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Time-dependent potentiation of insulin release induced by alpha-ketoisocaproate and leucine in rats: possible involvement of phosphoinositide hydrolysis

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Summary. The ability of the amino acid leucine and its keto acid, alpha-ketoisocaproate, to induce insulin release, to initiate phosphoinositide hydrolysis, and to amplify the subsequent insulin secretory response to glucose was assessed. In islets whose inositol-containing lipids were prelabelled with myo[2-^{3}H]inositol, the addition of either compound resulted in an increase in insulin output, an increase in ^{3}H efflux, rapid and significant increases in labelled inositol phosphate accumulation and a sustained increase in ^{3}H efflux after removal of the stimulant. Direct measurements of labelled inositol phosphate accumulation in islets previously stimulated with alpha-ketoisocaproate demonstrate that this sustained increase in ^{3}H efflux was the result of a persistent increase in phosphoinositide hydrolysis and was not simply a consequence of the hydrolysis of preformed inositol phosphates into more membrane permeable species. Prior exposure of islets to alpha-ketoisocaproate or leucine also resulted in an amplified secretory response to a subsequent glucose (10 mmol/l) stimulus. While peak first phase insulin release averaged 66 ± 4 (mean $\pm$ SEM, $n = 18$) pg·islet^{-1}·min^{-1} from control islets, this value increased to 204 ± 14 and 246 ± 11 pg·islet^{-1}·min^{-1} in the leucine or alpha-ketoisocaproate pretreated islets respectively. The duration of this amplified response paralleled the duration of the persistent increase in ^{3}H efflux. Prior alpha-ketoisocaproate exposure also amplified the subsequent insulin secretory response to tolbutamide and glyceraldehyde. While control (non-pretreated) islets in response to tolbutamide (200 μmol/l) released insulin at a rate of 50 ± 6 pg·islet^{-1}·min^{-1} ($n = 3$), this first phase response increased to 506 ± 38 pg·islet^{-1}·min^{-1} in prior alpha-ketoisocaproate treated islets. Peak first and second phase insulin responses to glyceraldehyde were increased 5-fold and 2-fold, respectively, by prior alpha-ketoisocaproate. These results suggest that events coupled to the hydrolysis of membrane inositol-containing phospholipids induced by leucine and alpha-ketoisocaproate participate not only in their acute insulin stimulatory action, but also in their ability to induce time-dependent potentiation (memory) in isolated islets.

Key words: Islets, phosphoinositides, memory, insulin secretion, leucine, alpha-ketoisocaproate, ^{3}H efflux.

It is well established that prior glucose stimulation of pancreatic B cells results in an amplified insulin secretory response to a second glucose challenge [1-4]. Some characteristics of this phenomenon, termed time-dependent potentiation (TDP) by Grill and associates [2, 3] have been established: (a) glucose must be metabolised to induce TDP; (b) TDP develops within minutes and persists long after removal of the initial stimulant; (c) intracellular accumulation of cAMP does not seem to play a major role in the process; and (d) glyceraldehyde and the gut hormone cholecystokinin [5] mimic to a large extent this sensitising effect of glucose. We recently reported that TDP noted with glucose [6], glyceraldehyde [6], or cholecystokinin [7], appeared to depend on agonist-induced increases in phosphoinositide (PI) hydrolysis, a response that persists despite stimulant removal. The duration of TDP parallels to a remarkable extent the duration of the sustained increase in PI hydrolysis and, moreover, conditions which abolish PI hydrolysis abolish TDP. In the present report, the capacity of leucine and alpha-ketoisocaproate (KIC) to promote PI hydrolysis and to induce TDP was assessed. These particular stimulants were chosen because they both augment insulin output from the B cell, an effect that seems to be related, at least in part, to their ability to increase PI metabolism [8-10]. The present results further emphasize the involvement of biochemical events in the inositol cycle in the induction of TDP by various stimulants.

Material and methods

Male Sprague-Dawley rats purchased from Charles River were used in all studies. The animals were fed ad libitum and weighed between

