Cyclic AMP-dependent protein phosphorylation and insulin secretion in intact islets of Langerhans

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Effects on insulin release, cyclic AMP content and protein phosphorylation of agents modifying cyclic AMP levels have been tested in intact rat islets of Langerhans. Insulin release induced by glucose was potentiated by dibutyryl cyclic AMP, glucagon, cholera toxin and 3-isobutyl-1-methylxanthine (IBMX); the calmodulin antagonist trifluoperazine reversed these potentiatory effects. Inhibition by trifluoperazine of IBMX-potentiated release was, however, confined to concentrations of IBMX below 50 μM ; higher concentrations, up to 1 mM, were resistant to inhibition by trifluoperazine. IBMX-potentiated insulin release was also inhibited by 2-deoxyadenosine, an inhibitor of adenylate cyclase. In the absence of glucose, IBMX at concentrations up to 1 mM did not stimulate insulin release and in the presence of 3.3 mM-glucose IBMX was effective only at a concentration of 1 mM; under the latter conditions trifluoperazine again did not inhibit insulin secretion. The maximum effect on insulin release was achieved with 25 μM -IBMX. Islet [cyclic AMP] was increased by IBMX, with the maximum rise occurring with 100 μM -IBMX. The increase in [cyclic AMP] elicited by IBMX was more rapid than that induced by cholera toxin. Trifluoperazine did not significantly affect islet cyclic AMP levels under any of the conditions tested. When islets were incubated with [^{32}P]P_i, radioactivity was incorporated into islet ATP predominantly in the γ -position. The rate of equilibration of label was dependent on medium P_i and glucose concentration and at optimal concentrations of these 100% equilibration of internal [^{32}P]ATP with external [^{32}P]P_i required a period of 3 h. Radioactivity was incorporated into islet protein and, in response to an increase in islet [cyclic AMP], the major effect was on a protein of M_r 15000 on sodium dodecyl sulphate/polyacrylamide gels. The extent of phosphorylation of the M_r -15000 protein was correlated with the level of cyclic AMP: phosphorylation in response to IBMX was inhibited by 2-deoxyadenosine but not by trifluoperazine. Fractionation of islets suggested that the M_r -15000 protein was of nuclear origin: the protein co-migrated with histone H3 on acetic acid/urea/Triton gels. In the islet cytosol a number of proteins were phosphorylated in response to elevation of islet [cyclic AMP]: the major species had M_r values of 18000, 25000, 34000, 38000 and 48000. Culture of islets with IBMX increased the rate of [^3H]-thymidine incorporation. These data indicate that the potentiation of insulin release by agents elevating [cyclic AMP] is accompanied by activation of cyclic AMP-dependent protein kinase and phosphorylation of islet proteins. However, the major substrate was tentatively identified as histone H3; this phosphorylation is unlikely to be related to exocytosis but could be relevant to effects of cyclic AMP on cell division. The findings with trifluoperazine suggest that calmodulin is more likely to play a role in secretion in mediating Ca^{2+} -dependent steps modulated by cyclic AMP rather than in the regulation of cyclic AMP concentration.

Abbreviations used: IBMX, 3-isobutyl-1-methylxanthine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Alterations in intracellular cyclic AMP concentration modify pancreatic B-cell function (for review, see Sharp, 1979). Thus in the presence of a

stimulatory concentration of glucose, addition to the medium of agents that activate adenylate cyclase or inhibit phosphodiesterase potentiate insulin release (Cooper *et al.*, 1973; Hellman *et al.*, 1974). Further, despite previous negative reports (Montague & Cook, 1971; Cooper *et al.*, 1973), glucose-stimulated insulin release is associated with an increase in intracellular [cyclic AMP] within the B-cell (Charles *et al.*, 1973; Zawulich *et al.*, 1975). Although less marked than for insulin release, insulin biosynthesis has also been observed in several (Lin & Haist, 1973; Malaisse *et al.*, 1974; Maldonado *et al.*, 1977), although not all (Ashcroft *et al.*, 1978), studies to be increased by phosphodiesterase inhibitors. In addition to these acute effects, indirect evidence implicates cyclic AMP in long-term regulation of B-cell function: thus B-cell replication is enhanced in B-cell monolayer cultures incubated with a phosphodiesterase inhibitor (Rabinovitch *et al.*, 1980). Finally, several reports indicate that phosphodiesterase inhibitors tend to restore towards normal the depressed insulin secretory responses of islets from starved animals (Voyles *et al.*, 1973; Hedekov & Capito, 1974; Capito & Hedekov, 1974).

The major, possibly the only, biochemical mechanism for cyclic AMP to exert its regulatory effects in eukaryotic systems is via the phosphorylation of specific proteins catalysed by the catalytic subunit of cyclic AMP-dependent protein kinase (for review, see Glass & Krebs, 1980). Therefore it is likely that the effects of cyclic AMP on B-cell function also involve protein phosphorylation. The presence and properties of cyclic AMP-dependent protein kinase in rat pancreatic islets have been studied (Howell *et al.*, 1974; Sugden *et al.*, 1979a). The presence of two isoenzymes similar to the type 1 and type 2 forms described for other tissues has been demonstrated and the physical and kinetic properties of these activities have been described (Sugden *et al.*, 1979a). Islets have also been shown to contain protein substrates for cyclic AMP-dependent protein kinase (Harrison & Ashcroft, 1982) but these have not been characterized.

The major aim of the present study was to investigate further the importance of cyclic AMP-dependent protein phosphorylation in intact islets. The ability of various agents to modulate islet cyclic AMP levels was determined and the effects of these agents on phosphorylation of islet proteins was compared with their effects on insulin release. We report also the effects of the calmodulin antagonist trifluoperazine on these parameters in order to assess the significance of the reported dependence on calmodulin of islet adenylate cyclase (Valverde *et al.*, 1979; Thams *et al.*, 1982) and phosphodiesterase (Sugden & Ashcroft, 1981).

