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ENHANCED GLUCOSE-INDUCED INSULIN RELEASE AND ENDOGENOUS Ca^{2+} IONOPHORETIC ACTIVITY IN NEONATAL RAT ISLETS FOLLOWING ISLET-ACTIVATING PROTEIN

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SUMMARY: Islet-Activating Protein, purified from the culture medium of *Bordetella pertussis*, enhanced glucose-induced insulin secretion from cultured neonatal rat islets in a calcium dependent manner. This effect was accompanied by an increase in lipid associated Ca^{2+} ionophoretic activity, as measured by passage of Ca^{2+} through multilamellar planar membranes containing islet lipid extracts. These findings suggest that the action of IAP in the neonatal islet may be mediated by enhanced entry of extracellular calcium following an effect on membrane lipid composition.

Islet-activating protein (IAP), a protein of 77,000 dalton purified from the culture medium of *Bordetella pertussis* , has been shown to increase glucose-induced insulin secretion in pancreatic islets from mature rats (1,2). IAP pretreatment also abolishes the inhibitory effect of epinephrine on glucose-stimulated insulin release (2,3). However, in IAP treated islets, epinephrine continues to exert an inhibitory action on insulin secretion in the absence of extracellular calcium. Because of this dependence on extracellular calcium, and because of demonstrated efects of IAP on islet calcium fluxes, Katada and Ui (2) initially suggested that IAP may act via an effect on native calcium ionophores in the β cell membrane. More recently, attention has been directed towards an action of IAP, demonstrated in several cell types, whereby hormone stimulated adenylate cyclase activity is increased, apparently via an effect of IAP on receptor-cyclase coupling (3-5). Increased ADP-

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ribosylation of a subunit of the nucleotide regulatory component of the adenylate cyclase complex may account for this effect (6).

We have recently demonstrated that the decreased insulin secretory response to glucose in neonatal rat pancreatic islets is associated with decreased endogenous lipid-associated calcium ionophoretic activity (7). In view of the original postulate of an effect of IAP on endogenous calcium ionophores in the islet, the following study was undertaken to determine if IAP could enhance the secretory responsiveness of neonatal islets, and whether this was accompanied by an effect on endogenous lipid associated ionophoretic activity.

MATERIALS AND METHODS

Maintenance of neonatal rat islets in culture.

Pancreas from neonatal Wistar strain rats, less than 24 h old were dispersed and cultured by the technique of Hellerstrom et al (8). Dispersed pancreatic fragments were suspended in culture medium RPMI 1640 containing glucose (11.1 mmol/l), penicillin (100 U/ml), streptomycin (0.1 mg/ml) and fetal calf serum (10g/l), buffered to pH 7.4 with N-2-hydroxyethylpiperazine N'-2 ethane sulphonic acid (Hepes) (15 mmol/l). After 26 h the medium was replaced by fresh medium containing either IAP (10 ng/ml in 115 mmol/l NaCl), or NaCl vehicle. Purified IAP was the generous gift of Dr M. Yajima, Kakenyaku Kako, Japan. Culture conditions were maintained for a further 24 h after which time islets formed the major component of the tissue in culture.

Glucose-stimulated insulin release.

Aliquots of 10 cultured islets were incubated in a Krebs-Ringer bicarbonate buffer (KRB) consisting of Na^{3}, 143.3 mmol/l; K^{+}, 5.0 mmol/l; Ca^{2+}, 2.5 mmol/l; Mg^{2+}, 1.2 mmol/; Cl^{-}, 123.5 mmol/l; HCO_3^{-}, 24.6 mmol/l; $H_2PO_4^{2-}$, 1.2 mmol/l and SO_4^{2-}, 1.2 mmol/l. The medium was supplemented with bovine serum albumin (5 mg/ml) and glucose (3.3 mmol/l). Ph 7.4 was maintained by saturation with 95% O_2 + 5% CO_2 at 37°C. When calcium deficient medium was prepared, Ca^{2+} was omitted and the buffer supplemented with EGTA (0.5 mmol/l). After 20 minutes the incubation medium was replaced by medium containing glucose (3.3 or 16.7 mmol/l). The time course of the secretory effect was studied by removal of 25 µl aliquots of medium at 1 minute intervals without replacement. Insulin content of the medium was determined by radioimmunoassay using rat insulin standards (9). Incremental insulin release was calculated from the difference of the cumulative insulin determination after correction for the volume change.

Islet phospholipid content.

Islets from multiple cultures were pooled, aspirated free of medium and frozen immediately at -20°C. Frozen islets were sonicated in chloroform: methanol, 2:1, v:v and placed at 4°C for 12 h. Extracted lipids were dried under N_2 at 37°C, sealed under N_2 and stored at -20°C. Phospholipid content was determined from the estimation of lipid phosphorus by the method of Bartlett (10).

