Cysteine Analogues Potentiate Glucose-Induced Insulin Release In Vitro

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SUMMARY

In rat pancreatic islets, cysteine analogues, including glutathione, acetylcysteine, cysteamine, D-penicillamine, L-cysteine ethyl ester, and cysteine–potentiated glucose (11.1 mM) induced insulin secretion in a concentration-dependent manner. Their maximal effects were similar and occurred at ~0.05, 0.05, 0.1, 0.5, 1.0, 1.0 mM, respectively. At substimulatory glucose levels (2.8 mM), insulin release was not affected by these compounds. In contrast, thiol compounds, structurally different from cysteine and its analogues, such as mesna, tiopronin, meso-2,3-dimercaptosuccinic acid (DMSA), dimercaprol (BAL), β-thio-D-glucose, as well as those cysteine analogues that lack a free-thiol group, including L-cystine, cystamine, D-penicillamine disulfide, S-carbocysteine, and S-carbamoyl-L-cysteine, did not enhance insulin release at stimulatory glucose levels (11.1 mM); cystine (5 mM) was inhibitory. These in vitro data indicate that among the thiols tested here, only cysteine and its analogues potentiate glucose-induced insulin secretion, whereas thiols that are structurally not related to cysteine do not. This suggests that a cysteine moiety in the molecule is necessary for the insulinotropic effect. For their synergistic action to glucose, the availability of a sulfhydryl group is also a prerequisite. The maximal synergistic action is similar for all cysteine analogues tested, whereas the potency of action is different, suggesting similarity in the mechanism of action but differences in the affinity to the secretory system. DIABETES 1986; 35:1390–96.

It has repeatedly been demonstrated that insulin release in response to glucose is related to the redox state of islet thiols. This view is based on observations that thiol oxidants that penetrate into islet cells inhibit glucose-induced insulin secretion,[1] whereas addition of thiols such as reduced glutathione (GSH),[2,3] L-cysteine, and dextran-linked cysteine[2] enhance the capacity for glucose-induced insulin release. It has also been suggested that critical membrane thiols are involved in the stimulus-secretion coupling of glucose-induced release of insulin.[4,5] Thus, it was hypothesized that the secretory response to a stimulatory concentration of glucose depends on the redox state of intracellular and/or membrane thiols of the β-cell, as was recently reviewed.[6] Little information is available on the relationship between the structure of sulfur-containing compounds and their possible synergistic effect on insulin release.

Therefore the aim of this study was to test the insulinotropic effect of cysteine analogues with free-thiol groups, of thiol compounds structurally different from cysteine, and of cysteine analogues without free-thiol groups in the presence of both a nonstimulatory and a stimulatory glucose concentration.

MATERIALS AND METHODS

Chemicals. Collagenase (Worthington, Freehold, NJ), D-glucose (Serva Feinbiochemica, Heidelberg, FRG), bovine serum albumin (Behringwerke, Marburg, FRG), reduced glutathione (GSH 98%, GSSG <1.5%, iron <10 ppm, specified and supplied by Boehringer, Mannheim, FRG), *S*-carbamoyl-L-cysteine, cystamine dihydrochloride, mecysteine, L-cysteine ethyl ester HCl, tiopronin, D-penicillaminedisulfide (Fluka, Buchs, Switzerland), acetylcysteine, cysteamine HCl, D-penicillamine, dimercaprol (BAL) (Serva), *S*-carbocysteine, meso-2,3-dimercaptosuccinic acid (DMSA) (EGA Chemie, Steinheim/Altbuch, FRG), L-cysteine (Merck, Darmstadt, FRG), mesna (Sigma, St. Louis, MO), β-thio-D-glucose (Sigma), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (Boehringer), rat insulin (Novo, Copenhagen, Denmark), insulin radioimmunoassay kit (INSIK-1) (CIS-SORIN, Italy, supplied by Isotopen Dienst West, Dreieich, FRG) were used. All other chemicals and reagents were of analytic grade and were obtained from Merck.