Materials and methods

Materials

Collagenase was from Serva, Heidelberg, Germany. IBMX, cholera toxin, glucagon, bovine serum albumin, 5'-nucleotidase and Hepes were from Sigma. ^{125}I -Insulin and [$\text{Me-}^3\text{H}$]thymidine were from Amersham International, Amersham, Bucks., U.K. [^{32}P]P_i and cyclic AMP radioimmunoassay kits were from New England Nuclear, Dreieich, Germany. QAE-Sephadex was from Pharmacia (G.B.), Hounslow, Middx., U.K. PEI-cellulose t.l.c. plates were from Merck, Darmstadt, Germany. Rat insulin standard was a gift from Dr. A. J. Moody, Novo Research Laboratories, Copenhagen, Denmark. Guinea-pig anti-insulin serum was from Wellcome Reagents, Beckenham, Kent, U.K. Trifluoperazine was a gift from Smith, Kline and French Laboratories, Welwyn Garden City, Herts., U.K. RPMI-1640 tissue culture medium was from Gibco Europe, Uxbridge, Middx., U.K. X-Omat AR and S film was from Kodak, Hemel Hempstead, Herts., U.K. Other reagents were from BDH Chemicals, Poole, Dorset, U.K.

Preparation of islets of Langerhans

Islets were prepared by a collagenase method (Coll-Garcia & Gill, 1969) from the pancreases of male Wistar rats fed *ad libitum* on standard laboratory diet. Islets were harvested by a wire loop under the dissecting microscope.

Insulin release

Batches of five islets were incubated in bicarbonate medium containing albumin (2g/litre) and the additions stated in the text or Tables. After incubation, insulin released into the medium was measured by radioimmunoassay (Ashcroft & Crossley, 1975).

Islet cyclic AMP content

Batches of five islets were pre-incubated at 37°C for 15 min in 20 μl of bicarbonate medium containing glucose (2mmol/litre) and albumin (2g/litre). Test substances were then added in 20 μl of medium and incubation continued for a further 10 or 60 min. Metabolism was arrested and cyclic AMP extracted by the addition of 100 μl of boiling acetate buffer, pH 6.2, followed by boiling for 5 min in a water bath. The boiled extract was sonicated for 5 s at position 1 on a Soniprobe (Dawe Instruments) and stored at -16°C until assayed. The cyclic AMP content of the sonicated material was assayed using a commercial radioimmunoassay kit according to the instructions of the manufacturer (New England Nuclear) for the

procedure with acetylation, except that half the recommended volumes were used.

Islet cyclic AMP phosphodiesterase

The cyclic AMP phosphodiesterase in islet extracts was measured as previously described in detail (Sugden *et al.*, 1979b). Briefly, islets were extracted with Tris buffer and incubated with [^3H]-cyclic AMP. The [^3H]AMP formed was converted into [^3H]adenosine by 5'-nucleotidase and the [^3H]adenosine was separated on QAE-Sephadex and quantified by liquid-scintillation spectrometry. Under the conditions used the extent of reaction is proportional to time of incubation and to amount of islet homogenate added.

Measurement of specific radioactivity of islet [γ - ^{32}P]-ATP after incubation with [^{32}P]P $_i$

Batches of 20 islets were incubated at 37°C in 100 μl of bicarbonate medium containing 20 mM-Hepes buffer, also containing albumin (2 g/litre), glucose (2 or 10 mM) and [^{32}P]P $_i$ (1.2 mM; 150 $\mu\text{Ci/ml}$). After incubation the islets were sedimented by centrifugation, 90 μl of medium was removed and 0.5 ml of ice-cold HClO_4 (3%, w/v) was added. The islets were disrupted by sonication, 25 μl of charcoal (50 mg/ml in water) was added and then the tubes were centrifuged for 1 min in an Eppendorf 3200 centrifuge. The pellet was washed four times with 0.5 ml of water. Bound radioactive nucleoside phosphates were eluted with 2×0.5 ml of methanol/ NH_3 /water (25:1:25, by vol.). The eluates were dried under vacuum and then redissolved in 40 μl of water. A portion (2 μl) was counted for total radioactivity by liquid-scintillation spectrometry. From each solution 20 μl was assayed for ATP using the luciferase method (Ashcroft *et al.*, 1973). T.l.c. was used to separate nucleoside phosphates. From each solution 10 μl was applied to PEI-cellulose plates (20 cm $\times$ 20 cm) and nucleoside phosphates were separated by ascending chromatography in 0.75 M- KH_2PO_4 , pH 3.4. Radioactive spots were located by autoradiography (using Kodak X-Omat AR paper) and the proportion of radioactivity incorporated into ATP determined by densitometric scanning of the autoradiograph using a Chromoscan 3 (Joyce-Loebl Instruments).

Phosphorylation of islet peptides

Batches of 20 islets were incubated for 3 h at 37°C in 100 μl of Hepes-buffered bicarbonate medium containing albumin (2 g/litre) glucose (10 mM), [^{32}P]P $_i$ (0.5–2.5 mCi/ml) and other additions as stated in the text. Incubation was terminated by the addition of 1 ml of ice-cold Hepes-buffered bicarbonate medium and islets were sedimented by gentle centrifugation (30 s at

600g). Medium was removed and the islets were washed with a further 1 ml of cold medium.

Sample preparation for sodium dodecylsulphate/polyacrylamide gels

Washed islets were suspended in 35 μl of 0.3 M-sucrose/50 mM-sodium phosphate (pH 7.0)/2 mM-EDTA/0.2 mM-EGTA/5 mM-NaF and sonicated for 5 s at 50 W. Sodium dodecyl sulphate (4 μl , 10%) and 4 μl of 0.1% Bromophenol Blue were added and the samples were boiled for 5 min. 2-Mercaptoethanol (2.5 μl) was added and the samples were run on sodium dodecyl sulphate/12.5% polyacrylamide gels (Laemmli, 1970). Dried gels were autoradiographed using Kodak X-Omat S or AR film. The extent of phosphorylation of individual bands was quantified by densitometry.

For fractionation studies, batches of 80 washed islets (as above) were homogenized in 200 μl of sucrose solution as above with 20 strokes of a motor-driven Teflon homogenizer. A 100 μl portion of the homogenate was retained and the remainder was centrifuged at 600g for 5 min at 4°C. The supernatant fraction was centrifuged at 190 000g for 30 min at 4°C. Pellets were resuspended in 100 μl of sucrose solution by sonication. From each fraction 50 μl was removed for assay of marker enzymes and the remainder subjected to sodium dodecyl sulphate polyacrylamide-gel electrophoresis as above.