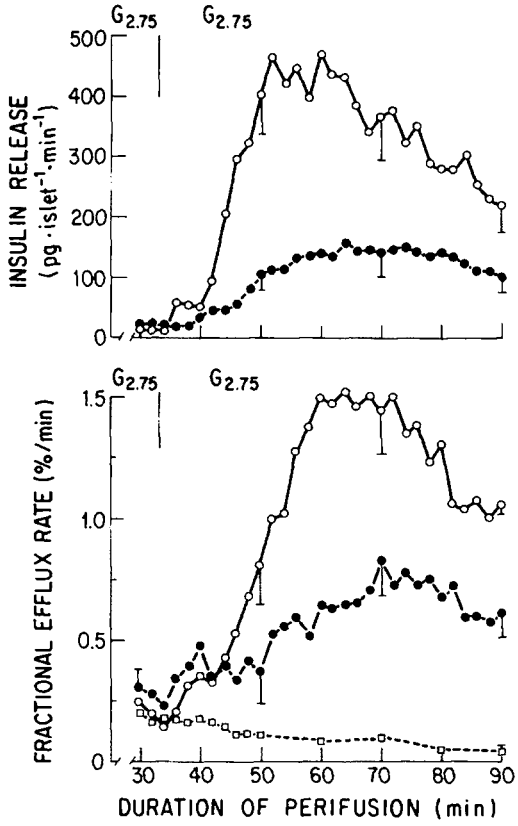

Fig. 1 a and b. Insulin release and phosphoinositide hydrolysis in response to alpha-ketoisocaproate (KIC) and leucine. **a** After a 2 h labelling period with myo [2-^{3}H] inositol, batches of islets were perifused with 2.75 mmol/l glucose for 30 min to establish stable basal rates of insulin secretion. They were then stimulated for 60 min with KIC, 15 mmol/l, (○) or leucine, 20 mmol/l, (●) with glucose still present. This and all subsequent figures have been corrected for the dead space (3 ml, equivalent to 3 min with a flow rate of 1 ml/min) in the perifusion system. Mean values are given ± selected SEM's of at least 4 experiments. **b** Fractional efflux rates of ^{3}H, used to monitor PI hydrolysis are depicted here in response to KIC (○) and leucine (●). Efflux rates in response to 2.75 mmol/l glucose alone (□) are also shown. These values were calculated according to the procedure of Borle et al. [25] and were obtained from the same islets whose insulin release profiles are given above

Table 1. Influence of leucine (20 mmol/l) and alpha-ketoisocaproate (15 mmol/l) on inositol phosphate accumulation in isolated perifused rat islets

Protocol (min)	IP_1	IP_2	IP_3
	(cpm/40 islets, mean ± SE)		
1. $G_{2.75}$ (50 or 60 min)	413 ± 30	111 ± 12	98 ± 9
2. $G_{2.75} \rightarrow G_{2.75}$ + Leucine (30) (20)	621 ± 62	168 ± 13	129 ± 7
3. $G_{2.75}$ (32)	406 ± 23	103 ± 13	88 ± 7
4. $G_{2.75} \rightarrow G_{2.75}$ + KIC (30) (2)	660 ± 38	179 ± 11	167 ± 12
5. $G_{2.75} \rightarrow G_{2.75}$ + KIC (30) (20)	1082 ± 139	527 ± 116	283 ± 61
6. $G_{2.75} \rightarrow G_{2.75}$ + LiCl (60) (20)	504 ± 41	95 ± 14	98 ± 18
7. $G_{2.75} \rightarrow G_{2.75}$ + KIC $\rightarrow G_{2.75}$ (30) (20) (10)	637 ± 29	182 ± 21	108 ± 17
8. $G_{2.75} \rightarrow G_{2.75}$ + KIC $\rightarrow$ (30) (20) $G_{2.75} \rightarrow G_{2.75}$ + LiCl (10) (20)	1540 ± 181	280 ± 28	197 ± 18

After labelling for 2 h with myo[2-^{3}H]inositol, groups of islets (n = at least 4 for each condition) were perifused as indicated. After the perifusion, inositol phosphates were extracted with 10% perchloric acid and separated as described [13, 14]. Statistical analysis is as follows: Protocol 1 vs Protocol 2 - $p < 0.05$ for all inositol phosphates, Protocol 1 vs Protocol 5 - $p < 0.05$ for all inositol phosphates, Protocol 1 vs Protocol 6 - no significant differences, Protocol 3 vs Protocol 4 - $p < 0.05$ for all inositol phosphates, Protocol 5 vs Protocol 7 - $p < 0.05$ for IP_1; IP_2, and IP_3, Protocol 6 vs Protocol 8 - $p < 0.05$ for all inositol phosphates

Abbreviations: IP_1 - inositol 1-phosphate; IP_2 - inositol 1,4-bisphosphate; IP_3 - inositol 1,4,5-trisphosphate; +1,3,4-trisphosphate; LiCl-lithium chloride, 10 mmol/l