Animals. Equal numbers of male and female Wistar rats from a local strain, weighing 200–300 g, were used for all ex-

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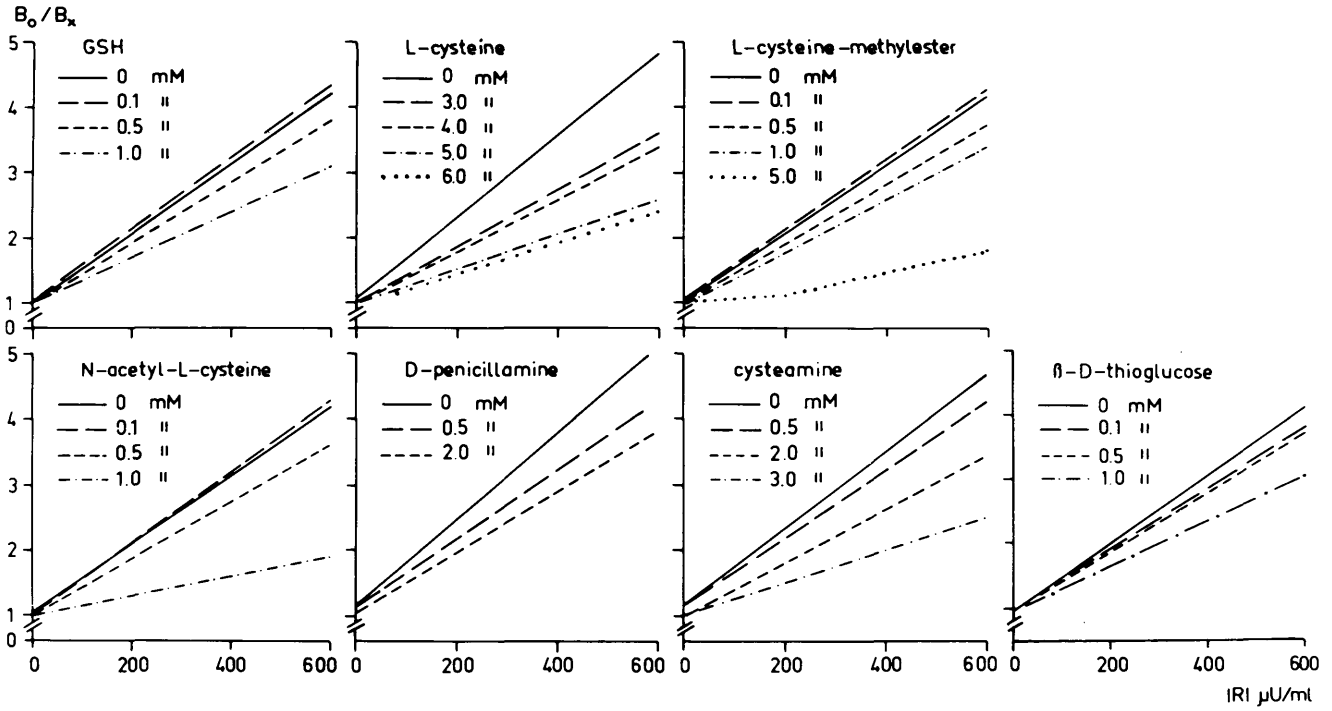

FIG. 1. Effect of thiol compounds on radioimmunological determination of insulin. Data show 1 representative experiment. Bo, bound radioactivity of labeled insulin at zero concentration of unlabeled insulin; Bx, bound radioactivity of labeled insulin at various concentrations of unlabeled insulin.

periments. They were kept on a standard pellet diet (Altromin, Lage/Lippe, FRG) and were given water ad libitum.

Isolation and incubation of islets. The rats were anesthetized with ether, and the pancreatic islets were prepared and harvested by the collagenase method of Lacy and Kostianovsky.[7] The medium employed for the incubation studies was Krebs-Ringer bicarbonate (KRB) buffer with 2% bovine serum albumin (pH 7.35) gassed before and during incubation with carbogen (95% O_2 + 5% CO_2). Because thiols are unstable in aqueous solutions, the thiol-containing media were prepared at 0°C immediately before incubation.

Islets were initially preincubated for 30 min in the medium described above containing 2.8 mM glucose. The islets were then washed three times with ice-cold Hanks' solution. After