Sample treatment for acid/urea-gel electrophoresis

Batches of 160 washed islets (incubated with [^{32}P]P $_i$ as above) were sonicated for 5 s at 50 W in 250 μl of 0.3 M-sucrose/50 mM-sodium phosphate (pH 7)/2 mM-EDTA/0.2 mM-EGTA/5 mM-NaF and centrifuged at 190 000g for 30 min at 4°C. The pellet was resuspended in 100 μl of 0.4 M-HCl, 2.5 μl of 2-mercaptoethanol was added and the pellet was extracted for 30 min on ice. Acid-insoluble material was removed by centrifugation for 1 min at 10 000g. Urea (75 mg), 0.7 mg of dithiothreitol and 0.5 μl of 0.2% phenolphthalein in ethanol were added and the solution was neutralized with NH_3 solution. Acetic acid (10 μl) and 1 μl of 1% Methylene Blue were added and the samples were run on acetic acid/urea/Triton gels as described by Bonner *et al.* (1980).

Preparation of histone fractions

Rat liver nuclei were prepared by a modification of the method of Chauveau *et al.* (1956) as described by Wang (1967). Histones were fractionated by the method of Johns (1964). Samples of each fraction were run on acetic acid/urea/Triton gels as described above.

Incorporation of [³H]thymidine into islet trichloroacetic acid-precipitable material

Batches of 20 islets were incubated for 70 h in RPMI 1640 medium/10% inactivated calf serum/penicillin (0.1 mg/ml)/streptomycin (0.1 mg/ml)/glucose (11 mM) at 37°C in an atmosphere of humidified air/CO₂ (19:1, v/v). To some batches, IBMX (0.1 mM) was added. After incubation, islets were removed in 0.5 ml of culture medium and washed in Hepes-buffered bicarbonate medium by gentle centrifugation. Trichloroacetic acid (250 µl, 10%) was added and islets were disrupted by sonication. The sonicated material was centrifuged at 10000g for 1 min and washed with 5 × 1 ml of 10% trichloroacetic acid. The pellet was redissolved in 250 µl of water and the radioactivity was measured by liquid-scintillation spectrometry.

Miscellaneous

The medium used for incubation of islets was a bicarbonate medium (Krebs & Henseleit, 1932) gassed with O₂/CO₂ (19:1) and containing the following ionic composition (mmol/litre): Na⁺, 141; K⁺, 5.9; Ca²⁺, 2.5; Mg²⁺, 1.2; H₂PO₄⁻, 1.2; SO₄²⁻, 1.2; HCO₃⁻, 25; Cl⁻, 105. Where Hepes-buffered bicarbonate medium is specified this refers to a medium of similar composition except that the bicarbonate concentration was reduced to 5 mM and 20 mM-Hepes was added. The gas phase was air.

The scintillant used for counting aqueous samples had the following composition: toluene/methoxyethanol (2:3, v/v)/naphthalene (80 g/litre)/5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (4 g/litre).

Lactate dehydrogenase was assayed spectrophotometrically by the rate of change of absorbance at 340 nm in a cuvette containing 50 mM-potassium phosphate (pH 7.5)/0.63 mM-sodium pyruvate/0.1 mM-NADH.

All data are means ± S.E.M. for the numbers of observations given in parentheses. The significance of observed differences was assessed by Student's *t*-test or by the Mann-Whitney U-test.

Results

Insulin release

The effects on insulin release of agents that may elevate islet [cyclic AMP] are shown in Table 1 for islets incubated in 0.6 ml of medium. In the absence of glucose, IBMX (5–1000 µM) had no effect on the basal rate of insulin secretion. In the presence of a non-stimulatory concentration of glucose (3.3 mM), IBMX was again ineffective at concentrations from 5 to 100 µM but 1 mM-IBMX significantly increased insulin release. Insulin

release was stimulated approx. 6-fold on raising the glucose concentration to 10 mM. IBMX significantly enhanced glucose-stimulated insulin release at a concentration of 5 µM and the maximum potentiating effect of IBMX was obtained with the drug present at a concentration of 25 µM. Insulin release in the presence of 10 mM-glucose was approximately doubled by the simultaneous presence of either cholera toxin or glucagon (5 µg/ml) or dibutyryl cyclic AMP (10 mM).

Because of the fact that for technical reasons islet cyclic AMP content was measured in islets after incubation in only 40 µl of medium it was considered necessary to assess the effect of incubation volume on the response of the islets to the above agents. Table 1 shows that although the secretory response to 10 mM-glucose was higher in islets incubated in 600 µl compared with that in 40 µl, the increment in response elicited by IBMX, cholera toxin or glucagon was little modified and the sensitivity to IBMX was independent of incubation volume.

Both adenylate cyclase and phosphodiesterase in islets have been reported to be calmodulin-dependent (Valverde *et al.*, 1979; Sugden & Ashcroft, 1981; Thams *et al.*, 1982); the effects of the calmodulin-antagonist trifluoperazine on these secretory responses were therefore tested. For islets incubated in 0.6 ml, trifluoperazine (20 µM) did not affect basal insulin release but caused a 60% inhibition of insulin release initiated by 10 mM-glucose. Trifluoperazine also inhibited insulin release in the presence of 10 mM-glucose plus 5, 10 or 25 µM-IBMX. Moreover the magnitude of the decrement induced by trifluoperazine in the presence of IBMX was greater than that attributable to inhibition of only the glucose-stimulated component of the release. Thus with 10 µM-IBMX, the net decrement in the presence of trifluoperazine was 312 µ-units/islet per h, whereas the decrement in glucose-induced insulin release was only 102 µ-units/islet per h. On the other hand, at higher concentrations of IBMX, trifluoperazine no longer inhibited insulin release to any greater extent than accountable for by inhibition of the glucose-dependent component. Trifluoperazine also markedly inhibited the potentiating effect on glucose-stimulated release of glucagon, cholera toxin and dibutyryl cyclic AMP. For islets incubated in 40 µl, trifluoperazine inhibited glucose-stimulated insulin release to a similar extent as before. However, effects on the potentiatory agents were attenuated. Thus only the potentiatory effects of 25 or 50 µM-IBMX were reduced by trifluoperazine and, although the effects of both cholera toxin and glucagon were reduced, only for glucagon did this action of trifluoperazine achieve statistical significance.