300-400 g. After nembutal-induced (50 mg/kg) anaesthesia, islets were isolated by collagenase digestion [11]. In some experiments, islets were directly perifused to assess secretory responsiveness. In other experiments batches of 40-80 islets were loaded onto nylon filters and placed in small glass vials. They were incubated for 2 h in 200 µl of a myo[2-^{3}H] inositol containing solution prepared by adding 10 µCi myo[2-^{3}H] inositol (initial specific activity 16.6 Ci/mmol) to 250 µl of incubation medium. The medium used for this incubation procedure was similar to that employed during the islet perifusion and consisted of 115 mmol/l NaCl, 5 mmol/l KCl, 2.2 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 24 mmol/l $NaHCO_3$, and 0.17 g % bovine serum albumin. The solution was gassed with 95% O_2/5% CO_2. Glucose (2.75 mmol/l) was also present during the incubation. After termination of the incubation the islets, still attached to the nylon filters, were washed with 5 ml nonradioactive medium and perifused. The pH of the perifusion medium was maintained at 7.4, the temperature at 37 °C, and the flow rate at 1 ml/min. Islets were usually perifused for 30 min to establish stable insulin secretory rates and then exposed to various agonists indicated in the figure legends. Perifusate samples were collected at time intervals indicated in the figures and 200 µl aliquots analysed for ^{3}H content when appropriate as well as insulin [12] using rat insulin (Lilly and Co., Indianapolis, Indiana, USA # 615-D63-12-3) as standard. In some experiments the nature of the ^{3}H-containing molecules was assessed in pooled perifusate samples collected during the final 10 min of the perifusion. In other experiments, the levels of free ^{3}H-inositol, glycerophosphoinositol (GPI) and labelled inositol phosphates were determined after extraction with 10% perchloric acid by methods previously detailed [13, 14]. Briefly, after neutralisation with 0.25-0.28 ml 6 N KOH, the further addition of 5 ml water, and centrifugation, the supernatant was applied to columns. These columns were prepared by adding (to achieve a length of 3 cm) anion exchange resin (AG # 1-8X Bio-Rad Labs, Richmond, Calif, USA) to Pasteur pipettes. Further additions to the column included 10 ml water (to elute free ^{3}H-inositol) and 5 ml 5 mmol/l Borax/60 mmol/l sodium formate (to elute GPI). Elution of the inositol phosphates was accomplished by the sequential addition of 10 ml 0.1 mol/l formic acid/0.2 mol/l ammonium formate (IP_1), 0.1 mol/l formic acid/0.4 mol/l ammonium formate (IP_2) and 0.1 mol/l formic acid/1 mol/l ammonium formate (IP_3). This procedure does not differentiate between various inositol trisphosphate isomers. Aliquots (0.4 ml) of the eluate were then analysed for radioactive contents. The radioisotope used to measure insulin release (^{125}I-insulin) was purchased from New England Nuclear (Boston, Mass, USA) and the myo[2-^{3}H]inositol from Amersham (Arlington Hts., Ill, USA). Leucine and alpha-ketoisocaproate (sodium salt), borax, formic acid and D-glyceraldehyde were purchased from Sigma Chemical Co.

(St. Louis, Mo, USA). Ammonium formate was purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Tolbutamide (sodium salt) was the generous gift of Upjohn Co. (Kalamazoo, Mich, USA).

Statistical analysis

Where appropriate, statistical significance was determined using the Student's t-test for unpaired data and a p value less than 0.05 was taken as significant. Values presented in the figures represent means ± SEM of the specified number of observations.

Results

After a 2 h incubation period with myo[2-^{3}H]inositol to label their inositol-containing phospholipids, batches of islets were perifused with 2.75 mmol/l glucose for 30 min to establish basal insulin secretory rates. Subsequent exposure to 15 mmol/l KIC or 20 mmol/l leucine (in the continued presence of 2.75 mmol/l glucose) was accompanied by an increased output of insulin (Fig. 1, top). The response to KIC (15 mmol/l) was significantly ($p < 0.05$) greater at most time points than that noted to leucine (20 mmol/l). Insulin release rates in response to both agonists tended to fall somewhat during the final minutes of the 60 min stimulation period.

A situation similar to that noted with insulin secretion, at least in quantitative terms, was observed when ^{3}H efflux rates were monitored in these same islets (Fig. 1, bottom). While prestimulatory efflux rates were comparable in the presence of 2.75 mmol/l glucose

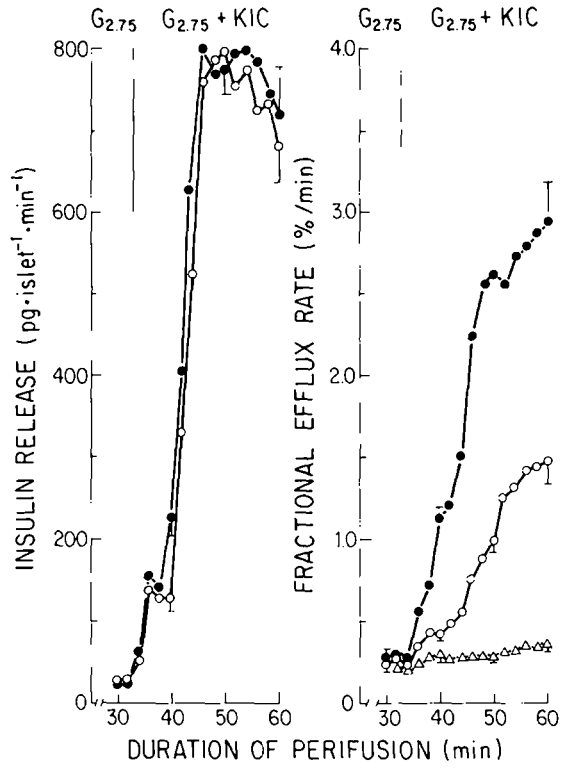

Fig. 2. Nonradioactive inositol increases ^{3}H efflux in response to KIC. Groups of 60–80 islets were incubated for 2 h with myo[2-^{3}H] inositol, washed with fresh medium and then perifused. Insulin secretory rates (left panel) and fractional rates of ^{3}H efflux (right panel) were measured in response to 15 mmol/l KIC alone (○) or KIC plus 1 mmol/l nonradioactive inositol (●). Fractional efflux rates of ^{3}H in response to 2.75 mmol/l glucose plus 1 mmol/l inositol are also depicted (△)