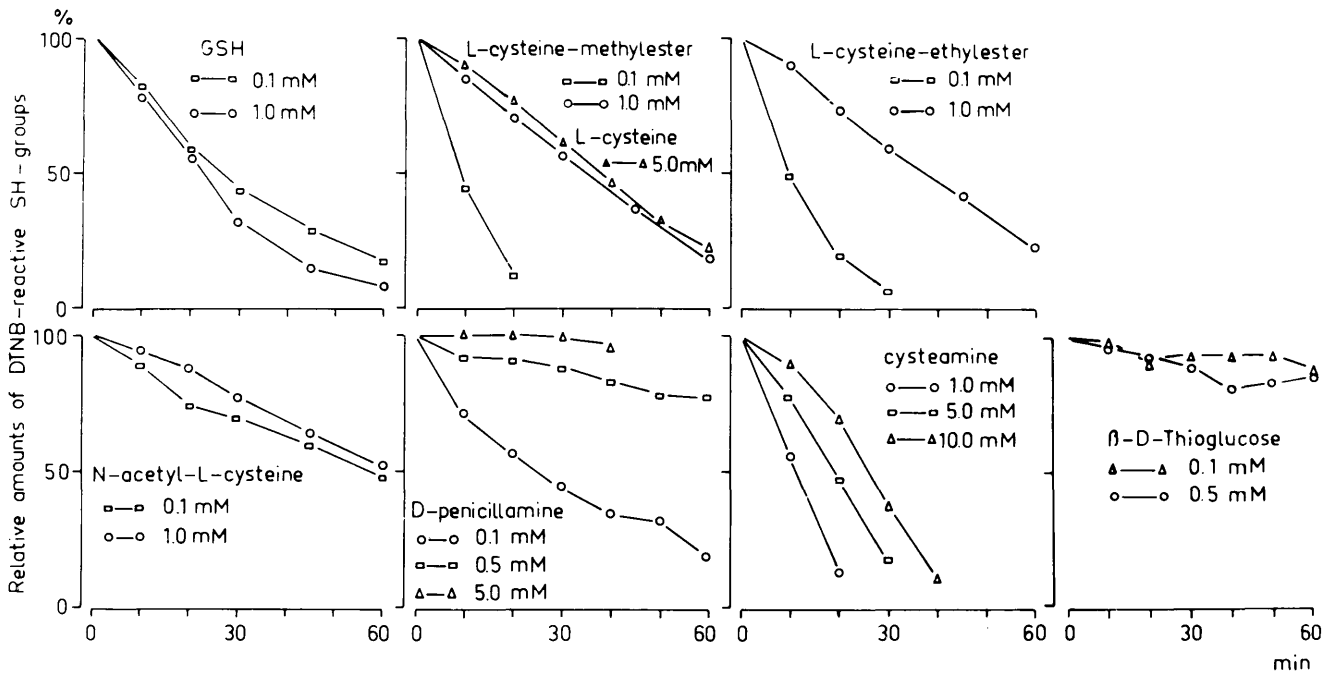

FIG. 2. Time course of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB)-reactive thiols during incubation in basal medium. Various concentrations of thiols were incubated at 37°C in Krebs-Ringer bicarbonate buffer containing 2% bovine serum albumin, and medium was gassed with carbogen (95% O_2, 5% CO_2). After various time periods, free-thiol groups were measured spectrophotometrically at 412 nm after reaction with DTNB. (1 of 3 identical experiments is shown.) Results with L-cysteine ethyl ester (data not shown) were similar to those obtained with mecysteine.

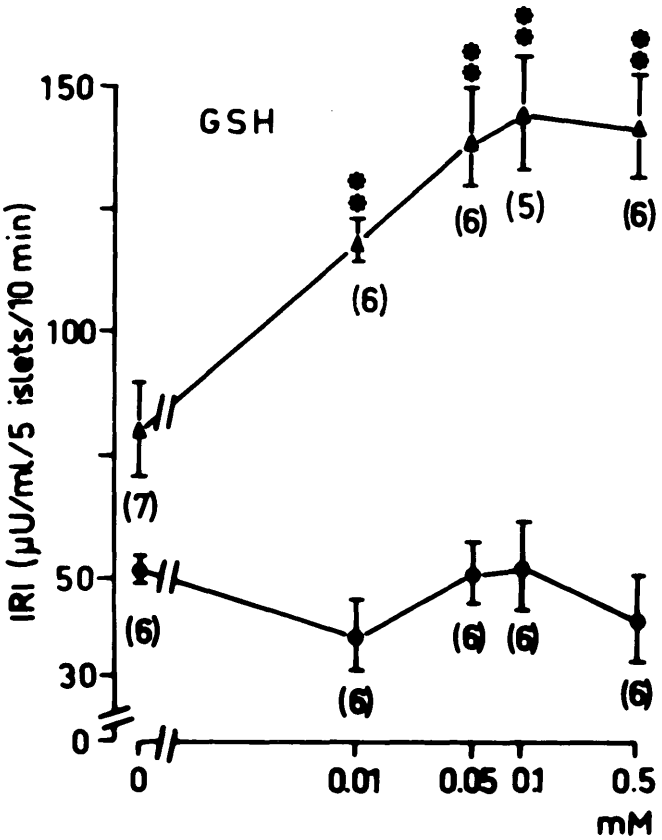

FIG. 3. Effect of GSH on insulin release from rat pancreatic islets. Five islets were incubated at 2.8 (●) or 11.1 (▲) mM glucose in presence of various concentrations of GSH for 10 min. Means ± SE; (*N*), number of experiments; IRI, immunoreactive insulin; **$P < .01$.

