Thiol-dependent and Non-thiol-dependent Stimulations of Insulin Release

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SUMMARY

The effects of reduced glutathione (GSH) and diamide (an oxidant of GSH) on insulin release induced by glucose, glyceraldehyde, leucine, tolbutamide, glibenclamide, Ca-ionophore A-23187, isoprenaline, and db-cAMP were studied using isolated rat pancreatic islets. In the absence or presence of low glucose (5.6 mM) neither GSH (0.1 mM) nor diamide (0.1 mM) affected insulin release. Insulinotropic action of glucose (11.1 mM) and glyceraldehyde (11.1 mM), and that of tolbutamide (0.1 mg/ml) and leucine (10 mM) both in the presence of 11.1 mM glucose was further augmented by GSH and inhibited by diamide. GSH (0.05–1 mM) and diamide (0.1 mM) failed to affect the insulin secretion evoked by glibenclamide (5 μg/ml) + glucose (11.1 mM), Ca-ionophore A-23187 (50 μg/ml) + glucose (5.6 mM), isoprenaline (1 μM) + glucose (5.6 mM), and db-cAMP (1 mM) + glucose (5.6 mM).

The data suggest that the insulin-releasing capacity of glucose, glyceraldehyde, tolbutamide, and leucine depends on the redox state of islet thiols, whereas the insulin-releasing effect of glibenclamide, Ca-ionophore, isoprenaline, and db-cAMP does not. The possibility that thiol dependency is associated with those compounds increasing Ca uptake but not with compounds acting as Ca-ionophores or only by increasing intracellular cAMP is discussed. DIABETES 33:251–257, March 1984.

It is well established that a wide variety of agents, such as carbohydrates, amino acids, hormones, and pharmacologic substances can stimulate the release of insulin.[1] Their mechanisms of action, however, might be in part different from each other and in most cases they are poorly understood. The process of glucose-induced insulin release has recently been suggested to be affected by the reducing equivalents of NADPH and GSH systems in the β-cell.[2,3] Based on the observations that the supplementation of reduced glutathione (GSH) to the isolated perfused rat pancreas increased the insulin secretory action of tolbutamide and/or glucose[4] and that the insulin release triggered by glucose, tolbutamide, p-chloromercuribenzoate, and leucine was inhibited by employing oxidants of GSH such as diamide, DIP [diazene dicarboxylic acid bis(N-'methylpiperazide)], and t-BHP (t-butyl hydroperoxide),[5–7] we have hypothesized that glucose, tolbutamide, and leucine will be more effective in releasing insulin during a more reduced state and less effective during a more oxidized state of islet thiols. The present investigations were undertaken to gain further insight whether or not thiol dependency is a common feature of insulin secretagogues or if there are also triggers of insulin release that do not depend on thiols.

MATERIALS AND METHODS

CHEMICALS

Collagenase (Worthington Biochemical Corp., Freehold, New Jersey), D-glucose (Serva Feinbiochemica, Heidelberg, West Germany), bovine serum albumin (Behringwerke, Marburg, West Germany), reduced glutathione (GSH 98%, GSSG <1.5%, iron <10 ppm, heavy metals as Pb <10 ppm, specified and supplied by Boehringer, Mannheim, West Germany), tolbutamide and glibenclamide (Farbwerke Hoechst, Frankfurt, West Germany), D(+)-glycerinaldehyde (Fluka, Buchs, Switzerland), Ca-ionophore A-23187 (Calbiochem-Behring Corp., La Jolla, California; Hoechst AG, Frankfurt, West Germany), isoprenaline sulfate (Boehringer, Ingelheim), rat insulin (Novo Lab., Copenhagen, Denmark), insulin radioimmunoassay kit (INSIK-1) (CIS-SORIN, Italy, supplied by Isotopen Dienst West, Sprendlingen, West Germany). Diamide [diazene dicarboxylic acid bis (N,N-dimethylamide)] was prepared and donated by HAG (Bremen, West

