

A New Phase Of Insulin Secretion

How Will It Contribute to Our Understanding of β -Cell Function?

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Although initially described two decades ago, biphasic insulin secretion has gradually been understood to reflect β -cell rate sensitivity, be important in minimizing overinsulinization in normal individuals, be defective in non-insulin-dependent diabetes mellitus (NIDDM), and be useful as an early predictor in prediabetic individuals. Recently, a third phase of insulin secretion has been observed in fully in vitro islets or pancreatic preparations. This phase is characterized as a spontaneous decline of secretion (desensitization) during 24 h of sustained exposure to glucose or other secretagogues and does not appear to be simply an artifact of in vitro preparations. The impaired secretion is localized to the final release process in that neither glucose-stimulated proinsulin synthesis nor its conversion to insulin is affected. The mechanisms responsible for the third phase of reduced secretion are unknown. Kinetic evidence suggests it is not caused by emptying of a single finite insulin storage compartment but does not exclude the possibility that the decreased release reflects depletion of threshold-sensitive β -cells recruited at a given secretagogue level. Alternatively, the third phase may reflect inhibition of a priming or terminal insulin-release process by metabolic feedback. Because several secretagogues cause similar third-phase impaired release, even in the absence of glucose, desensitization probably occurs at a common fundamental site in the secretory site (e.g., calcium metabolism). Preliminary studies indicate the third phase is not the result of a paracrine effect by other islet hormones or of a change in muscarinic regulation. Whether other neurologic effectors are involved requires further investigation. Experiments examining the effect of glucose-induced third-phase

desensitization on other secretagogues emphasize that priming (potentiation) and desensitization of insulin secretion are occurring simultaneously and that interpretations of results can be highly dependent on the design of the experiment. The possibility that the third phase of insulin secretion may involve mechanisms (waning of priming; desensitization) that relate to glucose-induced β -cell desensitization in NIDDM is discussed. Ultimate appreciation of the significance of the third phase of insulin secretion may develop, as was true for biphasic secretion, with our increasing understanding of the underlying processes in human diabetes. *Diabetes* 38:673–78, 1989

CLASSIC BIPHASIC INSULIN SECRETION: THE SLOW EMERGENCE OF ITS SIGNIFICANCE

Biphasic insulin release was initially observed in vitro and in humans over 20 years ago (1–4). This phenomenon occurs in response to a rapid-onset sustained stimulation and is characterized as a rate-sensitive (5) rapid, but transient, release of insulin (1st phase), followed by a progressively increasing release (2nd phase) over the 1–2 h typically studied. Because first-phase release is not observed when glucose is presented as a graded increase, which is typical of the rise in blood glucose after a meal (6), the physiologic relevance of the phenomenon was questioned for over a decade.

With time, however, awareness of the significance of biphasic secretion in normal and in diabetic subjects increased. Studies with closed-loop insulin-infusion devices showed that a fast release of insulin proportional to the rate of change of glucose concentration (1st phase) was required in the algorithms to prime the insulin-target tissues and prevent overinsulinization and secondary hypoglycemia (7,8). First-phase insulin release was found to be particularly important in regulating hepatic glucose output (9) rather than peripheral glucose utilization; perhaps this was not an unexpected observation, because first-phase release is diluted in the peripheral circulation.

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In addition, although impairment of first-phase release was reported two decades ago as a characteristic of the prediabetic subject (10,11), significance of this early marker of β -cell deficiency was not appreciated until a better definition of the prediabetic subject (e.g., islet cell antibodies) was developed (12). Current elegant studies show that decreased first-phase secretion (and appearance of insulin antibodies) is particularly useful to predict impending onset of insulin-dependent diabetes mellitus (IDDM; 13)*.

Finally, early kinetic analysis of first-phase secretion resulted in the hypothesis that β -cells differ from one another in their threshold sensitivities to a secretagogue and respond in a relatively all-or-none fashion when their threshold concentration of secretagogue is reached (6). This suggestion was consistent with concurrent observations that β -cells differed in electrical sensitivity to glucose (14) and predicted the recent experimental observations that a major contribution to the dose response in islets and the pancreas is the recruitment of additional β -cells as secretagogue levels are increased (15,16).

