Direct glucocorticoid inhibition of insulin secretion. An in vitro study of dexamethasone effects in mouse islets.

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Research Article

The direct effects of glucocorticoids on pancreatic beta cell function were studied with normal mouse islets. Dexamethasone inhibited insulin secretion from cultured islets in a concentration-dependent manner: maximum of approximately 75% at 250 nM and IC50 at approximately 20 nM dexamethasone. This inhibition was of slow onset (0, 20, and 40% after 1, 2, and 3 h) and only slowly reversible. It was prevented by a blocker of nuclear glucocorticoid receptors, by pertussis toxin, by a phorbol ester, and by dibutyryl cAMP, but was unaffected by an increase in the fuel content of the culture medium. Dexamethasone treatment did not affect islet cAMP levels but slightly reduced inositol phosphate formation. After 18 h of culture with or without 1 microM dexamethasone, the islets were perifused and stimulated by a rise in the glucose concentration from 3 to 15 mM. Both phases of insulin secretion were similarly decreased in dexamethasone-treated islets as compared with control islets. This inhibition could not be ascribed to a lowering of insulin stores (higher in dexamethasone-treated islets), to an alteration of glucose metabolism (glucose oxidation and NAD(P)H changes were unaffected), or to a lesser rise of cytoplasmic Ca2+ in beta cells (only the frequency of the oscillations was modified). Dexamethasone also inhibited insulin secretion induced by arginine, tolbutamide, or high K+. In this case also the […]

Direct Glucocorticoid Inhibition of Insulin Secretion

An In Vitro Study of Dexamethasone Effects in Mouse Islets

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Abstract

The direct effects of glucocorticoids on pancreatic β cell function were studied with normal mouse islets. Dexamethasone inhibited insulin secretion from cultured islets in a concentration-dependent manner: maximum of ~ 75% at 250 nM and IC_{50} at ~ 20 nM dexamethasone. This inhibition was of slow onset (0, 20, and 40% after 1, 2, and 3 h) and only slowly reversible. It was prevented by a blocker of nuclear glucocorticoid receptors, by pertussis toxin, by a phorbol ester, and by dibutyryl cAMP, but was unaffected by an increase in the fuel content of the culture medium. Dexamethasone treatment did not affect islet cAMP levels but slightly reduced inositol phosphate formation. After 18 h of culture with or without 1 μM dexamethasone, the islets were perifused and stimulated by a rise in the glucose concentration from 3 to 15 mM. Both phases of insulin secretion were similarly decreased in dexamethasone-treated islets as compared with control islets. This inhibition could not be ascribed to a lowering of insulin stores (higher in dexamethasone-treated islets), to an alteration of glucose metabolism (glucose oxidation and NAD(P)H changes were unaffected), or to a lesser rise of cytoplasmic Ca^{2+} in β cells (only the frequency of the oscillations was modified). Dexamethasone also inhibited insulin secretion induced by arginine, tolbutamide, or high K^+. In this case also the inhibition was observed despite a normal rise of cytoplasmic Ca^{2+}. In conclusion, dexamethasone inhibits insulin secretion through a genomic action in β cells that leads to a decrease in the efficacy of cytoplasmic Ca^{2+} on the exocytotic process. (*J. Clin. Invest.* 1997. 99:414–423.) Key words: glucocorticoids • pancreatic islets • insulin secretion • stimulus-secretion coupling • cytoplasmic calcium

Introduction

Glucocorticoids exert profound effects on glucose homeostasis under both normal and pathologic conditions. During periods of fasting, cortisol, in concert with other counterregulatory hormones, participates in the maintenance of blood glucose levels by decreasing utilization and increasing production of glucose (1–3). Patients with a glucocorticoid deficiency have a high insulin sensitivity and incur a risk of hypoglycemia, whereas glucocorticoid excess is associated with insulin resistance and tendency to hyperglycemia (1, 4).

Chronic administration of glucocorticoids increases fasting and stimulated plasma insulin levels in humans and animals (5). This rise is regarded as an adaptation of pancreatic β cell function to the insulin resistance that steroids cause in liver, muscle, and adipose tissues, and is likely to be mediated by the concomitant slight elevation of plasma glucose levels. Because glucocorticoid receptors are present in β cells, as demonstrated by both immunocytochemistry (6) and in situ hybridization (7), it is possible that glucocorticoids also exert direct effects on insulin secretion. This question is difficult to address in vivo because of the multiple influences that can impact on the endocrine pancreas. However, several lines of evidence indicate that glucocorticoids might initially inhibit insulin secretion in humans (8–10) and animals (11). Whether this effect is direct or indirect remains unresolved. Complex results have also been yielded by studies in which rats or mice were treated with glucocorticoids and insulin secretion was subsequently measured in vitro. Augmented (12–15), unchanged (16, 17), and decreased (14, 18, 19) insulin secretion has been reported. These differences are not easily explained and appear to depend on the dose of glucocorticoid, the length of administration, the propensity of the animal strain to develop diabetes, and the stimulus used in vitro.

Direct, in vitro effects of glucocorticoids on insulin secretion have been reported. A rapid (within minutes) inhibition of glucose-induced insulin secretion was produced by 0.1–1 μM corticosterone (20, 21) or 100 μM cortisone (22), and was attributed to an alteration of Ca^{2+} fluxes in β cells (23). In contrast, no rapid inhibitory effect was observed in several other studies (12, 16, 24, 25). A slower (within hours or days) inhibitory effect followed treatment of isolated rat or mouse islets with cortisol, prednisolone, or dexamethasone (25–27). The mechanisms underlying this inhibition are not known. In this study, therefore, normal mouse islets were treated with dexamethasone under various conditions and submitted to different tests in order to explore how glucocorticoids directly influence stimulus–secretion coupling in pancreatic β cells.

Methods

Preparation. All experiments were performed with islets isolated by collagenase digestion of the pancreas of male NMRI mice, killed by decapitation. After isolation, the islets were usually cultured for 18 h at 37°C in 35-mm petri dishes containing 3 ml RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The culture medium also contained dexamethasone or its solvent alone (DMSO 1:1000). Unless specifically indicated the concentration of glucose was 10 mM and no other substance was added.

Solutions. The medium used for islet isolation and for the experiments after islet culture was a bicarbonate-buffered solution which contained 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$,

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and 24 mM $NaHCO_3$. It was gassed with O_2/CO_2 (94:6) to maintain pH 7.4, and was supplemented with BSA (1 mg/ml). Ca^{2+}-free solutions were prepared by replacing $CaCl_2$ with $MgCl_2$. Dexamethasone or DMSO was added to the solutions used during the different experiments.