Table 2. Effect of alpha-ketoisocaproate (KIC) stimulation on islet and effluent levels of ^{3}H-inositol (INS), glycerophosphoinositol (GPI), inositol monophosphate (IP_1), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3)

Islets	INS	GPI	IP_1	IP_2	IP_3
treatment	(cpm/40 islets)				
A. $G_{2.75} \rightarrow G_{2.75}$	1422 ± 241	97 ± 8	415 ± 23	94 ± 10	69 ± 12
B. $G_{2.75} \rightarrow G_{2.75}$ + inositol$_1$	2084 ± 217	110 ± 13	340 ± 25	69 ± 18	57 ± 14
C. $G_{2.75} \rightarrow G_{2.75} + KIC_{15}$	3118 ± 193	98 ± 17	1143 ± 118	512 ± 38	266 ± 21
D. $G_{2.75} \rightarrow G_{2.75} + KIC_{15}$ + inositol$_1$	3978 ± 268	69 ± 18	676 ± 53	240 ± 13	122 ± 16
Effluent	INS	GPI	Total inositol phosphates		
			IP_1	IP_2	IP_3
			(cpm/40 islets/10 min)		
E. $G_{2.75}$	147 ± 16	39 ± 7		28 ± 6	
F. $G_{2.75}$ + inositol$_1$	317 ± 47	41 ± 6		35 ± 8	
G. $G_{2.75} + KIC_{15}$	896 ± 73	51 ± 6		44 ± 7	
H. $G_{2.75} + KIC_{15}$ + inositol$_1$	1294 ± 139	69 ± 12		46 ± 9	

After a 2 h incubation with (2-^{3}H)inositol to label the phosphoinositides, groups of 60–80 islets were washed and then perifused for 30 min with 2.75 mmol/l glucose to establish basal stable ^{3}H efflux and insulin secretion rates. Some islets (A) were maintained with 2.75 mmol/l glucose for an additional 30 min while other groups were exposed to 2.75 mmol/l glucose plus 1 mmol/l inositol (B), 2.75 mmol/l glucose plus 15 mmol/l KIC (C), or 2.75 mmol/l glucose plus 15 mmol/l KIC plus 1 mmol/l inositol (D). The numbers given in subscript indicate the millimolarity of the compound. At the termination of the perifusion, the islets were placed in 10% perchloric acid (PCA) and the various compounds measured as described in the methods section. Effluent samples were collected during the final 10 min of the perifusion with the substances indicated and pooled 5 ml samples acidified with 500 µl PCA. After neutralisation with 6 N KOH and centrifugation, samples were analysed for content of the various substances. At least 4 experiments were performed under each condition. Mean values ± SEM are given. Because the levels of the individual labelled inositol phosphates were so low in the effluent, they were extracted togehter by the addition of 10 ml 0.1 mol/l formic acid + 1 mol/l ammonium formate. Statistical analysis: line A vs line B - $p < 0.05$ for inositol and IP_1; line C vs line D - $p < 0.05$ for inositol, IP_1, IP_2 and IP_3; line E vs line F, $p < 0.05$ for inositol; line G vs line H, $p < 0.05$ for inositol