washing, batches of five islets were placed into plastic tubes fitted with a nylon mesh button.[8] These tubes were then transferred into reaction tubes with 1 ml medium at either 2.8 or 11.1 mM glucose. Incubation of the islets was started for 30 min at 37°C under continuous shaking (100 strokes/min) in the absence of thiol compounds to get the ice-cold washed islets adapted to the incubation medium and temperature. For the studies with thiol compounds, the nylon net tubes with the islets were then transferred into a second set of reaction tubes containing 1 ml basal medium with either 2.8 or 11.1 mM glucose and different concentrations of thiols, as described in detail in the figure legends. After a 10-min incubation at 37°C, aliquots of the medium were frozen until insulin assay. In our anesthetized rats, plasma GSH has been found to be ~0.01 mM (unpublished data). Thus, except for cysteine, the concentrations of the cysteine analogues used here were close to physiologic GSH levels in plasma.

For β-thio-D-glucose, incubation studies were also performed for 40 min without changing media to compare our data with the data of an earlier study.[9]

Radioimmunoassay of insulin. Insulin released into the incubation medium was assayed radioimmunologically by a double-antibody technique.[10] Because thiols added to the medium may nick the disulfide bonds of insulin and therefore may cause an underestimation of released insulin, the effect of used thiols on the calibration-curve values of insulin in the radioimmunoassay was tested. For this reason, different concentrations of thiol compounds and different concentrations of insulin (in absence of pancreatic islets) were incubated for 10 min in the incubation medium, as described above. The results are shown in Fig. 1. At 0.1 mM thiol, the calibration curves of insulin were not significantly or not at all affected by the tested compounds. However, these curves were shifted downward at higher concentrations. To exclude underestimation of insulin release in the presence of higher concentrations of thiols (>0.1 mM) in each case, the preparation of each standard curve was designed in such a way that the thiols were added at the same concentration to the standard insulin and were processed the same way as islet-containing media during incubation; thus, the data with pancreatic islets were corrected automatically. The data on insulin release from the use of the highest concentrations of L-cysteine, mecysteine, and L-cysteine ethyl ester are not shown because of the strong effect of the substances on radioimmunoassay.

Basal insulin release. In the presence of 2.8 mM glucose, insulin released into the medium varied between 17 and 51 μU/ml. We consider this release to be mainly due to leakage from some damaged cells and from some basal secretion. Under these circumstances, it cannot absolutely be ruled out that in case of relatively high basal medium insulin, some insulin secretion induced by test compounds may be masked.

Assay of free thiols in incubation media. Because thiol compounds may be rapidly oxidized in albumin-containing medium under carbogen gassing at 37°C,[11] it was necessary to determine the decline of the effective concentration of thiols in the incubation medium in a separate experiment without islets. Figure 2 shows the time course of DTNB-reactive thiols during incubation in basal medium. At various times, aliquots were taken to measure the free-thiol groups spectrophotometrically at 412 nm after reaction with DTNB.[12] These data show that in most cases the concentration of free thiols in compounds declines in a time-dependent manner. This loss is suggested to be due to disulfide formation in the O_2-saturated medium.[11] Because GSSG does not stimulate insulin release,[2] it was necessary to test the effects of thiol compounds on insulin secretion during a time period when most of the quantity of thiols added was still present in its reduced form. From Fig. 2, it is evident that most of the thiol compounds tested are sufficiently stable within the first 10 min of incubation. However, the stability was less at low thiol concentrations, and in one case (cysteamine) even during 10 min there was a rapid 55% decrease of free thiols.

TABLE 1
Approximate measurable synergistic effect of cysteine analogues with free-thiol group on glucose-induced insulin release

	Analogue concentration (mM)	Glucose effect (%)
Glucose (11.1 mM)		100
Glucose (11.1 mM) with		
GSH	0.05	327
Acetylcysteine	0.05	307
Cysteamine	0.1	287
D-Penicillamine	0.5	276
Mecysteine	1.0	212*
L-Cysteine ethyl ester	1.0	182*

*Maximal possible effect could not be determined due to interference with radioimmunoassay.

