Vol. 116, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1983 Pages 9-16

STIMULATION OF PHOSPHOINOSITIDE BREAKDOWN IN RAT PANCREATIC ISLETS BY GLUCOSE AND CARBAMYLCHOLINE

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Received June 15, 1983

SUMMARY : In the presence of Li^+, glucose, 2-ketoisocaproate and carbamylcholine induced the rapid formation of ^{3}H-inositol phosphates in rat pancreatic islets prelabelled with ^{3}H-inositol. The production of labelled inositol phosphates continued up to 20 min of incubation. Glibenclamide and ionophore A23187 had no significant effect on labelled inositol phosphate production. The effects of carbamylcholine and to a lesser extent, glucose were found to persist in the absence of added Ca^{2+}, but both were strongly inhibited by excess EGTA. In general, the rise in ^{3}H-inositol phosphate production was associated with a fall in lipid bound radioactivity, although the latter was found to occur more slowly, and was of a smaller magnitude than labelled inositol phosphate formation. The results suggest that nutrient secretagogues and cholinergic agonists stimulate hydrolysis of phosphoinositides in pancreatic islets by a phospholipase C mechanism. This effect is Ca^{2+}-dependent, but probably not triggered by increased Ca^{2+} uptake into the islet.

A number of studies have demonstrated enhanced ^{32}P-labelling of phospholipids in pancreatic islets in response to glucose (1-4). We have recently shown that carbamylcholine and cholecystokinin-pancreozymin also stimulate ^{32}P-labelling of phospholipids, specifically phosphatidic acid and the phosphoinositides (5). It is generally believed that a stimulation of cellular phospholipid labelling with ^{32}P reflects resynthesis of lipids following an initial breakdown of phosphatidylinositol or its phosphorylated derivatives the polyphosphoinositides (6-9), and a rapid fall in labelled phosphoinositides has been demonstrated in a number of tissues following stimulation (10-12). However, in our studies of the kinetics of ^{32}P-labelling in rat islets, we were unable to demonstrate an early fall in ^{32}P-labelled phospholipids preceeding enhanced labelling in response to either glucose (4) or car-

0006-291X/83 $1.50

bamylcholine (5). One possible explanation for this apparent absence of ^{32}P-labelled phospholipid breakdown as an early event in islet activation is that lipid breakdown may be limited to a relatively small phospholipid pool (e.g. that associated with the plasma membrane) and thus difficult to detect against a high level of background labelling in other cellular membranes (e.g. secretory granules, endoplasmic reticulum) which in islets comprise a large proportion of the total cellular membranes (13). In order to overcome this difficulty, we have adapted a technique described recently by Berridge et al. (14) which involves prelabelling the tissue with ^{3}H-inositol and subsequent measurement of ^{3}H-inositol phosphates, which are among the immediate products of phosphoinositide hydrolysis by a phospholipase C mechanism. This method relies on the use of Li^{+} which, by blocking inositol phosphate hydrolysis to free inositol (15), provides an extremely sensitive method for monitoring breakdown of phosphoinositides. We have thereby demonstrated that glucose, 2-ketoisocaproate and carbamylcholine cause a rapid increase in the formation of ^{3}H-inositol phosphates, together with a loss of lipid-bound radioactivity, in islets prelabelled with ^{3}H-inositol.

MATERIALS AND METHODS

Pancreatic islets were isolated from fed adult rats by collagenase digestion (16). Batches of 100-200 islets were preincubated in glass tubes in 1.0 ml gassed bicarbonate medium (17) containing 2.8 mM glucose, 0.25 % (w/v) bovine serum albumin and 6 µCi ^{3}H-inositol (16.4 Ci/mmol) for 2 hr at 37°C. The medium was then removed and replaced by 0.9 ml fresh medium containing 5 mM LiCl and 1 mM cold inositol but no radioactive material. Following the addition of 100 µl of the same medium containing test substances, the islets were further incubated for various lengths of time. Incubations were terminated by the addition of 3 ml $CHCl_3$:CH_3OH (2:1 v/v), the tubes vortex-mixed for 30 sec and subsequently centrifuged to separate the two phases. The upper, inorganic phase was removed, mixed with 2 ml H_2O and applied to a column containing 0.5 ml Dowex AG1X8 (200-400) in the formate form. Free ^{3}H-inositol was eluted with 4 x 2.5 ml H_2O and the ^{3}H-inositol phosphates with 2 x 1 ml 1 M ammonium formate/0.1 M formic acid. The radioactivity in each fraction was counted following the addition of 10 ml Aquasol scintillant. The lower organic phase from the extracted samples was acidified with 0.5 ml

