Tight links between adenine and guanine nucleotide pools in mouse pancreatic islets: a study with mycophenolic acid

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Glucose metabolism in pancreatic B-cells leads to an increase in the ATP/ADP ratio that might participate in the regulation of insulin secretion. Good correlations have also been observed between guanine nucleotide levels in isolated pancreatic islets and insulin secretion. To assess whether guanine nucleotides have a specific role in stimulus–secretion coupling, their concentration should be modified selectively. This was attempted by culturing mouse islets overnight in the presence of mycophenolic acid (MPA), an inhibitor of GMP synthesis at the level of IMP dehydrogenase. The drug (25–50 μg/ml) did not affect the insulin content but decreased the GTP content of the islets and inhibited insulin secretion during subsequent incubation in the presence of 15 mM glucose. However, MPA also decreased the ATP/ADP ratio in the islets. The addition of guanine to the culture medium (to stimulate the salvage pathway of GTP synthesis) restored normal GTP levels, corrected the ATP/ADP ratio and partly prevented the inhibition of insulin release. In contrast, attempts to stimulate ATP synthesis specifically (by provision of adenine or adenosine) failed to reverse any of the effects of MPA. It is concluded that guanine and adenine nucleotide pools are tightly linked and cannot be specifically affected by MPA in pancreatic islet cells, probably because of the activity of nucleoside diphosphate kinase and because of the role of GTP in several reactions leading to adenine nucleotide generation. Contrary to previous claims, MPA is not an adequate tool for evaluating a specific role of guanine nucleotides in the control of insulin secretion.

INTRODUCTION

GTP is essential for the regulation of numerous cellular functions by heterotrimeric or monomeric G-proteins [1–3]. It is also involved in a number of metabolic pathways and in protein biosynthesis [4]. However, cellular GTP concentrations are usually fairly stable and not rate-limiting in the above functions.

The control of insulin secretion from pancreatic B-cells is peculiar in that it requires metabolism of the major physiological stimulus, glucose [5–7]. In contrast with the situation in most other cell types, this metabolism leads to a rise in ATP levels and a fall in ADP levels [8,9]. These changes are currently thought to underlie the action of glucose (and other nutrients) on several steps of stimulus–secretion coupling.

It has also been reported that islet GTP levels increase on stimulation with glucose [9–13]. In a recent study we further showed that there exists a good correlation between the changes in guanine nucleotides and insulin secretion [9], which raises the possibility that these changes have a regulatory role. To address this question the concentration of guanine nucleotides should be modified selectively. This was attempted by Metz et al. [14], who treated rat islets with mycophenolic acid (MPA), a specific inhibitor of IMP dehydrogenase [15], the enzyme that converts IMP into GMP (Figure 1). This indeed resulted in a marked lowering in islet GTP levels, but also affected ATP levels and the insulin content of the islets [14]. Because of the potential role of ATP [8] and also of the presence of adenine and guanine nucleotides in insulin granules [16], the available results do not permit any firm conclusion about the specific role of GTP in the control of insulin release. Given the importance of the question we have re-evaluated the possibility of selectively modulating pancreatic B-cell GTP levels by culturing mouse islets with MPA and purine bases.

MATERIALS AND METHODS

Preparation and solutions

Islets were isolated by collagenase digestion of the pancreas of fed female NMRI mice (25–30 g), followed by hand picking. They were then cultured for 18 h at 37 °C in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 10 mM glucose. When so specified, the medium was supplemented with MPA and/or purine bases. At the end of the culture, some batches of islets were saved for measurement of insulin content. Other batches were used to measure insulin secretion during incubation. The medium used for isolation and incubation of the islets was a bicarbonate-buffered solution that contained 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 mM Na_2HPO_4 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). Insulin was measured by a double-antibody radioimmunoassay with rat insulin as a standard (Novo Research Institute, Bagsvaerd, Denmark).

Measurements of islet insulin content

After culture, batches of six islets were directly transferred into 1 ml of an acid/ethanol mixture [95% (v/v) ethanol, 750 ml; concentrated HCl, 15 ml; water, 235 ml). In some experiments,

Abbreviation used: MPA, mycophenolic acid.