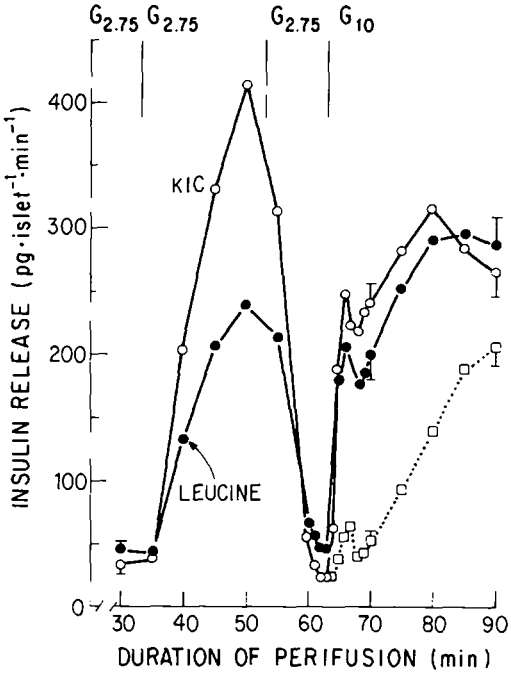

Fig. 3. Time-dependent potentiation (memory) induced by KIC and leucine. Freshly isolated islets were perifused for 30 min with 2.75 mmol/l glucose and for an additional 20 min with KIC, 15 mmol/l (○) or leucine, 20 mmol/l (●). After a 10 min washout period with 2.75 mmol/l glucose alone, the islets were stimulated for 30 min with 10 mmol/l glucose ($n = 5$ for each condition). The response of islets perifused with 2.75 mmol/l glucose alone (□) prior to stimulation with 10 mmol/l glucose is also depicted. The responses of control islets, maintained for 60 or 70 min with 2.75 mmol/l glucose prior to stimulation with 10 mmol/l glucose were virtually identical and pooled values for these experiments ($n = 18$) are presented here. The control responses presented in Figure 4 (left panel) were similarly calculated

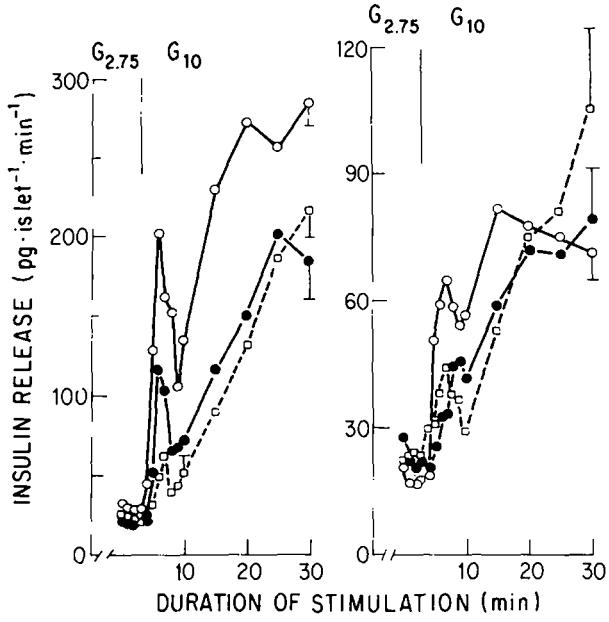

Fig. 4a and b. Duration of memory induced by KIC or leucine. **a** Similar to the protocol outlined in Figure 3, islets were stimulated with KIC (○) or leucine (●) for 20 min. After a 20 min washout with 2.75 mmol/l glucose, the islets were stimulated with 10 mmol/l glucose. Only the duration of this stimulatory period is shown. The control response of islets perifused for 60 or 70 min with 2.75 mmol/l glucose (□) prior to stimulation with the higher hexose level is also shown. **b** A stimulatory protocol similar to that described previously was employed except that the interval between KIC (○) or leucine (●) exposure and 10 mmol/l glucose stimulation was extended to 60 min. The response of islets maintained with low glucose for 110 min prior to stimulation is also shown (□)

(approximately 0.25%/min), the addition of KIC or leucine was accompanied by quantitatively disparate increases in perifusate ^3H. KIC (15 mmol/l) again proved to be significantly ($p < 0.05$) more effective than leucine (20 mmol/l). Also, and similar to insulin release rates, ^3H efflux rates fell somewhat during the final phase of stimulation with both compounds. A 20 min exposure to KIC or leucine also resulted in a significant increase ($p < 0.05$) in the levels of islet inositol phosphates (Table 1, lines 2 and 5). Consistent with the insulin release results and ^3H efflux data, the effect of KIC was significantly greater ($p < 0.05$) than leucine. KIC significantly ($p < 0.05$) increased islet levels of labelled inositol phosphates within 2 min after KIC exposure (Table 1, line 4).

In a previous report [15], we demonstrated that the inclusion of nonradioactive inositol (1 mmol/l) in the perifusion medium improved our ability to monitor glucose-induced increases in ^3H efflux. A similar situation occurs with KIC-stimulated islets (Fig. 2). While nonradioactive inositol had no effect on KIC-stimulated secretion (left panel), its inclusion with 15 mmol/l KIC (right panel) resulted in a significantly greater ^3H efflux response ($p < 0.05$), a response that was nearly as rapid in onset as insulin secretion. Furthermore, the inclusion of cold inositol significantly increased ($p < 0.05$) islet content of free ^3H-inositol but significantly reduced ($p < 0.05$) the levels of labelled inositol phosphates in response to KIC stimulation (Table 2). Analysis of effluent radioactivity demonstrated that free ^3H-inositol was the major radioactive moiety in the effluent of control and stimulated islets (Table 2). Approximately 70% of the label in the perifusate from control islets (perifused with 2.75 mmol/l glucose) was free ^3H-inositol, a value that increased to over 90% when islets were stimulated with KIC alone or in the presence of 1 mmol/l nonradioactive inositol.

