-6-phosphate sodium salt, and L-aspartic acid monopotassium salt (K aspartate) were obtained from Sigma Chemical Co. (St. Louis, MO). S-Nitroso-N-acetyl-DL-penicillamine (SNAP) and Ethylene Glycol Bis-N,N,N',N '-tetraacetic acid (EGTA) were purchased from Dojindo (Kumamoto, Japan). Streptolysin O (SLO) was obtained from Wellcome (Dartford, UK).

Isolation of Rat Pancreatic Islets and Dispersion of Islet Cells

Pancreatic islets were isolated from male Wistar rats weighing 250–300 g by collagenase digestion technique (Sutton, Peters, Mcshane, Gray, and Morris, 1986). Freshly isolated islets were used in the assessment of insulin secretory capacity. Another mass of islets were dispersed to obtain single β cells for electrophysiological study by means of the method previously reported (Tsuura, Ishida, Okamoto, Kato, Sakamoto, Horie, Ikeda, Okada, and Seino, 1993). In brief, islets were first incubated for 30 min at 37°C with Ca^{2+}-free Krebs-Ringer bicarbonate buffer (KRBB) medium containing 16.7 mM glucose and 0.2% bovine serum albumin (BSA). Afterward, islets were dispersed mechanically with a 1,000-µl micropipette, and were cultured on small cover glasses (15 × 4 mm) overnight using RPMI 1640 medium containing 11.1 mM glucose, 10% fetal calf serum (FCS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified incubator gassed with 95% air and 5% CO_2. The individual cover glasses were transferred to a test chamber placed on an inverted microscope (Nikon TMD, Tokyo, Japan) for patch clamp experiments.

Assessment of the Insulin Secretory Capacity

Insulin secretion from pancreatic β cells was determined by the batch incubation method (Tsuura et al., 1993). First, islets were preincubated for 30 min at 37°C in KRBB medium supplemented with 5.5 mM glucose and 0.2% BSA. After that, each batch of ~10 islets was incubated in the following 0.7 ml test solution containing 0.2% BSA: 2.8 mM glucose, 8.3 mM glucose, 8.3 mM glucose plus 10 µM SNP, 8.3 mM glucose plus 100 µM SNP, 8.3 mM glucose plus 1 mM SNP in the presence or absence of 10 µM hemoglobin, 10 mM glyceraldehyde, and 10 mM glyceraldehyde plus 1 mM SNP, as shown in Fig. 1. Incubations were performed for 30 min under shaded conditions to prevent ultraviolet exposure. SNP and hemoglobin were dissolved into solutions immediately before the incubation. Five batches were prepared for each test solution, and an aliquot (0.1 ml) was diluted from each batch at the end of incubation for measurement of insulin by RIA.

Cell Preparation from Rat Ventricular Myocytes

Single ventricular myocytes were isolated by the enzymatic dissociation technique (Horie, Hayashi, Yuzuki, and Sasayama, 1992). In brief, the heart of a male Wistar rat weighing ~300 g was dissected under artificial respiration after anesthetization with pentobarbital (40–50 mg/kg, i.p.). The aorta was cannulated and the heart was perfused using the Langendorff

apparatus with Tyrode's solution for 5 min followed with Ca^{2+}-free Tyrode's solution for 3 min. It was then perfused with Ca^{2+}-free Tyrode's solution containing collagenase (10 mg/dl, Nitta Zeratin, Tokyo, Japan) for 10 min, and was rinsed with a high-K^+, low-Ca^{2+} solution (KB solution). The ventricle, dissected into small pieces in the solution, was filtered through a stainless mesh (105 μm), and was stored in KB solution (4°C) for at least 1 h before use. A drop of cell suspension was dispersed in a test chamber placed on an inverted microscope (Nikon TMD, Tokyo, Japan) for patch clamp experiments.

Patch Clamp Technique with Nonpermeabilized Pancreatic β Cells

Single-channel recording in cell-attached and inside-out configurations was performed using pancreatic β cells. The composition for the bathing solution in cell-attached records was (in millimolar) 135 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgSO_4$, and 5 HEPES (pH 7.4 with NaOH). The solution, consisting of (in millimolar) 135 KCl, 0.1 $CaCl_2$, 2 $MgSO_4$, 1 EGTA, 0.0001 K_2ATP, and 5 HEPES (pH 7.2 with KOH) was used as bathing one in inside-out records. The common pipette solution in both configurations was composed of (in millimolar) 140 KCl, 2 $CaCl_2$, and 5 HEPES (pH 7.4 with KOH). When using SNP and hemoglobin, they were dissolved immediately before the experiments and were used under shading. Because SNAP is known not to be readily water soluble, the sonication was performed in an appropriate volume of bathing solution immediately before using. Larger single islet cells (with diameter >10 μm) were selected because smaller cells have been found to be non-β cells (Pipeleers and Pipeleers-Marichal, 1981). Patch pipettes with resistance of 2.5–5 mΩ were pulled from borocillicate glass capillaries. Data currents were recorded through a patch clamp amplifier (EPC-7, List Electronics [Darmstadt, Germany] and Axopatch 200A [Axon Instruments, Foster City, CA]) and were stored on video tape via a pulse code modulation system (PCM 501, Sony, Tokyo, Japan) for later analysis by computer (PC-98XL, NEC, Tokyo, Japan). Representative data were shown using a chart recorder (thermal array recorder RTA-1, Nihon Koden, Tokyo, Japan). The channel activities (I) were expressed as mean patch current ($N \times P_o \times i$) where N, P_o, and i represent the number of open channel, open probability, and unit amplitude, respectively. The mean patch currents were calculated as the average difference between baseline current (no channel open) and open-channel currents. The effect of SNP was estimated as relative values (I/I_c) where I_c represented channel activity in the absence of glucose. The time course of K_{ATP} channel activity was estimated in calculation of the mean patch current each 20 s for 180 s, as previously reported (Tsuura et al., 1993).

Patch Clamp Technique with Permeabilized β Cells

The experiments were performed in cell-attached configuration (open cell-attached configuration, [Dunne et al., 1986]). The bathing solution consisted of (in millimolar) 110 K aspartate, 30 KCl, 1 $MgSO_4$, 0.084 $CaCl_2$, and 0.5 EGTA (pH 7.4 with KOH, pCa ~7.5). The pipette solution throughout was composed of (in millimolar) 140 KCl, 2 $CaCl_2$, and 5 HEPES (pH 7.4 with KOH). SLO (0.02 U/ml) was dissolved into the bathing solution immediately before the experiments, and was used within 1 h. Before exposure to SLO, cell-attached patches were completed at a pipette potential of −60 mV. Continuous single-channel currents were illustrated using a chart recorder.

Patch Clamp Technique with Rat Ventricular Myocytes

In the experiments with ventricular myocytes, the bathing solution in cell-attached configuration was Tyrode's solution which consisted of (in millimolar) 143 NaCl, 0.3 NaH_2PO_4, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, and 5 HEPES (pH 7.4 with NaOH). The pipette solution contained (in

millimolar) 150 KCl, 0.5 EGTA, and 5 HEPES (pH 7.4 with KOH). DNP was prepared as a stock of 100 mM in dimethyl sulfoxide (DMSO) and was dissolved immediately before the experiment, which was performed under shading. A representative trace was demonstrated using the chart recorder (Thermal Array TA240, Gould-Electronics, Valley View, OH). The patch clamp experiments were conducted at room temperature (22–25°C).

Statistical Analysis

The statistical analyses of results and the significance of the differences were assessed by unpaired t test and $P < 0.05$ was considered significant. Numerical data were expressed as mean ± SE.

