Perifusion of Rat Pancreatic Tissue In Vitro: Substrate Modification of Theophylline-Induced Biphasic Insulin Release

IAN M. BURR, LUC BALANT, WERNER STAUFFACHER, and ALBERT E. RENOLD
with the technical assistance of DANIELLE DUVILLARD

From the Institut de Biochimie Clinique, University of Geneva, 1211 Geneva 4, Switzerland

ABSTRACT The immunoreactive insulin (IRI) release patterns produced by continuous theophylline stimulation of rat pancreas have been defined, using an in vitro perifusion system. In the presence of glucose, citrate, and pyruvate at concentrations which were nonstimulatory by themselves, continuous stimulation with theophylline produced a biphasic IRI release profile. In the absence of substrate, continuous theophylline stimulation produced only an abrupt and limited primary response. Of the substrates tested, only glucose significantly enhanced this primary response. With increasing theophylline concentrations, whether in the presence or absence of substrate, significant increases were noted in the primary response as estimated by either the maximum rate of IRI release attained or by the total amount of IRI released during this time. Similarly, the secondary responses to theophylline increased with theophylline concentration in the presence of either citrate or pyruvate. With glucose as substrate, however, increasing theophylline concentrations from 2.5 to 5, then 10 mM produced a progressive reduction in both indices of the secondary response, which was inversely related to the primary response. These findings suggest that cyclic AMP not only mediates IRI release in quantitative terms but is also implicated in the qualitative nature of the response pattern. They also indicate a possible metabolic basis for biphasic IRI release, the acute or primary response being dependent upon the basal state of the cell and the availability of endogenous energy sources, the secondary response upon the availability of exogenous substrate.

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INTRODUCTION

Since the advent of radioimmunoassay techniques for the assay of insulin in plasma (1), increasing emphasis has been assigned to the possible role of anomalies of dynamic aspects of isulin release in the pathogenesis of diabetes mellitus. It has been established in normal man (2–4) and in some animal preparations in vitro (5, 6) that continuous glucose stimulation produces a biphasic pattern of insulin release, and it has been suggested that this pattern could result from either intracellular feedback modulations (5, 7) or the presence of two storage pools of insulin with different release rates (8, 9). The apparent early delay in insulin release after glucose challenge in overt or chemical diabetics (1, 3, 10–13) and potential (those with presumed genetic predisposition) diabetics (3, 4) has been related to failure of the initial phase of insulin release, resulting in an inadequate and/or delayed response.

Recently, much data has been accumulated to implicate 3′,5′-cyclic AMP (cAMP) in the process of insulin release (14–17). Methylxanthines, which inhibit cAMP phosphodiesterase activity (18), potentiate insulin release in vitro (14, 15, 19, 20) and provoke rapid insulin release in vivo in rat (21), dogs (22), and in man (23). Utilizing a perifusion system previously shown to respond to pancreatic β-cell stimulation in a manner similar to that established for in vivo and other in vitro systems (24, 25), we have observed a biphasic pattern of immunoreactive insulin (IRI) release in response to theophylline. This has provided a basis for investigating some additional metabolic influences on the pattern of IRI release. This report describes the in vitro release profiles obtained by continuous perifusion of adult rat pancreas with differing concentrations of theophylline, in the absence of added substrate, and in the

presence of glucose, pyruvate, or citrate, at concentrations which by themselves were nonstimulatory.

METHODS

Pancreases were removed from male Wistar rats weighing 175–200 g, kept under light ether anaesthesia after an overnight (18–24 hr) fast. Preliminary studies had established that variation in the method of anaesthesia (i.e. removal of the pancreas while under nembutal anaesthesia and/or removal of the pancreas after decapitation while under nembutal anaesthesia) did not influence the subsequent in vitro pancreatic IRI responses. The pancreases were dissected free of fat, connective tissue, and ganglia. 100-mg fragments were briefly minced under buffer with sharp stainless steel scissors to provide pieces approximately 1 mm in diameter, which were immediately placed into an inverted cone perifusion chamber (capacity 0.7 ml). Two such chambers could be perifused simultaneously and in parallel, allowing for control and test experiments to be performed simultaneously on samples from the same pancreas.