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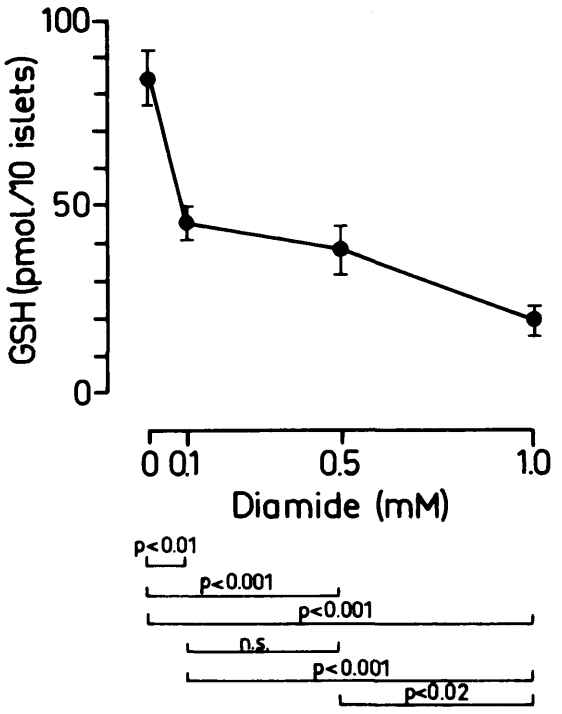

FIGURE 1. Effect of diamide on GSH content of rat pancreatic islets. Batches of 10 islets were incubated for 15 min in 1 ml medium containing 16.7 mM glucose. Values are mean ± SEM (N = 26–38).

Germany) through the courtesy of Dr. O. Vitzthum. All other chemicals and reagents of analytic grade were obtained from E. Merck (Darmstadt, West Germany).

PREPARATION AND INCUBATION OF ISLETS

Animals. Male and female Wistar rats from a local strain, weighing 200–300 g, were used. They were kept at standard pellet diet (Herilan, Eggersmann Corp., Inteln/Weser, West Germany) and were given tap water ad libitum.

Isolation of islets. The rats were anesthetized with ether and the pancreatic islets were prepared and harvested by the collagenase method of Lacy and Kostianovsky.[8] The following medium was employed for the incubation studies: Krebs-Ringer bicarbonate (KRB) buffer with 2% albumin, pH 7.35, gassed for 10 min with carbogen (95% O_2:5% CO_2). While being gassed with carbogen, islets were initially preincubated for 30 min in the medium described above, containing, in addition, 0.5 mg/ml glucose. After preincubation, islets were kept in ice for 3 min and washed three times with ice-cold Hanks' solution.

Main incubations as described later were performed under shaking islets in test tubes (100 strokes/min) in a metabolic incubator. Test tubes were shaken by hand before taking aliquots for insulin determinations.

INCUBATION OF ISLETS

Studies with diamide. For determination of insulin secretion groups of five islets, placed into 2-ml tubes each, were kept in ice until the beginning of incubation. The incubation was started by the addition to each group of five islets of 1 ml KRB buffer (pH 7.35) supplemented with 20 mg/ml albumin at 37°C under continuous gassing with carbogen containing the test substances with or without 0.1 mM diamide. After 40 min of incubation, aliquots of incubation medium (100 μl) were taken for measurement of the released insulin.

In order to investigate whether or not diamide at the concentration used for studying its effect on insulin release would also decrease islets' GSH levels, GSH content in islet tissue was determined according to the method of Hissin and Hilf,[9] as previously described by us in detail for use in pancreatic islets.[2] As shown in Figure 1 at the concentration used here (0.1 mM), diamide decreases the GSH level in the islets after exposure to 16.7 mM glucose for 15 min. In an earlier article it was demonstrated by us that 0.1 mM diamide does not affect the ATP content.[10] Thus, it appears that diamide at the concentration level used here decreases islets' GSH without affecting their viability.

Studies with exogenous GSH. In a previous study, we demonstrated that the effective concentration of the added reduced GSH can decline in albumin-containing media with time.[4] Therefore, a second series of control experiments was performed to measure the GSH disappearance in the incubation medium. The concentration of GSH in the incubation media was assayed spectrophotometrically by taking samples (20 μl) from incubation medium at the beginning and at the end of incubation with GSH. As can be seen in Figure 2 there is about 20% loss in the effective concentration of total GSH measured after 10 min incubation with islets. This loss may be due to binding of GSH at the islet tissue and/or serum albumin present in the incubation medium. Figure 3 shows the time course of disappearance of GSH from the incubation medium when 0.1 mM was added. Probably due to oxidation there was a time-dependent decline that exceeded 60% after 60 min incubation. Based on the results shown in Figures 2 and 3, GSH was added for only