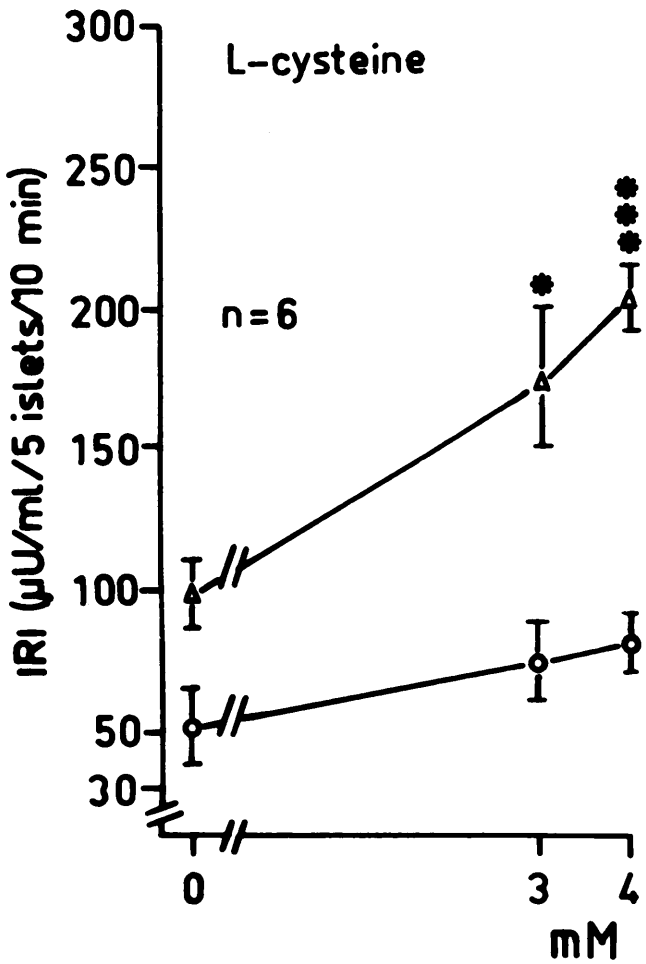

FIG. 4. Effect of L-cysteine on insulin release from rat pancreatic islets. Five islets were incubated at 2.8 (○) or 11.1 (▲) mM glucose in presence of various concentrations of L-cysteine for 10 min. Means ± SE; (*N*), number of experiments; IRI, immunoreactive insulin; *$P < .05$, ***$P < .001$.

To study the effect of thiols on insulin secretion, we therefore selected a time period that was as little as 10 min and that was just long enough to detect measurable amounts of released insulin.

Statistics. One-way analysis of variance and Student's *t* test were used for statistical evaluation of the results. Values are given as means ± SE; *N* is the number of experiments from separate preparations of islets.

RESULTS

Glutathione. Figure 3 shows the effect of GSH on insulin secretion at two different glucose concentrations. Although GSH had no effect at the substimulatory glucose level (2.8 mM), it augmented glucose-mediated (11.1 mM) insulin release. The half-maximal synergistic action of GSH was detectable with a little <0.01 mM, which is close to physiological levels in the plasma. The maximal effect was achieved at ~0.05 mM, showing an additional 227% increase in insulin secretion (Table 1) compared with the effect of glucose alone [100% represents actively secreted insulin, i.e., medium insulin concentration at 11.1 mM glucose minus that at basal glucose (2.8 mM)].

Cysteine. The effect of L-cysteine on insulin release at two different glucose concentrations was tested. At the substimulatory glucose level (2.8 mM), cysteine in the concentration range studied here did not significantly affect insulin secretion; however, it was effective at 11.1 mM glucose. In contrast to the effect of glutathione, the synergistic effect of cysteine appeared at much higher concentrations (>3 mM) than that of GSH. However, the maximal possible effect of cysteine could not be determined due to the interference of high concentrations with the radioimmunoassay (Fig. 4).