The basic buffer used throughout was Krebs-Ringer bicarbonate containing 0.5 g/100 ml bovine serum albumin (previously dialyzed against EDTA and then against twice-distilled water) and Trasylol, 500 IU/ml. The buffer was continuously gassed with 95% O_2: 5% CO_2 maintaining a pH of 7.35–7.45 throughout the duration of the experiments. Buffer from reservoir flasks was passed through the perifusion chambers, from below, using twin peristaltic pumps at flow rates of 2.2–2.8 ml/min. Effluent was collected in glass tubes mounted in a fraction collector, the tissue fragments being retained in the chambers by means of Millipore filter (8 μ) covers (Millipore Corp., Bedford, Mass.). The temperature of the buffer throughout the system was maintained at 37°C by thermostatic control of the temperature of water which was constantly recirculated through jackets surrounding the reservoir flasks, perifusion chambers, and connecting tubing. Collected effluent was immediately placed in an ice bath before transfer to a freezer where samples were stored until assay.

Perfusion of the chambers was commenced immediately after the tissue was placed in them and consisted of an initial wash period of 35 min followed by an experimental period of 58 min. Total time from removal of the pancreas to commencement of the perifusion averaged 7 min, tissue being immersed in oxygenated buffer for the majority of this period. Effluent was collected continuously throughout each study, at intervals of 1 min during the final 5 min of the wash, at intervals of 30 sec during the first 5 min of the stimulation, and every minute thereafter. The efficacy of this system and the factors involved in obtaining maximum insulin recovery have been described (24).

The IRI content of the effluent fractions was measured in all the basal (wash) samples, in all samples from the first 10 min of the test period, and in each fifth sample thereafter. The remainder of the samples were kept for confirmation of the patterns if needed. This optimally efficient pattern of collection and assay was derived from initial studies in which all samples were assayed. IRI was assayed by a modification of the method of Hales and Randle (26), in which rat insulin was used as standard, labeled porcine insulin as tracer, and guinea pig anti-procine insulin serum as first and rabbit anti-guinea pig serum as second antibodies. Precipitates were separated by centrifugation.

The following four experimental designs were utilized. In the first series, 2.4 mM glucose was added to the wash but no substrate was added to the stimulation buffer; theophylline in concentrations of 5 or 10 mM was added to the stimulation buffer only. In the second series, 2.4 mM glucose was added to the wash and 2.4 mM citrate to the stimulation buffer together with either 5 or 10 mM theophylline. In the third series, citrate was replaced by 2.4 mM pyruvate, and in the fourth series, 2.4 mM glucose was retained throughout both experimental periods and theophyl-

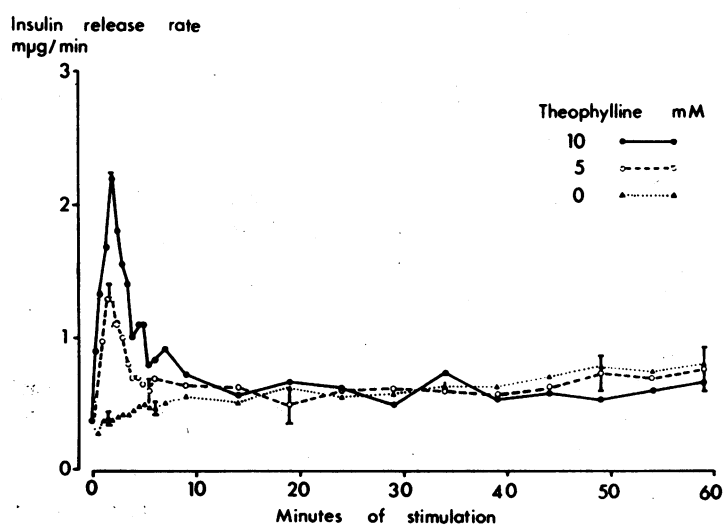

FIGURE 1 Insulin release patterns induced by continuous theophylline stimulation, in the absence of substrate. Samples were collected continuously using a fraction collector, at 30-sec intervals for the first 5 min of stimulation; then at 1 min intervals, all samples were assayed over the first 10 min of the test period then each 5th sample. Concentration times rate of flow provides release per minute.