Measurements of insulin secretion and content. Insulin secretion during the culture period was measured in aliquots of medium taken after 18 h. When the onset and the reversibility of the effects of dexamethasone were studied, aliquots of medium were also taken at different times during the culture period. Insulin secretion was also measured after culture with or without dexamethasone. The islets were first washed and preincubated for 60 min in the presence of 15 mM glucose. Batches of three islets were then incubated for 60 min in the presence of various test substances. At the end of the incubation, a portion of the medium was appropriately diluted before insulin measurement. To measure the dynamics of insulin secretion, batches of 20 or 30 islets (depending on the protocol) were placed in parallel perifusion chambers and perifused at a flow rate of 1 ml/min (28). Samples were collected every 1 or 2 min for insulin measurement. In one series of experiments the islets were not cultured but immediately preincubated for 60 min in the presence of 15 mM glucose and then perifused. In several experiments islets were recovered from the petri dishes or from the incubation tubes, and their insulin content was determined after extraction in acid ethanol (29). Insulin was measured by a double-antibody radioimmunoassay with rat insulin as the standard (Novo Research Inst., Bagsvaerd, Denmark).

Measurements of the concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$)[1] and of NAD(P)H. After culture, the islets to be used for $[Ca^{2+}]_i$ measurement were loaded with fura-2 during a 40-min preincubation in the presence of 10 mM glucose and 1 μM fura-2-acetoxymethylester (Molecular Probes, Inc., Eugene, OR). They were then transferred into a temperature-controlled perifusion chamber placed on the stage of an inverted microscope, and successively excited at 340 and 380 nm. The emitted fluorescence, filtered at 510 nm, was recorded with a CCD camera (Photonic Sciences Ltd., Turnbridge Wells, UK). The images were analyzed with the system MagiCal (Applied Imaging Intl. Ltd., Sunderland Tyne, UK). The technique has been described in detail previously (30). The islets to be used for NAD(P)H measurement were preincubated without dye. They were then transferred to the same recording system, but were excited at 360 nm and the emitted fluorescence was filtered at 470 nm (30). The method is known to record $[Ca^{2+}]_i$ changes in β cells with little interference from other cells (30–32). It should indeed be borne in mind that islets from normal mice are composed of about 80% β cells in number and almost 90% β cells in volume (because of their larger volume than that of non-β cells; 33).

Measurements of glucose oxidation. After culture and preincubation in 15 mM glucose, batches of 10 islets were incubated for 2 h at 37°C in 50 μl of medium supplemented with 1μCi [U-^{14}C]glucose. Oxidation of glucose was calculated from the production of $[^{14}C]CO_2$. Technical aspects of the method have been described previously (34).

Measurements of inositol phosphates and cAMP. After culture, the islets were loaded with [2-^{3}H]*myo*-inositol during 2 h in the presence of 15 mM glucose. After washing, batches of about 40 islets were incubated in 500 μl medium containing 1 mM cold inositol, 10 mM LiCl, and 15 mM glucose. After 30 min, 500 μl of solution containing an appropriate concentration of test substance were added for another 30 min. The experiments were stopped by addition of 3 ml of chloroform/methanol/concentrated HCl (200:100:1, by vol) and 100 μl EDTA (100 μM). The lipid and the water phases were separated by centrifugation. Free inositol and inositol phosphates dissolved in the water phase were separated by anion exchange chromatography (35). Phosphoinositides labeled with [2-^{3}H]*myo*-inositol and present in the lipid phase were measured after deacylation and separation of the glycerophosphoesters (36). Technical aspects of the methods have been reported previously (37). In the experiments aimed at measuring cAMP, batches of 10 cultured islets were quickly washed twice with ice-cold acetate buffer containing 0.5 mM isobutylmethylxanthine before being boiled and sonicated in the same buffer. Islet cAMP content was then determined with a commercially available kit (DuPont-New England Nuclear, Boston, MA) as described previously (38).

Materials. Dexamethasone was obtained from Certa Ltd. (Braine l'Alleud, Belgium) and dissolved (1 mM) in DMSO. Diazoxide was from Schering–Plough Avondale Corp. (Rathdrum, Ireland); ketoisocaproic acid, acetylcholine, yohimbine, PMA, dibutyryl cAMP, and pertussis toxin were obtained from Sigma Chemical Co. (St. Louis, MO). Compound RU 38486 (Mifepristone) was kindly provided by

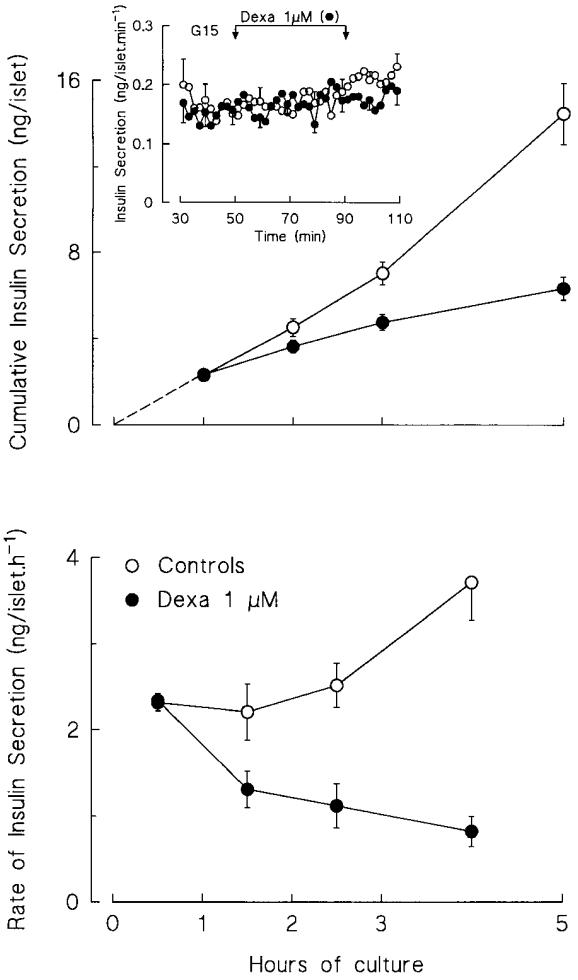

Figure 1. Onset of dexamethasone inhibition of insulin secretion by isolated islets. Batches of 10 islets were cultured for 5 h without or with 1 μM dexamethasone. *Top* shows the cumulative insulin secretion. *Bottom* shows the same results expressed as a secretion rate per hour, calculated from the difference between two successive measurements for each batch of islets. Values are means±SEM for 20 batches of islets from four separate experiments. *Inset* shows that 1 μM dexamethasone (dexa) had no immediate effect on insulin secretion by islets perifused with a bicarbonate-buffered medium containing 15 mM glucose (G) immediately after isolation and preincubation for 60 min in the presence of 15 mM glucose. Values are means±SEM for six separate experiments.