Table 1. *Effects of IBMX, glucagon, cholera toxin, dibutyryl cyclic AMP and trifluoperazine on insulin secretion*
 Batches of five islets were incubated for 2 h at 37°C in bicarbonate medium containing albumin (2 mg/ml) and the additions stated. Data in column (A) refer to islets incubated in 0.6 ml and those in (B) to an incubation volume of 0.04 ml. Insulin released into the medium was measured by radioimmunoassay. Results are means $\pm$ s.e.m. for the number of batches of islets given in parentheses. *Significantly greater than control at the same glucose concentration; ** significant inhibition by trifluoperazine ($P < 0.05$).

Incubation conditions					Insulin release (μ -units/islet per h)	
Glucose concn. (mM)	Agent	Concn.	Trifluoperazine at 20 μ M		(A)	(B)
0	None	—	—		32.7 $\pm$ 7.0 (15)	
3.3			—		23.9 $\pm$ 2.2 (52)	19.2 $\pm$ 4.2 (20)
10			—		159.7 $\pm$ 13.9 (20)	84.0 $\pm$ 7.6 (45)
3.3			+		32.3 $\pm$ 5.5 (15)	
10			+		58.2 $\pm$ 15.0 (5)**	43.5 $\pm$ 6.0 (15)**
0	IBMX	5 μ M	—		26.5 $\pm$ 6.9 (14)	
		10 μ M	—		20.0 $\pm$ 4.0 (15)	
		25 μ M	—		22.8 $\pm$ 6.5 (14)	
		50 μ M	—		14.7 $\pm$ 1.5 (15)	
		100 μ M	—		22.6 $\pm$ 4.3 (15)	
		1000 μ M	—		30.6 $\pm$ 3.9 (15)	
3.3	IBMX	10 μ M	—		15.1 $\pm$ 3.2 (10)	
		25 μ M	—		21.1 $\pm$ 6.2 (10)	
		50 μ M	—		16.1 $\pm$ 2.5 (9)	
		100 μ M	—		22.3 $\pm$ 3.1 (9)	
		1000 μ M	—		92.6 $\pm$ 13.0 (20)*	
		1000 μ M	+		141.1 $\pm$ 17.1 (9)	
10	IBMX	5 μ M	—		241.1 $\pm$ 39.6 (10)*	135.6 $\pm$ 17.7 (20)*
		5 μ M	+		114.2 $\pm$ 9.6 (10)**	121.2 $\pm$ 20.7 (20)
		10 μ M	—		514.7 $\pm$ 58.8 (10)*	234.2 $\pm$ 25.8 (20)*
		10 μ M	+		202.5 $\pm$ 18.2 (10)**	181.0 $\pm$ 25.8 (20)
		25 μ M	—		612.8 $\pm$ 43.6 (14)*	418.1 $\pm$ 56.7 (15)*
		25 μ M	+		377.7 $\pm$ 31.6 (15)**	236.9 $\pm$ 38.2 (15)**
		50 μ M	—		452.3 $\pm$ 42.8 (15)*	435.9 $\pm$ 55.2 (15)*
		50 μ M	+		342.1 $\pm$ 28.0 (15)	272.1 $\pm$ 31.3 (15)**
		100 μ M	—		456.0 $\pm$ 44.8 (15)*	498.2 $\pm$ 30.3 (25)*
		100 μ M	+		385.2 $\pm$ 22.3 (15)	411.5 $\pm$ 37.5 (15)
		1000 μ M	—		589.6 $\pm$ 58.2 (15)*	458.4 $\pm$ 58.0 (15)*
		1000 μ M	+		459.3 $\pm$ 55.9 (14)	426.5 $\pm$ 62.7 (15)
10	Glucagon	5 μ g/ml	—		217.8 $\pm$ 19.0 (10)*	151.3 $\pm$ 24.9 (15)*
		5 μ g/ml	+		92.8 $\pm$ 8.5 (10)**	94.0 $\pm$ 12.5 (15)**
10	Cholera toxin	5 μ g/ml	—		283.0 $\pm$ 30.9 (9)*	145.9 $\pm$ 21.0 (15)*
		5 μ g/ml	+		89.2 $\pm$ 11.4 (9)**	108.7 $\pm$ 7.4 (15)
10	Dibutyryl cyclic AMP	10 mM	—		279.9 $\pm$ 19.7 (14)*	
			+		169.8 $\pm$ 18.9 (15)**	

2-Deoxyadenosine produced a dose-dependent inhibition of insulin release stimulated by 10 mM-glucose plus 100 μ M-IBMX: thus the rates of release (μ -units/islet per h) in the absence of 2-deoxyadenosine and in the presence of 1, 5 and 10 mM-2-deoxyadenosine were 525 ± 51 , 349 ± 45 , 89 ± 11 and 56 ± 7 respectively ($n = 10$).

Islet cyclic AMP content

Control experiments were carried out to assess the validity of the procedure employed for extrac-

tion and assay of islet cyclic AMP. There was full recovery of exogenous cyclic AMP added to islets after incubation. Addition of phosphodiesterase to islet extracts resulted in the hydrolysis of 93% of measured cyclic AMP. Of the total cyclic AMP in extracts of islets plus medium, the tissue cyclic AMP accounted for 67% of the total.

Time-course studies of the islet cyclic AMP content are shown in Table 2. Basal cyclic AMP levels seen with 2 mM-glucose showed a tendency to increase during incubation for periods from 5 to

Table 2. *Time course of effects of IBMX and cholera toxin on islet cyclic AMP*

Batches of five islets were pre-incubated for 10 min at 37°C in 20 µl of bicarbonate medium containing glucose (2 mM) and albumin (2 g/litre). Additions were then made in a further 20 µl of medium to give the incubation conditions shown below. After incubation for the times shown cyclic AMP in the islets plus medium was extracted in acetate buffer and assayed by radioimmunoassay. Data are means $\pm$ S.E.M. for the numbers of batches shown in parentheses. *Significant effect of 10 mM-glucose at $P < 0.05$; **significantly greater than control with glucose alone at $P < 0.01$.