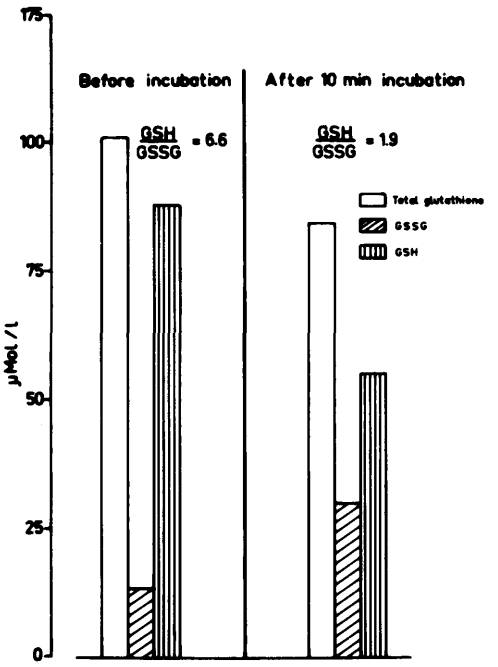

FIGURE 2. Changes of the concentration of added GSH in the incubation medium within 10 min. Krebs-Ringer bicarbonate buffer (pH 7.4) containing 2% bovine serum albumin supplemented with 0.1 mM reduced GSH, gassed with 0_2:CO_2 (95:5) at 37°C. Samples were taken after 10 min to measure total and oxidized GSH using the photometric method of enzymatic cycling.[11]

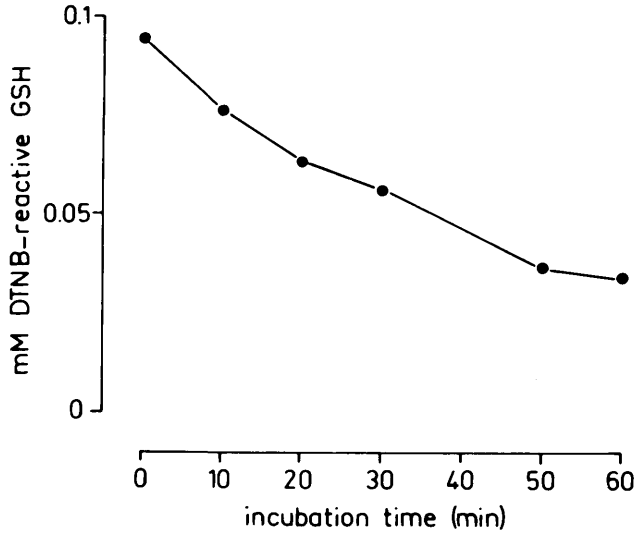

FIGURE 3. Time course of DTNB-reactive GSH (0.1 mM) during incubation in a medium used for islet incubations, i.e., Krebs-Ringer bicarbonate buffer containing 2% bovine serum albumin and GSH (0.1 mM) was incubated at 37°C and gassed with carbogen (O_2–CO_2; 95:5). Samples were taken at various periods of time to measure the free thiol groups spectrophotometrically at 412 nm after reaction with DTNB.[12] One of three identical experiments with principally the same results is shown.

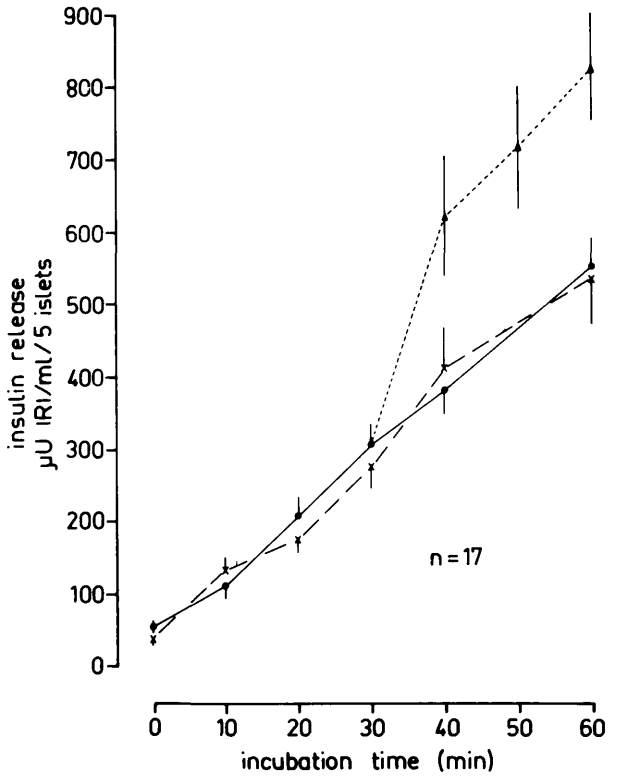

FIGURE 4. Time course of glucose (11.1 mM)- and glucose + GSH (0.1 mM)-induced insulin release from rat pancreatic islets. Batches of five islets were incubated in 1 ml Krebs-Ringer bicarbonate buffer containing 2% bovine serum albumin and glucose with and without GSH. Means ± SEM; number of experiments: 17; IRI = immunoreactive insulin. Glucose (11.1 mM) (●——●); glucose (11.1 mM) + GSH (0.1 mM) added at time 0 (X——X); glucose (11.1 mM) plus GSH (0.1 mM) added at 30 min (▲------▲).