1. *Abbreviations used in this paper:* ACh, acetylcholine; AVP, arginine vasopressin; $[Ca^{2+}]_i$, concentration of cytoplasmic Ca^{2+}; KIC, α-ketoisocaproate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin.

Roussel-Uclaf (Romainville, France). Most other reagents were from UCB Chemicals (Braine l'Alleud, Belgium).

Presentation of results. Results are presented as means±SEM for the indicated number of batches of islets or of experiments. The statistical significance of differences between means was assessed by Student's *t* test or by one-way analysis of variance followed by Newman-Keuls test when more than two groups were compared. A two-way analysis of variance was also used to analyze the results of insulin secretion by incubated islets. Differences were considered significant at $P < 0.05$.

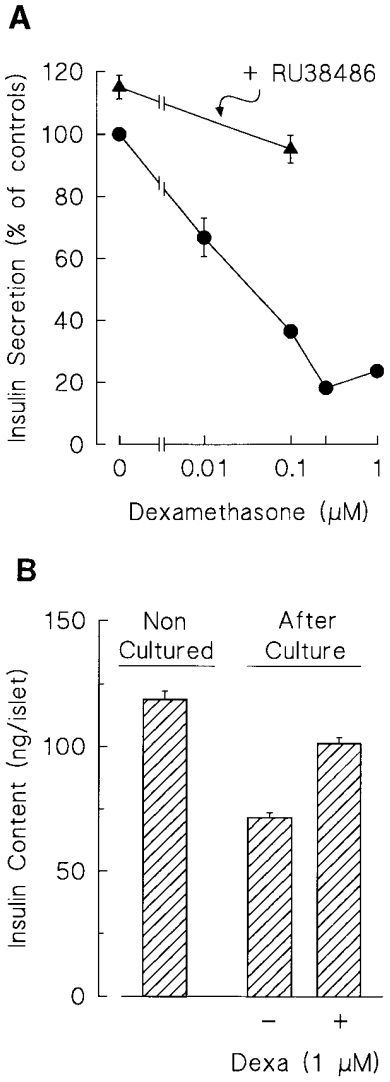

Figure 2. Insulin secretion by and insulin content of islets cultured for 18 h with dexamethasone. (*A*) Effect of various concentrations of dexamethasone on the amount of insulin released by islets cultured for 18 h in RPMI medium containing 10 mM glucose. Certain experiments were performed in the presence of 1 μM RU 38486. All values are expressed as a percentage of release by control islets from the same experiment and cultured without dexamethasone. Values are means±SEM for 4–23 experiments. (*B*) The insulin content of islets cultured without or with 1 μM dexamethasone is compared to that of noncultured islets from the same preparations. Values are means±SEM for 125 batches of islets from 23 experiments.

Results

Time and concentration dependence of the effects of dexamethasone on insulin secretion. Isolated mouse islets steadily released insulin during the initial hours of culture (Fig. 1). The secretion rate was fairly stable during the first 3 h and then slightly increased. Less insulin was secreted by islets cultured in the presence of 1 μM dexamethasone. However, the secretion rate was unaffected during the first hour and declined only thereafter, to a value of no more than 20–25% of that of controls between 3 and 5 h (Fig. 1, *bottom*). That dexamethasone had no acute, even transient, inhibitory effect was confirmed by monitoring the dynamics of insulin secretion from islets perifused with a bicarbonate-buffered medium containing 15 mM glucose (Fig. 1, *inset*).

The concentration dependence of the effect of dexamethasone was tested with islets cultured for 18 h. As shown in Fig. 2 *A*, the inhibition of insulin secretion increased with the concentration of dexamethasone to a maximum of ~ 80% at 250 nM. The IC_{50} was observed at ~ 20 nM. At the end of the 18-h culture, the insulin content of control islets was ~ 40% lower ($P < 0.001$) than that of noncultured islets from the same preparations (Fig. 2 *B*), whereas that of islets cultured with 1 μM dexamethasone was only 15% lower ($P < 0.05$).

Compound RU 38486 is a potent blocker of nuclear glucocorticoid receptors (39). Its addition to the culture medium slightly increased control insulin secretion over 18 h (by ~ 15%,

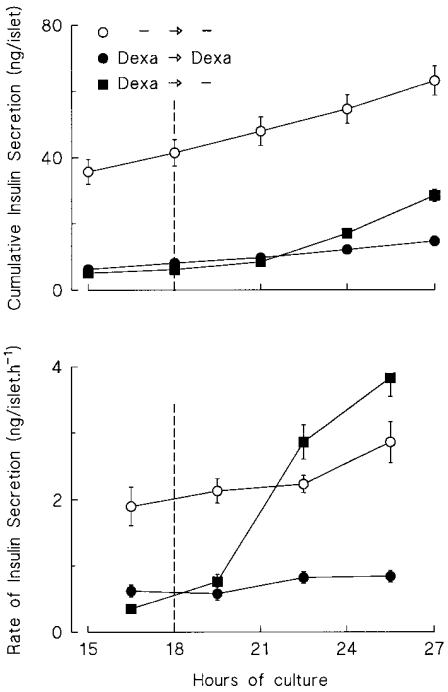

Figure 3. Reversibility of dexamethasone inhibition of insulin secretion by cultured islets. Batches of 10 islets were cultured without or with 250 nM dexamethasone. An aliquot of medium was taken at 15 and 18 h. After 18 h, the medium was replaced by fresh medium without or with dexamethasone as indicated, and the islets were cultured for another 9 h. *Top* shows the cumulative insulin secretion. *Bottom* shows the same results expressed as a secretion rate per hour for each 3-h period. Values are means±SEM for 25 batches of islets from five separate experiments.