The actual mechanism underlying first-phase insulin release is unclear. The possibilities that it reflects a compartment of readily releasable insulin or the transient rise and fall of a metabolic signal for insulin secretion have been reviewed (6). Both islet calcium and inositol triphosphate levels can parallel biphasic insulin secretion (17,18), suggesting that ion or phospholipid turnover (or an interrelationship of both) could provide the putative signal for secretion.

THE THIRD PHASE OF INSULIN SECRETION: A REPRODUCIBLE PHENOMENON

Until 1986, investigators did not continue to follow insulin secretion in any perfusion or perfusion system throughout the first 24 h after tissue isolation, even though studies with glucose-stimulated cultured islets indicated total secretion declined after the 1st day (19). Investigators from three independent laboratories recently showed that continued stimulation during the first 24 h caused a third (depressed) phase of secretion from perfused islets, batch-incubated islets, and the perfused pancreas (20–22). Although the time frames differed, results were essentially the same as reported from this laboratory (Fig. 1). After the initial first-phase release, insulin secretion during the second phase progressively increased over 2–3 h of constant glucose stimulation. This time-dependent increase in insulin release during constant stimulation reflects the ability of glucose and certain other secretagogues to amplify their own signal and is referred to as *time-dependent potentiation* or *priming* (6,23). At 1.5–3 h, the third phase of insulin secretion begins and is characterized by a spontaneous decline of secretion to 15–25% of peak release, a level that is subsequently sustained for ≥ 48 h (20). This phase of insulin secretion may be regarded as an onset of inhibition (desensitization) or as

*The current general use of a brief intravenous stimulation with glucose is adequate to measure the rate sensitivity of the β -cell. However, because the stimulus is rapidly decreasing, the insulin responses may not include the total first phase of secretion. Furthermore, this procedure does not evaluate second-phase secretion; therefore, no implications of a preferential decrease solely in first-phase release should be drawn.

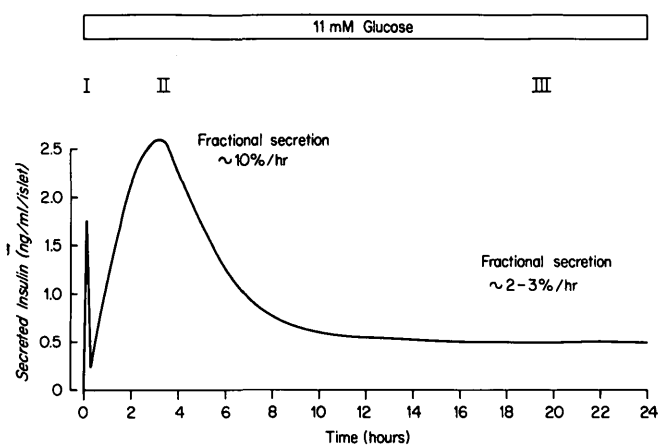


FIG. 1. Schematic representation of characteristic 3 phases of insulin secretion during 24-h constant stimulation of islets with glucose. Defined medium: HANA HB104. (From Bolaffi et al. [24]. © By The Endocrine Society.)

a waning of priming; the conceptual difference is subtle but could describe major mechanistic differences.

The reduced phase of secretion is not caused by impairment of islet cell function resulting from prolonged incubation alone. If, for example, glucose stimulation was delayed for hours, the subsequent temporal pattern of release followed by inhibition was similar, although of lesser magnitude, to that seen with fresh islets (21,24). Furthermore, islets or pancreas in the third phase of reduced secretion were capable of massive response to additional stimuli (20,21).

Finally, because isolated islets may or may not reflect normal β -cell function, it is important to emphasize that almost identical results were obtained in the perfused pancreas, in which the possible artifacts introduced by collagenase digestion or diffusion-related anoxia are not involved and in which the vascular system remains intact (20,22). Some impairment of β -cell function occurs simply because of time in culture, resulting in a decreased magnitude of the total potential response. For this reason, culture of islets for days or weeks probably represents not a third-phase secretion but a release more dependent on general culture conditions. Indeed, improved insulin secretion was resumed after 15 days of chronic glucose perfusion of islets seeded in a hollow-fiber apparatus (25). In contrast, prolonged culture in low glucose can decrease insulin synthesis and the capability of secretion (19,26–28).