Cysteine analogues. The effect of cysteine analogues on insulin release at two different glucose concentrations is shown in Fig. 5. The cysteine analogues mecysteine, L-cysteine ethyl ester, acetylcysteine, D-penicillamine, and cysteamine were tested. None of them stimulated the secretion of insulin in the presence of 2.8 mM glucose. However, in the presence of 11.1 mM glucose, acetylcysteine and cysteamine exhibited a potent synergistic effect, which was maximal with as little as ~0.05 mM acetylcysteine and 0.1 mM cysteamine (Fig. 5); there was a 207 and 187%, respectively, increase in glucose-induced insulin secretion (Table 1). It was not possible to establish complete dose-response curves for mecysteine and L-cysteine ethyl ester for reasons mentioned earlier. At 1.0 mM mecysteine and L-cysteine ethyl ester, the increase in glucose-induced insulin secretion was 112 and 82%, respectively (Table 1). The effect of D-penicillamine was maximal at ~0.5 mM (176% increase; Table 1).

Mono- and dithiols. None of the mono- and dithiols such as mesna, tiopronin, BAL, and DMSA in the concentration range between 0.1 and 0.5 or 1.0 mM stimulated insulin secretion in the presence of 2.8 or 11.1 mM glucose (Table 2). Higher concentrations of these compounds were not used because of their effect on the insulin standard curve. Also, at concentrations up to 0.5 mM, β-thio-D-glucose exhibited no significant action at either 2.8 or 11.1 mM glucose. Even when the effect of β-thio-D-glucose was retested in a static incubation similar to that of Hellman et al.,[9] no effect was observed (Fig. 6).

Thioether, thioester, and disulfides of cysteine and cysteine analogues. As shown in Table 3, neither thioether nor thioester (including *S*-carbocysteine nor *S*-carbamoyl-L-cysteine) in the dose range between 0.1 and 1.0 mM affected insulin secretion in the presence of 2.8 or 11.1 mM glucose. Of the disulfides tested, cystine decreased but cystamine and D-penicillamine disulfide did not influence glucose-induced insulin secretion at concentrations at which their corresponding thiol analogues maximally potentiated glucose-induced secretion (5, 2, and 0.5 mM, respectively).

DISCUSSION

Relationships between chemical structure and biological effect. Our data show that in the presence of 11.1 mM glucose, insulin release was augmented by cysteine and those analogues that possess free-thiol groups; in contrast, at similar concentrations the corresponding disulfides and other cysteine analogues that lack an SH-group (such as *S*-carbamoyl-L-cysteine and *S*-carbocysteine) did not increase insulin secretion. These data and the observation that cystine actually blocks the insulin-releasing capacity of glucose, as does GSSG,[2] favor a prerequisite role of a free-thiol group for the synergistic effect on insulin release. This agrees with the observation by Hellman et al.[2] employing GSH and dextran-linked cysteine. Our data indicate that a cysteine moiety

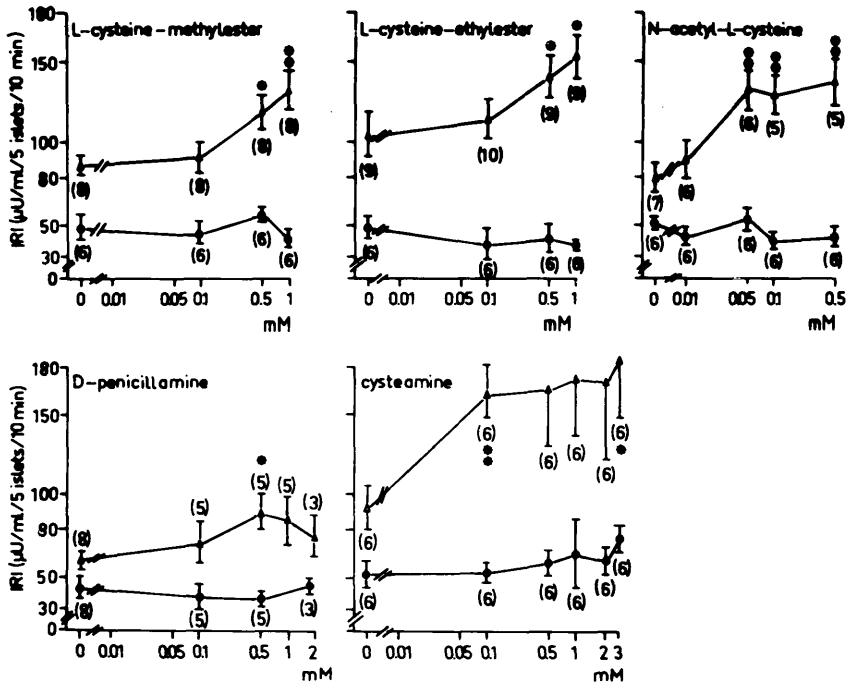

FIG. 5. Effects of cysteine analogues with free-thiol group on insulin release from rat pancreatic islets. Five islets were incubated at 2.8 (●) or 11.1 (▲) mM glucose in presence of various concentrations of cysteine analogues for 10 min. Means ± SE; (*N*), number of experiments; IRI, immunoreactive insulin; **P* < .05, ***P* < .01.

in the molecule is also a prerequisite for the synergistic action of thiol compounds because no insulin secretory action could be observed with several non-cysteine thiols. We were not able to confirm the earlier data for β-thio-D-glucose reported by Hellman et al.,[2] who observed a slight synergistic action of this compound.