CH_3OH:13N HCl (50:1 v/v), vortex-mixed to optimise lipid extraction, washed with 1 ml H_2O and dried in a vacuum oven. In most experiments, the extracts were dissolved in 10 ml aquasol and the radioactivity counted to provide an estimate of total lipid-bound radioactivity. In certain selected experiments, the lipid extracts were dissolved in 150 µl $CHCl_3$:CH_3OH (2:1 v/v) and the phosphoinositide classes separated by thin-layer chromatography using $CHCl_3$:CH_3OH:NH_3:H_2O (90:90:7:20 v/v) as solvent system (18). Authentic phosphoinositides were run in parallel as standards. The lipid spots were identified with iodine vapour, scraped into counting vials and the radioactivity counted following the addition of 2.5 ml MeOH and 10 ml aquasol.

Myo-[2-^{3}H]inositol (16.4 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. Carbamylcholine, 2-ketoisocaproate, phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were purchased from Sigma Chemical Co., St. Louis, Missouri, ionophore A23187 from Calbiochem Ltd. and glibenclamide from Hoechst Pharmaceuticals.

RESULTS

After 120 min preincubation, the incorporation of ^{3}H-inositol in islet lipids averaged 9.22 ± 1.15 fmol/islet (n = 6), as calculated by reference to the specific radioactivity of ^{3}H-inositol in the preincubation medium. In the presence of LiCl (5 mM), the basal rate of ^{3}H-inositol phosphate formation would correspond to a fractional turnover of labelled lipids close to 6.1 ± 1.7 %/30 min (n = 6).

Preincubation of rat pancreatic islets with ^{3}H-inositol and subsequent exposure, in the presence of 5 mM LiCl, to stimulatory concentrations of either glucose or 2-ketoisocaproate resulted in a pronounced increase in the formation of ^{3}H-inositol phosphates (Table 1). A similar stimulation of ^{3}H-inositol phosphate production occurred in response to carbamylcholine, although no significant effect was observed with glibenclamide or ionophore A23187 (Table 1). A stimulatory effect of carbamylcholine was found to occur in the absence of LiCl, although under these conditions, the production of labelled inositol phosphates was greatly reduced compared to that seen in the presence of LiCl (Table 1).

Study of the time courses of ^{3}H-inositol phosphate production revealed an increase within 4 min and 2 min of stimulation

Table 1

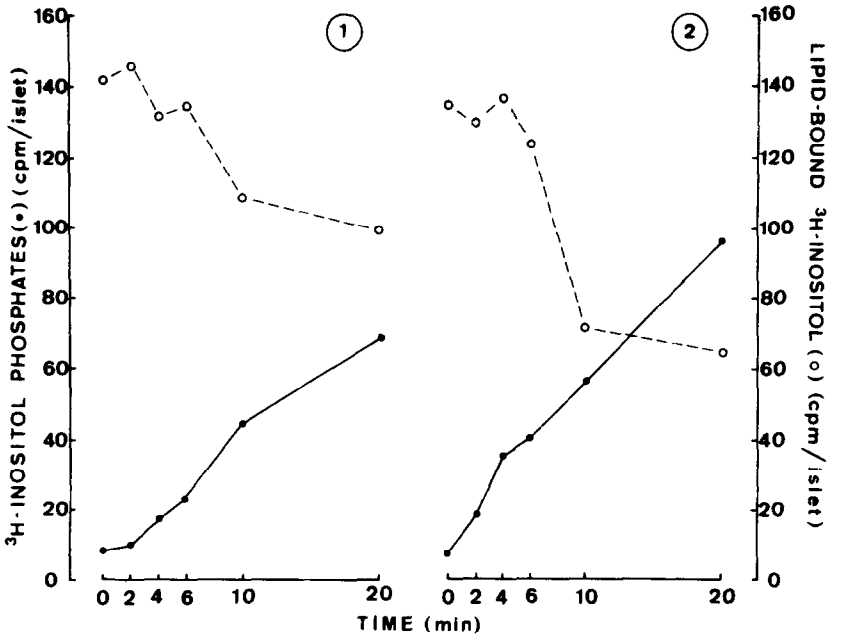
Effects of various secretagogues, in the presence or absence of Ca^{2+}, on ^{3}H-inositol phosphate accumulation and lipid-bound ^{3}H-inositol in rat islets in the presence or absence of LiCl