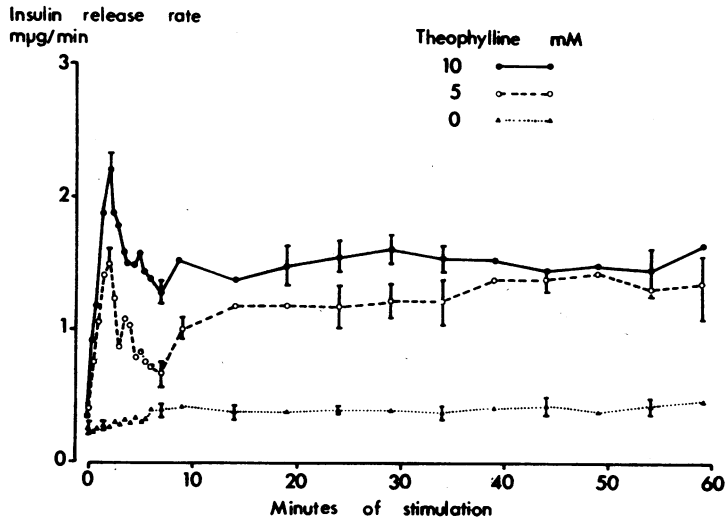

FIGURE 2 Insulin release patterns induced by continuous theophylline stimulation, in the presence of 2.4 mM citrate. Methods as for Fig. 1.

line added to the stimulation buffer at concentrations of 2.5, 5, or 10 mM. In each series, controls were run with the appropriate substrates in the absence of theophylline. Note that 2.4 mM glucose was present in all prestimulation wash buffers. Control experiments served to demonstrate that prolonged washing in the absence of substrate did not inhibit the ability of the perifused preparation to subsequently respond to substrate, since washing in substrate-free buffer for periods up to 1 hr did not materially affect subsequent substrate responses. Furthermore, when substrate-free buffer was used for the initial 35 min wash and the first 5 min of continuous theophylline stimulation, substrate added at that time produced normal secondary responses.

The maximum release rate achieved and the total amount of IRI released during both the primary and secondary responses were derived from the measured flow rates and the concentrations of IRI in the fractions. The total amount of IRI released during the primary response period was obtained by simple addition (all effluent over this period having been collected and assayed); the total amount of IRI released during the secondary response was calculated for each individual experiment from the area subtended by the plot of IRI released against time.

RESULTS

IRI release patterns obtained in control (substrate only) experiments and in experiments in which continuous theophylline stimulation was maintained are shown for each series in Figs. 1–4, in all of which the mean rate of IRI release for the appropriate group is plotted against the time after commencement of stimulation. Standard errors of the means were calculated for each point but only representative examples are shown in the interest of graphic clarity. It is evident that, at the concentrations used, none of the substrates stimulated IRI release, but that in the presence of theophylline together with any one of the three substrates (Figs. 2–4) a biphasic pattern of release resulted. In the absence of added substrate, continuous theophylline stimulation produced only the primary spike response, but no secondary response (Fig. 1). With either citrate or pyruvate as substrate, increases in theophylline concentration increased both the primary and the secondary responses (5 mM vs. 10 mM theophylline in Figs. 2 and 3). With glucose as substrate, increases in theophylline concentration from 2.5 to 10 mM were associated with enhancement of the primary but reciprocal reduction of the secondary IRI responses (Fig. 4).

Four indices served to estimate the mean individual responses to stimulation: the maximum IRI release rates (independent of time of occurrence) achieved during the primary and during the secondary responses (R_1 and R_2), and the corresponding total amounts of IRI released (T_1 and T_2) are shown in Table I. The values for R_1 and R_2 in this table are greater than those seen in Figs. 1–4; in a pooled time plot the maximum rate appears blunted due to slight asynchrony of individual peaks.