Time of incubation (min)	Islet cyclic AMP (fmol/islet)			
	2 mM-Glucose	10 mM-Glucose	10 mM-Glucose + 100 µM-IBMX	10 mM-Glucose + 5 µg of cholera toxin/ml
5	2.09 $\pm$ 0.65 (8)	3.34 $\pm$ 0.56 (9)	9.16 $\pm$ 1.67 (9)**	2.26 $\pm$ 0.54 (9)
10	2.07 $\pm$ 0.43 (9)	3.98 $\pm$ 0.81 (8)*	14.62 $\pm$ 2.97 (9)**	2.71 $\pm$ 0.53 (7)
30	3.78 $\pm$ 1.01 (9)	4.54 $\pm$ 1.41 (7)	27.16 $\pm$ 3.37 (8)**	5.91 $\pm$ 0.97 (9)
60	3.65 $\pm$ 0.41 (9)	5.17 $\pm$ 0.54 (9)*	22.38 $\pm$ 1.81 (7)**	12.51 $\pm$ 1.40 (7)**
120	4.42 $\pm$ 0.40 (8)	6.43 $\pm$ 1.12 (8)	28.27 $\pm$ 4.98 (8)**	10.77 $\pm$ 1.11 (8)*

Table 3. *Concentration-dependence of effects of IBMX on islet [cyclic AMP]*

Batches of five islets were pre-incubated for 15 min in bicarbonate medium containing 2 mM-glucose and 2 g of albumin/litre. The glucose concentration was raised to 10 mM and IBMX and trifluoperazine were added as indicated below. After incubation for 60 min cyclic AMP was extracted in acetate buffer and assayed by radioimmunoassay. Data are means $\pm$ S.E.M. for the numbers of observations shown in parentheses. * $P < 0.05$ versus control in the absence of IBMX; ** $P < 0.001$ versus control in the absence of IBMX.

Incubation conditions		Islet cyclic AMP (fmol/islet)
[IBMX] (µM)	Trifluoperazine at 20 µM	
—	—	5.78 $\pm$ 0.62 (33)
—	+	5.35 $\pm$ 0.70 (20)
5	—	5.20 $\pm$ 0.79 (16)
5	+	3.70 $\pm$ 0.67 (11)
10	—	5.97 $\pm$ 0.99 (17)
10	+	5.14 $\pm$ 1.13 (10)
25	—	10.00 $\pm$ 1.42 (21)*
25	+	13.31 $\pm$ 2.75 (12)*
50	—	16.95 $\pm$ 1.96 (21)**
50	+	25.45 $\pm$ 4.42 (12)**
100	—	23.15 $\pm$ 2.41 (17)**
100	+	31.40 $\pm$ 3.74 (17)**

120 min. A modest increase was observed on raising the glucose concentration to 10 mM, this effect of glucose achieving significance at the 10 and 60 min time points. IBMX (100 µM) elicited a marked and rapid increase in islet cyclic AMP with a stable maximum value being attained at 30 min. The effect of cholera toxin (5 µg/ml) was less marked and also clearly slower in onset than that of IBMX.

The concentration-dependence of the effect of IBMX is summarized in Table 3, the data being for islets incubated for 60 min. The lowest concentration of IBMX giving a significant increase in islet cyclic AMP was 25 µM. Shown also in Table 3 are data demonstrating that 20 µM-trifluoperazine did not affect basal islet cyclic AMP levels: the elevated cyclic AMP levels seen with 25–100 µM-IBMX showed a tendency to be increased by 20 µM-trifluoperazine but this effect did not achieve statistical significance.

Effects of IBMX on islet cyclic AMP phosphodiesterase

The inhibitory effect of 10, 100 and 1000 µM-IBMX on islet phosphodiesterase at two different cyclic AMP concentrations is given in Table 4. At either 5 or 100 µM-cyclic AMP, 10 µM-IBMX gave about 20% inhibition of phosphodiesterase. At 5 µM-cyclic AMP, over 90% inhibition required 1 mM-IBMX: at the higher cyclic AMP concentration, the 73% inhibition by 1 mM-IBMX reduced the phosphodiesterase activity to that seen at 5 µM-cyclic AMP in the absence of IBMX.

Equilibration of islet ATP with medium [32 P]P_i

Table 5 shows that ATP, ADP and GTP accounted for about 90% of the charcoal-bound radioactivity extracted from islets incubated with [32 P]P_i for up to 5 h in the presence of 10 mM-glucose. Whereas radioactivity in ATP was 69% of the total, that in ADP was only 3.9%. Hence over 90% of the label in ATP was in the γ -phosphate position. Similar relative distribution of label was found at 2 mM-glucose. However, glucose did markedly affect the rate at which equilibration of internal [32 P]ATP with external [32 P]P_i was achieved. Fig. 1 shows that, whereas in the presence of 10 mM-glucose equilibration was approached closely in 3 h, at 2 mM-glucose the extent

Table 4. *Effect of IBMX on islet cyclic AMP phosphodiesterase*

Portions of islet homogenate were incubated with [³H]cyclic AMP (5 or 100 μM) and with the given concentrations of IBMX for 60 min. [³H]AMP formed by phosphodiesterase was converted into [³H]adenosine, which was separated by ion-exchange chromatography and quantified by liquid-scintillation spectrometry. Data are means of closely agreeing duplicate determinations, each from two separate experiments.

[IBMX] (μM)	Phosphodiesterase activity (pmol/islet per h):	
	at 5 μM-cyclic AMP	at 100 μM-cyclic AMP
0	17.3	64.4
10	13.7	51.0
100	6.4	39.8
1000	1.4	17.1

Table 5. *Relative incorporation of [³²P]P_i into islet ATP, ADP and GTP*

Islets were incubated with [³²P]P_i (1.2 mM; 150 μCi/ml) for up to 5 h. After addition of HClO₄, nucleoside phosphates were absorbed on to charcoal and separated by t.l.c. Radioactive spots were located by autoradiography and identified by comparison with standards. The relative incorporation (expressed as a percentage of the total radioactivity eluted from the charcoal) was determined by densitometric scanning of the autoradiographs. Data are given as means ± S.E.M. for 10 determinations.

