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Failure of glucose to elicit a normal secretory response in fetal pancreatic beta cells results from glucose insensitivity of the ATP-regulated K^+ channels

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ABSTRACT **Fetal pancreatic beta cells demonstrate a deficient insulin release in response to glucose, but the underlying mechanism at the cellular level is unknown. By using beta cells from 21-day fetal rats we made an attempt to clarify the mechanism(s) behind this reduced glucose response. In addition to measuring insulin release, glucose metabolism, and cellular ATP content, ATP-regulated K^+ channels (G channels) and voltage-activated Ca^{2+} currents were investigated with the patch-clamp technique. It was thus demonstrated that the ATP-regulated K^+ channels in fetal beta cells were not sensitive to glucose but otherwise had similar characteristics as those of adult beta cells. Also, the characteristics of the voltage-activated Ca^{2+} currents were similar in adult and fetal beta cells. However, as judged from measurements of both glucose oxidation and glucose utilization, glucose metabolism was impaired in fetal beta cells. In addition, there was no increase in the ATP content, even when the cells were stimulated for 30 min. It is therefore concluded that the attenuated glucose-induced insulin release in fetal pancreatic beta cells is due to an immature glucose metabolism resulting in impaired regulation of the ATP-sensitive K^+ channels. These findings may be relevant to the understanding of the deficient stimulus–secretion coupling associated with non-insulin-dependent diabetes.**

In adult beta cells glucose promotes the closure of a K^+ channel that is regulated by intracellular ATP (G channel) (1–4), resulting in depolarization, opening of voltage-activated Ca^{2+} channels, and insulin release (1). The significance of the G channel is further emphasized by the finding that hypoglycemic sulfonylureas, drugs used in the treatment of non-insulin-dependent diabetes (type II diabetes), initiate insulin release by a direct closure of this channel (1, 5–7). Thus, one can postulate that failure of glucose to promote insulin release in type II diabetes might result from either the G channel or its regulation being defective. *In vitro* studies of the endocrine pancreas of both man and animals have demonstrated a poor coupling between ambient glucose concentration and fetal insulin discharge (8–20). So far it has not been possible to define the step(s) in the stimulus–secretion coupling responsible for the reduced glucose response. In the present study we intended to clarify whether the relative inability of glucose to promote insulin release from fetal pancreatic beta cells can indeed be explained in terms of an altered function of the G channel protein and/or a defect in G channel regulation. Information obtained from studies of the stimulus–secretion coupling in fetal beta cells is relevant also for the understanding of the mechanism(s) responsible for the defect in insulin release in type II diabetes.

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MATERIALS AND METHODS

Preparation and Dispersion of Fetal Pancreatic Islets as Well as Measurements of Insulin Release. Fetal rat islets were isolated from 21-day pregnant rats as in Hellerström *et al.* (21). The islets were collected after 1 day of culture in RPMI 1640 medium containing 11 mM glucose, 10% (vol/vol) fetal bovine serum, 100 international units of penicillin per ml, 100 μg of streptomycin per ml, and 50 μg of gentamycin per ml. Isolated islets were collected in groups of 50 and perifused at 37°C as described by Arkhammar *et al.* (1) using a Hepes buffer (pH 7.4), with Cl^- as the sole anion (22), containing 1.28 mM Ca^{2+} and 1 mg of bovine serum albumin per ml. The flow rate was 0.3 ml/min. Insulin contents of the effluents were determined according to Heding (23). Islets were dispersed to small clusters of various sizes as well as single cells with trypsin-EDTA and placed on coverslips for culture in the same type of medium.