RESULTS

The Effect of SNP on Insulin Secretory Capacity from Islets

Stimulation with 8.3 mM glucose enhanced the basal insulin secretion in the presence of 3.3 mM glucose, but it was markedly inhibited by SNP in a dose-dependent
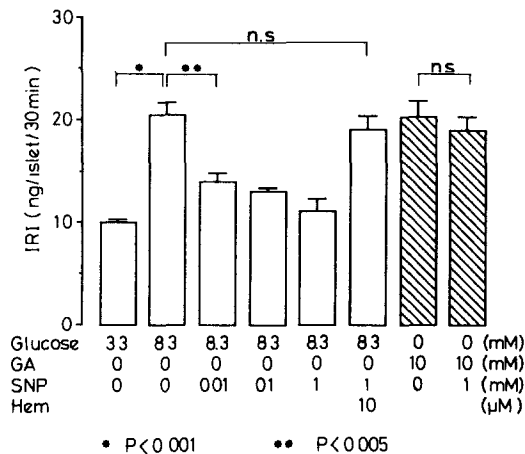

Glucose	3 3	8 3	8.3	8 3	8.3	8 3	0	0	(mM)
GA	0	0	0	0	0	0	10	10	(mM)
SNP	0	0	0.01	0.1	1	1	0	1	(mM)
Hem						10			(µM)

• P<0 001   •• P<0 005

FIGURE 1. The inhibitory effect of SNP on insulin secretion enhanced by glucose (*white bars*) and glyceraldehyde (*hatched bars*). GA and Hem represents glyceraldehyde and hemoglobin, respectively. The mean result is demonstrated in each column. The absolute values of the results represented in this figure were as follows (from left to right): 0.99 ± 0.02, 2.05 ± 0.12, 1.40 ± 0.09, 1.30 ± 0.05, 1.11 ± 0.12, 1.91 ± 0.13, 2.02 ± 0.15, and 1.89 ± 0.14 (ng/islet/30 min). These values were obtained from the results of five batches. The experiments of the same protocol were performed three times to ascertain the reproducibility, showing the similar results.

manner, and 1 mM SNP showed the complete suppression of glucose-induced insulin release to the basal level (Fig. 1). On the other hand, 10 µM hemoglobin completely reversed the inhibitory effect of SNP. Glyceraldehyde (10 mM) also elicited insulin secretion, but 1 mM SNP failed to inhibit the glyceraldehyde-induced insulin secretion, in contrast to the case of glucose stimulation (Fig. 1). In another series of experiments, 2 mM dibutyryl cGMP significantly potentiated the 8.3 mM glucose-induced insulin secretion from 1.50 ± 0.17 ng/islet/30 min ($n = 7$) to 2.48 ± 0.25 ng/islet/30 min ($n = 7$, $P < 0.05$). It is suggested from this result that the inhibition of SNP on glucose-induced insulin secretion is not attributable to cGMP-mediated mechanism.

The Effect of SNP on K_{ATP} Channel Activity of Pancreatic β Cells

As shown in Fig. 2 A, the K_{ATP} channel activity which had been reduced by raising the extracellular glucose concentration to 8.3 mM in cell-attached patches was reversibly activated by the addition of 1 mM SNP. This activating effect of SNP was almost completely eliminated by 10 μM hemoglobin in a reversible manner in all the cells tested (n = 4, Fig. 2 B). Hemoglobin itself, however, did not exhibit any effect on K_{ATP} channel activity in cell-attached configuration (data not shown). The unit
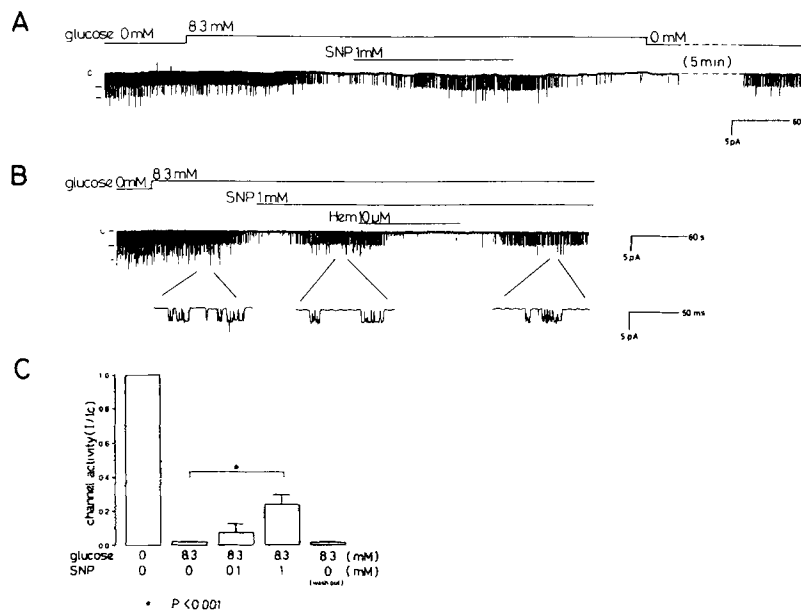
FIGURE 2. The effect of SNP on K_{ATP} channel activity inhibited by glucose in rat pancreatic β cells. The representative traces shown in A and B are recorded at 0 mV of pipette potential in cell-attached configuration. (A) Reversible activating effect of 1 mM SNP on K_{ATP} channel activity inhibited by 8.3 mM glucose. (B) The effect of hemoglobin (*Hem*) on the current activated by SNP. Expansions of activated channel records before and after adding 1 mM SNP and after washout of 10 μM Hem are shown in lower panels. (C) Concentration dependent effect of SNP on K_{ATP} channel activity inhibited by 8.3 mM glucose. 0.1 mM and 1 mM SNP raised I/I_c values from 0.02 $\pm$ 0.01 (n = 10) to 0.07 $\pm$ 0.03 (n = 6), and 0.24 $\pm$ 0.06 (n = 10), respectively. Washout of SNP lowered to 0.01 $\pm$ 0.00 (n = 10).

amplitude of K_{ATP} channels was not significantly different in the condition before the inhibition of the channel activity by 8.3 mM glucose, during the activation by SNP, or after recovering from the inhibition induced by hemoglobin (5.98 $\pm$ 0.16 pA [n = 10], 6.14 $\pm$ 0.13 pA [n = 13], and 6.10 $\pm$ 0.14 pA [n = 10], respectively) as shown in Fig. 2 B. The addition of 0.1 mM SNP slightly increased the reduced K_{ATP} channel activity by 8.3 mM glucose, and 1 mM SNP produced an obvious activation of K_{ATP} channel activity. After washout of 1 mM SNP, the channel activity returned to a very low level (Fig. 2, A and C).

The application of 300 μM SNAP, another potent NO donor (Southam et al., 1991; Kenneth, 1993), reversibly reopened K_{ATP} channels inhibited by 8.3 mM glucose in all three tested cells (Fig. 3), and this activating effect was almost completely eliminated by 10 μM hemoglobin. (Fig. 3, *A* and *B*), similarly to the case of SNP.

On the other hand, as shown in Fig. 4 *A*, 1 mM SNP failed to affect the K_{ATP} channel inhibition when applying 10 μM, 100 μM, and 1 mM ATP to the inner side of the patch membrane in inside-out configuration, comparing with that in the absence of SNP. Half-maximal inhibitory concentration of ATP for K_{ATP} channels of β cells in the presence of SNP was within the same range as that reported previously (Ashcroft, 1988; Tsuura et al., 1993; Fig. 4 *B*).

The addition of 5 mM glyceraldehyde almost completely abolished K_{ATP} channel activity of β cells in cell-attached configuration until the I/I_c value of 0.01 ± 0.01 ($n = 7$), but as shown in Fig. 5, the channels were not reopened by SNP in any of the cells examined (0.02 ± 0.01, $n = 7$).

The Effect of SNP on 2-deoxyglucose-activated K_{ATP} Channels in β Cells

To study the effect of SNP on glucose phosphorylation through glucokinase under the physiological condition, nonmetabolized sugar, 2-deoxyglucose was applied to the cell-attached patches, where the glycolytic enzymes are well preserved. This sugar is known only to be phosphorylated by glucokinase, similar to glucose (Felig, 1980; Hatanaka, Augl, and Gilden, 1970). Accordingly, it is expected that its phosphorylation activates K_{ATP} channel activity because ATP consumption occurs through this step in β cells. As shown in Fig. 6, *A* and *C*, the K_{ATP} channel activity on cell-attached patches was gradually increased after addition of 10 mM 2-deoxyglucose. On the other hand, 1 mM SNP failed to affect K_{ATP} channel activity in the absence of 2-deoxyglucose (Fig. 6 *C*), but even in the presence of SNP, 2-deoxyglucose activated the channel activity, and the activation tended rather to occur more rapidly than in the case of without SNP (Fig. 6, *B* and *C*).