10 min in all experiments. Addition of GSH to the incubation medium was not associated with changes of pH. Taking or adding an aliquot of 0.1 ml during incubation in our hands did not change insulin release. Figure 4 shows the time course of glucose-induced insulin secretion over a period of 60 min when GSH was added either just from the beginning of the experiments or was supplemented after a 30-min lasting incubation period with glucose alone. The data show that GSH, when added at the beginning, did not alter insulin secretion in response to glucose. However, its addition after 30 min significantly further augmented insulin release triggered by glucose.

Therefore, when the effect of incubation with 0.1 mM GSH on the release of insulin with various stimulators of insulin secretion was investigated, the incubations were performed by the addition to each group of five islets of 1 ml KRB buffer (pH 7.35) supplemented with 20 mg/ml albumin, at 37°C under continuous gassing with carbogen, containing the test substance. After 30 min incubation, a fraction of incubation medium (100 µl) was taken in order to maintain the same incubation volume and immediately replaced by a same volume (100 µl), of the same medium described above, containing the test substance, at the same concentration level, with or without 1 mM GSH to give a final concentration of GSH of 0.1 mM in 1 ml incubation medium. At the end of 40 min incubation, aliquots of the medium (100 µl) were taken for measurement of the released insulin. Since at the concentration level of 0.1 mM, GSH induced a maximal stimulating effect on the insulin release affected by 11.1 mM glucose (as shown in Figure 5), for all further experiments with various stimulators of insulin secretion 0.1 mM GSH was used. In addition, in those cases where 0.1 mM GSH was without effect, lower and higher concentrations of GSH were employed (Tables 1 and 2). Depending on our previous observations, in order to avoid the risk of feedback effect caused by the high levels of insulin secretion[13] due to either prolongation of incubation period or addition of GSH in pres-

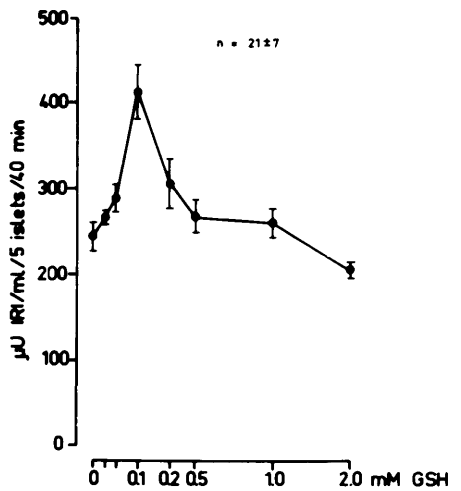

FIGURE 5. Effects of different concentrations of reduced GSH on glucose (11.1 mM)-induced insulin release. Batches of five islets were incubated for 40 min in 1 ml Krebs-Ringer bicarbonate buffer containing 2% albumin and 11.1 mM glucose. GSH was added at the last 10 min incubation. (Means ± SEM; IRI = immunoreactive insulin.)

TABLE 1
Effects of different concentrations of reduced GSH (0.05–1.0 mM) on Ca-ionophore A-23187 (50 μg/ml) + glucose (5.6 mM)- and glibenclamide (5 μg/ml) + glucose (11.1 mM)- induced insulin release from rat pancreatic islets

	Insulin release (μU IRI/ml/5 islets)				
	GSH (mM)				
	0.0	0.05	0.1	0.5	1.0
Glucose (5.6 mM) + Ca-ionophore (50 μg/ml)	A 267 ± 18 (9)	B 190 ± 33 (8)	C 306 ± 62 (9)	D 234 ± 68 (10)	E 259 ± 27 (9)
Glucose (11.1 mM) + Glibenclamide (5 μg/ml)	F 341 ± 29 (10)	G 317 ± 38 (10)	H 363 ± 61 (9)	I 452 ± 51 (10)	K 319 ± 29 (10)

Batches of five islets were incubated for 40 min in 1 ml Krebs-Ringer bicarbonate buffer containing 2% bovine serum albumin, glucose (5.6 mM or 11.1 mM), and the stimulator. GSH was added at the last 10 min incubation.
Mean ± SEM; number of experiments is given in parentheses; IRI = immunoreactive insulin.
Control: glucose, 5.6 mM: 117 ± 15 (7); glucose, 11.1 mM: 203 ± 27 (11).
Statistical evaluation: AB, AC, AD, AE: NS; FG, FH, FI, FK: NS.

ence of the secretagogue used, we have selected the 40-min incubation period for measurement of insulin release.