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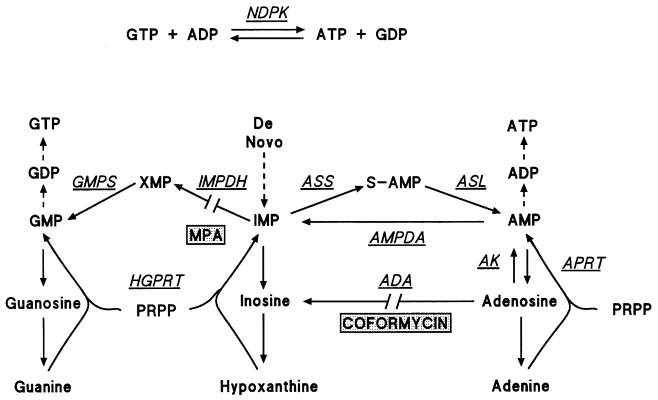

Figure 1 Pathways of metabolism of guanine and adenine nucleotides

Guanine, hypoxanthine, adenine and adenosine were used as precursors for the salvage pathway. MPA and coformycin were used as inhibitors of IMP dehydrogenase (IMPDH) and adenosine deaminase (ADA) respectively. Abbreviations: AK, adenosine kinase; AMPDA, AMP deaminase; APRT, adenine phosphoribosyltransferase; ASS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; GMPS, GMP synthase; HGPRT, hypoxanthine:guanine phosphoribosyltransferase; NDPK, nucleotide-diphosphate kinase; PRPP, phosphoribose diphosphate; S-AMP, adenylosuccinate.

the islets were first incubated for insulin release study before being transferred into acid/ethanol. After sonication the tubes were kept at −20 °C. An aliquot of the extract was eventually diluted in assay buffer before the measurement of insulin.

Measurements of insulin release from incubated islets

After culture, the islets were preincubated for 60 min at 37 °C in the bicarbonate-buffered solution described above and containing 15 mM glucose, a concentration that causes half-maximal stimulation of insulin release in mouse islets [8]. They were then distributed in batches of six and incubated for 60 min in 1 ml of bicarbonate buffer containing 3 or 15 mM glucose. The substances tested during the culture were usually also added to the preincubation solution but were omitted from the incubation solution. Exceptions are mentioned in the description of the results. At the end of the incubation, an aliquot (0.625 ml) of the medium was taken and diluted appropriately for measurement of insulin. The islets were then processed for measurement of insulin or nucleotide content.

Measurements of nucleotides in incubated islets

After the aliquot for insulin assay had been taken (while the tubes remained at 37 °C), the islets were incubated for another 5 min. The incubation was then stopped by the addition of 0.125 ml of trichloroacetic acid to a final concentration of 5% (w/v). The tubes were then vortex-mixed, left on ice for 5 min and then centrifuged for 5 min in a Microfuge (Eppendorf). A fraction (0.4 ml) of the supernatant was mixed with 1.5 ml of diethyl ether, and the ether phase containing trichloroacetic acid was discarded. This step was performed three times to ensure the complete elimination of trichloroacetic acid. The extracts were then diluted with 0.4 ml of buffer (20 mM Hepes/3 mM $MgCl_2$/KOH as required to adjust pH to 7.75). They were then frozen at −70 °C until the day of the assays, which were started by an appropriate further dilution with the same buffer.

All nucleotides were measured after enzymic conversion into ATP followed by luminometric measurement of the latter [17,18] as detailed previously [9]. Briefly, ATP was measured by the addition of an ATP-monitoring reagent containing luciferin and luciferase (Bioorbit, Turku, Finland). The light emitted was measured in a luminometer (1250 Luminometer system; Bioorbit). The sum ATP + ADP was measured after the conversion of ADP into ATP by pyruvate kinase in the presence of phosphoenolpyruvate. ADP was calculated by difference. Before the determination of GTP, the ATP and UTP present in the samples were removed by a 30 min incubation at 37 °C after mixing with buffer supplemented with 1 mM glucose, 1 unit/ml hexokinase, 1 mM NAD, 4.5 units/ml NAD-dependent glucose-6-phosphate dehydrogenase, 2 mM glucose 1-phosphate and 5 units/ml UDP-glucose pyrophosphorylase. This was followed by boiling for 1 min to denature the enzymes. GTP was then transformed into ATP by nucleoside diphosphate kinase in the presence of an excess of ADP, and the ATP formed was measured in duplicate aliquots by the luminometric method described above. Blanks and ATP or GTP standard curves diluted in bicarbonate-buffered solution were run through the entire procedure, including the extraction steps. Samples with known amounts of ADP were added to the assay of adenine nucleotides to verify complete transformation. Samples with known amounts of UTP and ATP were added to the assays of guanine nucleotides to verify their complete degradation.