The Effect of SNP on K_{ATP} Channels in the Permeabilized β Cells

As shown in Fig. 7, the application of 5 mM ATP from extracellular space did not affect K_{ATP} channel activity in cell-attached patches of nonpermeabilized β cells. However, the channel activity was gradually inhibited after the exposure of 0.02 U/ml SLO, resulting in almost complete closure of the K_{ATP} channels. We found the periods of SLO application ranging from 30–120 s for the channel closure in eight cells tested (90 s in a cell shown in Fig. 7). After washout of 5 mM ATP, the channels were shown to be reopened with a delayed time of 210–480 s in 7 cells examined, but reapplication of 5 mM ATP abolished the channel activity promptly. In three tested cells, including the one shown in Fig. 7, the third application of 5 mM ATP also was capable of inhibiting K_{ATP} channels which had been reopened after the second washout of ATP. In a cell shown in Fig. 7, the K_{ATP} channel activity was controllable by extracellular ATP during more than 20 min. Here, we used the cells exposed for 120 s (the maximum period) to 0.02 U/ml SLO as permeabilized β cells.

The application of 10 mM fructose-6-phosphate to nonpermeabilized cells exhibited no effect on the K_{ATP} channel activity in all cells tested ($n = 4$), as shown in Fig.
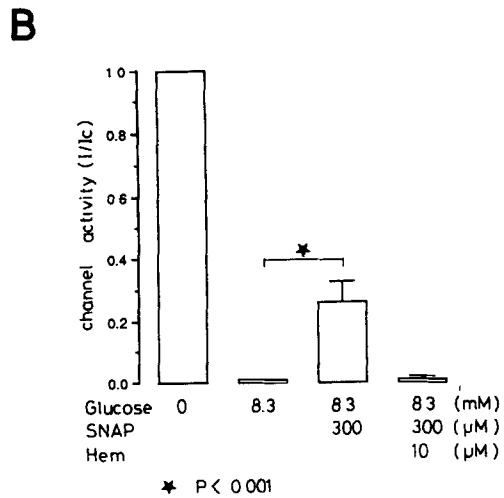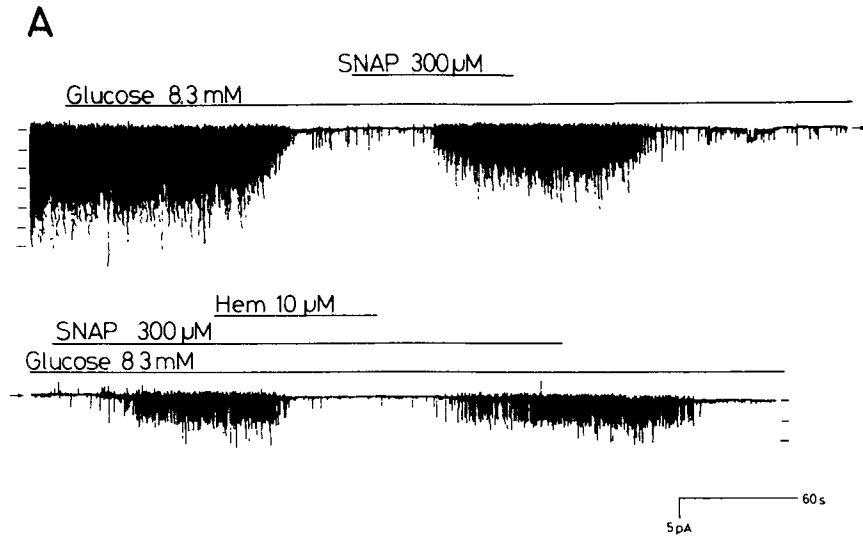
A

B

FIGURE 3. The effect of SNAP on K_{ATP} channels inhibited by 8.3 mM glucose. (A) A trace recording at 0 mV of pipette potential in cell-attached configuration is illustrated. Hem represents hemoglobin. The upper trace leads to the lower trace continuously. (B) The I/I_c values obtained from three experiments. 300 μM SNAP raised from 0.01 ± 0.00 in the presence of 8.3 mM glucose alone to 0.26 ± 0.07 with significant difference. The value in the presence of hemoglobin were 0.01 ± 0.00.

A

B
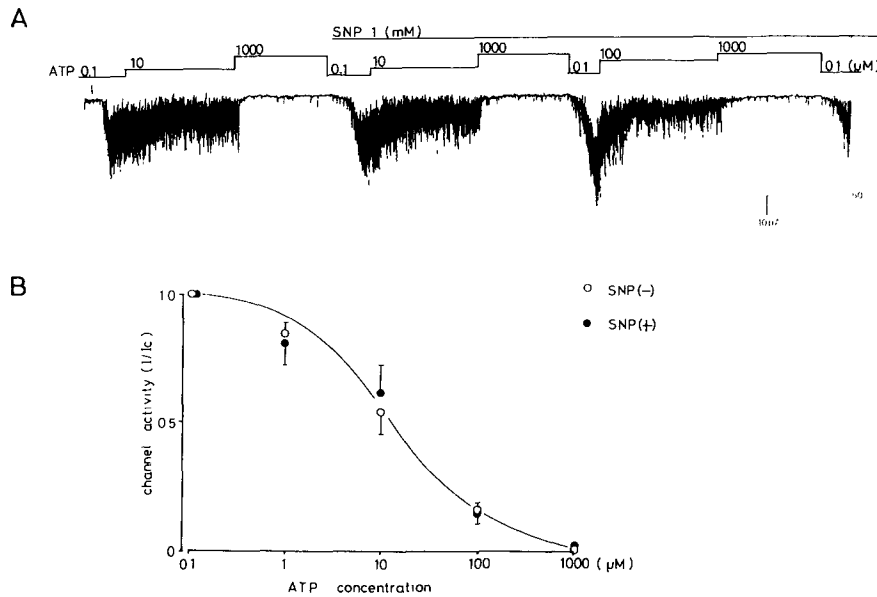
FIGURE 4.   The effect of SNP on K_{ATP} channels inhibited by ATP in pancreatic β cells. (*A*) A representative trace showing the inhibition of K_{ATP} channels by ATP in the presence or absence of SNP. The trace is recorded at -60 mV of pipette potential in inside-out patch. Patch membrane was excised at time indicated by arrow. (*B*) The concentration dependent inhibition of ATP for K_{ATP} channels in the absence (*open circles*) or presence (*closed circles*) of 1 mM SNP. The inhibition curve was well fitted by the Hill equation:

$$I/I_c = 1/(1 + [X/K_i]^h)$$

where K_i ($=15$ μM) is the concentration of ATP causing half-maximal inhibition, h ($=0.9$) is the Hill coefficient, and X is the ATP concentration. The I_c values were 16.4 ± 4.3 pA ($n = 7$) in the absence of SNP and 18.7 ± 5.0 pA ($n = 6$) in the presence of SNP. The number of observations was 3–5 at the given concentrations.

8. Similarly, neither 10 mM glucose-6-phosphate nor 10 mM fructose-1,6-bisphosphate inhibited the channels (data not shown). After permeabilizing β cells using SLO, as shown in Fig. 8, 10 mM fructose-6-phosphate reversibly and almost completely suppressed the channel activity in 16 of 20 cells tested (80%) until the I/I_c value of 0.04 ± 0.01. In addition, 10 mM glucose-6-phosphate and 10 mM
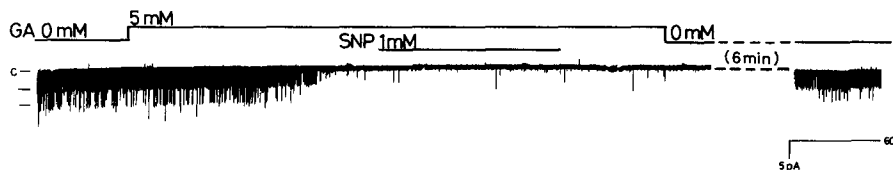