The significance of the differences in these indices which were induced by changes in theophylline concentration within each substrate series are indicated in Table II. In the absence of theophylline, no change from basal IRI release rates was observed with any of the substrates in the concentrations used. Both in the absence and in the presence of any one of the three substrates, theophylline produced a rapid initial (primary) release of IRI, during which both the maximum rate of IRI release and the total amount of IRI released were increased in a dose-dependent fashion. Theophylline did

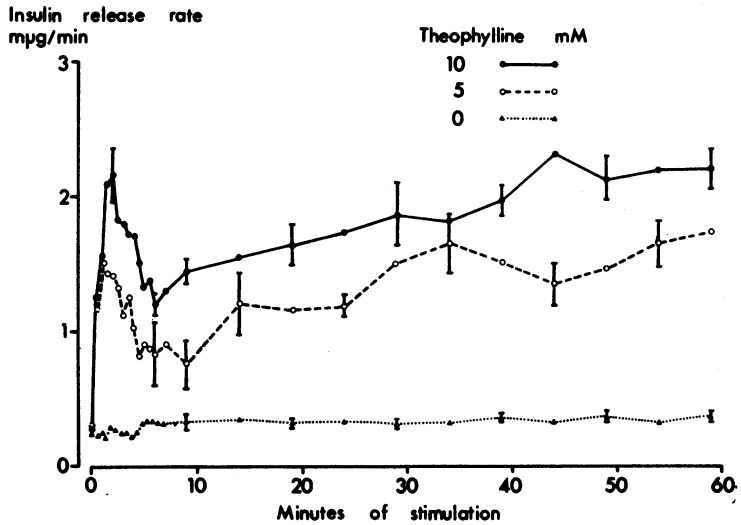

FIGURE 3 Insulin release patterns induced by continuous theophylline stimulation, in the presence of 2.4 mM pyruvate. Methods as for Fig. 1.

not provoke a secondary response in the absence of substrate. In the presence of either citrate or pyruvate, the secondary response (like the primary) was increased with an increase in theophylline concentration; though for citrate, R_2 was not significantly greater with 10 mM than with 5 mM theophylline. With glucose as substrate, theophylline produced a marked secondary response at 2.5 mM, a lesser response at 5 mM ($P < 0.05$ for T_2 and $P < 0.05$ for R_2), and a further significant decrease at 10 mM ($P < 0.01$ for T_2 and $P < 0.01$ for R_2), i.e., a pattern reciprocal to that observed in the primary response to theophylline in the presence of glucose.

Substrate-induced differences in the IRI release responses to theophylline are analyzed in Table III, which clearly shows that glucose enhanced but pyruvate and citrate did not affect the primary IRI response to either 5 or 10 mM theophylline. All three substrates enhanced the secondary response to both theophylline concentra-

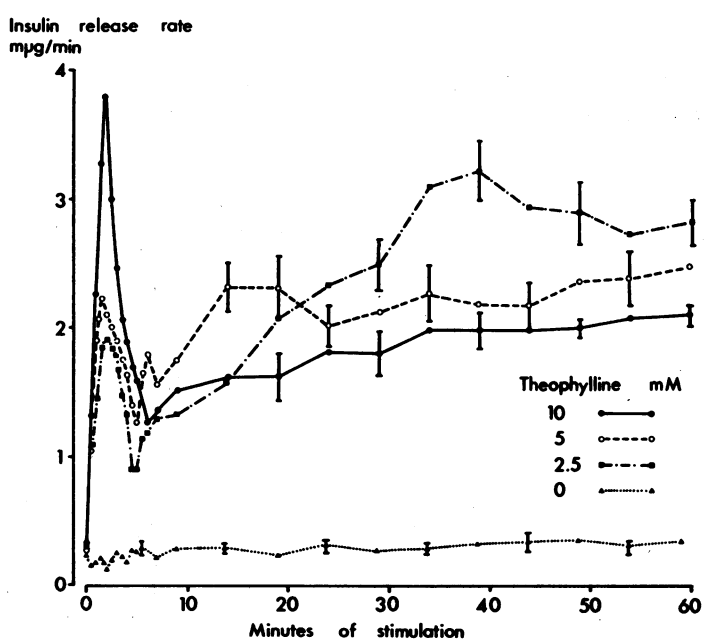

FIGURE 4 Insulin release patterns induced by continuous theophylline stimulation, in the presence of 2.4 mM glucose. Methods as for Fig. 1.