Since the time of action of GSH, because of the reasons discussed above, was only 10 min, but the time of action of diamide was 40 min, only qualitative comparisons of the effects of both compounds on insulin release are allowed.

Radioimmunoassay of insulin. Insulin released into the incubation medium was assayed radioimmunologically using the double antibody technique.[14] Since thiols attack the disulfide bridges of insulin and, therefore, can cause an underestimation of the amounts of insulin released,[4,15] the effect of different concentrations of reduced GSH on the standard curves of insulin radioimmunoassay was studied. As shown in Figure 6 the standard curves of radioimmunoassays of insulin were shifted by 1 mM GSH downward; however, 0.1 mM of either GSH or diamide, which are used throughout the experiments here, has an insignificant effect on radioimmunoassays. For measuring insulin release in the presence of higher concentrations of GSH the experiments were therefore designed in such a way that the standard insulin was handled exactly like islets after incubation, and GSH was added at equal concentrations to the standards in order to obtain the same conditions.

Statistics. Student's *t* test was used for statistical evaluation of the results. Values are given as mean ± SEM; N = number of experiments from separate preparations of islets.

RESULTS

Effect of GSH and diamide on the insulin release induced by glucose, glyceraldehyde, and leucine (Figure 7). In the absence or presence of 5.6 mM glucose both GSH and diamide failed to affect the insulin secretion. The insulin secretion induced by glucose (11.1 mM) and glyceraldehyde

TABLE 2
Effects of different concentrations of reduced GSH (0.05–1.0 mM) on isoprenaline (1 μM)- and db-cAMP (1 mM)-induced insulin release in the presence of glucose (5.6 mM) from rat pancreatic islets

	Insulin release (μU IRI/ml/5 islets)				
	GSH (mM)				
	0.0	0.05	0.1	0.5	1.0
Glucose (5.6 mM) + Isoprenaline (1 μM)	A 227 ± 46 (10)	B 205 ± 53 (10)	C 283 ± 60 (9)	D 272 ± 37 (9)	E 256 ± 71 (8)
Glucose (5.6 mM) + db-cAMP (1 mM)	F 332 ± 36 (10)	G 319 ± 51 (9)	H 278 ± 49 (10)	I 318 ± 50 (9)	K 258 ± 48 (10)

Batches of five islets were incubated for 40 min in 1 ml Krebs-Ringer bicarbonate buffer containing 2% bovine serum albumin, glucose (5.6 mM), and the stimulator. GSH was added at the last 10 min incubation.
Mean ± SEM; number of experiments is given in parentheses; IRI = immunoreactive insulin.
Control: glucose, 5.6 mM: 117 ± 15 (7).
Statistical evaluation: AB, AC, AD, AE: NS; FG, FH, FI, FK: NS.

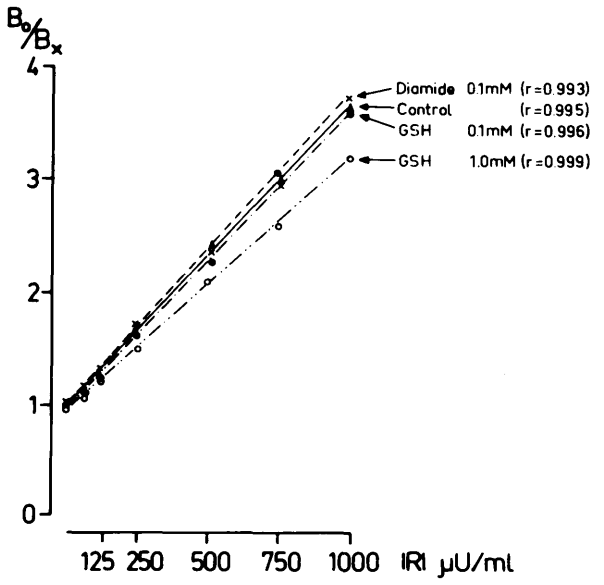

FIGURE 6. Effect of reduced GSH and diamide on the radioimmunoassay of insulin. One of six identical experiments with effectively the same results is shown. B_0 = bound radioactivity of labeled insulin at zero concentration of unlabeled insulin. B_x = bound radioactivity of labeled insulin at various concentrations of standard unlabeled insulin.