Our previous results with other B-cell stimulants [5-7, 16] support the concept that events in the inositol cycle play a crucial role in the induction and maintenance of TDP. Since both KIC and leucine activate PI hydrolysis (Fig. 1, Table 1) [8-10], we next investigated whether both compounds heighten the secretory response to a subsequent glucose stimulus. The results are shown in Figure 3. In these experiments, freshly isolated islets were perifused for 30 min with 2.75 mmol/l glucose before being provoked for 20 min with KIC (15 mmol/l) or leucine (20 mmol/l). In response to either compound, insulin output rose. Following a 10 min period with 2.75 mmol/l glucose

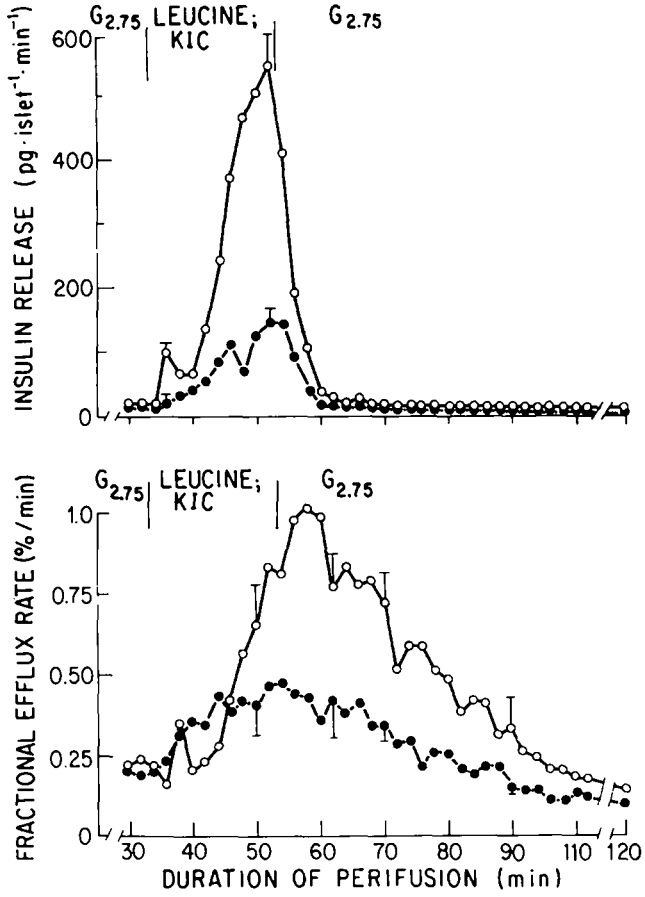

Fig. 5. Sustained effect of KIC or leucine on ^3H efflux. After a 2 h prelabelling period, islets were perifused for 30 min with low glucose and then stimulated for 20 min with KIC, 15 mmol/l (○) or leucine, 20 mmol/l (●). The perifusion was continued for an additional 50 min with 2.75 mmol/l glucose alone ($n = 4$ for each condition). Insulin release (top) and ^3H efflux rates (bottom) were measured

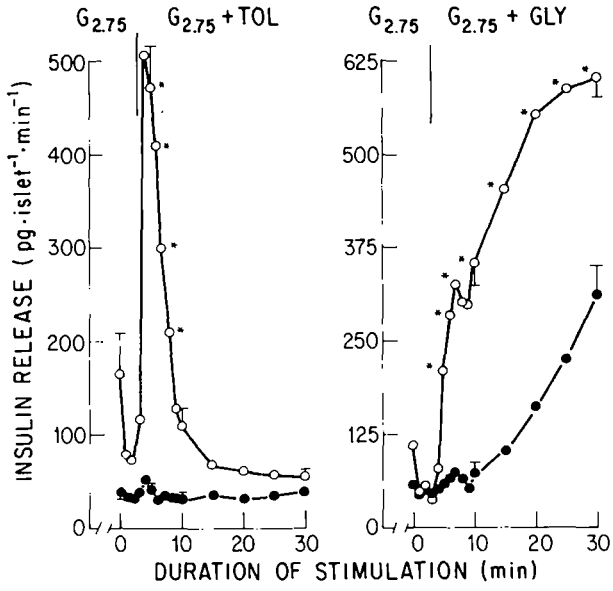

Fig. 6. Time-dependent potentiation induced by KIC to other agonists. Islets were stimulated for 20 min with KIC, 15 mmol/l (○), perifused for an additional 10 min with 2.75 mmol/l glucose alone, and then stimulated with 200 μmol/l tolbutamide (left panel) or 5 mmol/l glyceraldehyde (right panel). Control responses of islets not previously stimulated with KIC are also depicted (●). Only the 30 min stimulatory period with tolbutamide or glyceraldehyde is depicted here. Selected time points were analysed for statistical significance, *$p < 0.05$. At least 3 experiments were performed under each condition