[Glucose] (mM)	Relative incorporation of radioactivity (%)		
	ATP	ADP	GTP
2	61.0 ± 2.7	6.7 ± 0.3	11.4 ± 0.5
10	69.0 ± 0.9	3.7 ± 0.3	15.6 ± 0.4

of equilibration was never more than 60% even after incubation for 5 h. It should be noted that this finding may pose problems in the design of experiments to test the effect of glucose in itself on phosphorylation. However, for the present study it was convenient to use a standard glucose concentration of 10 mM throughout. Fig. 2 suggests that the rate-limiting step for equilibration of [³²P]ATP with [³²P]P_i is uptake of [³²P]P_i into the cells. At the normal extracellular concentration of P_i, islet ATP reached equilibrium in 2 h (note that in this experiment ATP was not separated from other charcoal-bound radioactivity and hence specific radioactivity will be overestimated: the true extent of equilibration in 2 h is about 60%). Lowering the P_i concentration to 0.1 mM reduced the rate of equilibration markedly; about 30% apparent equilibration was found in 2 h (about 20% true equilibration). Further control experiments

Fig. 1. *Effect of glucose on the rate of equilibration of medium [³²P]P_i with islet [³²P]ATP*

Batches of 20 islets were incubated in HEPES-buffered bicarbonate medium containing [³²P]P_i (1.2 mM; 150 μCi/ml), albumin (2 g/litre) and glucose (2 mM; ○; 10 mM; ●). At the times indicated islets were disrupted by sonication in HClO₄ and nucleoside phosphates were extracted by charcoal adsorption. ATP in eluates was measured by the luciferase assay; ATP was separated from other nucleoside phosphates by t.l.c. on PEI-cellulose and the radioactivity in ATP determined by autoradiography and densitometry. The specific radioactivity of the [³²P]-ATP was thus calculated and expressed relative to that of the medium [³²P]P_i. Results are plotted as means ± S.E.M. for six determinations.

showed that at normal extracellular [P_i] and with 10 mM-glucose the rate of equilibration of islet [³²P]ATP with P_i was not affected by the marked elevation of cyclic AMP content induced by 100 μM-IBMX.

In initial studies of protein phosphorylation in islets, the above findings led us to adopt a protocol of incubating the islets for 3 h with [³²P]P_i (1.2 mM) and 10 mM-glucose, i.e. to isotopic equilibrium and then to add IBMX or other test agent. However, it became apparent that the procedure gave the same pattern of protein phosphorylation if the IBMX were present throughout. Hence to minimize experimental manipulation of the relatively high amounts of radioactivity used, the standard procedure adopted for study of islet protein phos-

Fig. 2. Effect of medium P_i concentration on the equilibration of medium $[^{32}P]P_i$ with islet $[^{32}P]ATP$

The same protocol was used as given in the legend to Fig. 1 except that ATP was not separated from other nucleoside phosphates before determination of its radioactivity. The medium phosphate concentration was 0.1 mM (○) or 1.2 mM (●). Results are means $\pm$ S.E.M. for three observations.

phorylation was a single incubation in which $[^{32}P]P_i$ and the test agent were present throughout.

Phosphorylation of islet peptides

Islets incubated for 3 h with $[^{32}P]P_i$ incorporated radioactivity into a large number of peptides as evidenced by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and autoradiography. Fig. 3 shows densitometric traces from typical experiments and demonstrates the effects of IBMX, theophylline, glucagon, cholera toxin and dibutyl cyclic AMP. The major effect of these agents was on a protein of M_r 15000. Table 6 expresses the data from several experiments as mean peak height of the peak corresponding to the M_r -15000 protein. The effects of the phosphodiesterase inhibitors and of dibutyl cyclic AMP were considerably greater than those of glucagon and cholera toxin.

Table 6 also shows the concentration-dependence of the effect of IBMX on phosphorylation of the M_r -15000 protein. A significant effect was elicited by 5 μ M-IBMX and there was an increase up to 8-fold with an increase of IBMX concentration up to 100 μ M. The effect of 100 μ M-IBMX

Fig. 3. Cyclic AMP-dependent protein phosphorylation in intact islets of Langerhans

Batches of 20 islets were incubated for 3 h in Hepes-buffered bicarbonate medium containing $[^{32}P]P_i$ (1.2 mM; 0.5–2.5 mCi/ml)/albumin (2 g/litre)/glucose (10 mM) and the following additions: A, none; B, glucagon (5 μ g/ml); C, cholera toxin (5 μ g/ml); D, theophylline (5 mM); E, IBMX (100 μ M); F, dibutyl cyclic AMP (10 mM). Islets were disrupted by sonication and subjected to electrophoresis on sodium dodecyl sulphate/12.5% polyacrylamide gels. Radioactive peptides were visually detected by autoradiography and densitometric scans of autoradiographs are shown from typical experiments. The position of the M_r -15000 protein peak is indicated by the arrow.

on phosphorylation of the M_r -15000 protein was inhibited by 2-deoxyadenosine (10 mM) but not by trifluoperazine (20 μ M).

When islet homogenates were fractionated before analysis of phosphoproteins, it was found that the M_r -15000 protein band appeared exclusively in the fraction sedimented by centrifugation at 600g for 10 min in 0.3 M-sucrose. To exclude the possibility that the homogenization was inadequate, resulting in sedimentation of whole islets or cells, the fractions were assayed for the cytosolic enzyme lactate dehydrogenase. The 600g pellet contained $14.8 \pm 2.3\%$ ($n = 6$) of the total homogenate lactate dehydrogenase and the 190000g supernatant contained $74.2 \pm 3.2\%$. The results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the phosphoproteins in the 600g pellet and the 190000g supernatant are shown as densitometric traces in Fig. 4. The marked effect of IBMX on the 600g pellet fraction is evident. The absence of the

Table 6. Phosphorylation of an M_r -15000 protein in response to agents modifying islet cyclic AMP content. Batches of 20 islets were incubated for 3 h in bicarbonate medium containing [32 P]P_i/albumin (2g/litre)/glucose (10mM) and the agents stated. Islet proteins were extracted and gels prepared for autoradiography as described in the legend to Fig. 3. The extent of phosphorylation of the M_r -15000 protein was assessed by densitometry of the autoradiographs. Results are means $\pm$ s.e.m. or as means of closely agreeing duplicates and are expressed relative to the control with glucose alone. *Significant increase in phosphorylation at $P < 0.05$.