Materials

Pyruvate kinase, MPA, adenine, guanine and hypoxanthine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); adenosine, ATP, ADP, GTP, UTP and all the enzymes used for the assays [9] were from Boehringer Mannheim (Mannheim, Germany). Coformycin was from Warner Lambert (Ann Arbor, MI, U.S.A.). Other reagents were of analytical grade and were usually obtained from UCB (Brussels, Belgium).

Presentation of results and statistical analysis

Results are presented as means ± S.E.M. for the indicated number of batches of islets. The statistical significance of differences between means was assessed by Student's *t* test for unpaired data or by an analysis of variance followed by a Newman–Keuls test for multiple comparisons.

RESULTS

Effects of MPA on glucose-induced changes in nucleotide contents and on insulin release

The concentration of MPA and the duration of treatment with the drug were selected after the results of a previous study [14]. Islets were cultured for 18 h in RPMI medium with or without 50 μg/ml MPA before being incubated in a bicarbonate-buffered solution containing 3 or 15 mM glucose. A high glucose concentration increased the GTP content by 54% and more than doubled the ATP/ADP ratio as a result of a 36% rise in ATP levels and a 35% decrease in ADP levels ($P < 0.001$). These changes were attended by a strong stimulation of insulin release (Table 1). After culture with MPA and incubation with 3 mM glucose, the ATP and GTP contents were similarly decreased by approx. 20% compared with control islets, whereas the ADP content was unaffected. MPA treatment impaired the rises in GTP and ATP, and attenuated the decrease in ADP content otherwise produced by 15 mM glucose. Hence the ATP/ADP ratio increased only slightly and the stimulation of insulin release was strongly inhibited. None of these changes in insulin release

Table 1 Effects of MPA on glucose-induced changes in nucleotide levels and in insulin release from mouse islets

The islets were cultured for 18 h and preincubated for 60 min in the absence or presence of 50 μg/ml MPA before being incubated for 60 min in a medium containing 3 or 15 mM glucose without MPA. The amount of insulin accumulated in the medium and the nucleotide content of the islets were measured at the end of the incubation. Values are means ± S.E.M. for 12 (nucleotides) or 20 (insulin) batches of islets from three or five separate experiments. $^{*}P < 0.05$, $^{**}P < 0.001$, compared with control islets cultured without MPA and incubated at the same glucose concentration.

Addition during		Nucleotide content after incubation (pmol per islet)				Insulin release during incubation (ng/h per islet)
Culture	Incubation	GTP	ATP	ADP	ATP/ADP	
None	3 mM glucose	3.5 ± 0.2	13.7 ± 0.7	3.3 ± 0.1	4.2 ± 0.3	0.6 ± 0.1
None	15 mM glucose	5.4 ± 0.3	19.2 ± 0.3	2.0 ± 0.1	9.3 ± 0.5	4.3 ± 0.2
MPA	3 mM glucose	2.5 ± 0.1**	10.6 ± 0.3**	3.3 ± 0.1	3.2 ± 0.2*	0.4 ± 0.1
MPA	15 mM glucose	3.2 ± 0.2**	12.4 ± 0.4**	2.5 ± 0.1*	5.0 ± 0.2**	1.4 ± 0.1**

Table 2 Effects of MPA and purine bases on the insulin content of mouse islets

Islets were cultured for 18 h in the absence or presence of 25 or 50 μg/ml MPA and the indicated base. Results are expressed as percentages of the insulin content of control islets within the same experiment. For all controls the insulin content averaged 107 ± 5 ng per islet. Values are means ± S.E.M. for *n* (in parentheses) batches of islets. $^{*}P < 0.01$ compared with controls.