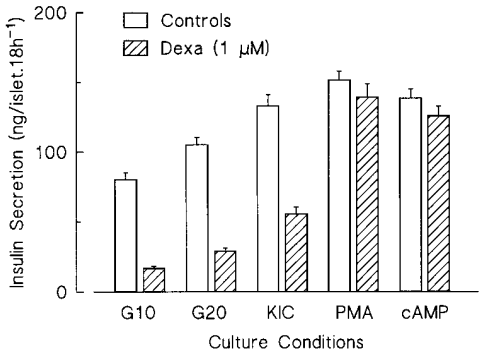

Figure 4. Effect of different agents on dexamethasone inhibition of insulin secretion during culture. Batches of 10 islets were cultured for 18 h in RPMI medium without or with 1 μM dexamethasone. The medium contained 10 or 20 mM glucose (G), or 10 mM glucose plus 10 mM ketoisocaproate (KIC), 25 nM PMA or 0.5 mM dibutyryl-cAMP (cAMP). Values are means±SEM for 12–26 batches of islets from seven separate cultures.

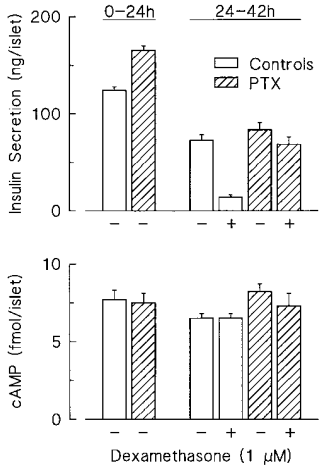

Figure 5. Effect of pertussis toxin on dexamethasone inhibition of insulin secretion during culture and on cAMP levels in islets after culture. *Top*: batches of 10 islets were cultured for 24 h without or with 200 ng pertussis toxin (PTX)/ml. Dexamethasone (1 μM) was then added to half of the batches from each group and the culture was continued for 18 h. The insulin content of the culture medium was measured after 24 and 42 h and the amount of insulin released during the last 18 h was determined by difference for each batch of islets. Values are means±SEM for 20 batches of islets from five separate cultures. *Bottom*: after the first 24 h of culture without or with pertussis toxin, certain batches of islets were processed for measurement of cAMP content. The other batches were then cultured without of with dexamethasone as indicated, before being processed for cAMP measurement after 42 h. Values are means±SEM for 14 batches of islets from four separate cultures.

$P < 0.05$) and largely prevented the inhibitory effect of 0.1 μM dexamethasone (Fig. 2 *A*).

The reversibility of the inhibition of insulin secretion was studied after 18 h of culture in the presence of 250 nM dexamethasone (Fig. 3). Insulin secretion by test islets maintained in the presence of dexamethasone throughout was steadily inhibited. When the culture medium was changed to fresh medium without dexamethasone, the secretion rate increased to higher values than in control islets, but this rise was delayed by about 3 h (Fig. 3).

Attempts to modify the inhibitory effect of dexamethasone during culture. The islets were usually cultured in RPMI medium containing 10 mM glucose. Raising the concentration of glucose to 20 mM or combining 10 mM glucose and 10 mM α-ketoisocaproate (KIC) increased the amount of insulin secreted over 18 h by control islets (Fig. 4). However, this did not affect the inhibitory action of dexamethasone which still amounted to ~ 60% in the presence of KIC. In contrast, addition of either 25 nM PMA or 0.5 mM dibutyryl cAMP to the culture medium containing 10 mM glucose increased insulin release and prevented the inhibition by dexamethasone (Fig. 4).

It has recently been reported that glucocorticoids upregulate the expression of α_2-adrenoceptors in two insulin-secreting cell lines (40). Because activation of these receptors potently inhibits insulin secretion (41) we tested whether their blockade might affect the inhibitory action of dexamethasone. Addition of 10 μM yohimbine to the culture medium, a concentration that abolishes clonidine inhibition of insulin secretion in our model (42), had no significant effect on insulin secretion from control islets (86±5 vs. 78±6 ng/islet, $18h^{-1}$ with and without the drug). However, it slightly attenuated the inhibitory action of dexamethasone (42±3 vs. 18±2 ng/islet, $18h^{-1}$, i.e., 51 and 77% inhibition, respectively, with and without yohimbine, $P < 0.001$).

Because the effects of α_2-adrenoceptors are mediated by pertussis toxin-sensitive G proteins (43), we looked for a possible influence of pertussis toxin on the action of dexamethasone. In preliminary experiments we found that it was necessary to culture mouse islets for about 42 h in the presence of 200 ng/ml pertussis toxin to abolish the inhibition of insulin release by epinephrine, and this control subsequently served to ascertain the efficacy of pertussis-toxin in all experiments (data not shown). Fig. 5 (*top*) shows that control islets cultured with pertussis toxin secreted ~ 30 and ~ 13% more insulin than did control islets during the first 24 and subsequent 18 h, respectively. When dexamethasone was added to the medium for the last 18 h, it produced its usual ~ 80% inhibition of insulin secretion from control islets, but did not significantly affect secretion from pertussis toxin-treated islets (Fig. 5, *top*). Parallel experiments showed that cAMP levels were not significantly different in control islets and islets treated with the toxin, and that dexamethasone did not lower cAMP in either group (Fig. 5, *lower panel*).

Effects of glucose in islets cultured with dexamethasone. The functional characteristics of mouse islets cultured for 18 h in the presence of 1 μM dexamethasone were studied in experiments carried out with a bicarbonate-buffered medium (supplemented with dexamethasone for test islets). In the interpretation of the secretory data, it should always be borne in mind that the insulin content of dexamethasone-treated islets was ~ 30% higher than that of control islets (Fig. 2).

Basal insulin secretion from islets incubated in the presence of 3 mM glucose was not significantly affected by dexamethasone treatment, whereas the responses to 15 and 30 mM glucose were clearly inhibited (Table I).

Glucose oxidation by control cultured islets was fivefold higher in the presence of 15 mM rather than 3 mM glucose in the incubation medium (Fig. 6, *left*). Similar changes were measured in islets cultured with 0.1 and 1 μM dexamethasone.

Table I. Effects of 18 h of Culture in the Presence of 1 μM Dexamethasone on Insulin Secretion by Mouse Islets Incubated with Various Test Agents

Line	Test agents during incubation	*n*	Insulin secretion (ng/islet.h^{-1})		Change induced by dexamethasone
			Controls	Dexamethasone	
	mM				
1	Glucose 3	40	0.29±0.02	0.24±0.02	−15%
2	Glucose 15	100	2.10±0.10	1.38±0.08*	−35%
3	Glucose 30	30	3.62±0.22	1.54±0.11*	−56%
4	G3 + KIC 10	30	3.72±0.33	1.66±0.23*	−58%
5	G3 + Ca 0 + Ba 2.5	20	6.84±0.55	4.52±0.46*	−33%
6	G3 + K30 + Dz 0.25	20	4.42±0.36	2.10±0.25*	−52%
7	G15 + K30 + Dz 0.25	40	7.04±0.44	3.80±0.40*	−48%
8	G15 + Arginine 10	25	15.5±0.79	11.1±0.95*	−29%
9	G15 + Tolbutamide 0.025	20	4.68±0.42	2.29±0.26*	−50%
10	G15 + Dibutyryl-cAMP 1	31	11.8±0.65	11.9±0.65	−0%
11	G15 + PMA 0.025	25	13.5±0.57	15.0±0.92	+10%

The islets were first cultured for 18 h in RPMI medium containing 10 mM glucose and supplemented or not with 1 μM dexamethasone. Batches of three islets were then incubated for 1 h in 1 ml of bicarbonate-buffered medium containing the indicated test substances. At the end of the incubation, an aliquot of the medium was taken for measurement of insulin secretion. Values are means±SEM for *n* batches of islets from 4–20 different cultures. *$P < 0.05$ or less vs. controls by two-way analysis of variance.