TABLE I

Insulin Release Responses to Citrate, Pyruvate, or Glucose with or without Theophylline in Perifused Rat Pancreas

Substrate added	Theophylline	(n)	Primary response		Secondary response	
			R_1	T_1	R_2	T_2
	mM		*mμg/min**	*mμg**	*mμg/min**	*mμg**
None	0	(6)	0.5 ±0.05	2.3 ±0.25	0.8 ±0.06	31.8 ±2.3
	5	(7)	1.57 ±0.07	4.6 ±0.33	0.86 ±0.12	32.5 ±4.4
	10	(6)	2.37 ±0.15	7.0 ±0.78	0.71 ±0.21	32.1 ±7.9
Citrate, 2.4 mM	0	(6)	0.4 ±0.04	1.7 ±0.18	0.47 ±0.04	20.6 ±1.92
	5	(6)	1.65 ±0.15	5.3 ±0.4	1.55 ±0.16	61.0 ±5.0
	10	(8)	2.44 ±0.13	8.2 ±0.43	1.71 ±0.08	76.3 ±4.4
Pyruvate, 2.4 mM	0	(6)	0.32 ±0.05	1.43 ±0.2	0.4 ±0.03	17.2 ±1.4
	5	(6)	1.7 ±0.1	5.85 ±0.5	1.7 ±0.1	68.5 ±4.7
	10	(8)	2.6 ±0.1	8.6 ±0.4	2.4 ±0.13	92.3 ±5.4
Glucose, 2.4 mM	0	(6)	0.3 ±0.06	1.28 ±0.12	0.37 ±0.03	15.5 ±1.6
	2.5	(5)	2.02 ±0.07	7.4 ±0.41	3.24 ±0.12	123.2 ±3.69
	5	(7)	2.47 ±0.12	8.84 ±0.5	2.71 ±0.1	108.9 ±3.2
	10	(6)	4.32 ±0.24	11.5 ±0.5	2.08 ±0.09	92.1 ±3.1

R_1 and R_2 are the maximum release rates measured during the primary and secondary response periods irrespective of the time within that period that they were recorded.
T_1 and T_2 are the total amounts of insulin released during the respective periods.
* All responses are recorded as the mean ±SEM of (n) experiments.

tions; at 5 mM theophylline, glucose was the most effective substrate, while at 10 mM theophylline, glucose provided no advantage over pyruvate.

The relationship between the primary and secondary responses in each experimental series, as reflected by the relationship between R_1 and T_2 are illustrated in Figs. 5 *a*, *b*, and *c*. In these figures all values from each series are plotted individually and without regard to theophylline concentration. Positive correlations were observed for citrate ($r = 0.766$, $P < 0.01$) and pyruvate ($r = 0.776$, $P < 0.01$) as substrates, but a clear negative corelation was seen with glucose as substrate ($r = -0.740$, $P < 0.001$). The correlations observed in all series when T_1 and T_2 were compared (for citrate, $r = 0.752$, $P < 0.01$; for pyruvate, $r = 0.715$, $P < 0.01$; and for glucose, $r = -0.674$, $P < 0.01$), were quite similar to those for

TABLE II

Significance of Theophylline-Induced Differences in Insulin Release Responses Shown in Table I

Substrate	Theophylline	S	Primary response		Secondary response	
			R_1	T_1	R_2	T_2
	mM					
None	5	*	<0.01	<0.01	NS	NS
	10	‡	<0.05	<0.02	NS	NS
Citrate, 2.4 mM	5	*	<0.01	<0.01	<0.01	<0.01
	10	‡	<0.01	<0.01	NS	≤0.05
Pyruvate, 2.4 mM	5	*	<0.01	<0.01	<0.01	<0.01
	10	‡	<0.01	<0.01	<0.02	<0.01
Glucose, 2.4 mM	2.5	*	<0.01	<0.01	<0.01	<0.01
	5	§	<0.05	NS	<0.05	<0.05
	10	‡	<0.01	<0.01	<0.01	<0.01

Calculations by Student's *t* test.
R_1, R_2, T_1, and T_2 as for Table I.
* Significance of difference from response in the absence of theophylline.
‡ Significance of difference from response in the presence of 5 mM theophylline.
§ Significance of difference from response in the presence of 2.5 mM theophylline.