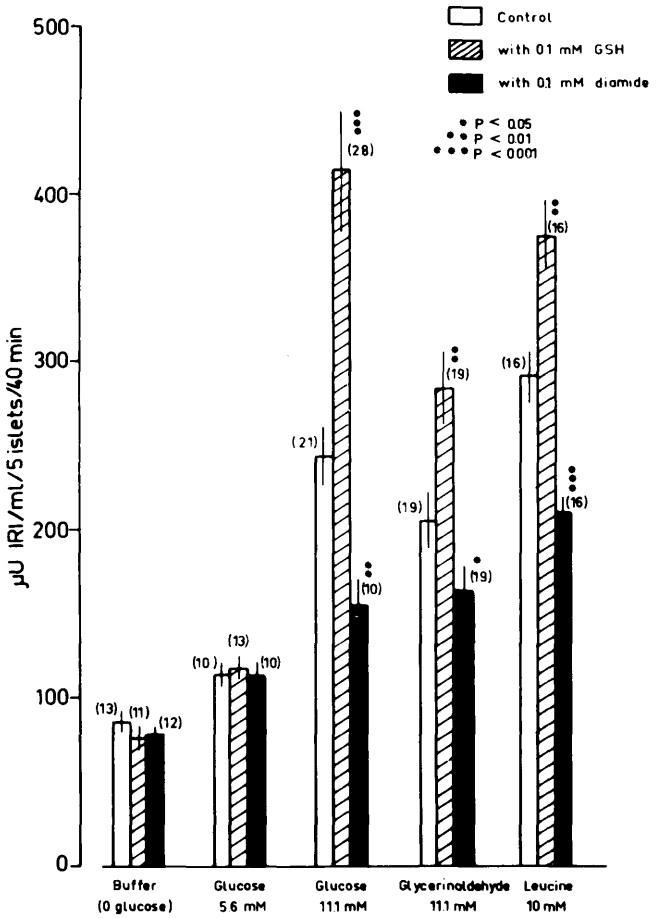

FIGURE 7. Effect of GSH (0.1 mM) and diamide (0.1 mM) on the insulin release induced by glucose, glyceraldehyde, and leucine.

(11.1 mM) was further augmented by GSH and inhibited by diamide. The insulin secretion induced by leucine (10 mM) in the presence of 11.1 mM glucose was further increased by GSH and inhibited by diamide.

Effect of GSH and diamide on the insulin release induced by tolbutamide, glibenclamide, and Ca-ionophore A-23187 (Figure 8 and Table 1). Tolbutamide (0.1 mg/ml) and glibenclamide (5 µg/ml) significantly increased the insulin release evoked by 11.1 mM glucose. Whereas the effect of tolbutamide was increased further in the presence of 0.1 mM GSH and inhibited by diamide, the effect of glibenclamide was not affected by GSH (0.05–1 mM) and diamide. The secretory rate of 5.6 mM glucose was also increased when the islets were exposed to Ca-ionophore A-23187 (50 µg/ml). However, no alteration in the insulin-triggering action of Ca-ionophore was observed due to incorporation of GSH or diamide in the incubation medium.

Effect of GSH and diamide on the insulin release induced by isoprenaline and db-cAMP (Figure 9 and Table 2). Isoprenaline (1 µM) and db-cAMP (1 mM) significantly stim-