The effect of glucose on β cell metabolism was also measured by recording the NAD(P)H fluorescence of the islets (44). Raising the concentration of glucose from 3 to 15 mM markedly and reversibly increased the signal in both control and dexamethasone-treated islets (Fig. 6, *right*). Because no absolute quantification of the signal is possible, the changes are expressed relative to basal values in 3 mM glucose. We, therefore, cannot exclude the possibility that the apparently larger response of dexamethasone-treated islets might be caused by a slightly lower basal signal. Nevertheless, a conservative conclusion of both series of experiments is that glucose metabolism is not impaired in islets cultured with dexamethasone.

The kinetics of insulin secretion changes was then studied with perifused islets. Decreasing the glucose concentration from 15 to 3 mM in the perifusion medium was followed by a marked drop of insulin secretion from control islets, whereas the subsequent return to 15 mM triggered a biphasic increase in insulin secretion (Fig. 7 *A*). Qualitatively similar changes were observed in dexamethasone-treated islets. However, the rate of secretion was ~ 50% lower during stimulation with 15 mM glucose, while the basal rate recorded in the presence of 3 mM glucose was not different from that in control islets (Fig. 7 *A*).

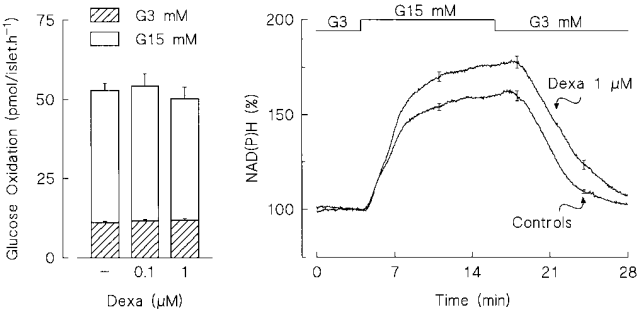

Figure 6. Glucose metabolism by islets cultured for 18 h with dexamethasone. *Left*: glucose oxidation by islets cultured without or with 0.1 and 1 μM dexamethasone. The measurements were carried out during 2 h of incubation in the presence of 3 or 15 mM glucose (G). Values are means±SEM for 15 batches of islets from three separate experiments. *Right*: changes in reduced pyridine nucleotides (NAD[P]H) in islets stimulated by an increase of glucose concentration from 3 to 15 mM. Values are means±SEM for 15 islets from three separate cultures.

Glucose-induced $[Ca^{2+}]_i$ changes in β cells were studied with single intact islets. Raising the glucose concentration from 3 to 15 mM induced the characteristic triphasic response (Fig. 7 *B*): an initial small decrease followed by a large, peak-shaped rise, and, eventually, oscillations in $[Ca^{2+}]_i$. This response was altered in several ways in islets cultured with 1 μM dexamethasone. The initial decrease was usually not seen because of the earlier occurrence of the $[Ca^{2+}]_i$ rise. During steady-state stimulation, two patterns of $[Ca^{2+}]_i$ changes were observed with a similar frequency: large and slow oscillations (Fig. 7 *C*) or a sustained elevation with sometimes small and rapid oscillations (Fig. 7 *D*). Analysis of the $[Ca^{2+}]_i$ changes in different subregions of the islets (30) did not reveal any desynchronization of the signal after dexamethasone treatment (results not shown). Basal $[Ca^{2+}]_i$ was not different in control (82±3 nM) and dexamethasone-treated islets (84±3 nM), but average $[Ca^{2+}]_i$ between 10 and 14 min of stimulation was slightly higher in test islets (167±6 nM vs. 140±5 nM, $n = 30$, $P < 0.05$).

The reversibility of these changes was tested in a separate series of experiments in which the islets were cultured in the presence of 250 nM dexamethasone. Again the treatment (24–27 h) induced two types of alterations in the $[Ca^{2+}]_i$ response to 15 mM glucose: slow oscillations in 7/18 islets and a sustained elevation without oscillations in 11/18 islets. No recovery of the control oscillatory pattern was observed in 18 islets that had been cultured for 6–9 h in a control medium after 18 h in the presence of dexamethasone. Average $[Ca^{2+}]_i$ was 176±7