TABLE III

Significance of Substrate-Induced Differences in Insulin Release Responses Shown in Table I

Substrate	Theophylline	S	Primary response		Secondary response	
			R_1	T_1	R_2	T_2
2.4 mM	*mM*					
Citrate	5	*	NS	NS	<0.05	<0.05
	10	*	NS	NS	<0.01	<0.01
Pyruvate	5	*	NS	NS	<0.05	<0.01
		‡	NS	NS	NS	NS
	10	*	NS	NS	<0.001	<0.001
		‡	NS	NS	<0.02	NS
Glucose	5	*	<0.01	<0.001	<0.01	<0.001
		‡	<0.05	<0.05	<0.05	<0.01
		§	<0.05	<0.05	<0.05	<0.001
	10	*	<0.01	<0.001	<0.001	<0.001
		‡	<0.05	<0.05	<0.001	<0.05
		§	<0.05	<0.05	NS	NS

Calculations by Student's *t* test.

R_1, R_2, T_1, and T_2 as for Table I.

* Significance of difference from response in the absence of substrate.

‡ Significance of difference from response in the absence of citrate.

§ Significance of difference from response in the presence of pyruvate.

R_1 and T_2. Preference was given to the latter relationship for illustration because the primary maximal rate best represents the primary secretory response while the total IRI secreted best represents the magnitude of the secondary response. The total amount of IRI released in any experiment did not exceed 10% of the initial content of the tissue used.

DISCUSSION

Theophylline, a methylxanthine, is a potent inhibitor of cAMP phosphodiesterase in several tissues (18) and has been shown to increase intracellular levels of cAMP in isolated pancreatic islets (27). It is not known to what extent theophylline effects on phosphorylase phosphatase (28), on phosphorylase (29), or on other intracellular activities may contribute to, or modify the cAMP phosphodiesterase-mediated action. It is nevertheless reasonable to assume that the effects of theophylline on IRI release are mediated, at least in part, by changes in the cAMP content of β-cells. The present study establishes that biphasic patterns of IRI release may be obtained in vitro in response to theophylline, in the presence of substrate. A biphasic IRI release into the portal vein of rats had also been demonstrated in vivo by Turtle, Littleton, and Kipnis (21) using a single injection of theophylline, although these authors attributed the biphasic pattern of the response to intravascular mixing effects. Our observations suggest that this biphasic pattern may well be related to the nature of the methylxanthine stimulation as such. Furthermore, the data reported here indicate that the relationship of the primary to the secondary IRI release responses during continuous theophylline stimulation may be modified by the nature of the substrate present during stimulation, thereby suggesting that cAMP not only mediates IRI release in quantitative terms but may also be implicated in the qualitative nature of the response pattern.

Results of in vitro studies with isolated islets from adult rat pancreas have led to the suggestion that the presence of exogenous substrate or intense in vivo glucose preloading was necessary for the manifestation of a methylxanthine effect of IRI release (14). Lambert, Junod, Staffacher, Jeanrenaud, and Renold, utilizing cultured explants of fetal rat pancreas demonstrated that added substrate was not necessary for methylxanthine-induced IRI release (30), but these explants had been cultured for some days in a high glucose medium. In the present study, using pancreases from fasted adult rats, a primary brisk IRI response to theophylline was observed in the absence of added substrate, while exogenous substrate was required for the secondary phase of IRI release. These data suggest that, in adult rat pancreas maintained in vitro, rapid but limited IRI release may be stimulated by increased availability of cAMP, independently of exogenous substrate. The possibility that glycogen may be a significant energy source for this reaction is supported by electron microscopic and chemical evidence for the presence of glycogen in adult β-cells (31, 32).