Treatment during culture	Insulin content (% of controls)
Controls	100 ± 3 (38)
+ 30 μM adenine	89 ± 6 (12)
+ 250 μM adenine	80 ± 3 (16)*
+ 30 μM guanine	98 ± 4 (24)
+ 100 μM guanine	104 ± 7 (10)
MPA	108 ± 7 (38)
+ 30 μM adenine	113 ± 6 (12)
+ 250 μM adenine	104 ± 4 (16)
+ 30 μM guanine	93 ± 5 (24)
+ 100 μM guanine	97 ± 8 (10)

or nucleotide content could be ascribed to differences in B-cell granulation because the insulin content of the islets was not affected by the presence of MPA during culture (Table 2).

Influence of purine bases on effects of MPA

In an attempt to correct the nucleotide changes brought about by MPA, the islets were provided with exogenous purine bases. Guanine can be converted into guanine nucleotides by hypoxanthine:guanine phosphoribosyltransferase, and adenine can be converted into adenine nucleotides by adenine phosphoribosyltransferase (Figure 1).

In a first series of experiments the bases were added during culture and preincubation but not during the final incubation in the presence of 15 mM glucose (Figure 2). Neither guanine nor adenine (each at 30 μM) influenced GTP, ATP or ADP levels, the ATP/ADP ratio or insulin release in control islets. The effects of MPA (here used at 25 μg/ml) were similar to those shown in Table 1 and were totally unaffected by adenine. In contrast, guanine restored GTP levels and also corrected ATP levels and the ATP/ADP ratio. However, the reversal of the inhibition of insulin release was only partial (Figure 2). Guanine also reversed the effect of 50 μg/ml MPA on nucleotides while incompletely preventing its inhibitory action on insulin release (results not shown). None of these changes could be ascribed simply to changes in B-cell granulation (Table 2).

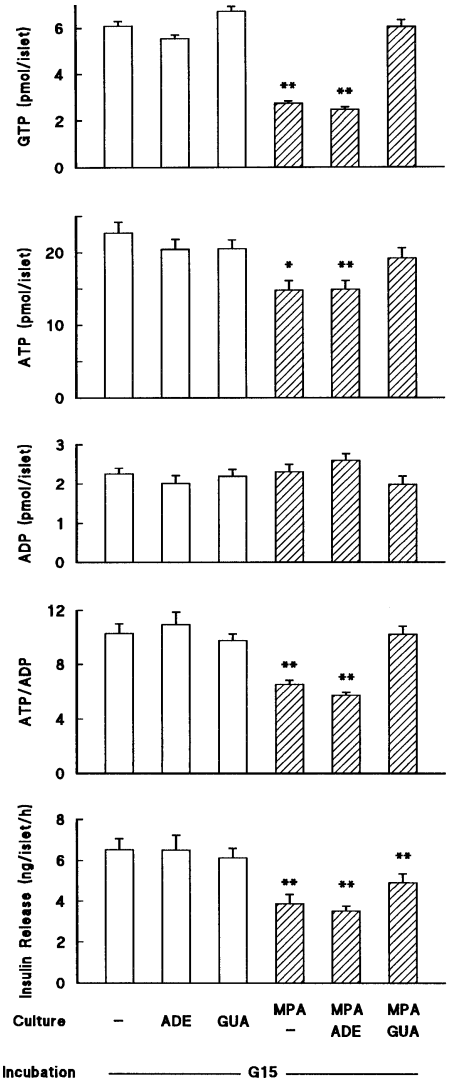

Figure 2 Influence of culture with adenine and guanine on the effects of MPA in mouse islets

The islets were cultured and preincubated in the absence or presence of 25 μg/ml MPA. The culture and preincubation media also contained 30 μM adenine (ADE) or guanine (GUA) as indicated. The subsequent incubation was performed in 15 mM glucose (G15) without further addition. Values are means ± S.E.M. for 12 (nucleotides) or 24 (insulin) batches of islets from three or six separate experiments. $^{*}P < 0.01$, $^{**}P < 0.001$, compared with controls cultured without MPA and incubated under the same conditions.