Test agent(s)	Phosphorylation of M_r -15000 protein (% of control)
None	100 (control)
Glucagon (5 μ g/ml)	167 $\pm$ 21 (6)*
Cholera toxin (5 μ g/ml)	207 $\pm$ 46 (6)*
Dibutylryl cyclic AMP (10mM)	720 $\pm$ 93 (4)*
Theophylline (10mM)	413 $\pm$ 31 (4)*
IBMX (5 μ M)	194 $\pm$ 15 (4)*
IBMX (10 μ M)	250 $\pm$ 14 (4)*
IBMX (25 μ M)	353 $\pm$ 36 (4)*
IBMX (50 μ M)	381 $\pm$ 24 (3)*
IBMX (100 μ M)	758 $\pm$ 66 (12)*
IBMX (100 μ M) + 2-deoxyadenosine (10mM)	118 (2)
IBMX (100 μ M) + trifluoperazine (20 μ M)	674 (2)

M_r -15000 band from the 190000g supernatant fraction can be seen; however, it is evident that fractionation before analysis reveals several bands of M_r 18000, 25000, 34000, 38000 and 48000 whose phosphorylation is enhanced by IBMX. Since the above data suggested that the M_r -15000 protein band may be of nuclear origin its possible identity as a histone was examined. If islet homogenates in sucrose were sonicated then the M_r -15000 protein was no longer sedimented at 600g but could be sedimented by centrifuging at 190000g for 30 min. Addition of 0.4M-HCl to such sonicated preparations solubilized the M_r -15000 protein completely; partial solubilization was achieved with 0.5M-NaCl. The addition of acetone to the HCl-solubilized extracts precipitated the M_r -15000 protein. These properties supported the possibility that the M_r -15000 protein may be a histone; the mobility of the M_r -15000 band on sodium dodecyl sulphate/polyacrylamide gels was identical with that of a commercial sample of histones. However, individual histones are not clearly resolved on sodium dodecyl sulphate/polyacrylamide gels and there may be interspecies variability to consider. Therefore, acetic acid/urea/Triton-gel electrophoresis of the acid extracts described above was carried out using rat histones

Fig. 4. Stimulation by IBMX of protein phosphorylation in intact islets of Langerhans. Islets were incubated for 3 h in Hepes-buffered bicarbonate medium containing [32 P]P_i (1.2mM; 0.5–2.5Ci/mol)/albumin (2g/litre)/glucose (10mM) and in the absence of IBMX (lower traces in a and b) or in the presence of 100 μ M IBMX (upper traces in a and b). After incubation, islets were washed and then homogenized in 0.3M-sucrose. Homogenates were centrifuged at 600g for 10 min, the pellet was sonicated in sucrose solution and the supernatant was centrifuged at 190000g for 30 min. Fractions were subjected to electrophoresis on sodium dodecyl sulphate/12.5% polyacrylamide gels. In (a) densitometric scans of the autoradiographs of the 600g pellet are shown. The arrow indicates the position of the M_r -15000 protein. In (b) scans of the autoradiograph of the 190000g supernatant are shown. The arrows mark the positions of the major bands whose phosphorylation was enhanced by IBMX and their M_r values ($\times 10^{-3}$) are indicated above the arrows.

prepared from rat liver nuclei as standards. Fig. 5 shows that the mobility of the M_r -15000 band coincided with that of histone H3.

Effect of IBMX on incorporation of [3 H]thymidine by islets into trichloroacetic acid-precipitable material

In incubations of islets for periods similar to those used for the studies described above, insuf-

Fig. 5. Acid/urea-gel electrophoresis of histone and the M_r -15000 protein

The Figure shows acetic acid/urea/Triton-gel electrophoresis of rat histones and of phosphorylated islet proteins. Lanes (1)–(5) are gels stained with Coomassie Blue as follows: (1), rat liver nuclei; (2), fraction F_1 (histone H1); (3), fraction F_{2a} (histones H2a and H4); (4), fraction F_{2b} (histone H2b); (5), fraction F_{2b} (histone H3). Lanes (6) and (7) are autoradiographs of islet proteins labelled as described in the legend to Fig. 3. The unlabelled arrow marks the position of the M_r -15000 protein.

ficient radioactivity was incorporated from [^3H]thymidine into trichloroacetic acid-precipitable material by islets to permit analysis. Therefore islets were subjected to culture for a prolonged period (70 h). The inclusion of $100\ \mu\text{M}$ -IBMX in the culture medium increased [^3H]thymidine incorporation from 3754 ± 365 to 5068 ± 562 d.p.m./20 islets ($n = 8$; $P < 0.01$).

Discussion

In studies of protein phosphorylation in tissues pre-incubated with [^{32}P]P_i, it is not always essential to be at isotopic equilibrium; however, it is important to know the rate at, and the extent to, which this state is achieved under the conditions used. There are no previous data on this point in islets of Langerhans. It is clear that equilibration of islet [^{32}P]ATP with external [^{32}P]P_i is relatively slow. Since islet ATP turnover is rapid (Ashcroft *et al.*, 1973; Hutton & Malaisse, 1980), the rate of equilibration is likely to reflect the slow rate of entry of P_i into the cells; this conclusion is consistent with the observed dependence of the equilibration rate on external P_i concentration. The rate of equilibration is also markedly dependent on extracellular glucose concentration. The

practical consequence of these findings is that for experimental protocols where it is necessary to have achieved isotopic equilibration, the medium P_i concentration must be physiological, i.e. not trace [^{32}P]P_i, and the glucose concentration must be high. Even under these conditions at least 3 h pre-incubation with [^{32}P]P_i is necessary. These findings are relevant to studies not only of protein phosphorylation but also of, e.g., lipid phosphorylation.

In agreement with previous observations (Cooper *et al.*, 1973; Hellman *et al.*, 1974), the present study shows that an increase in islet [cyclic AMP] is not a sufficient condition for eliciting insulin release. However, insulin release already stimulated by glucose is markedly sensitive to cyclic AMP. Qualitatively there is a good correlation between the ability of agents to alter islet AMP content (whether by inhibition of phosphodiesterase or by activation or inhibition of adenylate cyclase) and their effects on glucose-stimulated insulin release. Although there are some quantitative discrepancies, e.g., the apparently lower threshold for effects of IBMX on release compared with cyclic AMP content, these in our view are minor and perhaps attributable to the difficulty of measuring a small change in cyclic AMP: as little as $5\ \mu\text{M}$ -IBMX

elicited detectable inhibition of phosphodiesterase (M. R. Christie, unpublished work). It is clear from the data that the maximum effect of IBMX on insulin release occurs at a concentration well below that giving a maximum rise in [cyclic AMP]. Since there are reports that IBMX may have effects on islets at high concentrations independent of cyclic AMP (Sehlin, 1970; Capito & Hedekov, 1974; Sugden & Ashcroft, 1978) the use of such concentrations, 1 mM in many experiments, should be viewed with caution; this is especially relevant to consideration of the effects of trifluoperazine as discussed below.