Measurements of Single-Channel and Whole-Cell Currents. The gigaseal patch-clamp technique (24) was used for recording single-channel and whole-cell currents as described (4). During the experiments the current and voltage signals were stored on magnetic tape using a dc tape recorder (4D, Racal, Hythe, England). All filter settings are given as the −3-decibel point. The standard extracellular solution contained (in mM) 140 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, and 10 Hepes-NaOH (pH = 7.4). In the cell-attached and inside-out patch measurements the pipette solution (i.e., that adjacent to the extracellular face of the plasma membrane) contained (in mM) 145 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, and 10 Hepes-KOH (pH = 7.4). During the inside-out patch experiments the "intracellular" solution (i.e., the medium of the bath) contained (in mM) 120 KCl, 30 KOH, 10 EGTA, 2 $CaCl_2$, 1 $MgCl_2$, and 5 Hepes-KOH (pH = 7.15). When ATP was included, the concentration of Mg^{2+} was increased in parallel to ensure that it was always present in a 1 mM excess. In the whole-cell recordings the pipette solution consisted of (in mM) 150 *N*-methyl-D-glucamine, 10 HCl, 10 EGTA, 4 $MgCl_2$, 2 $CaCl_2$, 5 Hepes-HCl (pH = 7.15), and 3 ATP. The free concentrations of Ca^2 and Mg^{2+} were calculated to be 0.06 μM and 1.2 mM, respectively, by using the binding constants of Martell and Smith (25).

Measurements of Glucose Metabolism and ATP Content. Islets isolated from 21-day fetal rats were collected after 1 day or 7 days in culture in RPMI 1640 medium, as described above. The effects of 1.7 and 17 mM glucose on the rates of glucose oxidation and utilization (26) were studied by incubating batches of 10 islets each for 90 min at 37°C in 100 and 15 μl of KRB-Hepes buffer, respectively, containing [6-^{14}C]glucose (0.8–8 Ci/mol; 1 Ci = 37 GBq) (glucose oxidation) or D-[5-^{3}H]glucose (4–40 Ci/mol) (glucose utilization) and unlabeled glucose to yield the concentrations indicated. Glucose oxidation rates were calculated from the formation

of $^{14}CO_2$ during the incubation period, and glucose utilization rates were similarly determined from the formation of ^3H_2O. For the ATP measurements, the islets were transferred to dishes containing KRB supplemented with 10 mM Hepes, 1 mg of bovine serum albumin per ml, and 1.7 mM glucose and incubated for 30 min at 37°C in 5% CO_2. The glucose concentration was then increased to 17 mM in some of the dishes. All of the dishes were then incubated further for 30 min. After the incubation period, islets in groups of 10 were rapidly transferred to 100 μl of ice-cold 0.89 M perchloric acid containing 2 mM EDTA. The samples were briefly sonicated, neutralized by the addition of 100 μl of 0.89 M NaOH/2 mM EDTA and diluted 100 times with 0.1 M Tris-acetate buffer (pH 7.75) supplemented with 2 mM EDTA. The ATP contents of the samples were subsequently measured at room temperature using a bioluminescence procedure by adding 20 μl of an ATP reagent (LKB) to 80 μl of the sample, which provided a constant light signal. The light signal was quantified in a LKB 1250 luminometer.

RESULTS AND DISCUSSION

Insulin Release and Appearance of Fetal Beta-Cell Aggregates. When investigating the kinetics of insulin release from fetal pancreatic beta cells in a perfusion system (Fig. 1*A*), there was an increase in insulin release when raising the glucose concentration from 1.7 to 17 mM. However, this release was different from that obtained in adult beta cells (27), and in accordance with what has recently been demonstrated by Hole *et al.* (28) there was no biphasic response pattern. When depolarizing the fetal beta cells with high concentrations of K^+, there was a marked increase in insulin release. It has previously been demonstrated that there is a predominance of beta cells in the fetal islets (21), and Fig. 1*B* demonstrates aggregates of various sizes of such cells after isolation. It is known that adult beta cells have to be coupled to respond properly to glucose with an increased insulin release (27). Hence, when investigating metabolic effects on channel activity, small beta-cell aggregates, 5–10 cells, were used.