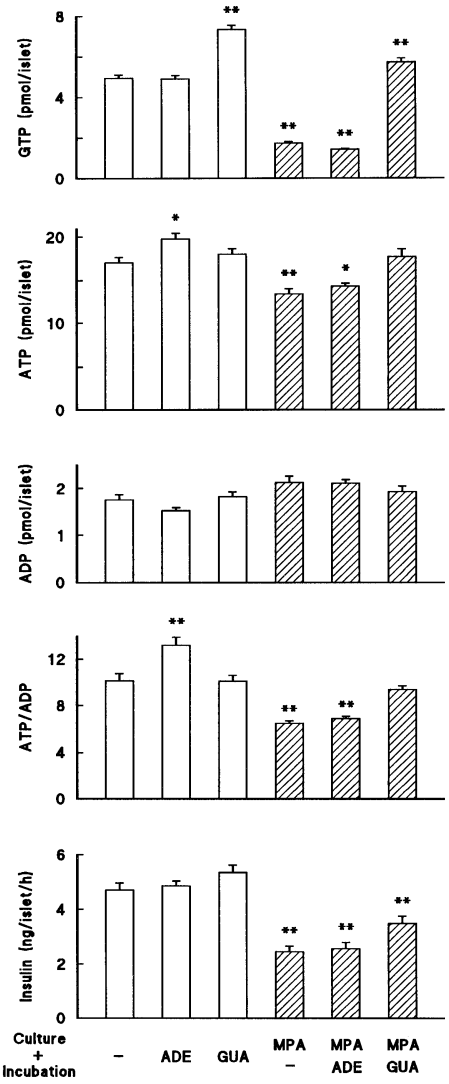

Figure 3 Influence of culture and incubation with adenine and guanine on the effects of MPA in mouse islets

The islets were cultured, preincubated and incubated in the presence or absence of 50 μg/ml MPA, with or without 250 μM adenine (ADE) or 100 μM guanine (GUA). Values are means $\pm$ S.E.M. for 16 batches of islets from three separate experiments. $^{*}P < 0.01$, $^{**}P < 0.001$, compared with controls without MPA.

In view of the lack of effect of adenine, adenosine (250 μM) was also tested. The medium was supplemented with 0.1 μM coformycin to inhibit adenosine deaminase (Figure 1) [19,20] and avoid the possibility that the metabolism of inosine formed from adenosine influenced B-cell function [20,21] and obscured the picture. Adenosine failed to correct the depleting effects of MPA on GTP levels, ATP levels and the ATP/ADP ratio, and the inhibitory effect on insulin release (results not shown).

In a second series of experiments, purine bases and MPA also remained present during the final incubation with 15 mM glucose (Figure 3). Guanine (at 100 μM) increased GTP levels but did not affect adenine nucleotide levels or insulin release in control islets. It also prevented the lowering by MPA of GTP and ATP levels and of the ATP/ADP ratio, but incompletely reversed the inhibition of insulin release (Figure 3). Adenine (here used at the higher concentration of 250 μM) increased the ATP/ADP ratio by raising ATP levels without changing ADP levels. Insulin release was unaffected (Figure 3). It should, however, be noted that the insulin content of these islets was 20 % lower than that of controls (Table 1), which might contribute to the increase in the ATP/ADP ratio [8]. In contrast, the inhibitory effects of MPA were not corrected by adenine (Figure 3).

Hypoxanthine (250 μM), which can serve as a precursor of purine nucleotides (Figure 1) in rat islets [22], had no effect on the nucleotide content of control islets and did not reverse any of the effects of MPA (results not shown).

DISCUSSION

Measurements of purine nucleotide changes in pancreatic islets meet with the problem that a substantial proportion of these nucleotides is present within insulin granules [23,24]. We have recently shown that the metabolically important pools of nucleotides are better estimated when the inert granular pool is decreased by overnight culture of the islets [8]. By selectively permeabilizing the plasma membrane of cultured islet cells, the proportion of nucleotides present within organelles (non-diffusible) could be measured [16]. It amounted to approx. 20 % for ATP, approx. 50 % for ADP, approx. 10 % for GTP and approx. 80 % for GDP. We therefore decided not to measure GDP levels because changes induced by MPA would be largely obscured by the non-cytoplasmic pools. The compartmentation of nucleotides also complicates the interpretation of the results when the insulin content of the islets has been significantly modified by the treatment. Fortunately, this was not the case in the present study, except in one single experimental condition.