alone, during which time insulin release rates rapidly fell to prestimulatory values, the islets were restimulated, this time with 10 mmol/l glucose. The response of control islets maintained fro 60 or 70 min (Fig. 3 legend) with 2.75 mmol/l glucose alone before being provoked with 10 mmol/l glucose is also shown. When compared to the insulin response from these control islets, prior exposure to leucine or KIC was accompanied by a greater insulin secretory response to 10 mmol/l glucose. Particularly dramatic is the first phase response. In control islets, peak first phase release averaged 66 ± 4 ($n = 18$) pg·islet^{-1}·min^{-1}. This value increased to 204 ± 14 and 246 ± 11 pg·islet^{-1}·min^{-1} in the leucine-and KIC-pretreated groups respectively. Release rates measured 25–30 min after the onset of stimulation were also significantly greater ($p < 0.05$) in the KIC-and leucine-treated islets. If the interval between the exposure to KIC or leucine and 10 mmol/l glucose was extended to 20 or 60 min, TDP was observed only in the 20 min interval group (Fig. 4, left panel). In the case of KIC, both first and second phase responses were amplified. With leucine preexposure, only the first phase insulin secretory response was significantly increased above control release rates. Extending the interval between stimulant presentations to 60 min resulted in the abolition of TDP (Fig. 4, right panel).

We next examined the impact of a 20 min stimulatory period with either KIC or leucine on the efflux of ^3H. The results are given in Figure 5. While acute exposure to either compound was accompanied by insulin output (Fig. 5, top), insulin secretion rates rapidly subsided within 10 min after stimulant removal. In these same islets, ^3H efflux rates slowly increased with agonist addition (Fig. 5, bottom) but, unlike insulin secretory rates, remained elevated long after KIC or leucine removal from the medium. In the case of KIC, efflux rates remained significantly elevated above prestimulatory values for 40 min. In the case of leucine, efflux rates remained elevated above prestimulatory rates for 28 min after stimulant removal. Of particular relevance to the notion that the persistent increase in PI hydrolysis regulates TDP, the duration of the increase in ^3H efflux paralleled to a large extent the duration of TDP (see Fig. 4).

The concept that prior KIC exposure results in a persistent increase in PI hydrolysis was substantiated further by direct measurements of labelled inositol phosphates in prior KIC-stimulated islets (Table 1). First, KIC rapidly and significantly increased ($p < 0.05$) the levels of all inositol phosphates measured (Table 1,

compare lines 1 and 3 with lines 4 and 5). These levels fell after a 10 min perifusion with 2.75 mmol/l glucose alone (Table 1, line 7), but, in the case of IP_1 and IP_2, remained significantly higher than control values ($p < 0.05$) (line 1). In other experiments control (Table 1, line 6) or prior KIC-treated islets (Table 1, line 8) were perifused for an additional 20 min with 2.75 mmol/l glucose plus 10 mmol/l lithium chloride. Lithium chloride, by blocking phosphatase action on inositol phosphates [17], allows these compounds to accumulate intracellularly. In control islets treated with 2.75 mmol/l glucose alone (Table 1, line 6), this manipulation has no significant effect on levels of these compounds, indicating that with low glucose PI hydrolysis is low. However, in the islets previously exposed to KIC, dramatic elevations of inositol phosphates, particularly in IP_1, were noted (Table 1, line 8). Since we had already established that these inositol phosphate levels decrease after removal of KIC from the perifusion medium (Table 1, line 7), a reasonable explanation for this dramatic increase in these inositol phosphates is a sustained activation of PI hydrolysis induced by prior KIC exposure. Despite these dramatic increases in islet inositol phosphate levels under this condition (prior KIC and subsequent exposure to LiCl), there was no parallel increase in insulin output from these islets (data not shown). We attribute this observation, at least in part, to the inhibitory effect of lithium chloride on insulin output [6].

Finally, experiments were designed to test the specificity of TDP induced by KIC. The results from these studies (Fig. 6) demonstrate that a prior 20 min exposure to KIC results in an amplified secretory response to either 200 μmol/l tolbutamide (with 2.75 mmol/l glucose) or 5 mmol/l D-glyceraldehyde.

Discussion

In a series of preceding publications [5-7, 16], we have explored the possible involvement of biochemical events set into motion by an increase in PI hydrolysis in the induction of B-cell memory to glucose. The phenomenon of memory has been known for some time but its biochemical basis has yet to be firmly established. Our previous results with cholecystokinin [5], glucose [6], glyceraldehyde [6], tolbutamide [16] and a report by Sorenson [18] with the phorbol ester TPA, all support the concept that events in the inositol cycle are somehow intimately involved in this process. In the present study, we have expanded the list of compounds that induce memory to glucose and, again, PI hydrolysis seems to be an integral component of this phenomenon.

In the present experiments, we employed KIC and leucine to activate the B cell secretory apparatus. Previous studies [8, 9] have established that both compounds increase the incorporation of ^{32}P into islet inositol containing phospholipids. KIC (10 mmol/l) has also been shown to elevate the levels of labelled inositol phosphates from islets prelabelled with [2-^{3}H] inositol [10]. A previous report by Clements et al. [19], however, failed to document a stimulatory effect of leucine or KIC on recovery of ^{3}H inositol prelabelled PI. This observation may be attributable, at least in part, to the low rates of ^{3}H incorporated into PI and the low levels (8-10 mmol/l) of leucine or KIC used in these studies. Even at 20 mmol/l, the impact of leucine on PI hydrolysis is modest. The present studies indicate that the quantitative impact of KIC (15 mmol/l) on both insulin release and PI hydrolysis is significantly greater than leucine (20 mmol/l).