FIGURE 5.   The effect of SNP on K_{ATP} channels inhibited by 5 mM glyceraldehyde. A trace is recorded at 0 mV in cell-attached configuration.
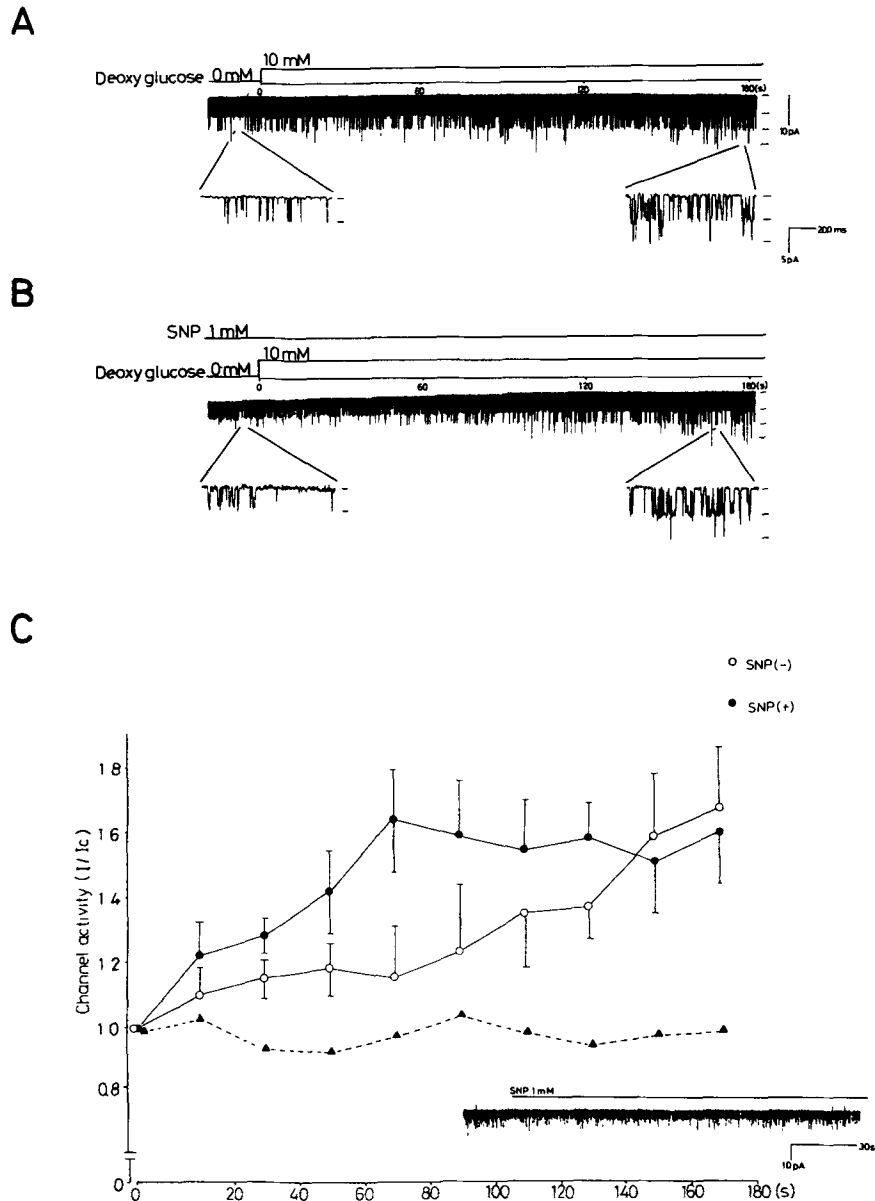
FIGURE 6. The effect of SNP on K_{ATP} channels activated by 10 mM 2-deoxyglucose. The traces are recorded at 0 mV pipette potential in cell-attached mode. The cells remained for at least 5 min in the glucose-free solution before these experiments. The upper trace (A) is recorded in the absence of SNP and the lower (B) is recorded in the presence of 1 mM SNP from the beginning. The left columns of expansions of each panel are recorded before applying deoxyglucose, and the right columns are recorded ~ 180 s after applying it. In A, the mean patch currents increased from 0.88 pA to 2.02 pA, and in B, they increased from 0.97 pA to 2.27 pA. (C) The time course of K_{ATP} channel activity for 180 s after adding 10 mM 2-deoxyglucose. The time course in the absence of SNP are shown as open circle, and in the presence of SNP are as closed circle. Each was calculated from data of eight experiments. The time course after adding 1 mM SNP in the absence of 2-deoxyglucose is illustrated as closed triangle (average of four experiments). A representative trace is shown in inset of C. In the absence of 2-deoxyglucose, the I/I_c value remained 0.98 ± 0.10 180 s after applying SNP. However, it attained to 1.67 ± 0.20 and 1.60 ± 0.17 in the absence and presence of SNP, respectively, after applying 2-deoxyglucose, which were not significantly different.
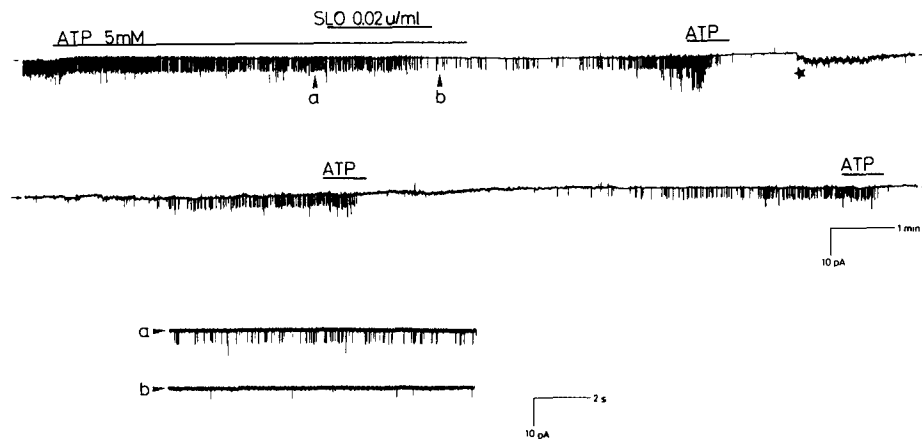
FIGURE 7. The control of 5 mM ATP from extracellular space on K_{ATP} channel activity after exposing to 0.02 U/ml SLO. The trace is recorded at -60 mV of pipette potential in cell-attached mode. Exposure to SLO was continued for ~ 90 s (until closure of almost channels). The upper panel leads to the lower panel continuously. At the point indicated with an asterisk, the patch was loosed temporarily. The expansions of a and b are the record before applying SLO (a) and after washout of SLO in the presence of 5 mM ATP (b).

fructose-1,6-bisphosphate also inhibited the channels in five of six cells (83%) and all eight cells (100%) until I/I_c level of 0.05 ± 0.03 and 0.03 ± 0.01, respectively.

In permeabilized β cells, SNP reopened the K_{ATP} channels which had been inhibited by 10 mM glucose-6-phosphate in four of six cells (Fig. 9 C). Moreover, the inhibited channel activity by 10 mM fructose-6-phosphate was shown to be reversed and rather enhanced by 1 mM SNP in all nine cells tested (Fig. 9, A and C). After washout of SNP, the channel activity was found to be gradually decreased in four cells examined (Fig. 9A). On the other hand, 1 mM SNP failed to reopen the K_{ATP} channels inhibited by 10 mM fructose-1,6-bisphosphate in all seven cells (Fig. 9, B
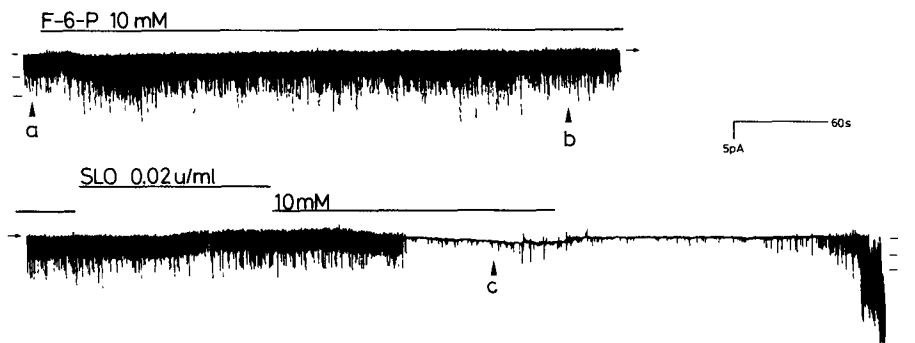