In contrast to the effects of theophylline on this preparation and in contrast to the effects of glucose in *perfusion* systems (5, 7, 8), previous work had shown that stimulation with even large concentrations of glucose resulted in only small primary IRI responses with vigorous secondary increases in the rate of IRI release (24, 25). The combination of minimal primary responses to glucose stimulation and brisk or enhanced responses to agents increasing intracellular cAMP are not exclusive characteristics of our perifusion system, or indeed of in vitro systems. A relayed IRI response to glucose, possibly secondary to an absent or diminished primary response, has been described in diabetic and potentially diabetic subjects in vivo (1, 3, 4, 10–13) while such patients may respond with brisk insulin release to glucagon (33, 34) and theophylline (23) administration.

Similarly, an overall depressed response to glucose has been reported for fetal and perinatal pancreatic tissue both in vitro (20, 30, 35) and in vivo (36, 37) with the in vitro tissue response being enhanced in the presence of cAMP phosphodiesterase inhibitors (20). Possible reasons for such an anomaly in our preparation might result from changes in the basal metabolic state and the reactivity of insulin release mechanisms of pancreatic β-cells obtained from animals fasted overnight and isolated from all other tonic or facilitating influences originating elsewhere in the organism, such as humoral regulators of gastrointestinal origin (33, 34, 38–41), hormones originating in other endocrine glands (15, 42, 43), and the autonomic nervous system (28, 44–52). The absence of such factors may result in failure to maintain critical concentrations of cAMP or of other as yet hypothetical cofactors required for full effectiveness of substrate stimulation. It is evident that accumulation of cAMP to a critical level would be facilitated by the presence of an inhibitor of its degradation, such as theophylline.

In the presence of substrate, theophylline not only induces a primary response but also a secondary IRI release at substrate concentrations which are by themselves nonstimulatory. The secondary response may also be induced by an increase in the level of substrate alone