Affinity and biological response to cysteine and its analogues. For reasons discussed earlier, it was not possible in each case to establish complete dose-response curves on the synergistic effect of cysteine and its analogues on glucose-induced (11.1 mM) insulin secretion. Nevertheless, the shapes and locations of our dose-response curves allow tentative conclusions regarding the affinity and biological activity of thiols in this system. If the concentration range of the synergistic effects of L-cysteine and various cysteine analogues is considered, the following rank of effectiveness (from most to least effective) can be established: glutathione > acetylcysteine > cysteamine > D-penicillamine > mecysteine > L-cysteine ethyl ester > L-cysteine.

There are no major differences among all tested cysteine analogues in maximal insulinotropic effect. Thus, our data suggest similarities in the mechanism of action but differences in affinity to the secretory system.

It has previously been reported that nonpermeable thiol

TABLE 2
Effects of thiols structurally different from cysteine and its analogues on glucose-induced insulin release from isolated rat pancreatic islets

Compound	Concentration (mM)	Insulin release (μU IRI · 5 islets^{-1} · 10 min^{-1}) 2.8 mM glucose	11.1 mM glucose
None		51 ± 7 (7)	98 ± 15 (9)
Mesna	0.01	45 ± 8 (5)	87 ± 12 (7)
	0.10	38 ± 11 (5)	91 ± 10 (10)
	0.50	49 ± 6 (5)	109 ± 15 (10)
Tiopronin	0.01	39 ± 4 (5)	84 ± 9 (7)
	0.10	33 ± 4 (5)	79 ± 14 (10)
	0.50	52 ± 12 (5)	118 ± 21 (10)
None		36 ± 11 (5)	65 ± 11 (4)
BAL	0.10	43 ± 5 (5)	49 ± 6 (5)
	1.00	61 ± 9 (5)	45 ± 7 (5)
DMSA	0.10	48 ± 13 (4)	44 ± 13 (4)
	1.00	20 ± 14 (4)	48 ± 6 (4)
None		17 ± 3 (4)	94 ± 17 (4)
β-Thio-D-glucose	0.01	16 ± 6 (4)	82 ± 14 (4)
	0.05	25 ± 7 (4)	88 ± 9 (4)
	0.1	18 ± 6 (4)	78 ± 18 (4)
	0.5	18 ± 4 (4)	80 ± 16 (4)

Five islets were incubated for 10 min in Krebs-Ringer buffer containing 2.8 or 11.1 mM glucose in presence of above mono- and dithiols. Means ± SE; (*N*), number of experiments; IRI, immunoreactive insulin. No significant synergistic effects were observed at any concentration compared with control. BAL, dimercaprol; DMSA, meso-2,3-dimercaptosuccinic acid.

TABLE 3
Effects of cysteine analogues lacking free-thiol group on glucose-induced insulin release from isolated rat pancreatic islets

Compound	Concentration (mM)	Insulin release (μU IRI · 5 islets^{-1} · 10 min^{-1}) 2.8 mM glucose	11.1 mM glucose
None		34 ± 8 (6)	63 ± 7 (6)
S-carbocysteine	0.1	27 ± 8 (6)	62 ± 13 (6)
	1.0	38 ± 5 (6)	62 ± 16 (6)
S-carbamoyl-L-cysteine	0.1	26 ± 5 (6)	60 ± 3 (6)
	1.1	37 ± 6 (6)	73 ± 14 (6)
None		50 ± 12 (6)	101 ± 7 (6)
Cystine	5.0	44 ± 11 (6)	66 ± 6 (6)
Cysteamine	2.0	60 ± 13 (6)	97 ± 11 (6)
D-Penicillamine disulfide	0.5	52 ± 5 (6)	101 ± 21 (6)

Five islets were incubated for 10 min in Krebs-Ringer buffer containing 2.8 or 11.1 mM glucose in presence of above compounds. Means ±SE; (*N*), number of experiments; IRI, immunoreactive insulin. Except for cystine at 11.1 mM glucose ($P < .05$), there were no significant changes observed in presence of cysteine analogues.

reagents, including bis(*N*^{1}-methylpiperazide)iodide and *p*-chloromercuribenzoate, stimulate secretion of insulin. However, their mechanism of action appears to be different from that of cysteine analogues, as reviewed previously.[6] Thus, these compounds have been shown to cause the release of insulin even in the presence of substimulatory glucose.