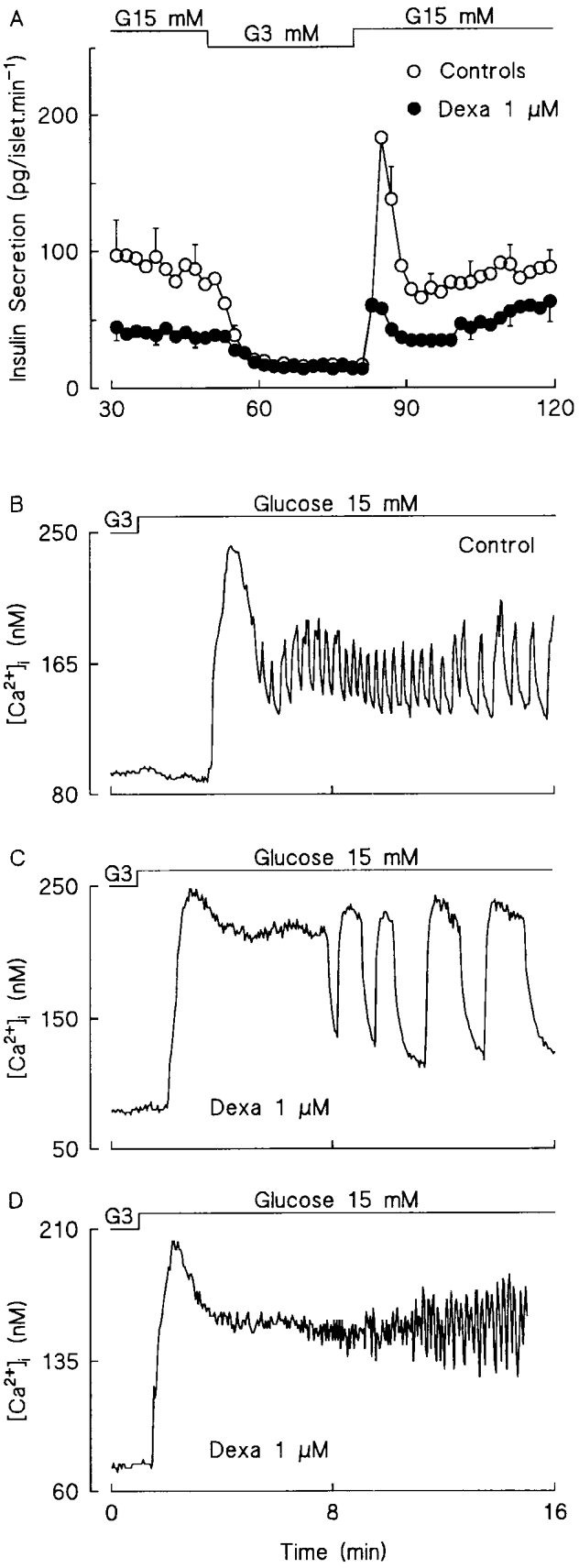

Figure 7. Glucose-induced insulin secretion and $[Ca^{2+}]_i$ changes in islets cultured for 18 h without or with 1 μM dexamethasone. (*A*) Insulin secretion from batches of 20 control or test islets perifused with a medium containing 15 or 3 mM glucose (G) as indicated on top of the panel. Values are means±SEM for six experiments. (*B–D*) Increase in $[Ca^{2+}]_i$ in islets stimulated by a rise of the glucose concentration from 3 to 15 mM in the perifusion medium. The traces without and with dexamethasone are representative of recordings obtained with 30 islets from seven separate cultures.

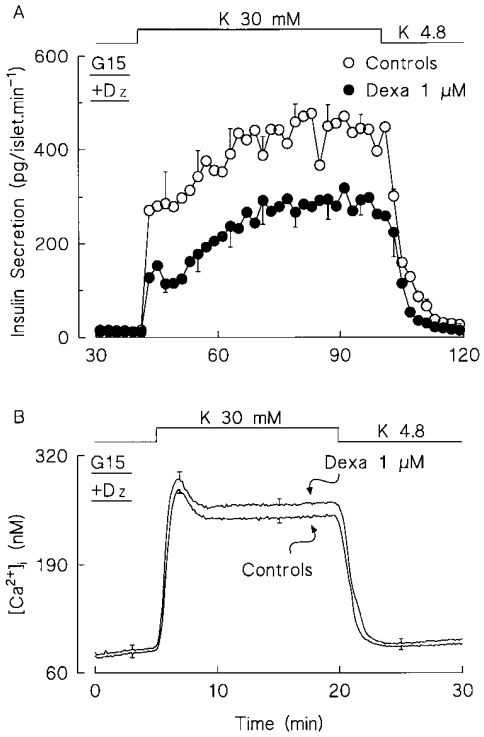

Figure 8. K^+-induced insulin secretion and $[Ca^{2+}]_i$ changes in islets cultured for 18 h without or with 1 μM dexamethasone. (*A*) Insulin secretion from batches of 20 control or test islets stimulated by high K^+. The perifusion medium contained 15 mM glucose (G) and 250 μM diazoxide (Dz) throughout whereas the concentration of K^+ was raised from 4.8 to 30 mM between 40 and 100 min. Values are means±SEM for six experiments. (*B*) Increase in $[Ca^{2+}]_i$ in islets stimulated by a rise in K^+ concentration from 4.8 to 30 mM between 5 and 20 min. Values are means±SEM for 18 islets from three separate cultures.

nM in control islets, 246±8 nM in islets cultured with dexamethasone throughout, and 237±8 nM in islets cultured with and then without dexamethasone (n = 18 for each group). The higher $[Ca^{2+}]_i$ in this second experimental series may be due to the use of a different calibration table and to a lower proportion of islets displaying $[Ca^{2+}]_i$ oscillations after treatment.

Effects of various agents in islets cultured with dexamethasone. The inhibitory effect of dexamethasone was not restricted to glucose-induced insulin secretion. Stimulation with KIC, a leucine-derivative that is metabolized by mitochondria only, induced a smaller secretory response in dexamethasone-treated than control islets (Table I, *line 4*). The inhibition was also observed when insulin secretion was stimulated by non-metabolized agents like barium or high K^+ in the presence of diazoxide (to open ATP-sensitive K^+ channels). Interestingly, the effect of dexamethasone was similar (∼ 50% inhibition) whether high K^+ was applied in the presence of low or high

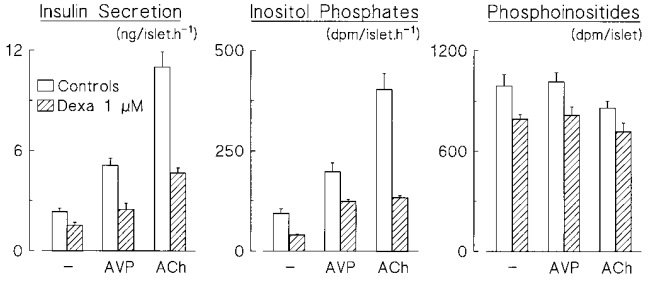

Figure 9. Insulin secretion and inositol phosphates production by islets cultured for 18 h with 1 μM dexamethasone. After culture, the islets were incubated in a medium containing 15 mM glucose and supplemented or not with 100 nM arginine vasopressin (AVP) or 1 μM acetylcholine (ACh). Inositol phosphates and phosphoinositides were measured after labeling with [^{3}H]*myo*-inositol as described in Methods. Values are means±SEM for 25 batches of islets from five cultures for insulin release and for 5–8 batches of islets from six cultures for inositol phosphates and phosphoinositides.