FIGURE 8. The inhibitory effect of fructose-6-phosphate (F-6-P) on K_{ATP} channels before and after exposing to 0.02 U/ml SLO. The trace is recorded at -60 mV in cell-attached mode. The upper trace leads to the lower trace continuously. The mean patch currents near a–c were 2.41, 2.59, and 0.08 pA, respectively.
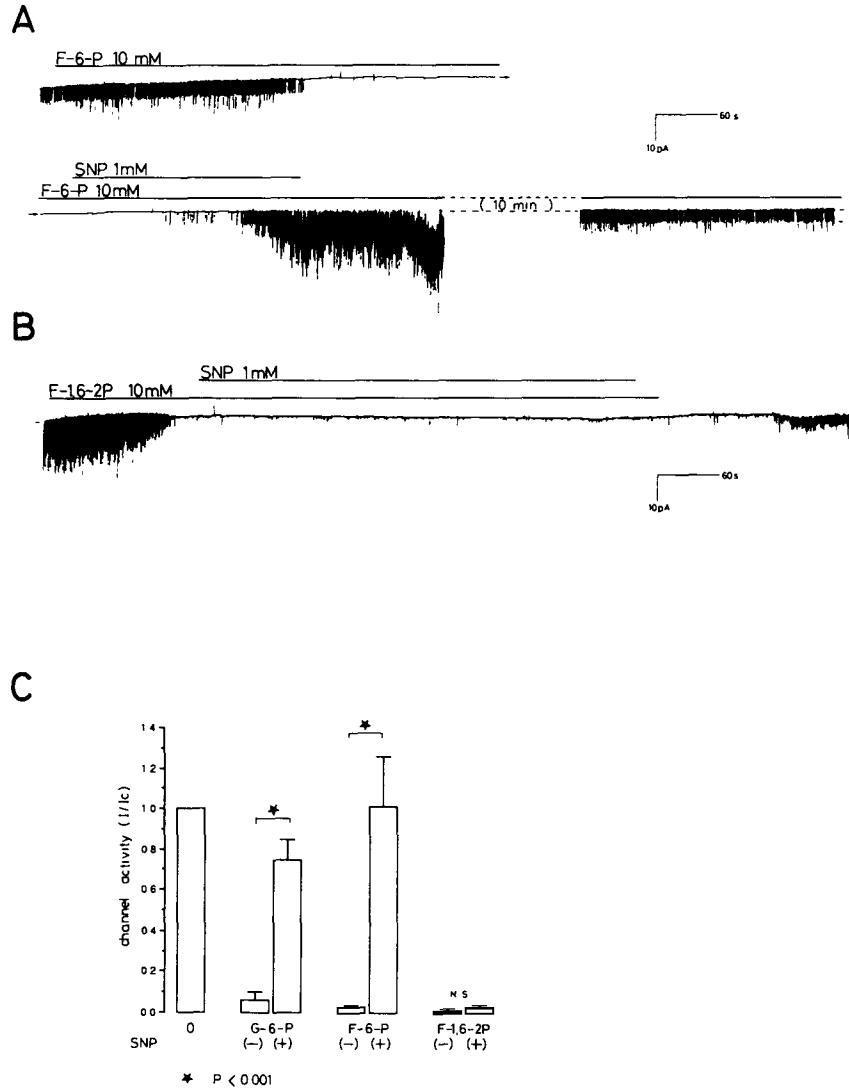
FIGURE 9.   The effect of SNP on K_{ATP} channels abolished by 10 mM glucose-6-phosphate (G-6-P), 10 mM fructose-6-phosphate (F-6-P) or 10 mM fructose-1,6-bisphosphate (F-1,6-2P). Traces indicated the effect of SNP on the channels inhibited by F-6-P (A) and F-1,6-2P (B) are shown. The traces are recorded at −60 mV in open cell-attached mode, and the recordings were started after exposing to 0.02 U/ml SLO for 120 s. The upper and lower traces of A are recorded continuously. (C) The summary of the effect of SNP on the channels. In the cases of G-6-P and F-6-P, SNP activated K_{ATP} channel activity from 0.06 ± 0.04 to 0.75 ± 0.10 (n = 4), and from 0.03 ± 0.01 to 1.01 ± 0.25 (n = 9), but in F-1,6-2P, SNP did not affect the channel activity (from 0.01 ± 0.01 to 0.03 ± 0.01 [n = 7]).
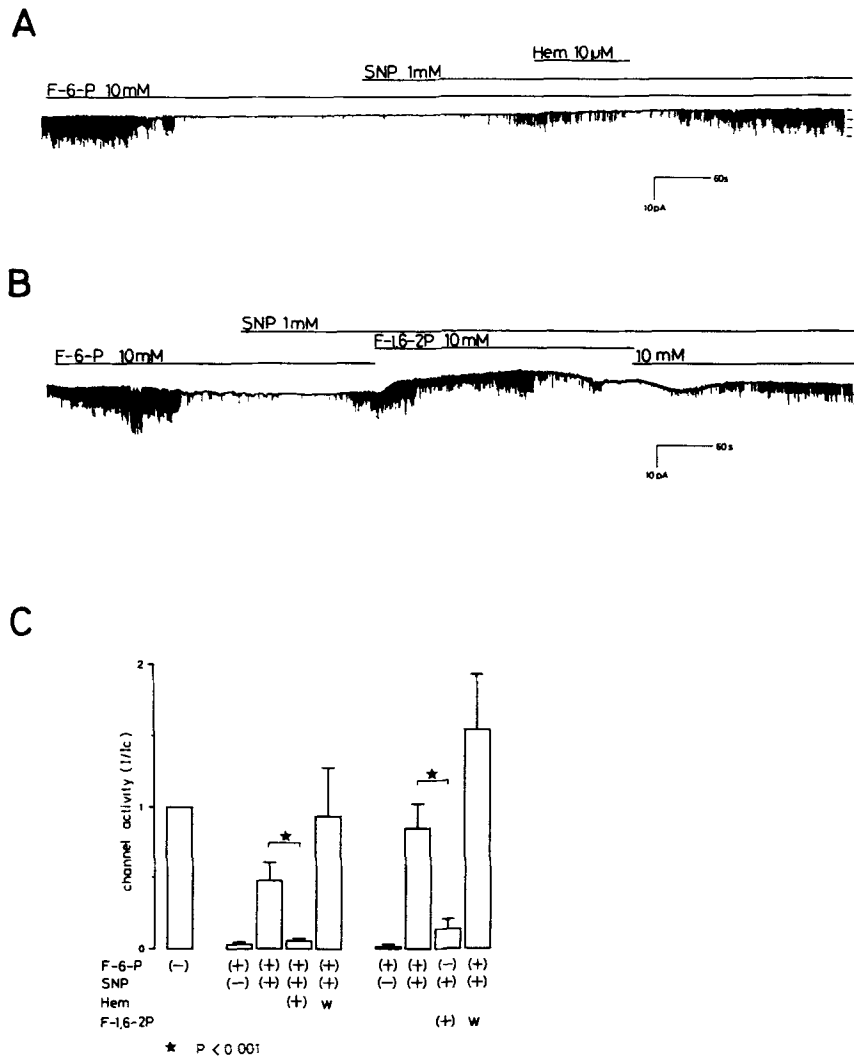
Figure 10. (*A*) The influence of 10 μM hemoglobin (*Hem*) on K_{ATP} channels reopened by SNP. The trace are recorded at −60 mV in open on-cell mode, and the recording were started from after exposing to SLO. (*B*) The effect of changing extracellular fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-2P) on K_{ATP} channels activated by SNP. The continuous single-channel recording was performed at −60 mV in open on-cell mode. The traces are illustrated after exposure to SLO. (*C*) Hem inhibited K_{ATP} channels from 0.47 ± 0.13 to 0.06 ± 0.01 (*n* = 2). The channels were temporarily inhibited through converting F-6-P to F-1,6-2P from 0.84 ± 0.18 to 0.14 ± 0.07 (*n* = 7). The letter of *w* represents washout.

and *C*). The activating effect of SNP on the channel activity was reversibly eliminated by 10 μM hemoglobin in two cells examined (Fig. 10, *A* and *C*). Furthermore, the K_{ATP} channels activated by addition of SNP in the presence of 10 mM fructose-6-phosphate were reversibly inhibited by changing extracellular fructose-6-phosphate

to fructose-1,6-bisphosphate (10 mM) in seven cells examined, as shown in Fig. 10, *B* and *C*.