K^+ Channels in Fetal Pancreatic Beta Cells. Fig. 1*C* shows a recording from a cell-attached patch on a fetal beta cell recorded at the cell's membrane potential. Two types of channels are active within the patch (trace A). Whereas the small channel spends most of the time in an open state, indicated by the current level i_1, with only brief returns to the baseline zero current level (0), the larger channel ($i_1 + i_2$) opens infrequently. The small channel dominated most cell-attached patches on fetal beta cells and occasionally as many as four or five channels were active simultaneously within the same patch. The openings of the large channel are clustered together in bursts, as has previously been reported for the G channel of adult beta cells (1, 4), display flickery behavior, and decay exponentially with time (trace B). The decrease (40–50%) can be explained by a depolarization of 20–30 mV (see Fig. 1*D*). The waveform superimposed on the second group of openings is due to an action potential elicited by the inward channel current. The current (*i*)–voltage (*V*) relationships for the large (squares) channels in fetal (open symbols) and adult (filled symbols) beta cells (Fig. 1*D*) yielded the single-channel conductances (γ) of 57 ± 3 pS (n = 7) and 60 ± 2 pS (n = 4), respectively. These values are similar to those reported for the G channel in rat (2, 3) and mouse beta cells (1, 4). The corresponding values for γ of the smaller channel (circles) were 21 ± 4 pS (n = 6) and 25 ± 1 pS (n = 3) for fetal (open