It should be emphasized that the rationale for using ^{3}H efflux rates as the index of PI hydrolysis is based on several pertinent observations previously reported. First, ^{3}H-inositol is exclusively incorporated into inositol-containing phospholipids [20, 21]. Second, an increase in perifusate ^{3}H is paralleled by a decrease in labelled inositol-containing phospholipids [20]. Third, it is possible to simultaneously monitor the kinetics of PI hydrolysis and insulin output in the same islets with this approach [14-16, 20]. Fourth, the functional integrity (insulin release) of the islets can be conveniently established [14-16, 20], an issue not often addressed in many previous reports on PI hydrolysis in islets. Along these lines, for example, lithium chloride is often included in the medium to facilitate the measurements of inositol phosphates generated during agonist-induced PI hydrolysis. We recently reported [6], in agreement with other studies [22, 23] that the level (10 mmol/l) of lithium commonly employed in many of these studies actually inhibits insulin output, thus making the extrapolation of results obtained in the presence of this cation to the dynamic insulin secretory response tenuous at best. Finally, and most importantly, the ^{3}H efflux data were substantiated, both qualitatively and quantitatively, by direct measurements of labelled inositol phosphate accumulation in perifused islets. This finding adds further credence to the concept that ^{3}H efflux from ^{3}H-inositol prelabelled islets is indeed a valid index of an increase in cellular PI hydrolysis.

One possible problem with using ^{3}H efflux rates to monitor PI hydrolysis in islets is the fact that while inositol phosphates accumulate rapidly in response to KIC (Table 1, line 4), significant increases in ^{3}H efflux lag somewhat behind both this response and the increase in insulin output. A similar situation occurs with glucose-stimulated islets [15, 20]. However, we recently reported that the inclusion of nonradioactive inositol improved our ability to kinetically monitor PI hydrolysis using this efflux methodology. Similar studies were conducted with KIC-stimulated islets and the following major observations were made: (a) nonradioactive inositol has no appreciable effect on insulin output

(Fig. 2, left panel); (b) however, its inclusion results in a markedly amplified ^3H efflux response to KIC stimulation (Fig. 2, right panel); (c) nonradioactive inositol increases the levels of free ^3H-inositol in both control and KIC-stimulated islets (Table 2); (d) finally, nonradioactive inositol attenuates the increase in islet levels of labelled inositol phosphates noted with KIC stimulation (Table 2). These findings suggest that an increase in ^3H efflux (primarily free ^3H-inositol, Table 2) most accurately reflects increases in PI hydrolysis provided cold inositol is included in the medium. We further suggest that cold inositol competes with labilised free ^3H-inositol (derived from ^3H-PI) for reincorporation back into PI. The fact that nonradioactive inositol increased islet levels of free ^3H-inositol, increased ^3H efflux but reduced levels of labelled inositol phosphates agrees with this suggestion. By competing with this rapidly expanding pool of free ^3H-inositol for reincorporation into PI, nonradioactive inositol increases the pool of free ^3H-inositol allowing more of this moiety to efflux from the islet and appear in the effluent. In the absence of nonradioactive inositol, however, reincorporation of ^3H-inositol back into PI reduces efflux of the label from the islet but contributes to the islets' ability to maintain sustained increases in levels of labelled inositol phosphates (and perhaps insulin secretion as well) after stimulant presentation (Table 1 and 2).

The most cogent data supporting the concept that exposure to KIC results in a persistent increase in PI hydrolysis was obtained by directly measuring the accumulation of labelled inositol phosphates during and after KIC stimulation (Table 1). The results demonstrate that KIC rapidly increases IP_1, IP_2 and IP_3 levels in islets. Removal of KIC is paralleled by a reduction (although not to prestimulatory values) in the islet content of these compounds. Part of the persistent ^3H efflux response observed from these prior KIC stimulated islets may be the result of the degradation of these preformed inositol phosphates and/or the efflux of intracellular free inositol (Table 2). However, if these previously KIC-treated islets are subsequently perifused with lithium chloride, cellular levels of these inositol phosphates are elevated above those noted in control, non-KIC treated islets. The results obtained in the presence of lithium (Table 1) support the concept that prior KIC stimulation results in a persistent increase in PI hydrolysis via biochemical mechanisms that remain to be elucidated. When taken together with our other reports on the same topic, one is left with the impression that PI hydrolysis participates in agonist-induced insulin output and primes the B cell to any subsequent stimulation. Considering the inate complexity of events associated with PI hydrolysis [24], it is not yet possible to define precisely how insulin secretion and TDP are induced by PI-derived second messenger molecules. This promises to remain a fertile area for future studies.

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