WHAT DOES INSULIN PRODUCTION CONTRIBUTE TO THIRD-PHASE INSULIN SECRETION?

Although synthesis and secretion of insulin are regulated by glucose metabolism, desensitization (or the 3rd phase of insulin release) appears to be restricted to the secretion mechanism and not to a step in glucose metabolism common to both processes. In contrast to the three phases of insulin release produced by 11 mM glucose over 24 h, the rate of glucose-stimulated insulin synthesis measured as [3 H]leucine incorporation into proinsulin was unchanged (29,30). (In these [3 H]leucine pulse experiments, translational effects of glucose on proinsulin synthesis were maximized by a short prior exposure to glucose.) These conclusions are consistent with observations by others,

which, with some variation, generally confirm there is little change in insulin synthesis or insulin mRNA levels at stimulating glucose concentrations during a 24-h period (27,28).

One benefit of an open perfusion or perfusion system is that secreted insulin can be protected and assayed; thus, net insulin production can be determined simply by measurement of total insulin recovery in media and islets compared with measurement of initial islet insulin content. Under previously described conditions, 24 h of stimulation of islets at 11 mM glucose almost doubled insulin recovery (20,24). Because secretion was being stimulated concurrently and islet content declined only slightly, most of the insulin production was accounted for in the media. As noted above, the insulin synthetic rate was constant during this period (29); therefore, synthesis per hour could be calculated as ~3–4% of original islet content. Secretion rates can be three times the synthetic rate during the priming period (phase 2), but they later (phase 3) decline toward the synthesis rate (Fig. 1). Similar observations have been observed with the perfused pancreas (22) and suggest, but do not prove, that with time, secretory rates may be controlled by insulinogenesis, even though total islet insulin content is only mildly depleted. These observations made *in vitro* further support the general concept that during glucose challenge in humans, secretion of available insulin initially predominates, whereas, with time, insulin synthesis becomes the major regulator of the insulin-release capability. (The insulin production rate found for islets is much lower than that reported for the perfused pancreas, in which turnover of total insulin content was calculated to occur within 90 min of stimulation, i.e., 65%/h [22].) Although the perfused intact pancreas may be expected to synthesize insulin more efficiently than islets, estimates of insulin synthesis based on total recovery were highly dependent on the difference between assays of islet content before and after the experiment. Whereas aliquots of islets can serve as baseline controls, initial pancreatic content must be taken as a mean from multiple extracted pancreases. Regardless of the actual synthetic rates reported, relationships between secretion and synthesis are in essential agreement.

It is now recognized that glucose can stimulate conversion of proinsulin to insulin if the elevated glucose is present at least several hours before newly synthesized proinsulin reaches the granule (for review, see refs. 30 and 31). Increased conversion is dependent on glucose concentration, becomes maximized during the 3-h priming period and is sustained at the same high rate during the third phase of decreased insulin secretion (29,30). Thus, like synthesis, glucose-regulated conversion from proinsulin to insulin is not desensitized. The ability of islets incubated for 24 h to sustain glucose-regulated synthesis of proinsulin and its conversion to insulin further argues against the possibility that the third phase of diminished insulin secretion is simply an artifact resulting from a decline in functional capacity of islets with time. Instead, this ability indicates a specific inhibition at the insulin-release mechanism.

POSSIBLE EXPLANATIONS OF THIRD-PHASE INSULIN SECRETION

Can third-phase insulin secretion reflect compartmental depletion? The third phase is not the result of depletion of

total stored islet insulin because the ~80% decreased secretion rate was associated with only a 20–30% decrease in islet cell insulin content. This drop in efficiency of insulin secretion is expressed by the decline of fractional secretion as a percentage of total insulin content from ~10 to 2.5% (Fig. 1).