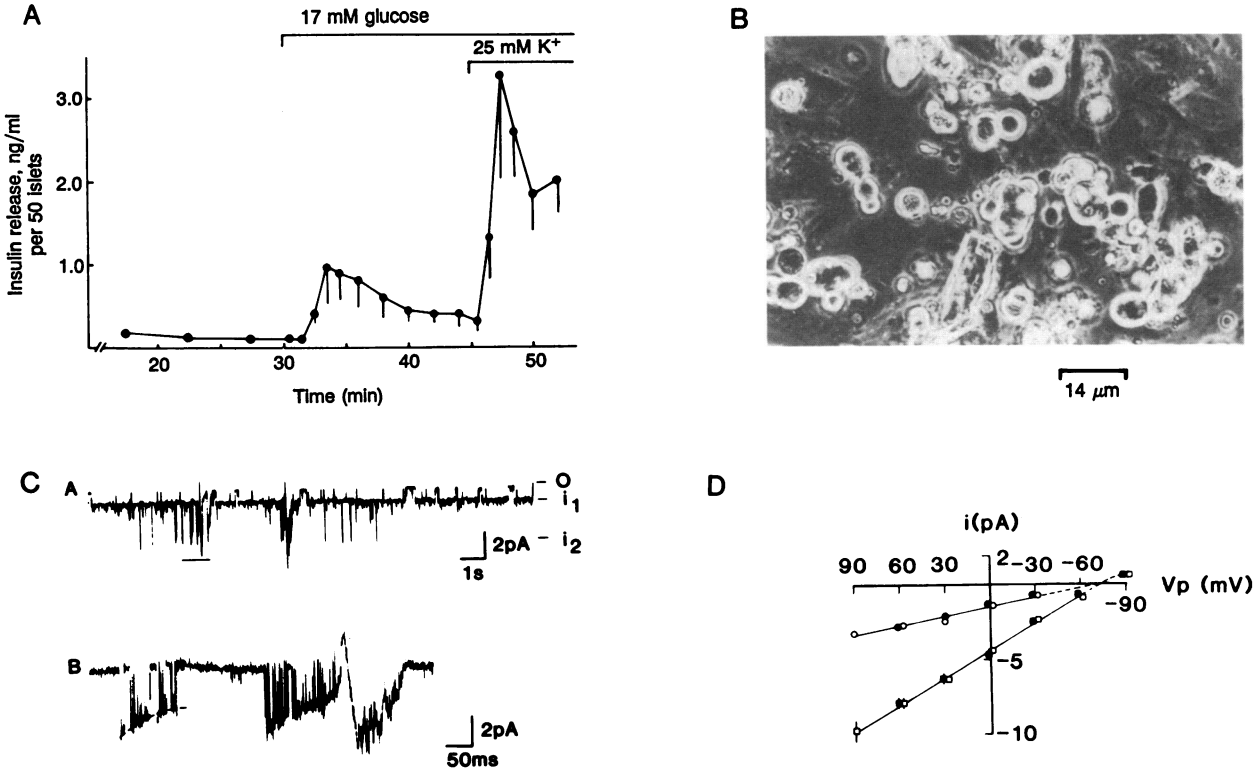

FIG. 1. Dynamics of insulin release and channel activity. (*A*) Insulin release from fetal pancreatic islets perifused as shown. The basal glucose concentration under these conditions was 1.7 mM. Mean values ± SEM for four different experiments are shown. (*B*) Small cell clusters of various sizes as well as single cells were photographed 4 hr after being placed on coverslips, using a phase-contrast microscope. The actual magnification is given by the inserted scale. (*C*) Trace A, a record from a cell-attached patch on a fetal beta cell. Trace B, expanded record of the part underlined in trace A. In traces A and B the current signal was filtered at 500 Hz and V_p corresponded to 0 mV. (*D*) *i*–*V* relationships of the small (circles) and large (squares) channels of fetal (open symbols) and adult (filled symbols) beta cells. Potentials indicate a shift in patch potential with respect to the resting potential (*Vr*) of the cell. For example, −30 mV indicates a patch potential 30 mV more negative than *Vr*.

symbols) and adult (filled symbols) beta cells, respectively. All channels had reversals around a pipette potential of +70 mV, as expected for K^+ channels in a cell assuming an internal K^+ concentration of 120 mM and a membrane potential of −70 mV (29, 30). Furthermore, also the intraburst kinetics were almost identical for G channels of fetal and adult beta cells (data not shown). Whereas adult rat beta cells respond with an inhibition of G-channel activity subsequent to glucose stimulation (2, 3), openings were observable for at least 20 min in the fetal beta cell (Fig. 2), a few cells even demonstrating an increase of the activity. The amplitude of the channel openings can be used to indirectly monitor changes in the membrane potential. If the cells depolarize, corresponding to more negative values of pipette potential (V_p) in Fig. 1*D*, the size of the channel openings will decrease. As can be noted in Fig. 2*B*, no such changes seemed to occur, suggesting that the fetal beta-cell membrane potential does not change. As a consequence, action potentials are not induced by glucose in these cells. The experiment shown here is representative for a total of nine experiments performed on aggregates comprising 5–10 cells. Noteworthy is that 2 cells in intact islets, the diameter being >100 μm, exhibited a response to glucose. Since previous studies have demonstrated that fetal beta cells mature during culture (31), this variability in glucose sensitivity can be attributed to the cells having reached a more mature state. As is the case for adult beta cells (1, 5–7, 32), the activity of the G channel of fetal beta cells was suppressed by the antidiabetic sulfonylurea tolbutamide (Fig. 2*C*). In 30% of the cells the cessation of channel activity was paralleled by the appearance of action potentials (Fig. 2*C*, trace a), appearing as biphasic current deflections (Fig. 2*C*, trace b), resulting from the activation of voltage-gated Ca^{2+} channels (compare Fig. 4). The small 21-pS K^+ channel was neither affected by glucose nor tolbutamide. However, the K^+ current through these channels might be large enough to maintain a polarized state, which should then account for the fact that tolbutamide failed to elicit action potentials in the remaining 70% of the fetal beta cells. As in adult beta cells, the G channel of the fetal beta cells could be activated by the hyperglycemic sulphonamide diazoxide at 400 μM (1, 5–7). Assuming that the glucose unresponsiveness in fetal beta cells is accounted for by an inability of the sugar to promote depolarization, this might result from either the channel protein or its regulation being defective. Since intracellular ATP serves as the coupling factor between glucose metabolism and membrane depolarization, this probably means that either the G channel in the fetal beta cell is insensitive to ATP or glucose stimulation fails to increase the ATP concentration enough to promote a closure of a majority of these channels. To investigate the first possibility, the ATP sensitivity of fetal G channels was investigated using inside-out patches (Fig. 3). From continuous recordings (traces a and b) it is apparent that the channel activity decreases with increasing ATP concentrations. Expanded sections of the record in Fig. 3*A* are shown in Fig. 3*B*, underlined, and marked a and b. In *C* the dose–inhibition curve is shown for the G channel of both fetal and adult beta cells. To construct these curves, the average current during exposure to ATP (I) was calculated and compared with that observed in the absence of ATP, the latter (I_{max}) taken as unity. The ratios I/I_{max} were plotted against the respective ATP concentrations and the values were fitted to the equation