The present study shows that there is a good correlation between islet cyclic AMP and protein phosphorylation; thus concentrations of IBMX sufficient to inhibit islet phosphodiesterase and increase islet cyclic AMP lead to increased phosphorylation of islet protein together with potentiation of insulin release stimulated by glucose. The predominant protein phosphorylated has an M_r of 15000 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. That phosphorylation of the M_r -15000 protein is mediated by cyclic AMP-dependent protein kinase is supported by the finding that glucagon, theophylline, cholera toxin and dibutyryl cyclic AMP also caused enhanced phosphorylation of the protein. Moreover, 2-deoxyadenosine, an inhibitor of islet adenylate cyclase (Campbell & Taylor, 1982), decreased insulin release and blocked IBMX-stimulated phosphorylation of the M_r -15000 protein.

Subcellular fractionation suggested that the M_r -15000 protein is associated with the nuclear fraction. The protein was extracted by high salt or by mineral acid and was precipitated by acetone. On sodium dodecyl sulphate/polyacrylamide gels the protein migrated with histone and on acid/urea gels it was found to co-migrate with rat histone H3. These data suggest that the M_r -15000 protein is histone.

This conclusion makes it unlikely that the actions of cyclic AMP on the release system itself are mediated by the M_r -15000 protein. A role in regulation of protein synthesis seems possible and indeed effects of IBMX on biosynthesis of proinsulin have been documented (Lin & Haist, 1973; Maldonado *et al.*, 1977), although these have in general been rather small effects. Since it has been reported that IBMX causes increased mitosis of B-cells (Rabinovitch *et al.*, 1980) we examined the effect of IBMX on incorporation of [3 H]thymidine into islet nucleic acid as measured by incorporation into trichloroacetic acid-precipitable material. This was increased significantly and the possibility exists that phosphorylation of the M_r -15000 protein may be related to this mitotic activity of IBMX.

These data do not explain, therefore, the effect of cyclic AMP to amplify insulin secretory responses. However, the subcellular fractionation studies revealed a number of proteins that were phosphorylated in response to elevation of [cyclic AMP]. These appeared predominantly in the soluble fraction with the principal species having M_r values of 18000, 25000, 34000, 38000 and 48000.

Previous studies on protein phosphorylation in intact islets or B-cells are scarce. Suzuki *et al.* (1981) showed that elevation of glucose led to enhanced phosphorylation of approximately 30 polypeptides in rat islets. However, the effects were small (the maximum change was 22%) and under the conditions employed our data on [32 P]-ATP specific radioactivity raise the possibility that the effects of glucose observed by Suzuki *et al.* (1981) may have been wholly or in part related to effects on ATP turnover. In a later study (Suzuki *et al.*, 1983) the same group showed that glucagon stimulated the phosphorylation of 15 islet polypeptides; one of the largest effects they found was on a protein of M_r 15000. In rat insulinoma cells Schubart (1982) showed that the major protein phosphorylation response to glucagon was into two proteins each of M_r 16000. In contrast with our findings, these small polypeptides were found in the soluble fraction. However, Schubart (1982) homogenized the insulinoma cells in hypo-osmotic buffer before subcellular fractionation; this may have released organelle-bound protein to the soluble fraction.

Similar studies in other secretory systems are showing enhanced phosphorylation of proteins occurring with cyclic AMP-stimulated secretion. Thus a protein of M_r 32000 was increased in exocrine pancreas (Roberts & Butcher, 1983), parotid (Kanamori & Hayakawa, 1980; Jahn & Soling, 1981a; Spearman *et al.*, 1981) and lacrimal gland (Jahn & Soling, 1981b). Other substrates demonstrated include a protein of M_r 29000 in exocrine pancreas (Freedman & Jamieson, 1981) and of 20000 in parotid (Spearman *et al.*, 1981). To date, however, in none of these systems has it been possible to ascribe a functional role to such cyclic AMP-dependent phosphorylations.

The results obtained with trifluoperazine require comment. There is growing evidence to implicate calmodulin in insulin secretion. The presence of calmodulin in islet tissue is established (Sugden *et al.*, 1979b; Hutton *et al.*, 1981) and a functional role for calmodulin in stimulus-secretion coupling has been supported by demonstration of inhibition of insulin secretion in response to various stimuli by antagonists of calmodulin such as trifluoperazine (Gagliardino *et al.*, 1980), pimozone (Henquin, 1981) and W7 (Gagliardino *et al.*, 1980; Niki *et al.*,

1981). It has been suggested therefore that calmodulin mediates the actions of Ca^{2+} on the exocytotic discharge of insulin (Ashcroft, 1980; Gagliardino *et al.*, 1980); a possible mechanism is via activation of a Ca^{2+} -calmodulin-dependent protein kinase (Harrison & Ashcroft, 1982). This view has been opposed by Wollheim & Sharp (1981) because trifluoperazine failed to inhibit insulin release in the presence of IBMX in rat islets (Krausz *et al.*, 1980; Janjic *et al.*, 1981) and of glucagon in insulinoma cells (Schubart *et al.*, 1980). They proposed that calmodulin plays no role in insulin release after the elevation of cytosolic $[\text{Ca}^{2+}]$. In our view our present findings make this conclusion premature. It is clear that trifluoperazine can, under appropriate conditions, inhibit effects of cyclic AMP on insulin secretion. Thus in contrast with results with insulinoma cells (Schubart *et al.*, 1980), trifluoperazine opposed the action of glucagon on insulin release from normal rat islets. Moreover, although we confirm the insensitivity of insulin release with high concentrations of IBMX to trifluoperazine, when lower concentrations are used a pronounced inhibition by trifluoperazine is observed. The concentration of IBMX used by Wollheim & Sharp (1981) was 1 mM, which we feel may be too high a concentration to permit reliable conclusions to be drawn.

Finally, the failure of trifluoperazine to oppose the increase in cyclic AMP concentration elicited by IBMX casts some doubt on the physiological importance of the reported effects of calmodulin on islet adenylate cyclase (Valverde *et al.*, 1979; Thams *et al.*, 1982).

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