Ca^{2+} Ionophoresis.

$^{45}Ca^{2+}$ permeability due to endogenous islet lipids was determined in multilamellar planar membranes of egg yolk phosphatidylcholine and cholesterol prepared as described by Setaka et al (11). L α phosphatidylcholine and cholesterol (Sigma Chemical Co. St. Louis) were combined in the molar ratio 5:1 at a concentration of 20 mg/ml in ethanol:petroleum ether 3:2, v:v. When present dried lipid extracts of cultured islets were incorporated at this time. Lipids were resuspended by gentle mixing to avoid micelle formation. An aliquot of the lipid mixture (100 µl) was added to the surface of a dampened sheet of natural cellulose gel with low molecular weight cut-off (Spectropore, Spectrum Industries, LA). The solvent was evaporated and the membrane preparation covered with a further cellulose sheet. The prepared membrane was held in place across a tapered holder by a polypropylene collar and suspended in buffer (Tris HCL, 25 mmol/l, pH 8.0) for orientation of the phospholipid molecules. After 24 h at room temperature (23°C) the holder and membrane were removed to a vial containing buffered $^{40}CaCl_2$ (2.5 µmol/l), the soaking solution was removed from the chamber above the membrane and replaced by $^{40}CaCl_2$ (2.5 mmol/l) containing trace $^{45}CaCl_2$ so that no hydrostatic pressure difference was present across the membrane. A magnetic stirrer was used to circulate the solution in the outer container and aliquots removed for determination of $^{45}CaCl_2$ by liquid scintillation spectrometry. Transfer of $^{45}CaCl_2$ to the outer container across the membrane was taken to indicate ionophoretic activity.
The amount of lipid in the membrane was determined by comparison of membrane phosphorus content with that of the phospholipid mixture.

RESULTS

Insulin secretion following glucose is shown in Figure 1. It can be seen that in cultured neonatal islets insulin release was biphasic. Culture in the presence of IAP resulted in significantly increased glucose-stimulated insulin release. The peak level of insulin secretion in the first phase 0.19 ± 0.04 ng/10 islets from islets in control culture was significantly different from that of islets preexposed to IAP, 0.30 ± 0.04 ng/10 islets ($p < 0.01$). IAP pretreatment increased cumulative glucose-

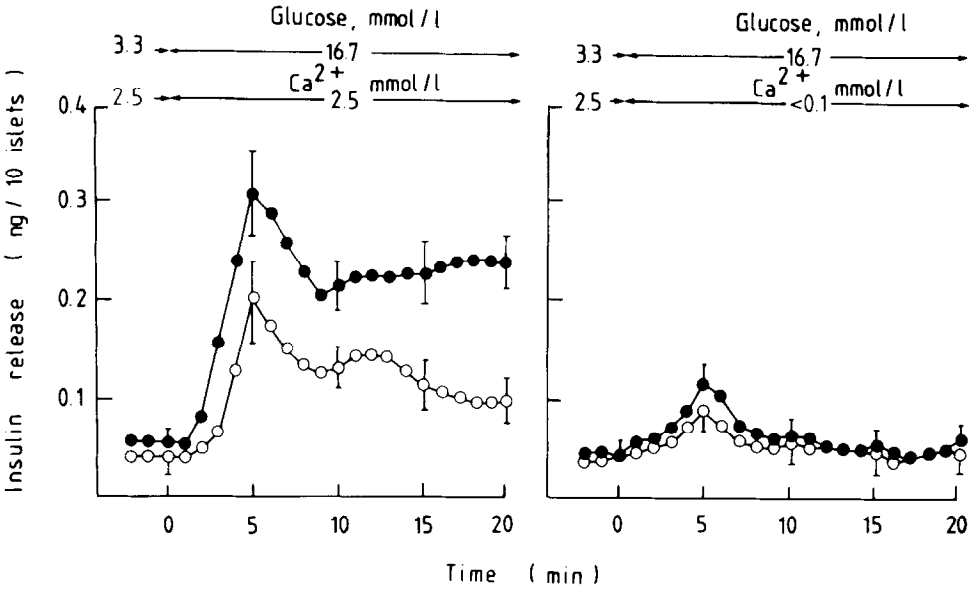
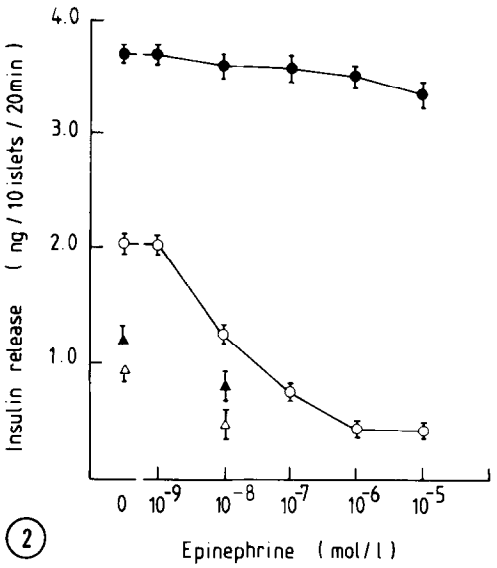