$$I/I_{max} = 1/[1 + ([ATP]/K_i)^H], \quad [1]$$

where K_i is the concentration of ATP reducing channel activity by 50% and H is the Hill, or cooperativity, coefficient. The values for K_i and H were, respectively, 14 ± 5 μM and 0.68 ± 0.18 in fetal beta cells and 42 ± 14 μM and 0.98 ± 0.25 in adult beta cells. These findings indicate that the glucose insensitivity of fetal G channels is not due to a decreased sensitivity to ATP. The fact that the values for the Hill coefficient approach 1 for the G channels of both fetal and adult beta cells indicates that one molecule of ATP is required to block one channel. Recent studies on adult beta cells have demonstrated that the G channel is actually not solely regulated by intracellular ATP but rather by the ATP/ADP ratio, the presence of ADP reducing the sensitivity of the G channel to ATP (1, 33–35). In fetal beta cells 2 mM ADP is able to relieve the block induced by 1 mM ATP (data not shown).

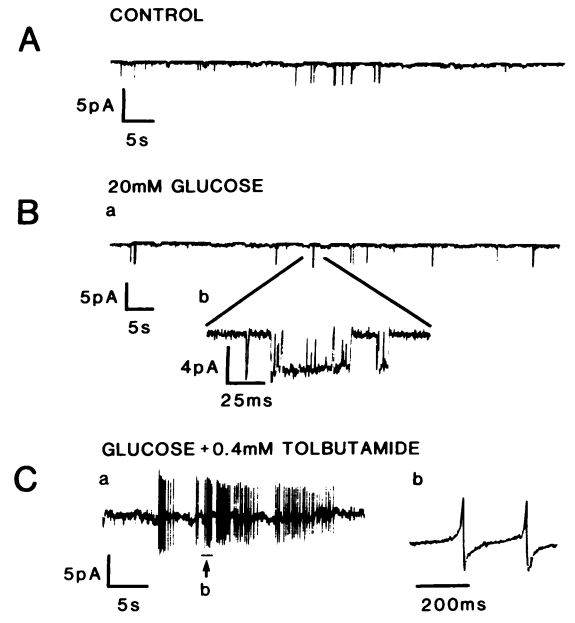

FIG. 2. Effects of glucose and tolbutamide on channel activity recorded from a cell-attached patch on a fetal beta cell. Glucose and tolbutamide were included as indicated. (*A*) Openings observed under basal (0 mM glucose) conditions. (*B*) Trace a, >10 min after addition of 20 mM D-glucose. Note that channel activity persists. Trace b (inset), expanded opening showing typical flickery behavior of channel. (*C*) Trace a, a few minutes after adding 0.4 mM tolbutamide to a cell while maintaining glucose at 20 mM, the cell starts generating biphasic current deflections, which are interpreted as action potentials and are displayed with an expanded time scale (trace b). The part underlined in trace a is shown. V_p corresponded to 0 mV. The filter was 125 Hz except in *B*, trace b, where it was 1 kHz.