However, it is established that newly synthesized insulin is preferentially secreted from islets (32,33), indicating that insulin can be released from more than one islet storage compartment. Thus, the third phase of insulin release could result from depletion of a finite storage compartment of insulin. However, islets stimulated with different concentrations of glucose still produce temporal patterns of insulin secretion similar in time-to-peak and time-to-50% inhibition to those shown in Fig. 1, and they differ only in the amount of insulin released during each stimulation (24). These studies do not support an explanation of third-phase insulin release based on depletion of a single finite small compartment. They are consistent with the possibility that this phase of insulin secretion reflects depletion of threshold-sensitive β -cells recruited in increasing numbers with increasing glucose (6,16). In a group of islets or an intact pancreas, this phenomenon displayed, as expected, similar temporal depletion characteristics at increasing concentrations of secretagogue with increasing amounts of total insulin released.

Can third-phase secretion reflect metabolic desensitization of terminal release?

Alternatively, the third phase could reflect a metabolic feedback inhibition (desensitization) in which the secretory event itself generates a linked inhibition of secretion. A large number of secretagogues cause desensitization or third-phase insulin release with temporal kinetics similar to that of glucose, although the time of onset can differ (Table 1). For example, commencement of the desensitization phase for arginine is more rapid than that for glucose, whereas glyburide plus 5 mM glucose causes a 1- to 2-h extension of the priming period before the third phase begins (34). In addition, the secretagogues α -ketoisocaproic acid (α -KIC) and phorbol ester cause stimulation-desensitization patterns in the absence of glucose (24). These observations argue against the involvement of a feedback signal related directly to glucose metabolism or signal initiation by any particular secretagogue but suggest the feedback may occur at some common terminal portion of the secretion process. The remarkable ability of islets to decrease sensitivity with constant exposure even extends to inhibitors. After 3–5 h, islets escape from inhibition caused

TABLE 1
Secretagogues that cause desensitization similar to glucose by continuous presentation to islets

Isobutylmethylxanthine (10^{-5} M) + glucose (11×10^{-3} M)
Forskolin (2.5×10^{-6} M) + glucose (11×10^{-3} M)
Fetal calf serum (10%) + glucose (11×10^{-3} M)
Glyburide (5×10^{-7} M) + glucose (5×10^{-3} M)
Phorbol ester (10^{-9} M)
α -Ketoisocaproic acid (2.5×10^{-2} M)
Arginine (1×10^{-2} M) + glucose (5×10^{-3} M)
Carbachol (10^{-5} M) + glucose (11×10^{-3} M)
Verapamil (10^{-5} M) + glucose (11×10^{-3} M) (escape from inhibition)

by continuous incubation with verapamil (Table 1), whereas they retain sensitivity to single 1-h test pulses interspersed throughout the 24 h (J. Bolaffi and G.M.G., unpublished observations).

Recent studies suggest a primary role of phosphoinositol metabolism and activation of C-kinases in priming and the final secretion processes that regulate insulin release (35). However, neither phorbol ester (24; Table 1) nor the addition of *myo*-inositol (21) reversed the inhibited third phase of insulin secretion. Despite these observations, the possibility that the diacylglycerol-inositol triphosphate pathway could still be implicated requires additional investigation. Furthermore, some other obvious regulators of insulin secretion may not be involved; levels of fuel and redox nucleotides did not change during third-phase insulin secretion (36). Finally, known and unknown factors in 10% fetal calf serum had no effect on the secretion patterns (24).

Since the initial observation (37), it has been established that calcium is required for insulin secretion and possibly priming (23 and reviewed in refs. 38 and 39), regardless of the stimulus. Prolonged exposure to glucose has been shown to decrease stored calcium, ⁴⁵Ca uptake, and sensitivity to extracellular calcium (38–41). Thus, the third phase of insulin secretion could reflect a change in calcium-channel activity or compartmental redistribution of β -cell calcium as a general feedback-inhibitory phenomenon in response to various secretagogues.

HOW DO OTHER ISLET HORMONES CHANGE, AND IS THIRD-PHASE INSULIN SECRETION CAUSED BY AN ISLET PARACRINE EFFECT?