Figure 1. The effect of culture with IAP on glucose-induced insulin secretion in the presence and absence of extracellular calcium. Basal medium contained Ca^{2+} (2.5 mmol/l) and glucose (3.3 mmol/l). At time 0 medium was replaced by mediums containing glucose (16.7 mmol/l) with Ca^{2+} (2.5 mmol/l), left panel or Ca^{2+} (< 0.1 mmol/l) and EGTA (0.5 mmol/l), right panel. The values shown are mean ± SEM (10 determinations) for control islets (o) or islets cultured in the presence of 10ng/ml IAP (●).

induced insulin secretion over 20 minutes from 2.05 ± 0.42 ng/10 islets to 3.70 ± 0.40 ng/10 islets ($p < 0.001$).

The dependency of glucose induced insulin secretion on extracellular calcium is shown in Figure 1. When glucose addition (16.7 mmol/l) was simultaneous with the removal of medium calcium insulin release from both control cultured and IAP preexposed neonatal islets was significantly reduced 0.97 ± 0.4 ng/10 islets/20 min and 1.22 ± 0.6 ng/10 islets/20 min, respectively (both $p < 0.05$). In particular, the enhanced second phase insulin secretion seen after IAP was abolished.

Figure 2 demonstrates the inhibitory effect of epinephrine on insulin release in cultured neonatal islets. Half maximal inhibition was seen at epinephrine concentration 10^{-8} mmol/l in control islets. By contrast epinephrine was without significant effect in cultured islets preexposed to IAP. However in the



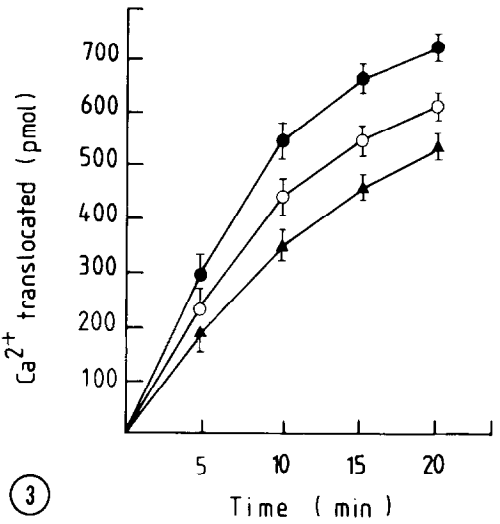


Figure 2. Effect of epinephrine on glucose-induced insulin secretion in cultured neonatal islets. Control (o) or IAP cultured (●) islets were incubated with glucose (16.7 mmol/l) and Ca^{2+} (2.5 mmol/l) in the presence of epinephrine (10^{-9}-10^{-5} mol/l). The effect of removal of extracellular calcium (Ca^{2+}, < 0.1 mmol/l; EGTA, 0.5 mmol/l) is indicated for control (△), and IAP cultured islets (▲). Values shown are mean ± SEM for 10-12 determinations at each point.

Figure 3. Time course for the increase in Ca^{2+} translocation across multilamellar bilayer membranes by islet lipid extracts. Phospholipid-sterol membranes (1.76 cm^2 area) were prepared with 0.11 mg lipid/cm^2. Calcium translocation from [^{45}Ca], 2.5 mmol/l to [^{40}Ca], 2.5 µmol/l was determined for the basal membrane (▲) and following the inclusion of lipid extracts from control (o) and IAP cultured islets (●). Values shown are mean ± SEM for 10 determinations.

absence of medium calcium, epinephrine (10^{-8} mol/l) inhibited insulin secretion in both control cultured and IAP preexposed islets.

Net calcium transport across multilamellar bilayer membranes is indicated in Figure 3. Calcium was translocated through these membranes in a time dependent manner. Under these experimental conditions barrier function of phospholipid-sterol containing membranes was low. In a membrane with highest barrier function (0.11 mg lipid/cm^2) calcium translocation was significantly enhanced by the addition of lipid extract from cultured isletes.