Voltage-Activated Ca^{2+} Currents. To exclude that the immature response to glucose in fetal beta cells can be accounted for by a defect in the voltage-activated Ca^{2+} channels, we have investigated this possibility by taking advantage of the whole-cell configuration of the patch-clamp technique (24) (see Fig. 4). In Fig. 4 *A* and *B* the rising and falling parts of the Ca^{2+}-current *I–V* are demonstrated. The current becomes detectable at potentials beyond −50 mV and peaks around zero. Equimolar replacement of Ca^{2+} by Ba^{2+} increases the current and removes inactivation (not shown). Fig. 4*C* shows the *I–V* relationships of the currents carried by Ca^{2+} (filled circles) with respect to Ba^{2+} (open circles) through the Ca^{2+} channels. Apart from increasing the current, Ba^{2+} also shifts activation by ≈20 mV, toward more negative potentials (36, 37). In experiments, not demonstrated here, we observed that 0.2 mM Cd^{2+} completely blocks the Ca^{2+} current, as can be expected for L-type Ca^{2+} channels (38). Hence, when comparing these data with results obtained from studies on adult beta cells (39), there are no indications suggesting that fetal beta cells are equipped with defective voltage-activated Ca^{2+} channels.

Glucose Metabolism. Previous studies have demonstrated a higher rate of oxygen consumption in fetal compared to adult

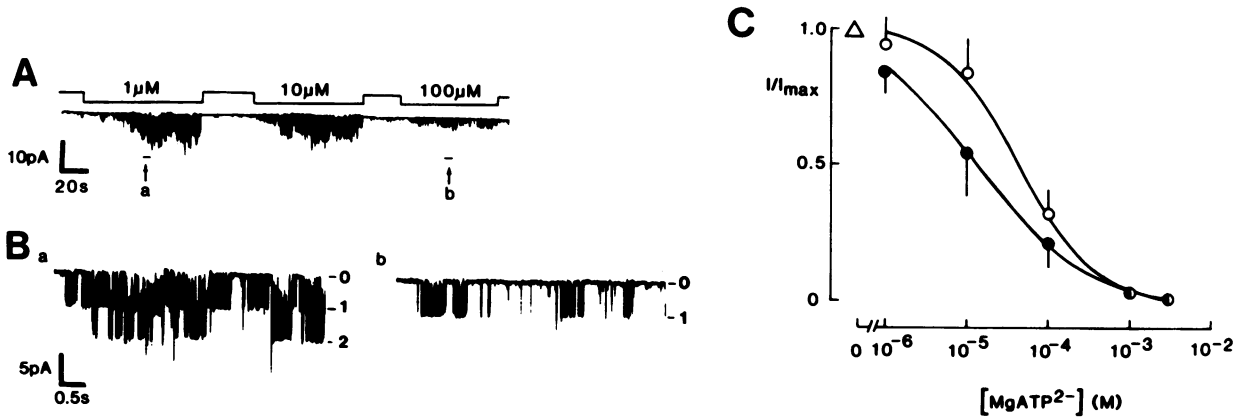

FIG. 3. Comparison of ATP sensitivity of channel activity in inside-out patches from fetal and adult beta cells. (*A*) Continuous record from a fetal beta cell. ATP (6 mM) was present throughout to minimize rundown. The ATP concentration was lowered as indicated (see staircase above recording). (*B*) Traces a and b, expanded records taken from the trace shown in *A*. Trace a, registration in the presence of 1 μM ATP. Current levels equivalent to zero to two simultaneous openings are indicated. Trace b, record obtained in the presence of 100 μM ATP. Activity was significantly reduced. The current level corresponding to zero to one openings is indicated. Note different ordinate and abscissa scales in *A* and *B*. The current signals were filtered at 125 Hz and the membrane potential was −70 mV. (*C*) Dose–inhibition curves for adult (open circles) and fetal (closed circles) beta-cell channel activity. The fraction of current remaining relative to that in the absence of ATP (I/I_{max}) is shown against the respective ATP concentrations. Concentrations of ATP refer to the amounts of MgATP added to the solution. The triangle gives the current amplitude in the absence of ATP, which was taken as unity. To calculate the average current in the ATP inhibition experiments, currents of 30–60 s length were filtered to 0.2 kHz and digitized at 1 kHz. The level of baseline was visually set to zero, and the average current was calculated by integration. Values are means ± SEM for four experiments (adult beta cells) or three experiments (fetal beta cells). The solid curves were drawn by fitting the observed values to Eq. 1, using an iterative least-squares nonlinear regression analysis.