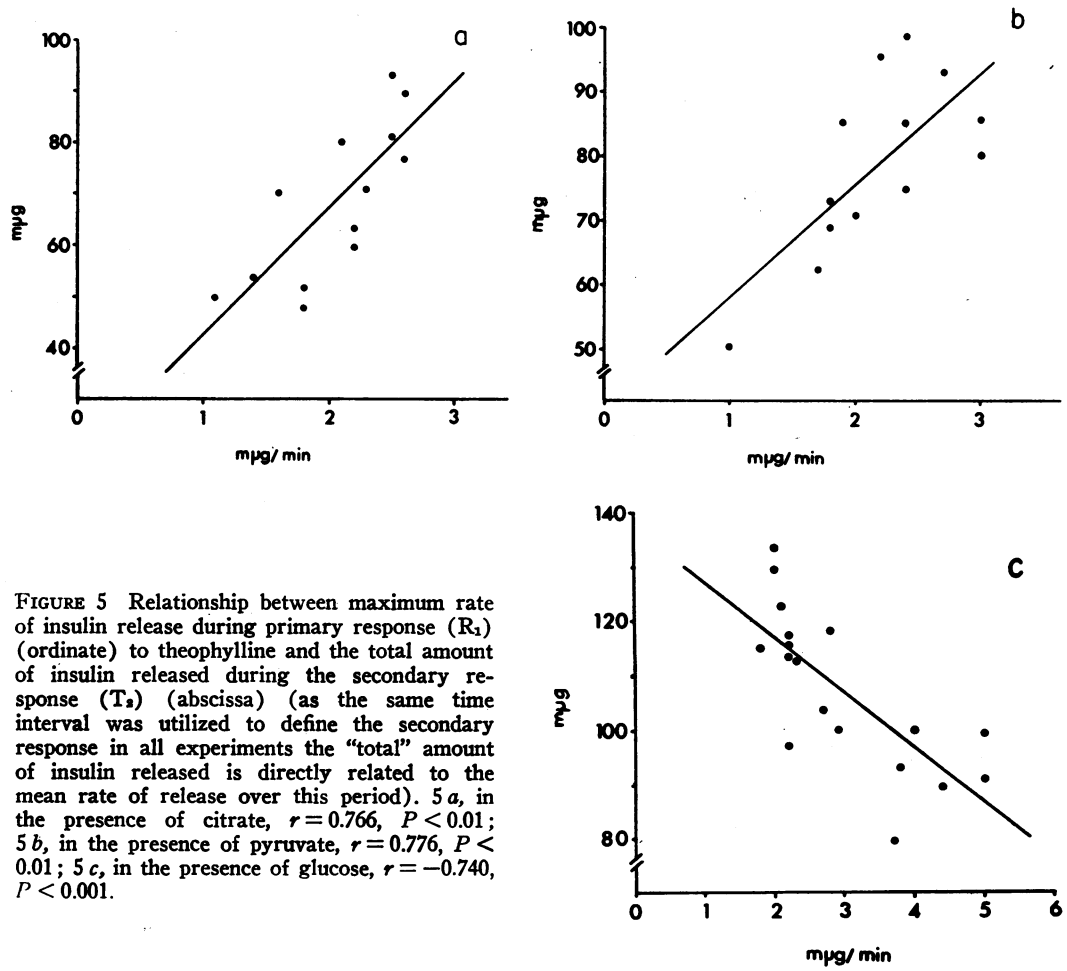

FIGURE 5 Relationship between maximum rate of insulin release during primary response (R_1) (ordinate) to theophylline and the total amount of insulin released during the secondary response (T_2) (abscissa) (as the same time interval was utilized to define the secondary response in all experiments the "total" amount of insulin released is directly related to the mean rate of release over this period). 5 *a*, in the presence of citrate, $r = 0.766$, $P < 0.01$; 5 *b*, in the presence of pyruvate, $r = 0.776$, $P < 0.01$; 5 *c*, in the presence of glucose, $r = -0.740$, $P < 0.001$.

(24, 25). Finally, the magnitude of the secondary response is influenced by the nature of the substrate present in the medium. Clearly, therefore, the secondary IRI release response is related to the metabolism of substrates by the pancreatic β-cell.

In our opinion, the best interpretation of the biphasic nature of IRI release is a metabolic one, the acute or primary response being modulated by the basal state of β-cells and the availability of endogenous energy sources with the delayed or secondary response being to a much greater extent dependent on the availability of exogenous substrate. The observation that the exogenous availability of glucose but not of pyruvate or citrate considerably enhanced the primary response to theophylline supports the notion of a specific role of glucose in the initiation of IRI release in the rat. This observation is unlikely to be due to emphasis on anaerobic glycolysis secondary to relative anoxia of this preparation, as normal physiological responsiveness and histological appearance of the tissue are maintained after more than 2 hr of perifusion. More efficient energy production from glucose is also unlikely as intracellular changes in substrate concentration, and enzyme activity in β-cells in response to glucose are relatively sluggish (31).

While the present observations are compatible with the concept of differential release from two separate insulin storage pools (8, 9), these pools are not necessary to explain these observations, which may equally well be explained on metabolic grounds, as already discussed. Furthermore, the "two-pools concept" does not help in understanding the remarkable reciprocal relationship between primary and secondary IRI release responses when glucose was the available substrate and the theophylline concentration was varied (Fig. 4 and Table I). This reciprocal relationship was equally striking whether the maximum rate of primary IRI release or the total amount of IRI released during the primary phase was related to the amount released during the secondary phase, although the most significant inverse relationship was obtained when using the maximum primary release rate. It is noteworthy that the inverse relationship between primary and secondary IRI release applied only when glucose was the substrate not when it was pyruvate or citrate. Since it is reasonable to assume that increasing theophylline concentrations led to increased accumulation of cAMP, the relationship suggests that the existence of a cAMP related feedback effect (direct or indirect) on early glycolysis or on another pathway of glucose metabolism, which is not shared by pyruvate or citrate, but which is involved in glucose-mediated IRI release. Whether the negative feedback after acute stimulation is metabolic only or whether it involves the influence of one insulin pool upon a second pool is immaterial; in both cases the implication is that the primary and secondary phases of insulin may influence each other.

Although we have repeatedly implied in this discussion that the major variable influenced by theophylline and/or substrates in the present studies was the β-cell concentration of cAMP, it should be clearly understood that this implication remains hypothetical for the present. As yet, preparations providing for isolation of islets or, better, of β-cells and suitable for kinetic studies of insulin release profiles are not available, while the measurement of intermediates, including cAMP, in mixed tissue samples would not yield interpretable data.

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