The Effect of SNP on K_{ATP} Channel Activity of Ventricular Myocytes

In cell-attached patches of ventricular myocytes, inwardly rectifying K^+ channels (iK_{rec}) were active regardless of the presence or absence of glucose (Fig. 11). K_{ATP} channels in cell-attached patches were usually inactive even in the absence of glucose (data not shown), but K_{ATP} channels began to activate in the continued presence of 50 μM DNP, an inhibitor of mitochondrial oxidative phosphorylation (Fig. 11). Accordingly, in order to detect K_{ATP} channel activity in the cell-attached condition, 50 μM DNP was perifused throughout the experiments. Washout of glucose led to the activation of the channels in all the cells tested ($n = 23$). In most myocytes, the channel activation and rigor contracture occurred simultaneously, but in a few myocytes, the K_{ATP} channel activity was considerably decreased through reapplication of 11.1 mM glucose, as shown in Fig. 11 *A* ($n = 5$). After that, the channel activity was not influenced by the addition of 1 mM SNP in any of the cells ($n = 5$). Under the condition of 2.8 mM glucose rather than 11.1 mM glucose, SNP also did not affect the K_{ATP} channel activity in all six cells examined (Fig. 11 *B*). The I/I_c value in the presence of 1 mM SNP was 1.16 ± 0.18 ($n = 6$), and 1.16 ± 0.15 after washout of SNP ($n = 6$), compared to that before application of SNP. Similarly, 300 μM SNAP failed to affect K_{ATP} channels in the presence of 2.8 mM glucose (data not shown).

DISCUSSION

The present study shows that SNP inhibits glucose-induced insulin secretion in a dose-dependent manner (Fig. 1). The inhibitory effect of SNP is thought to be mediated by NO, because SNP is known to be a potent NO donor (Southam et al., 1991; Knowles et al., 1990) and the inhibition is apparently reversed by hemoglobin, a known NO scavenger (Southam et al., 1991). NO also has been known to activate guanylyl cyclase and to generate cGMP (Lowenstein et al., 1992), but the hypothesis that cGMP is involved in the mechanisms of NO inhibition of glucose-induced insulin secretion seems to be unlikely because we found that dibutyryl cGMP can enhance the insulin release by 8.3 mM glucose, in agreement with reports of others (Laychock, 1987; Laychock et al., 1991).

In electrophysiological investigations, we found that SNP activates the K_{ATP} channel activity which has been reduced by glucose in a concentration-dependent manner (Fig. 2, *A* and *C*), and that hemoglobin again reverses the channel activation (Fig. 2 *B*). From these results, it is strongly suggested that NO derived from SNP activates K_{ATP} channel activity, leading to the suppression of glucose-induced insulin secretion. This conclusion is consistent with the recent report of a study using [86]Rb^+ efflux (Antoine, Hermann, Herchuelz, and Lebrun, 1993).

There has been reported no direct electrophysiological evidence that NO opens K_{ATP} channels in pancreatic β cells. Accordingly, activation of the channels by SNP might be attributable to another factor derived from SNP. However, 300 μM SNAP, another potent NO donor, is thought to release NO to the same extent as 1 mM SNP, because of the amount of cGMP production (Southam et al., 1991), and it
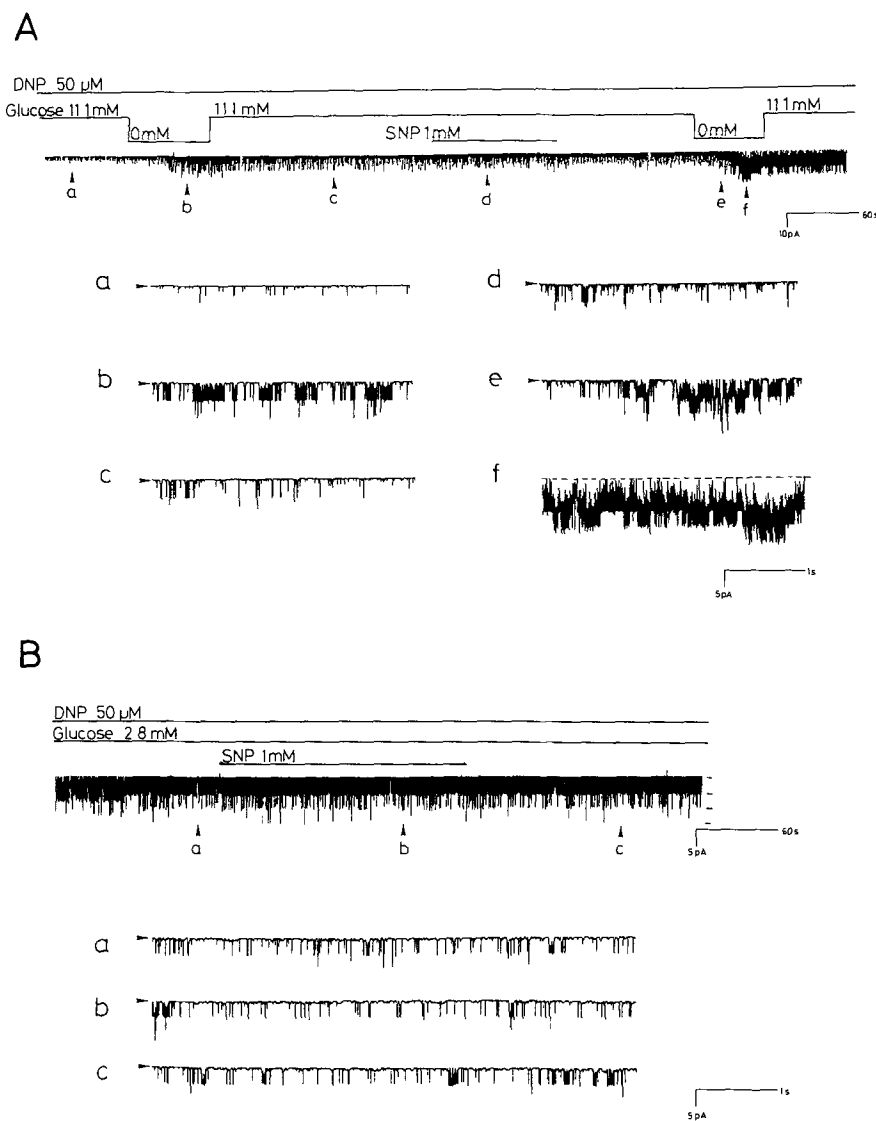
FIGURE 11. The effect of SNP on K_{ATP} channel activity in rat ventricular myocytes. The chart records are shown at 0 mV of pipette potential in cell-attached patch. 50 μM DNP was perifused throughout courses of experiments. (A) A trace shown the effect of washout of 11.1 mM glucose and SNP in the presence of 11.1 mM glucose for the channels. Expansions of record (a–f) are shown in lower panels. The mean patch currents at brief periods indicated with the letter of a–f were as follows: (a) 0.01 pA; (b) 2.28 pA; (c) 0.26 pA; (d) 0.31 pA; (e) 6.99 pA; and (f) 14.01 pA. Arrows (a–e) and dotted lines (f) indicate 0 current level. Time and current calibrations in lower panels are the same as in f. After the second washout of glucose, the cell fell into rigor contracture. (B) The effect of SNP on K_{ATP} channels in the presence of low concentration of glucose (2.8 mM). The mean patch currents near to the periods indicated with the letters of a–c were 0.65, 0.55, and 0.54, respectively.

opens K_{ATP} channels in β cells, similarly to 1 mM SNP (Fig. 3). This fact confirms that the channels open by NO released from SNP, in conjunction with the reversible inhibition of K_{ATP} channels by hemoglobin (Fig. 3).