Table 1. Values for Ca^{2+} ionophoretic activity of lipid extracts of control or IAP cultured islets. Activity was determined using multilamellar bilayers as described in Figure 3. Islets were preincubated in the presence or absence of epinephrine.

Islets	Epinephrine 10^{-7} mol/l	Ca^{2+} translocated pmol/10 islets/15 min
Control Culture	-	91.6 ± 10.4
	+	112.0 ± 11.4
IAP Culture	-	212.0 ± 14.2[a]
	+	207.4 ± 14.9[a,b]

Values shown are mean ± SEM for 10 determinations.
[a] Significant diference from control without epinephrine ($p < 0.001$).
[b] Significant difference from control plus epinephrine ($p < 0.001$) Student's t test.

Table 1 indicates that following IAP exposure there is a significant increase in lipid associated $^{45}Ca^{2+}$ ionophoretic activity, measured immediately following culture, although it could be shown that lipid content was similar in both control and IAP exposed islets at this time (total phospholipid 3.2 ± 0.6 and 3.4 ± 0.6 nmol/10 islets, respectively). Preliminary experiments (results not shown) had determined that significant loss of ionophoretic activity follows prolonged incubation in KRB buffer. Thus the effects of epinephrine on ionophoretic activity in incubated islets could not be compared. However, Table 1 indicates that when epinephrine (10^{-7} mol/l) was incubated in culture medium one hour prior to islet isolation, there is no significant effect on the lipid associated $^{45}Ca^{2+}$ ionophoretic activity of either control or IAP cultured islets.

DISCUSSION

The present study demonstrates that, as with mature islets, IAP enhances glucose-stimulated insulin release in neonatal cultured islets. It also reduces the degree of epinephrine induced inhibition of glucose stimulated insulin release.

Extracellular calcium is required for the enhancement of insulin secretion by IAP to be apparent, and in its absence, IAP fails to prevent the inhibitory action of epinephrine on insulin secretion. These observations suggest that the action of IAP in the neonatal islet may be mediated in part or in whole by enhancement of entry of extracellular calcium into the islet. A beneficial effect on insulin secretion of such an action would be consistent with our recent demonstration that the impaired insulin secretory response to glucose in neonatal islets is associated with deficient lipid associated native calcium ionophoretic activity (7). These observations led to a direct study of the effect of IAP on lipid associated ionophoretic activity in the neonatal islet. For this purpose, calcium passage across an artificial phospholipid/cholesterol multilamellar membrane was studied. In the general models proposed for phospholipid-cholesterol bilayers, sterol and phospholipid are considered uniformly distributed in bilayers when cholesterol concentration is less than or equimolar. It could be calculated using the average cross sectional area of a molecule in the phosphatidylcholine-cholesterol membrane as 80 A^2, that membranes of greater than 200 bilayer thickness were formed by this method (12). It was shown that islet lipid extracts enhanced the passage of calcium across this model membrane, and thus assay of endogenous ionophoretic activity in these islets was possible.

Using this technique, it was apparent that culture with IAP markedly increased the lipid associated calcium ionophoretic activity of the islets, without altering total lipid content. This is the first direct demonstration of an action of IAP on native calcium ionophores. Epinephrine had no effect on ionophoretic activity, with or without IAP pretreatment. The unmasking of the inhibitory action of epinephrine in IAP treated

islets by incubation in the absence of extracellular calcium ions suggests that its action may be due to inhibition of intracellular calcium mobilization, perhaps secondary to inhibition of cyclic AMP accumulation.

The present study showing an enhancement of lipid associated ionophoretic activity must be related to recent studies in several cell types showing that IAP pretreatment enhances hormone stimulated adenylate cyclase activity. It is possible that both effects are secondary to IAP-induced changes in lipid fluidity in the cell membrane. There are several lines of evidence indicating that adenylate cyclase activity is affected by membrane lipid composition and ordering (13-16). Changes in bilayer fluidity are reflected directly in the activity of adenylate cyclase in the presence of GTP (16). Further, reconstitution experiments have shown that association of catalytic protein and guanine nucleotide regulatory protein of adenylate cyclase requires specific lipid entities, in particular phosphatidylcholine (17,18). Enhanced Ca^{2+} translocation has been observed in multilamellar liposomes (19) following alteration of lipid composition by changing phosphatidylcholine molecular species. Thus, altered lipid composition could explain both effects of IAP.

Further investigation will be required to elucidate the changes in islet membrane lipid composition which allows enhanced Ca^{2+} translocation following IAP and enhanced adenylate cyclase activation. However, the present study highlights the potential role of islet lipids as ionophores, and their contribution to Ca^{2+} fluxes across islet membranes which are essential for insulin release.

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