Addition	^{3}H-inositol phosphate accumulation (% of control)	Lipid-bound ^{3}H-inositol (% of control)
+ 5 mM LiCl		
None	100	100.0
22.2 mM glucose	819 ± 37[a]	60.7 ± 9.9[a]
22.2 mM glucose, no added Ca^{2+}	251 ± 19[a,b]	63.5 ± 11.1
22.2 mM glucose + 10 mM EGTA	176 ± 8[b]	87.0 ± 5.1
10 mM 2-ketoisocaproate	975 ± 25[a]	46.9 ± 2.2[a]
2 mM carbamylcholine	842 ± 18[a]	76.4 ± 2.0[a]
2 mM carbamylcholine, no added Ca^{2+}	808 ± 17[a]	80.9 ± 9.4
2 mM carbamylcholine + 10 mM EGTA	422 ± 31[a,b]	72.9 ± 5.2
50 µg/ml glibenclamide	126 ± 1	105.0 ± 0.3
10 µM A23187	84 ± 13	101.0 ± 3.8
No LiCl		
None	100	100.0
2 mM carbamylcholine	199 ± 4[a]	83.2 ± 5.1

All incubations were for 30 min. Incubation media contained 1 mM Ca^{2+} unless otherwise stated. The values represent mean ± SEM of three separate incubations. The symbols a and b denote statistical significance ($P < 0.025$) of differences attributable either to a given secretagogue relative to basal value (a) or to changes in Ca^{2+} concentration (b).

with glucose and carbamylcholine respectively (Figs. 1 & 2). The formation of labelled inositol phosphates continued to rise upto 20 min in both cases.

The stimulation by either glucose or carbamylcholine of ^{3}H-inositol phosphate production was associated with a reduction in lipid-bound radioactivity (Figs. 1 & 2). However, this fall in lipid-bound ^{3}H-inositol, particularly in response to carbamylcholine, appeared to occur more slowly than the production of ^{3}H-inositol phosphates. Furthermore, the magnitude of the latter was, in general, greater than that of the change in lipid-bound



Figs. 1 & 2. Effect of 22.2 mM D-glucose (left) and 2.0 mM carbamylcholine (right) on ^{3}H-inositol phosphate production (closed circles, solid line) and lipid-bound ^{3}H-inositol (open circles, dotted line) in rat pancreatic islets. Each point represents the mean value derived from two separate experiments.

radioactivity. In experiments where the phosphoinositide classes were separated,the label was distributed between phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in the approximate proportions 85:4:11. In general, any changes in lipid-bound ^{3}H-inositol were apparent in all of the phosphoinositide species to a similar extent.

The enhanced formation of labelled inositol phosphates in response to carbamylcholine and to a lesser extent glucose, was found to persist in the absence of added Ca^{2+} in the incubation medium (Table 1). However, the addition of 10 mM EGTA strongly inhibited ^{3}H-inositol phosphate production with either secretagogue (Table 1).