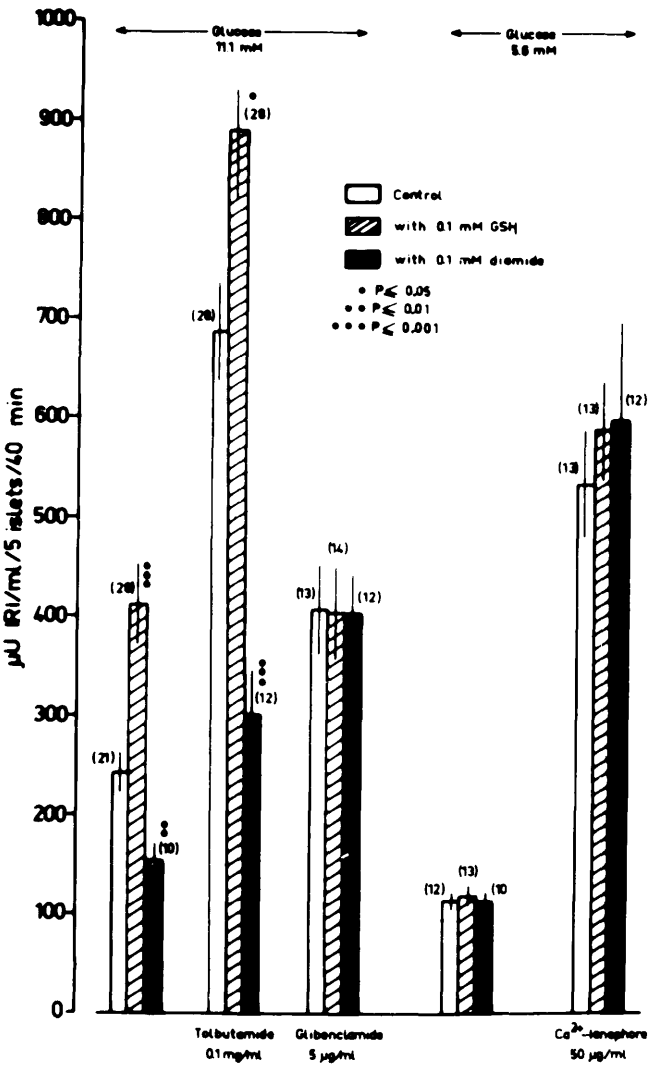

FIGURE 8. Effect of GSH (0.1 mM) and diamide (0.1 mM) on the insulin release induced by tolbutamide, glibenclamide, and Ca-ionophore A-23187.

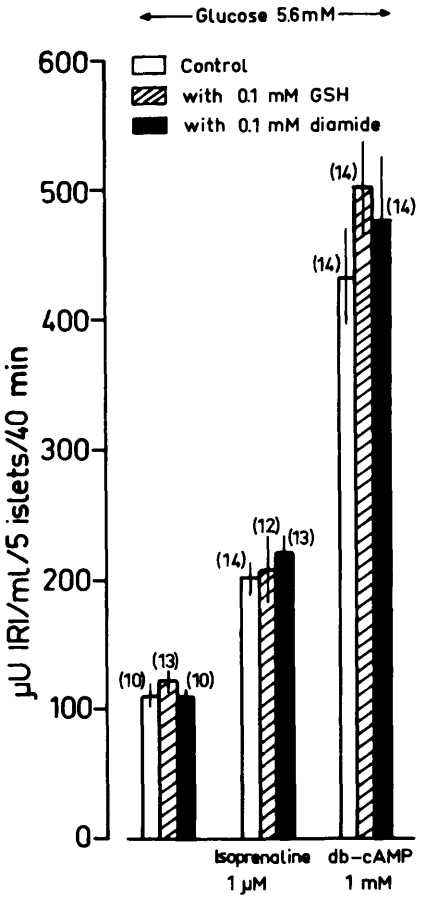

FIGURE 9. Effect of GSH (0.1 mM) and diamide (0.1 mM) on the insulin release induced by isoprenaline and db-cAMP.

ulated insulin secretion in the presence of 5.6 mM glucose. We have never observed any significant alteration in insulin release evoked by the two substances due to supplementation of GSH or diamide at all concentrations used.

DISCUSSION

The present study suggests the possible involvement of the redox state of the GSH system as a regulatory factor in the sequence of metabolic events leading to insulin secretion. Our data clearly demonstrate that the insulin-releasing capacity of glucose, glyceraldehyde, and the combined action of glucose with leucine or tolbutamide is further increased by addition of GSH and is inhibited by the thiol oxidant diamide. On the other hand, since insulin secretion induced by Ca-ionophore A-23187 and the combined action of glibenclamide with 11.1 mM glucose as well as the combined action of isoprenaline and db-cAMP with 5.6 mM glucose is neither increased by the addition of GSH nor inhibited by diamide, one may suggest that insulin release in response to the latter compounds does not depend on thiols.

Previous studies involving microdissected pancreatic islets of ob/ob mice have demonstrated that certain thiol compounds, e.g., 1-thio-D-glucose, reduced GSH, and dextran-linked cystein, potentiated the insulin-releasing action of 10 mM D-glucose without affecting glucose oxidation,[15] and it has been claimed that such compounds stimulate insulin release, either by establishing disulfide bridges with the β-cell membrane or by splitting certain disulfides. So far, the function of thiols and their possible involvement in the physiologic mechanism of insulin release are still obscure. It has been known for some years that the membrane thiol-disulfide redox status can be readily modified by addition of GSH.[16,17] In addition, it has been shown that perfusion of rabbit corneae with bicarbonate-Ringer's solution containing GSH raised the intracellular GSH levels.[18] It may be assumed that application of GSH can convert the membrane Prot-SSG, which acts as a pool of intracellular GSSG,[17] to Prot-SH, which in turn can react with the intracellular GSSG leading to increased intracellular GSH.