The possibility that the third phase of insulin release may result from a paracrine effect caused by decreasing release of islet glucagon (a known potentiator of insulin secretion) or, conversely, an increasing secretion of islet somatostatin (an inhibitor of the process) has been explored. Preliminary studies with J. Bolaffi indicate that both hormones decrease over 24 h of stimulation, indicating that a third phase of inhibited secretion may be characteristic of most, if not all, islet cell hormones. Because somatostatin decreases during the third phase of insulin secretion, the hormone cannot be responsible for the β -cell desensitization. Also, there was no evidence that β -cells in cultured islets during the third phase increase their sensitivity to somatostatin; pulses of somatostatin introduced during the peak of priming or during the third phase caused a similar percent inhibition of insulin secretion. The observed fall in glucagon could have contributed to the decreased insulin secretion of the third phase. However, coprifusion of glucagon at 1000 times the maximum secreted level increased secretion; but similar to isobutylmethylxanthine and forskolin (Table 1), it failed to influence onset of the third phase.

WHAT IS THE ROLE OF NEUROENDOCRINE MEDIATORS IN THIRD-PHASE INSULIN SECRETION?

Waning of the priming effect could produce the third phase secondary to a loss of receptors to, or decreasing availability of, positive neuroendocrine islet mediators resulting from denervation of the *in vitro* systems. Muscarinic agents may not be responsible, because the continuous presence of carbachol did not prevent third-phase insulin secretion (Ta-

ble 1). Furthermore, the introduction of carbachol at different periods during a 24-h exposure to glucose was found, (in agreement with others [39]), to increase, not decrease, with prolonged time in culture, indicating the sensitivity of the β -cell to muscarinic stimulation had not declined. Similar experiments are required to determine whether a change in adrenergic agents or their effectiveness on the β -cell is a contributing factor to this phenomenon.

HOW DOES GLUCOSE-INDUCED IMPAIRED INSULIN SECRETION PRODUCED DURING THE THIRD PHASE AFFECT SENSITIVITY TO OTHER ISLET SECRETAGOGUES?

A major concern relating to non-insulin-dependent diabetes mellitus (NIDDM) is whether β -cell desensitization caused by hyperglycemia is specific to glucose only. Although prolonged exposure to glucose has been shown to cause the desensitized third phase of insulin secretion, conclusions vary regarding the effects of other secretagogues (20,21,24,42,43). Two experimental designs have been used: one compares the responses to an alternate secretagogue before and after culture in a high concentration of glucose; the other compares the responses to an alternate secretagogue after 24 h culture with high or low concentrations of glucose. In a series of experiments (24), α -KIC was used as a test secretagogue because, with the buffers used, it was a direct stimulus of insulin release and did not require the potentiating presence of small amounts of glucose (Fig. 2). Despite the decreased response to glucose during the third phase of insulin secretion, response to α -KIC at 24 h was actually greater than that to the initial α -KIC stimulus. Also, α -KIC response at 24 h was greater after incubation with glucose than without. This observation might suggest

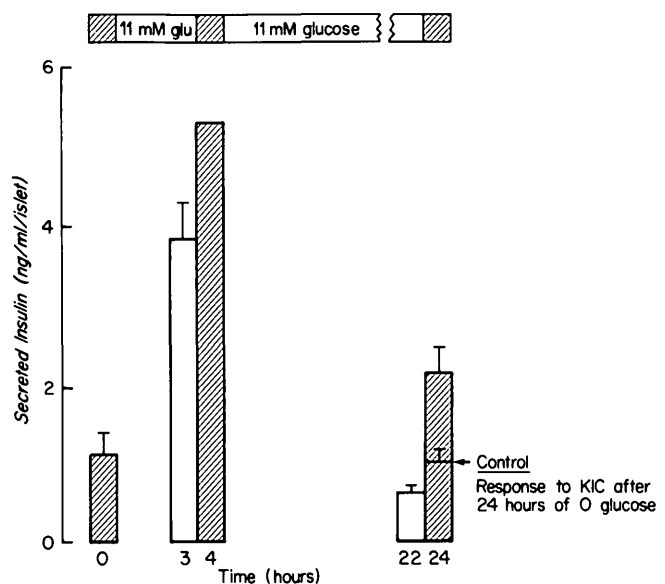


FIG 2. Effect of continuous glucose on potentiation of α -ketoisocaproic acid (α -KIC)-stimulated insulin secretion. Islets were batch incubated. After 1 h of preincubation at 0 mM glucose, islets were challenged with α -KIC alone for 1 h (hatched bars) and then incubated in hourly changes of 11 mM glucose (open bars). When indicated, islets were quickly washed, again preincubated for 1 h at 0 mM glucose, rechallenged with α -KIC alone for 1 h, and reincubated with 11 mM glucose. (From Bolaffi et al. [24]. © by The Endocrine Society.)