MPA has often been used as a tool to assess the potential role of GTP in various cellular processes: the reversal of the observed changes by exogenous guanine or guanosine has been taken as evidence for the specificity of action of the drug [25–28]. MPA has already been used in studies of pancreatic islet biochemistry by Metz et al. [13,14,22]. The results of the present study concur with their reports showing that MPA lowers GTP levels in islets and that guanine reverses that decrease [13,14], presumably by providing substrate for hypoxanthine:guanine phosphoribosyltransferase (Figure 1). However, our results disagree with the conclusion that GTP changes can be dissociated from changes in adenine nucleotide levels. We observed that, in addition to lowering GTP content, MPA treatment of the islets also lowered the ATP/ADP ratio because of a fall in ATP and sometimes an increase in ADP levels. Supplementation of the culture and incubation medium with adenine (or adenosine and hypoxanthine) failed to correct the decrease in GTP levels, ATP levels and ATP/ADP ratio observed in the presence of MPA. Conversely, decreases in both GTP and ATP levels were corrected by guanine. The metabolisms of adenine and guanine nucleotides are tightly linked [29] and the above results support the contention that changes in ATP content are secondary to changes in GTP content.

Several mechanisms might explain the parallel changes in adenine and guanine nucleotide levels. First, a lowering of GTP levels might provoke a lowering of adenine nucleotide levels by a dual action: a decreased synthesis of AMP through adenylosuccinate synthetase, for which GTP serves as cofactor [30], and a greater degradation of AMP by AMP deaminase, of which GTP is an inhibitor [31] (Figure 1). The enzymes of this purine nucleotide cycle are known to be active in islet cells [32]. Secondly, an interplay exists between the syntheses of ATP and GTP: GDP is involved in the citric acid cycle and hence in ATP production, whereas ATP is required for the synthesis of GMP from IMP at

the level of GMP synthase. Thirdly, adenine and guanine nucleotide pools might rapidly equilibrate because of the high activity of nucleoside diphosphate kinase (Figure 1) present in islet cells [33]. This might account for the parallel changes in ATP/ADP and GTP/GDP ratios that we observed in islets stimulated by various glucose concentrations [9].

In contrast with our results, Meredith et al. [13] have reported dissociations between the changes in ATP/ADP and GTP/GDP ratios in islets treated with MPA. However, two problems jeopardize the interpretation of their results. First, the insulin content of their islets cultured in the presence of MPA was consistently decreased [14], which should affect the estimations of the ATP/ADP and GTP/GDP ratios [8]. Secondly, after lowering of the guanine nucleotide pool by approx. 70% with MPA, the remaining GDP was most probably present in non-cytosolic compartments [16].

In agreement with Metz et al. [14] we observed a marked inhibition of insulin secretion from MPA-treated islets, no effect of adenine on this inhibition and at least a partial reversal by guanine. We do not believe, however, that this reversal is a decisive argument in favour of a direct role of guanine nucleotides in stimulus–secretion coupling. The reversibility of the inhibitory action of MPA by guanine simply supports the contention that the drug is an inhibitor of GTP synthesis. The inhibition of secretion might have resulted indirectly from the changes in GTP concentration because of the associated changes in the ATP/ADP ratio or because of the decreased synthesis of unidentified important proteins. MPA is known to inhibit protein synthesis [27], probably secondarily to the decrease in GTP content [4]. In addition, dissociations of GTP levels from insulin secretion have been observed under different conditions. The addition of guanine to the culture and incubation medium of control islets clearly increased GTP levels without influencing insulin secretion. The inhibition of insulin secretion by MPA observed by Metz was concentration-dependent, whereas the fall in GTP was very marked and not dependent on concentration [14]. Changes of a similar magnitude (-70%) do not occur in the absence of MPA, even when the islets are incubated in the absence of glucose [9].

In conclusion, in mouse islets MPA lowers GTP levels but this effect cannot be dissociated from changes in the ATP/ADP ratio, which indicates that a tight interplay exists between the pools of adenine and guanine nucleotides. The drug also inhibits insulin secretion, which supports the current hypothesis that nucleotides have an important role in stimulus–secretion coupling but does not suffice to establish any specific role of GTP. Other approaches will be necessary to assess whether the good correlations between GDP levels or the GTP/GDP ratio and insulin secretion [9] are purely coincidental or indicative of a specific, regulatory, role of guanine nucleotides.

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