rat islets (31). In addition, glucose failed to increase oxygen consumption in fetal islets, under conditions where the adult islets demonstrated a marked increase. In adult rat islets an increased concentration of glucose not only increases the total rate of hexose utilization but, relative to total utilization, also favors mitochondrial oxidative processes. Interestingly, in the clonal insulin-producing tumor cells RINm5F, which do not release insulin in response to glucose, there is no preference for mitochondrial oxidative processes (40). As evident from the present study, the glucose utilization in the presence of 17 mM glucose, the glucose oxidation, and the ratio of glucose oxidation to glucose utilization were all lower in fetal islets cultured for 1 day compared to those cultured for 7 days (Table 1). The ratios of glucose oxidation to glucose utilization on day 7 were still lower than those observed in adult islets (41). A disturbed glucose oxidation/glucose utilization ratio is also observed in islets from glucose-intolerant rats (41), suggesting that the immature glucose metabolism prevailing in fetal beta cells can be accounted for, at least in part, by an altered mitochondrial function. That the glucose metabolism of the fetal beta cells is defective is further supported by the measurements of the total ATP content. Glucose failed to increase the total ATP content in fetal islets cultured for 1 day, even when the incubation period was

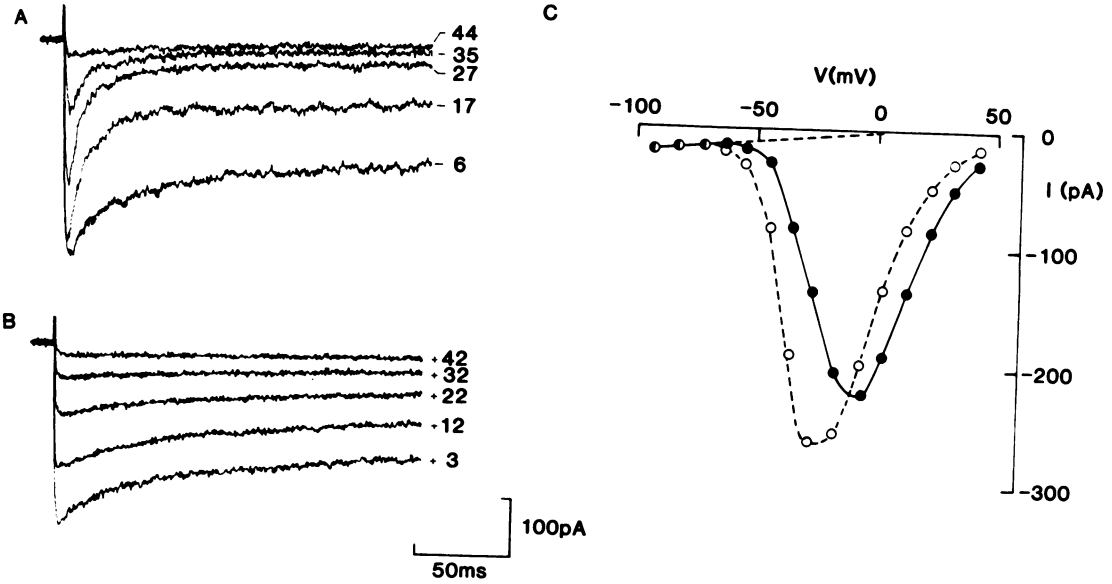

FIG. 4. Whole-cell recordings of Ca^{2+} currents in a fetal beta cell. When recording the Ca^{2+} currents, the Ca^{2+} concentration of the extracellular solution was raised to 10.2 mM and sometimes equimolarly substituted by Ba^{2+}. (*A* and *B*) Inward membrane currents recorded in the presence of 10.2 mM Ca^{2+} in the bath. The holding potential was −70 mV and voltage pulses to potentials indicated to the right of the traces were applied at a rate of 0.5 Hz (filter, 2 kHz). (*C*) Whole-cell current–voltage relationships for the Ca^{2+} current in the presence of Ca^{2+} (filled circles) or Ba^{2+} (open circles). Replacement of Ca^{2+} with Ba^{2+} resulted in an increase in the current and a shift of the peak current from −6 to −27 mV. The cell capacitance was 5.7 pF and the input resistance was 13.1 GΩ.