DISCUSSION

The present study demonstrates phosphoinositide hydrolysis in rat pancreatic islets in response to two types of stimulus, namely nutrient secretagogues including glucose and 2-ketoisoca-

proate, and the neurotransmitter carbamylcholine. By the use of Li^+, we were able to directly measure production of labelled inositol phosphates from the breakdown of ^{3}H-inositol-labelled phospholipids, suggesting that the route of phospholipid hydrolysis involved the action or activation of a phospholipiase C. This technique is thus highly suitable for the study of enhanced phosphoinositide metabolism, an integral component of the so-called phosphatidylinositol cycle, in pancreatic islets. However, the measurement of total inositol phosphate production does not permit a distinction between the various phosphoinositide species, and so we are unable to assess which of these is hydrolysed to release the water-soluble phosphates. In the absence of Li^+, the amounts of labelled inositol phosphates measured were greatly reduced, suggesting that these substances are very quickly broken down to free inositol. This may explain why Clements and Rhoten (19) found only a small increase in ^{3}H-inositol phosphates upon exposure of ^{3}H-inositol-prelabelled islets to glucose. These authors were also unable to demonstrate an effect of 2-ketoisocaproate on islet phosphoinositide breakdown (20).

In the present study, we failed to detect any phosphoinositide hydrolysis upon stimulation of islets with glibenclamide, or ionophore A23187, suggesting that the effects of glucose, 2-ketoisocaproate and carbamylcholine were not simply a secondary result of enhanced secretory activity. Furthermore, phosphoinositide hydrolysis in response to these secretagogues was found to occur in the absence of Ca^{2+} in the incubation medium, suggesting that the effect was not due to increased Ca^{2+} uptake into the islets. This conclusion is substantiated by the lack of effect of ionophore A23187. We did find, however, that the addition of excess EGTA strongly inhibited both glucose- and carbamylcholine-induced phosphoinositide hydrolysis, suggesting that this step

may be Ca^{2+}-dependent. These observations are consistent with our previous studies of ^{32}P-labelling of islet phospholipids (4, 5) which suggested that enhanced turnover of the phosphatidylinositol cycle in rat islets is a calcium-dependent process, though probably not triggered by increased calcium uptake into the islet cells.

The studies of the time courses of ^{3}H-inositol phosphate formation upon stimulation with glucose or carbamylcholine suggest that phosphoinositide breakdown may be an early event in the response of the islet to these secretagogues. These observations do not offer proof, however, that phosphoinositide breakdown is the initial step in enhanced phospholipid turnover upon stimulation. For example, increased breakdown of phospholipids might also, theoretically, result from an initial stimulation of phospholipid synthesis.

In general, increased ^{3}H-inositol phosphate production was accompanied by a fall in lipid-bound radioactivity after 20-30 min of incubation. This would be expected since in the presence of Li^{+}, the ^{3}H-inositol is trapped as phosphate derivatives and cannot therefore be recirculated for the resynthesis of phosphoinositides. However, it is noteworthy that this fall in lipid-bound radioactivity appeared to follow rather than precede labelled inositol phosphate production. This implies that synthesis of phosphoinositides from residual, unincorporated ^{3}H-inositol in the islet occurs very soon after, or even before phosphoinositide breakdown. Thus, it is only after the free ^{3}H-inositol has been consumed and/or diluted sufficiently by the added unlabelled inositol that a fall in lipid-bound radioactivity can be detected.

In conclusion, by using Li^{+} to trap the water-soluble products of phosphoinositide hydrolysis, we have shown that the

nutrient secretagogues glucose and 2-ketoisocaproate, and also carbamylcholine, are able to evoke a rapid breakdown of phosphoinositides in rat islets by phospholipase C mechanism. The time course characteristics and calcium-dependency of ^{3}H-inositol phosphate production were very similar to those previously observed measuring enhanced ^{32}P-labelling of islet phospholipids (4, 5) suggesting that these two phenomenon are closely related. Further work is necessary to determine whether a stimulation of phospholipid hydrolysis or of synthesis is the initial response to stimulation.

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