that glucose-induced desensitization is specific and does not affect response to another stimulus. It is clear, however, that at 3 h, during the maximum priming period initiated by glucose, the α -KIC response was several times higher than the initial response and greater than that seen at 24 h. These results suggest that the third phase represents a period of decreased priming capacity by glucose or that priming is maintained but obscured by inhibition of the release process. Response to another secretagogue at 24 h therefore represents the summation of any residual positive priming and the negative effects caused by inhibition of release. Because the peak of priming can occur relatively rapidly, depending on the buffer used (20,21), final comparison of 0- and 24-h test responses without appraisal of response during the priming period may be highly dependent on the buffers used, the duration of the test procedures, the priming character of the alternate test agent itself, and the time the islets have been at low glucose during the isolation and purification procedures.

WHAT IS OR WHAT WILL BE THE PHYSIOLOGIC SIGNIFICANCE OF THIRD-PHASE INSULIN SECRETION?

The physiologic significance or application to human diabetes of the third phase of insulin secretion is as obscure today as the first phase was in the early 1970s. In human NIDDM, in the early stages of IDDM, or in the genetically diabetic Chinese hamster (44), impaired insulin secretion is exacerbated by prolonged hyperglycemia, and insulin secretion can be improved by insulin, sulfonylureas, or diet (for review, see refs. 45 and 46). Conclusions on whether this glucose-induced impairment is specific only for glucose or causes impairment of response to other secretagogues are highly dependent on testing procedures (e.g., ambient and prior glucose concentration and phase of insulin secretion tested) and are a matter of debate (47). There is some evidence that in humans, as described for perfused islets (Table 1), desensitization may be a general phenomenon; the prolonged administration of a nonglucose secretagogue (sulfonylurea; 48) results in specific desensitization to that secretagogue's effectiveness.

It is tempting to speculate that the third phase of secretion measured in a fully in vitro system may relate to and be used to clarify the desensitization process in humans. Others have used for the same purpose combined in vivo/in vitro models in which the effects of hyperglycemia produced by early streptozocin, partial pancreatectomy, or glucose infusion are studied by extirpation and testing of islets or the perfused pancreas (46,49,50). In these models, insulin secretion is relatively insensitive to increased glucose, and secretagogues that normally require potentiation by glucose (e.g., arginine) are highly effective in the absence of glucose. In contrast to onset of the third phase of decreased secretion by 1–3 h (Fig. 1), decreased sensitivity to glucose occurs only after 48 h of hyperglycemia (51). Impairment can be reversed by culture of islets from these animal models in low glucose, indicating the process results from the original hyperglycemia produced in vivo (49). The phenomenon is complex, because at milder hyperglycemia, response to arginine may actually decrease, and results may be dependent on dietary changes (52). Nevertheless, many of these observations suggest that islets from these models have become

maximally primed by glucose, perhaps by building a glycogen reservoir that is mobilized and provides glucose equivalents during the in vitro testing procedures (53,54).

Hyperglycemia produced in animals or normal humans by prolonged glucose infusion usually results in sustained high blood insulin levels that do not spontaneously decline (3,46,55). This phenomenon further suggests that maximum priming (for a given blood glucose) had been achieved. However, whether priming by glucose can be sustained in human NIDDM is not clear.

In summary, it is possible that studies of the third phase of insulin secretion may prove particularly important in characterizing the priming phenomenon and in determining what causes it to become exhausted or desensitized. Current results suggest the third phase 1) is an event occurring at the insulin-release mechanism and relatively independent of insulin production, 2) may reflect depletion of recruitable threshold-sensitive β -cells or a metabolic feedback inhibition, 3) is not limited to glucose but is produced by various nonglucose secretagogues, 4) is probably not caused by a paracrine effect of other islet hormones, and 5) can still permit partial priming and enhanced secretion by alternate secretagogues, depending on temporal and test conditions.

Almost 20 years were required for the biologic significance of biphasic insulin release to be established. Only further investigation will determine whether, with the third phase, investigators are dealing with a "passing phase" or a "phase for the future."

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