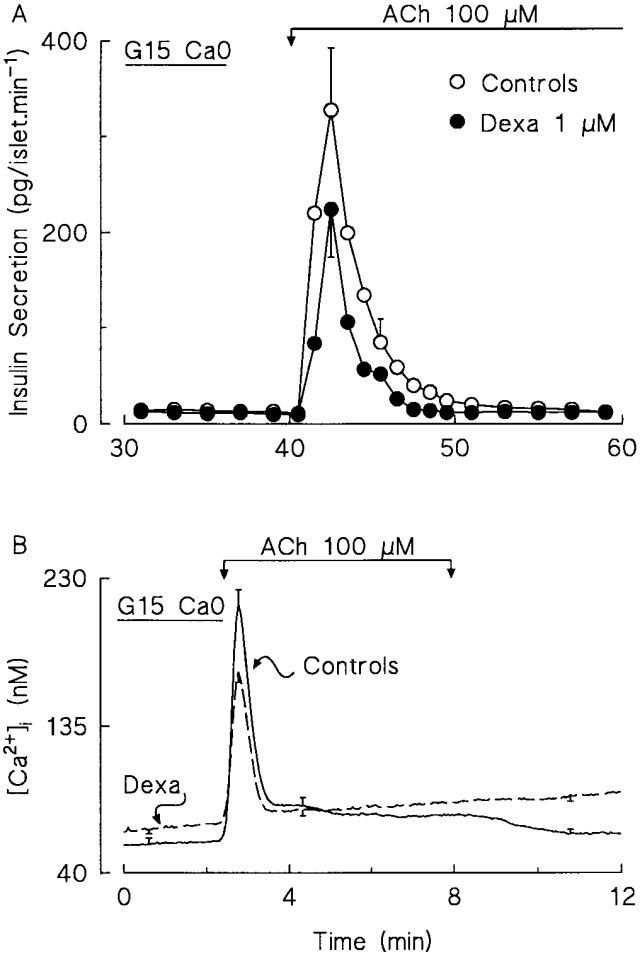

Figure 10. Acetylcholine-induced mobilization of intracellular Ca^+ and stimulation of insulin secretion in islets cultured for 18 h without or with 1 μM dexamethasone. (*A*) Insulin secretion from batches of 30 control or test islets stimulated by 100 μM ACh from 40 min. The perifusion medium contained 15 mM glucose (G) and no calcium (Ca 0). Values are means±SEM for five experiments. (*B*) Increase in $[Ca^{2+}]_i$ in islets stimulated by 100 μM ACh in a medium with 15 mM glucose and without calcium. Values are means±SEM for 24 islets from four separate cultures.

glucose (Table I, *lines 6 and 7*). As shown by Fig. 8 *A*, the inhibition of insulin release induced by high K^+ in the presence of 15 mM glucose (and diazoxide) did not involve any change in the time course of release. Most importantly, it occurred in spite of a similar rise of $[Ca^{2+}]_i$ in β cells of dexamethasone-treated or control islets (Fig. 8 *B*). Arginine and tolbutamide also raise $[Ca^{2+}]_i$ in β cells and do so by different mechanisms (30). None of them reversed the inhibition of insulin secretion in dexamethasone-treated islets (Table I, *lines 8 and 9*). In contrast, this inhibition was totally prevented when dibutyryl cAMP or PMA was added to the medium containing 15 mM glucose (Table I, *lines 10 and 11*).

We next evaluated the effects of arginine vasopressin (AVP) and acetylcholine (ACh), two agents whose action on insulin secretion is mediated (at least partially for ACh) by a stimulation of phosphoinositide metabolism (37). In control islets, AVP (100 nM) and ACh (1 μM) potentiated glucose-induced insulin secretion 2.5 and 5.5-fold, respectively (Fig. 9). Their effect was markedly reduced in islets cultured with dexamethasone. In parallel experiments, the islets were loaded with [2-^{3}H]*myo*-inositol to label their phosphoinositides before being incubated. Although the different inositol phosphates and phosphoinositides were separated, only total values are presented as they carry the whole information. Lower levels of inositol phosphates accumulated in dexamethasone-treated islets after incubation with 15 mM glucose alone or with AVP or ACh (Fig. 9, *middle*). The difference can only partially be ascribed to a smaller incorporation of the tracer in phosphoinositides. Thus, this decrease in [2-^{3}H]*myo*-inositol incorporation reached approximately 20% (Fig. 9, *right*), which was clearly less than the inhibition of inositol phosphates accumulation (40–65% depending on the conditions).

When control islets were perifused with a medium without $CaCl_2$, $[Ca^{2+}]_i$ in islet cells was low and stable despite the presence of 15 mM glucose (Fig. 10 *B*). Addition of ACh triggered a large peak of $[Ca^{2+}]_i$ followed by a small (10–15 nM) sustained elevation. In islets cultured with dexamethasone, basal $[Ca^{2+}]_i$ was marginally higher ($P < 0.05$) and the rise above basal brought about by ACh was smaller (99±6 nM vs. 153±7 nM; $P < 0.01$) than in controls. Another difference was that no decrease in $[Ca^{2+}]_i$ was observed after removal of ACh (Fig. 10 *B*). In parallel experiments, ACh induced a single peak of insulin secretion that was 50% smaller (over 10 min) when the islets had been cultured with dexamethasone (Fig. 10 *A*).

Discussion

This in vitro study shows that dexamethasone exerts a direct influence on pancreatic β cells, which results in a marked inhibition of insulin secretion during stimulation by glucose and a number of other agents. The effect of dexamethasone was of slow onset and reversibility, and was prevented by compound RU 38486, an inhibitor of the nuclear glucocorticoid receptor (39) that is known to be present in β cells (6, 7). These characteristics point to a classic genomic action of the glucocorticoid. Whether this involves increased synthesis of inhibitory proteins or reduced synthesis of proteins necessary for secretion, or both, is unknown. An inhibition of insulin synthesis cannot

account for the inhibition of insulin secretion because the insulin content of dexamethasone-treated islets was greater than that of control islets. Moreover, both in vivo (45, 46) and in vitro (47, 48) treatment with glucocorticoids has been reported to increase proinsulin mRNA in islets.

Alterations of β cell metabolism impede insulin secretion. Previous observations have raised the possibility that dexamethasone might impair glucose metabolism in β cells. First, treatment of rats with massive doses of dexamethasone decreased the expression of GLUT 2 glucose transporter in their β cells (49) and in vitro treatment of islets showed this to occur at a posttranslational level (50). Second, treatment of *ob/ob* mice with dexamethasone accelerated a futile glucose cycling (glucose→glucose 6P→glucose) in their islets (19) by increasing glucose-6-phosphatase activity (51, 52). Even if such changes occurred after in vitro treatment of the islets, glucose oxidation estimated by CO_2 production and rise in NAD(P)H was not altered. Glucose cycling could theoretically impair insulin secretion by increasing ATP consumption to such an extent that the ATP concentration drops. Unfortunately, ATP measurements would not solve the issue. Thus, differences in insulin stores, such as those observed here, are inevitably accompanied by differences in total adenine nucleotides (also present in insulin granules), which preclude reliable comparison of the metabolically important pools of ATP (29). Anyhow, we do not believe that an impairment of glucose metabolism underlies the dexamethasone-induced inhibition of insulin secretion for three further reasons: first, in normal mouse islets glucose cycling is quantitatively too small (∼ 3%; 19) to explain the degree of inhibition of secretion; second this inhibition was also seen with fuels other than glucose; and, third, several steps of stimulus-secretion coupling subsequent to glucose metabolism were not altered.