Table 1. Rates of glucose oxidation and utilization and ATP contents in fetal pancreatic islets

Culture time, days	Glucose utilization, pmol per islet per 90 min		Glucose oxidation, pmol per islet per 90 min		Glucose oxidation/glucose utilization, %		ATP content, pmol per islet	
	1.7 mM	16.7 mM	1.7 mM	16.7 mM	1.7 mM	16.7 mM	1.7 mM	16.7 mM
1	57 ± 13	109 ± 31	1.3 ± 0.2	2.0 ± 0.5	2.9 ± 0.6	3.1 ± 1.3	11.5 ± 1.8	12.8 ± 1.5
7	70 ± 33	256 ± 105*	3.7 ± 0.7†	14.5 ± 2.2‡	8.7 ± 1.4†	10.3 ± 2.2§	7.3 ± 1.2	9.3 ± 1.7¶

The results for glucose oxidation and utilization are given as means ± SEM for 6 experiments in pmol of glucose metabolized per islet per 90 min. The ratio of glucose oxidation/glucose utilization was determined in each experiment and is given as a percentage. For the ATP contents the results are given as means ± SEM for 6–10 experiments.
*$P < 0.05$ when compared with 1-day cultured fetal islets using a paired Student's t test.
†$P < 0.01$ when compared with 1-day cultured fetal islets using Student's t test.
‡$P < 0.001$ when compared with 1-day cultured fetal islets using Student's t test.
§$P < 0.05$ when compared with 1-day cultured fetal islets using Student's t test.
¶$P < 0.05$ when compared with 1.7 mM glucose incubation using a paired Student's t test.

extended to 30 min (Table 1). Since fetal pancreatic islets mature during culture (31), it is not astonishing that islets cultured for 7 days demonstrated a prompt increase in their ATP content after 30 min of glucose stimulation (Table 1). However, even after this prolonged culture period, the fetal islets failed to increase their ATP content when the stimulation period was only 5 min (data not shown). Control experiments performed on adult rat islets demonstrated a prompt increase (<5 min) of about 25% in the islet ATP content in response to 17 mM glucose (data not shown). Since the G channels directly determine the resting conductance of the beta cell, it is easy to envisage why an impaired generation of ATP leads to a defective depolarization and thereby no opening of the voltage-activated Ca^{2+} channels, no increase in the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), and no insulin release. That the impaired glucose-induced insulin release in fetal beta cells is due to a defect in the G channels and/or voltage-activated Ca^{2+} channels *per se* is ruled out not only from the patch-clamp experiments in the present study, revealing similar channel characteristics in both fetal and adult beta cells, but also from direct measurements of $[Ca^{2+}]_i$. Hence, whereas glucose fails to increase $[Ca^{2+}]_i$ in fetal pancreatic beta cells, both tolbutamide and high concentrations of extracellular K^+ do (data not shown).

Conclusions. We would like to propose that the failure of glucose to promote a proper stimulation of insulin release in fetal beta cells is accounted for by an immature oxidative metabolism of the sugar. Hence, an increased extracellular glucose concentration fails to generate the proper signal for the initiation of the stimulus–secretion coupling. In adult beta cells this signal corresponds to an increased ATP/ADP ratio (1–4), resulting in a closure of the G channels. Identification of the exact step(s) responsible for the immature glucose response will require more elaborate experiments, which may turn out to be rewarding also to the understanding of the secretory defect prevailing in the type II diabetic beta cell.

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