Although the mechanisms of the insulin-releasing action of all compounds tested in this study are still incompletely understood, it appears that all of them should finally lead to an increase of cytosolic calcium. Accumulation of Ca^{2+} in the submembrane cytoplasmic space in the β-cells can be achieved by (1) Ca uptake as induced by glucose,[19] glyceraldehyde,[20] leucine,[21] and tolbutamide;[22] (2) inhibition of Ca^{2+} efflux as found in the case of glucose;[23] (3) ionophoric Ca transport as induced by the antibiotic compound A-23187;[24] and (4) intracellular translocation as described for substances acting via cAMP system, e.g., glucagon, isoprenaline, and db-cAMP.[25,26]

Recently, we have shown that Ca^{2+} uptake induced by glucose can be inhibited by thiol oxidants including t-butylhydroperoxide, diamide, and DIP, suggesting that Ca^{2+} uptake into pancreatic islets is somehow related to islet thiols.[27]

It is interesting to note that those substances used in this study whose insulinogenic action depends on thiols were found by others to stimulate the Ca^{2+} uptake into pancreatic islets. These findings strengthen our suggestion that the modulation of the redox state of islet thiols may alter mainly the process(es) leading to entry of Ca into the islet cells. Irrespective of whether this speculation proves correct, it has the merit of being susceptible to further experimental testing.

The actions of glucose and glyceraldehyde as potent insulin secretagogues have many significant features in common.[20] If it is true that the signal of glucose-induced insulin secretion is provided by glucose metabolism at the level of the trioses, our data concerning thiol dependency of the action of glucose and glyceraldehyde seem to indicate that the insulinogenic action of such a signal also depends on thiols.

The insulin-releasing action of leucine has been shown to be accompanied by increase of NADPH/NADP and GSH/GSSG ratios in the islet cells.[6,7] Our results showing an increase of the insulinotropic action of leucine by GSH and its decrease by diamide seem to be compatible with our previous observations.

It is also interesting to note that we have never observed any alteration in the insulin-triggering action of Ca-ionophore A-23187 due to the presence of GSH or diamide in the incubation medium. This suggests that the insulin secretion due to ionophore is not thiol dependent. Previously we have observed that Ca uptake into pancreatic islets induced by Ca-ionophore A-23187 is not inhibited by the thiol oxidant DIP. Thus, it appears that the redox state of islet thiols is indeed necessary for insulin release after physiologic uptake of Ca but not for insulin secretion after entry via an ionophoric action. On the other hand, from our studies with the Ca-

ionophore A-23187[27] it appears that once the calcium is accumulated in cytosol, the further process of insulin secretion may not be affected by changes of redox state of islet thiols.

Malaisse and his co-workers[28,29] ascribe the insulinotropic activity of sulfonylureas to be similar to the action of the Ca-ionophore A-23187. Our data concerning the thiol independency of the insulin-releasing action of glibenclamide seem to be consistent with this hypothesis. On the other hand, this does not hold for the insulinogenic action of tolbutamide. Notwithstanding the indirect nature of this piece of evidence, tolbutamide appears to have a limited ability, if any, to enter the β-cells, whereas the uptake of glibenclamide was apparently a linear function of its concentrations.[30] In addition, stimulation of the electrical activity[31] and inhibition of Rb efflux[20] would be difficult to explain if tolbutamide were acting simply as a Ca-ionophore. Therefore, if tolbutamide increases intracellular Ca not by acting as a Ca-ionophore but rather by stimulating the physiologic Ca uptake, then it is reasonable to assume that tolbutamide-induced insulin secretion is thiol dependent similar to the action of glucose.

Isoprenaline and db-cAMP are thought to cause a translocation of Ca from an organelle-bound pool into the cytoplasm[25,26] through accumulation of intracellular cAMP. Since the insulin release in response to isoprenaline and db-cAMP is not thiol dependent as shown in Figure 9 and Table 2, it is possible that the modulation of the redox state of islet thiols does not affect the Ca-translocating capacity induced by such compounds.

In conclusion, we are of the opinion that, at least at the concentration levels used and under our experimental conditions, the insulin secretagogues tested here can be divided into thiol-dependent and non-thiol-dependent stimulators. It is conceivable that those stimulators that act by increasing physiologic Ca uptake depend on thiols, whereas compounds acting as Ca-ionophores and those acting via cAMP do not depend on thiols.

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