Glucose metabolism in β cells results in a closure of ATP-sensitive K^+ channels, membrane depolarization, influx of Ca^{2+} and increase in the cytoplasmic Ca^{2+} concentration. None of these steps of stimulus-secretion coupling is likely to be markedly perturbed by dexamethasone because the rise in $[Ca^{2+}]_i$ brought about by glucose was not smaller in test than control islets. Subtle modifications in this $[Ca^{2+}]_i$ response (faster initial rise, changes in the pattern of the oscillations) were, however, observed, for which we have no mechanistic explanations. We can simply rule out the possibility that they result from a loss of synchronicity between β cells. The impact of these modifications on insulin secretion is difficult to evaluate but it is unlikely that they explain the effect of dexamethasone on insulin secretion for two reasons. First, during steady state stimulation with glucose, $[Ca^{2+}]_i$ oscillations were differently affected, whereas insulin secretion was invariably inhibited. Second, transfer of the islets to a medium without dexamethasone corrected the inhibition of insulin secretion without restoring a normal oscillatory pattern of $[Ca^{2+}]_i$.

The results suggest that dexamethasone inhibits insulin secretion by a very distal action, by decreasing the efficacy of Ca^{2+} on the secretory process. This interpretation is in keeping with the observation that a variety of agents (KIC, barium, high K^+, arginine, tolbutamide) known to increase $[Ca^{2+}]_i$ by different mechanisms were all less effective in inducing insulin secretion from dexamethasone-treated than control islets. It is made inescapable by the experiments showing that the secretory response to high K^+ was clearly impaired despite a normal rise of $[Ca^{2+}]_i$ in β cells. The impairment of β cell function caused by administration of massive doses of dexamethasone to rats for several days abolished the ability of glucose to induce insulin secretion but augmented that of arginine (14, 49). It is thus mediated by a mechanism other than that described here.

The effects of cytoplasmic Ca^{2+} on the secretory process can be amplified by activating protein kinases A and C. Exogenous permeant cAMP antagonized the inhibitory action of dexamethasone on insulin secretion both when added during or only after culture with the corticoid. Interestingly, cAMP was also shown to prevent the inhibition of insulin gene expression that dexamethasone causes in dispersed islet cells (48), and the lack of negative effect of dexamethasone on the insulin gene regulation in reaggregated cells was ascribed to a higher cAMP content. However, neither this assumption nor a possible effect of dexamethasone on cAMP levels was directly tested (48). A major difference between insulin synthesis (or at least insulin mRNA levels) and insulin secretion is that the inhibitory action of dexamethasone on secretion is seen in intact islets. In addition, because dexamethasone did not lower islet cAMP levels, its effects on secretion cannot be explained by a decrease in adenylate cyclase or an increase in phosphodiesterase activity. Their reversal by an excess cAMP suggests that protein kinase A exerts actions that compensate for that of dexamethasone.

Corticoids upregulate α_2-adrenoceptor expression and signaling in β cell lines (40). Although it is not known whether a similar effect occurs in normal β cells, we evaluated the possibility of its intervention in dexamethasone inhibition of insulin secretion. Blockade of α_2-adrenoceptors by yohimbine during culture with dexamethasone did not prevent inhibition of insulin secretion, which suggests that it did not result from a hypersensitivity to noradrenaline that might be present in nerve endings remaining in isolated islets (53). Unexpectedly, treatment of the islets with pertussis toxin abrogated the inhibition by dexamethasone through a mechanism other than a change in cAMP levels. Glucocorticoids variably influence the expression of G proteins in different tissues (54, 55) and did not affect $G_{i1\text{-}3}$ in a β cell line (40). Other PTX-sensitive G proteins inhibiting insulin secretion are present in β cells (56) and could be involved in the inhibition of insulin secretion by dexamethasone.

It has been reported that 10–12 d of dexamethasone administration to normal rats upregulates the expression of neuropeptide-Y in pancreatic β cells (15, 57). Because immunoneutralisation of the peptide increased the already exaggerated secretion of insulin from islets of rats treated with dexamethasone, it was suggested that neuropeptide-Y serves to constrain insulin secretion by a paracrine action (15). This is unlikely to be the explanation for the inhibition that dexamethasone produced in our model because addition of the peptide to the culture medium was without effect on insulin secretion from control islets (unpublished data).

Direct activation of protein kinase C (PKC) by PMA during or after culture also antagonized the inhibitory action of dexamethasone on insulin secretion. In addition, inositol phosphate accumulation was less in dexamethasone-treated than control islets, whether after incubation with glucose alone or together with AVP or ACh. Admittedly, the incorporation of the tracer into phosphoinositides was also reduced after dexamethasone treatment, but this reduction was smaller than that of inositol phosphate production. The suggestion that the acti-

vation of the phospholipase C (PLC)–PKC pathway is somewhat impaired by dexamethasone thus seems warranted. It is supported by the observation of a smaller mobilization of intracellular Ca^{2+} by ACh after dexamethasone treatment. Because PKC activation is necessary for sustained potentiation of insulin secretion by AVP and ACh (37), defects in this pathway might thus contribute to the impaired secretory response to AVP and ACh after dexamethasone. On the other hand, the PLC–PKC pathway does not play a major role in glucose-induced insulin secretion from normal mouse β cells (58). Another pathway is thus likely to be affected by glucocorticoids.

In conclusion, glucocorticoids exert direct effects in pancreatic β cells, which result in a generalized inhibition of insulin secretion. This inhibition is brought about by a decrease in the effectiveness of cytoplasmic Ca^{2+} on the secretory process. It can be antagonized by direct activation of protein kinases A or C, two kinases known to increase Ca^{2+} efficacy (59), and prevented by pertussis toxin. Identification of the biochemical events underlying this previously unrecognized mode of action of a physiological agent will be important for our understanding of β cell pathophysiology. It could also help to elucidate how glucose itself amplifies the effectiveness of Ca^{2+} on insulin secretion (60).

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