Possible localization of thiol action. It has been suggested that thiol groups in the membrane of the β-cell are related to the insulin-triggering action of glucose and that these thiols are kept in the reduced state by the intracellular glutathione system.[4,5] If such is the case, intracellular GSH must be transferred to the membrane disulfides. Intracellular reduced glutathione can permeate the membrane and then be linked to the γ-glutamyl cycle (13). If this also holds for a possible reducing activity of such membrane thiols, which are related to insulin secretion, then it may be speculated that a dehydrogenase that uses glutathione as a substrate might be located in the membrane close to the related disulfide bridges. In fact, a thiol-protein-disulfide oxidoreductase was recently described in β-cell membranes.[14] We have previously observed that exogenous GSH in a concentration employed for the studies here (0.1 mM) does not penetrate into islet cells (unpublished data), and Hellman et al.[2] have shown that dextran-bound cysteine, although it also does not penetrate into pancreatic islets, augments glucose-mediated insulin release. Therefore, the surface of islet cells is apparently the predominant place where at least GSH and possibly also other cysteine analogues act. L-Cysteine and some of its analogues, including mecysteine, which penetrate liver cells[15,16] and β-cells (unpublished observations), might also serve as precursors for intracellular GSH synthesis or might release protein-bound GSH. However, this possibility is unlikely to be relevant under the conditions of this study because the onset of action of these compounds is very rapid and it is unlikely that 0.1 mM acetylcysteine or cysteamine contribute significantly to increases in intracellular GSH in such a short time. In addition, cysteamine is not a substrate for glutathione synthesis. However, we cannot dismiss the possibility that the above-mentioned cysteine analogues act on insulin release independently from the reactive sulfhydryl group and/or in an indirect manner.

In conclusion, our in vitro data indicate that of the several thiols tested, only cysteine and its analogues potentiate glucose-induced (11.1 mM) insulin secretion; thiols not structurally related to cysteine do not. For this action, the availability of a free-thiol group appears to be a prerequisite. The maximal synergistic action seems to be similar among all cysteine analogues tested, whereas there are differences in their potency of action: GSH and acetylcysteine are more potent than the others. GSH and other cysteine analogues may at least partly act synergistically with glucose on insulin release by affecting membrane disulfide-thiol systems. At which step in the cascade of stimulus-secretion coupling exogenous cysteine analogues potentiate the insulin-triggering action of glucose remains to be elucidated.

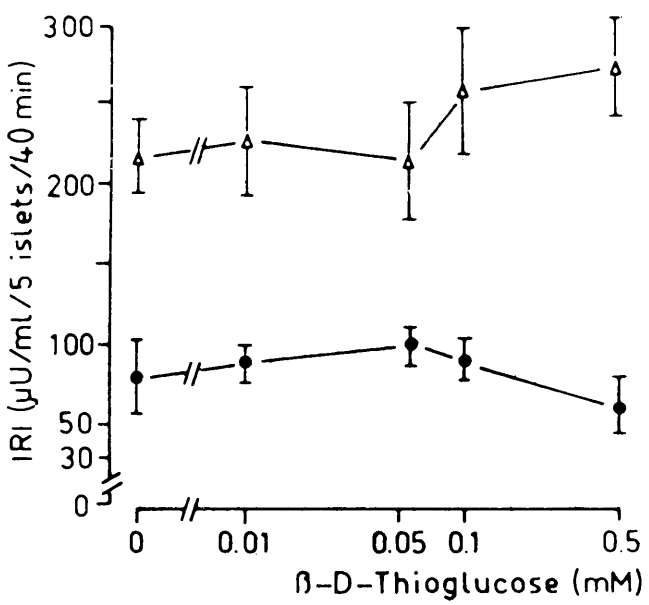

FIG. 6. Effect of β-thio-D-glucose on insulin secretion from rat pancreatic islets. Five islets were incubated at 2.8 (●) or 11.1 (△) mM glucose in presence of various concentrations of β-thio-D-glucose for 40 min. Means ± SE; 5 independent experiments; IRI, immunoreactive insulin.

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