Intracellular mechanisms underlying NO-induced activation of K_{ATP} channels in pancreatic β cells deserve consideration. The increase of channel activity is thought to be caused by the decreased ATP production, which is probably due to impaired glucose metabolism for three reasons. First, SNP failed to affect the K_{ATP} channel inhibition by ATP applied to intracellular membrane (Fig. 4), and the unit amplitude of K_{ATP} channels on cell-attached patches activated by SNP was not altered when compared to that observed in the absence of glucose (Fig. 2 B). These results suggest that NO does not affect the K_{ATP} channel directly. Second, the activation of channels by SNP and SNAP was accompanied with delayed time of 30–60 s (Fig. 2, A and B and Fig. 3 A). It is evident, therefore, that the channel activation is not due to a direct action of NO on the K_{ATP} channels. Third, NO failed to reopen K_{ATP} channels suppressed by glyceraldehyde, differently from the case of glucose (Fig. 5). This strongly suggests that NO decreases the intracellular production of ATP by disturbing the glycolytic pathway before glyceraldehyde-3-phosphate in pancreatic β cells. On the other hand, there have been some reports that NO might inhibit glyceraldehyde-3-phosphate dehydrogenase activity in brain and muscle through auto-ADP-ribosylation (Zhang and Snyder, 1992; McDonald and Moss, 1993). But this inhibition is likely to be physiologically insignificant in pancreatic β cells because NO elicited no effect on glyceraldehyde-induced insulin release or K_{ATP} channel inhibition (Fig. 1).

It has been reported that K_{ATP} channel activity is temporarily increased when exposed to glucose before its inhibition in pancreatic β cells (Ribalet, Eddlestone, and Ciani, 1988; Hayashi, Horie, Tsuura, Ishida, Okada, Seino, and Sasayama, 1993). This phenomenon has been thought to be due to ATP consumption in the early steps of glycolysis. Because the metabolism of 2-deoxyglucose is known to stop in the step of 2-deoxyglucose-6-phosphate (Felig, 1980), it is possible that K_{ATP} channel activity is increased in the presence of 2-deoxyglucose alone, because ATP is converted to ADP through 2-deoxyglucose phosphorylation by glucokinase. As shown in Fig. 6, SNP did not affect this increment of the channel activity in β cells. Glucokinase is known to be a rate-limiting enzyme in pancreatic β cells (Lenzen, 1992), and inhibition of its activity has been reported to lead to activate K_{ATP} channels (Misler, Falke, Gillis, and McDaniel, 1986). However, the result of a study with 2-deoxyglucose seems to be inconsistent with the hypothesis that NO inhibits glucokinase activity in β cells. Conversely, there may be a possibility that NO activates glucokinase activity, because 2-deoxyglucose tended to activate the channel activity more rapidly in the presence of SNP.

To identify the target enzyme of NO in glycolysis between glucose-6-phosphate and glyceraldehyde-3-phosphate, it was necessary to study the effect of SNP on K_{ATP} channels eliminated by glucose-6-phosphate, fructose-6-phosphate, or fructose-1,6-bisphosphate. In the present study, we tried to devise a permeabilized β cell for open cell-attached patch which could maintain intracellular metabolic events to allow such nonpermeable intermediates to enter the cells and to inhibit K_{ATP} channels through their metabolism. In RINm5F cells permeabilized by digitonin which reported

previously (Dunne et al., 1986), K_{ATP} channel activity on open cell-attached patch showed an immediate response to extracellular ATP concentration within several seconds. But, in this typical model of open cell-attached configuration, it seems difficult to maintain intracellular materials including glycolytic enzymes. In our trial, a much smaller volume of SLO (0.02 U/ml) was used when compared to previous reports (0.4 U/ml; Aridor, Rajmilevich, Beaven, and Sagi-Eisenberg, 1993; 1.5 U/ml; Li, Regazzi, Balch, and Wollheim, 1993). However, as shown in Fig. 7, K_{ATP} channel activities were consistently suppressed throughout the exposure for 90 s, and were under control of extracellular ATP for more than 20 min. Moreover, after exposure to SLO for 120 s, the K_{ATP} channels were found to be eliminated by nonpermeable intermediates in almost all the cells examined (Fig. 8). From these observations, the permeabilized β cells we have devised for this study should be an appropriate tool for evaluating the metabolic modulations of nonpermeable intermediates, including fructose-6-phosphate, which can be referred to as a new method of very mildly open cell-attached configuration, different from previous conventional mode.

The evidence that SNP reopened K_{ATP} channels inhibited by 10 mM glucose-6-phosphate in permeabilized β cells suggests that the disturbed metabolic step is not located before glucose-6-phosphate and that glucokinase is not a target of NO in β cells, consistently with the result of study using 2-deoxyglucose. Moreover, K_{ATP} channel activity was suppressed similarly by both 10 mM fructose-6-phosphate and 10 mM fructose-1,6-bisphosphate. The addition of SNP, however, reversed only the channel activity which had been inhibited by fructose-6-phosphate, but not that inhibited by fructose-1,6-bisphosphate (Fig. 9). Because this effect of SNP was eliminated by the NO scavenging hemoglobin, the target enzyme of NO in the glycolytic pathway is thought to be phosphofructokinase, an enzyme converting fructose-6-phosphate to fructose-1,6-bisphosphate. Phosphofructokinase has been known to be a rate-limiting enzyme in β cell lines, as well as glucokinase (Simizu, Parker, Najafi, and Matschinsky, 1988). Accordingly, the inhibition of this enzyme can affect intracellular glucose metabolism and open K_{ATP} channels in pancreatic β-cells. Since it has been reported that nitric oxide synthase is equipped with islet cells (Schmidt et al., 1992), NO may regulate glucose metabolism via inhibition of phosphofructokinase activity under the physiological conditions found in β-cells.

It remains unknown how NO inhibits phosphofructokinase activity. Because the activity is regulated allosterically by ATP or ADP (Hellman, Idahl, and Taljedal, 1975), NO may affect the allosteric regulator, not enzyme protein itself. On the other hand, another conceivable interpretation of this results is that a local increase in ADP concentration through an accelerated conversion of ATP to ADP by stimulation of this enzyme might lead to activate K_{ATP} channels, because the decrease of ATP/ADP ratio is known to open the channels (Ashcroft, 1988), although it seems intuitively less likely.

In cardiac myocytes, K_{ATP} channel activity is known to be dependent on glycolytic activity (Weiss and Lamp, 1987). In fact, in the present study, K_{ATP} channels were activated by washout of glucose in the presence of DNP, an uncoupler of mitochondrial oxidation (Fig. 11 A). Since the most important site of glycolysis control in cardiac myocytes is considered to be phosphofructokinase (Lawson and Uyeda, 1987), it is possible that inhibition of this enzyme by NO leads to activation of the

cardiac channels. Furthermore, it has been reported that H_2O_2 activates K_{ATP} channels by suppressing glycolytic and oxidative metabolism in guinea pig ventricle (Goldhaber, Scott, Lamp, and Weiss, 1989). However, SNP (Fig. 11B) and SNAP failed to increase K_{ATP} channel activity in rat ventricular myocytes even under the condition of low glucose. It is known that peroxynitrite anion might be produced from NO, (J. Beckman, T. Beckman, Chen, Marshall, and Freeman, 1990), and thus, it is suggested that the target of NO and peroxynitrite would be distinguishable from that of H_2O_2. From the results of Fig. 11, it is suggested that the regulatory mechanism of phosphofructokinase is distinct in β-cells and ventricular myocytes. A precise explanation for this discrepancy is not available at present, but it could be based on a difference in isozymes of the enzyme among tissues. Three types of isozyme has been identified as muscle type, liver type, and brain type (Gekakis, Gehnrich, and Sul, 1989), but the sort of isozyme in β-cells and cardiac myocytes remains to be learned. The further studies would be necessary to clarify the difference in the isozymes and their regulatory mechanisms in heart and pancreatic β cells.

In conclusion, SNP inhibits glucose-induced insulin secretion through the activation of K_{ATP} channel activity in pancreatic β cells. Since this effect can be reversed by hemoglobin, this inhibition is thought to be mediated by NO. In addition, the activation of the K_{ATP} channel is probably caused by a reduced level of intracellular ATP production, which is attributable to inhibition of phosphofructokinase in the glycolytic pathway.

REFERENCES

Antoine, M. H., M. Hermann, A. Herchuelz, and P. Lebrun. 1993. Sodium nitroprusside inhibits glucose-induced insulin release by activating ATP-sensitive K^+ channels. *Biochemica et Biophysica Acta.* 1175:293–301.

Aridor, M., G. Rajmilevich, M. A. Beaven, and R. Sagi-Eisenberg. 1993. Activation of exocytosis by the heterotrimeric G protein G_{i3}. *Science.* 262:1569–1572.

Ashcroft, F. M., 1988. Adenosine 5'-triphosphate-sensitive potassium channels. *Annual Review of Neuroscience.* 11:97–118.

Ashcroft, F. M., and P. Rorsman. 1989. Electrophysiology of the pancreatic β-cell. *Progress in Biophysics and Molecular Biology.* 54:87–143.

Beckman, J. S., T. W. Beckman, J. Chen, P. A. Marshall, and B. A. Freeman. 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of National Academy of Sciences, USA.* 87:1620–1624.

Boquist, L. 1989. Effects of sodium nitroprusside on blood glucose concentration, B-cell morphology and islet glutamate dehydrogenase activity in mice. *APMIS.* 97:971–980.

Dunne, M. J., I. Findlay, O. H. Petersen, and C. B. Wollheim. 1986. ATP-sensitive K^+ channels in an insulin-secreting cell line are inhibited by D-glyceraldehyde and activated by membrane permeabilization. *Journal of Membrane Biology.* 93:271–279.

Eizirik, D. L., E. Cagriero, A. Bjorklund, and N. Welsh. 1992. Interleukin-1β induces the expression of an isoform to that observed in activated macrophage. *FEBS Letters.* 308:249–52.

Felig, P. 1980. Diabetes Mellitus. *In* Metabolic Control and Disease. P. K. Bondy and L. E. Rosenberg, editor. W. B. Saunders Co., Philadelphia, PA. 307–356.

Gekakis, N., S. C. Gehnrich, and H. S. Sul. 1989. Phosphofructokinase isozyme expression during myoblast differentiation. *Journal of Biological Chemistry.* 264:3658–3661.

Goldhaber, J. I., S. Ji, S. T. Lamp, and J. N. Weiss. 1989. Effects of exogenous free radicals on electromechanical function and metabolism in isolated rabbit and guinea pig ventricle. Implications for ischemia and reperfusion injury. *Journal of Clinical Investigation.* 83:1800–1809.

Hatanaka, M., C. Augl, and R. V. Gilden. 1970. Evidence for a functional change in the plasma membrane of murine sarcoma virus-infected mouse embryo cells. *Journal of Biological Chemistry.* 245:714–717.

Hayashi, S., M. Horie, Y. Tsuura, H. Ishida, Y. Okada, Y. Seino, and S. Sasayama. 1993. Disopyramide blocks pancreatic ATP-sensitive K^+ channels and enhances insulin release. *American Journal of Physiology.* 265:C337–C342.

Hellman, B., L. A. Idahl, J. Sehlin, and I. B. Taljedal. 1975. Influence of anorexia on glucose metabolism in pancreatic islets: lack of correlation between fructose-1,6-diphosphate and apparent glycolytic flux. *Diabetologia.* 11:495–500.

Horie, M., S. Hayashi, Y. Yuzuki, and S. Sasayama. 1992. Comparative studies of ATP-sensitive potassium channels in heart and pancreatic β cells using Vaugham-Williams class Ia antiarrhythmics. *Cardiovascular Research.* 26:1087–1094.

Izumi, Y., D. B. Clifford, and C. F. Zorumski. 1992. Inhibition of long-term potentiation by NMDA-mediated nitric oxide release. *Science.* 257:1273–1276.

Jones, P. M., S. J. Persaud, T. Bjaaland, J. D. Pearson, and S. L. Howell. 1992. Nitric oxide is not involved in the initiation of insulin secretion from rat islets of Langerhans. *Diabetologia.* 35:1020–1027.

Kenneth, D. C. 1993. Evidence for an antiviral effect of nitric oxide. *Journal of Clinical Investigation.* 91:2446–2452.

Knowles, R. G., M. Palacios, R. M. J. Palmer, and S. Moncada. 1990. Kinetic characteristics of nitric oxide synthase from rat brain. *Biochemical Journal.* 269:207–210.

Lawson, J. W. R., and K. Uyeda. 1987. Effects of insulin and work on fructose-2,6-bisphosphate content and phosphofructokinase activity in perfused rat hearts. *Journal of Biological Chemistry.* 262:3165–3173.

Laychock, S. G. 1987. Effect of guanosine 3',5'-monophosphate on glucose utilization in isolated islets of Langerhans. *Endocrinology.* 120:517–524.

Laychock, S. G., M. E. Modica, C. T. Cavanaugh. 1991. L-arginine stimulates cyclic guanosine 3',5'-monophosphate formation in rat islets of Langerhans and RINm5F insulinoma cells: evidence for L-arginine: nitric oxide synthase. *Endocrinology.* 129:3043–3052.

Leahy, J. L., S. Bonner-Weir, and G. C. Weir. 1992. β-cell dysfunction induced by chronic hyperglycemia. *Diabetes Care.* 15:442–455.

Lenzen, S. 1992. Glucokinase: signal recognition enzyme for glucose-induced insulin secretion. *In* Nutrition Regulation of Insulin Secretion. P. R. Flatt, editor. Portland Press, London. 101–124.

Li, G., R. Regazzi, W. E. Balch, and C. B. Wollheim. 1993. Stimulation of insulin release from permeabilized HIT-T15 cells by a synthetic peptide corresponding to the effector domain of the small GTP-binding protein rab3. *FEBS Letters.* 327:145–149.

Lowenstein, C. J., and S. H. Synder. 1992. Nitric oxide, a novel biologic messenger. *Cell.* 70:705–707.

McDonald, L. J., J. Moss. 1993. Stimulation by nitric oxide of an NAD linkage to glyceraldehyde-3-phosphate dehydrogenase. *Proceedings of National Academy of Sciences, USA.* 90:6238–6241.

Misler, S., L. C. Falke, K. Gillis, and M. L. McDaniel. 1986. A metabolite-regulated potassium channel in rat pancreatic B cells. *Proceedings of National Academy of Sciences, USA.* 83:7119–7123.

Pipeleers, D. G., and M. A. Pipeleers-Marichal. 1981. A method for the purification of single A, B and D cells and for the isolation of coupled cells from isolated rat islets. *Diabetologia.* 20:654–663.

Ribalet, B., G. T. Eddlestone, and S. Ciani. 1988. Metabolic regulation of the K(ATP) and a Maxi-K(V) channel in the insulin-secreting RINm5F cell. *Journal of General Physiology.* 92:219–237.

Schmidt, H. H. H. W., T. D. Warner, K. Ishii, H. Sheng, and F. Murad. 1992. Insulin secretion from pancreatic B cells caused by L-arginine-derived nitric oxides. *Science.* 255:721–723.

Simizu, T., J. C. Parker, H. Najafi, and F. M. Matschinsky. 1988. Control of glucose metabolism in pancreatic β-cells by glucokinase, hexokinase, and phosphofructokinase. Model study with cell lines derived from β-cells. *Diabetes.* 37:1524–1530.

Southam, E., and J. Garthwaite. 1991. Comparative effects of some nitric oxide donors on cyclic GMP levels in rat cerebellar slices. *Neuroscience Letters.* 130:107–111.

Sutton, R., M. Peters, P. Mcshane, D. W. R. Gray, and P. Morris. 1986. Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation (Baltimore).* 20:689–691.

Tsuura, Y., H. Ishida, Y. Okamoto, S. Kato, K. Sakamoto, M. Horie, H. Ikeda, Y. Okada, and Y. Seino. 1993. Glucose sensitivity of ATP-sensitive K^+ channels is impaired in β cells of the GK rat, a new genetic model of NIDDM. *Diabetes.* 42:1446–1453.

Weiss, J. N., and S. T. Lamp. 1987. Glycolysis preferentially inhibits ATP-sensitive K^+ channels in isolated guinea pig cardiac myocytes. *Science.* 238:67–69.

Zhang, J., S. H. Snyder. 1992. Nitric oxide stimulates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase. *Proceedings of National Academy of Sciences, USA.* 89:9382–9385.