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RELATIONSHIPS BETWEEN ENERGY LEVEL AND INSULIN SECRETION IN ISOLATED RAT ISLETS OF LANGERHANS

A STUDY AT VARIOUS pH VALUES

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Abstract—To define better the role of [ATP]/[ADP] in insulin release from pancreatic islets, changes in the adenine nucleotide ratios elicited by alterations in external pH were correlated with the secretion profiles produced by administration of two metabolic secretagogues, 16 mM glucose and 10 mM α-ketoisocaproic acid. Experiments were carried out in buffers with and without bicarbonate, in the pH range 6.5–7.7. Insulin release was dependent on pH_e irrespective of the secretagogue used. Secretion profiles for α-ketoisocaproic acid were the same both with and without bicarbonate; the release was decreased below pH 7.1 but maintained at 7.4–7.7. The same pattern was seen with glucose in media buffered with Hepes. With bicarbonate present, secretion caused by high glucose showed a bell-shaped dependence on $[H^+]$, with reductions at the acid and alkaline sides of pH 7.1–7.4. [ATP] and [ADP] were higher when Hepes was the buffer, at all pH values studied. The [ATP]/[ADP] declined with increasing pH under both basal and stimulated conditions; the values were always larger after stimulation although at pH 7.7 with bicarbonate present and glucose as the stimulant the difference was very small. It is concluded that: (i) the [ATP]/[ADP] in pancreatic islets is markedly dependent on pH_e; (ii) there is no straight-forward correlation between either [ATP] or the absolute value for [ATP]/[ADP] and insulin secretion; and (iii) a rise in [ATP]/[ADP] is necessary for glucose-stimulated insulin release although it is not always the rate-determining event.

Glucose-stimulated insulin secretion is a complex sequence of events which occurs in three stages: enhancement of metabolism is followed by depolarization which, in turn, causes Ca^{2+} influx and exocytosis [1, 2]. According to a recent theory, the link between the metabolic (first stage) and ionic (second stage) events is provided by increased [ATP] which closes ATP-dependent K^+ channels and decreases membrane potential [3, 4].

We have shown previously [5] that when the energy level of islets is reduced by perifusion with a buffer containing lowered $[O_2]$, stimulated insulin secretion is attenuated markedly. Our earlier studies [6] have also demonstrated that cellular [ATP]/[ADP] is dependent on pH in the range 6.7–7.6, while other investigators have reported that glucose-elicited insulin release declines at both acid and alkaline sides of pH 7.2–7.4 [7]. Since the mechanism of these decreases remains unknown, the goal of the current study was to determine to what extent pH-dependent changes in [ATP]/[ADP] might contribute to the observed behavior of glucose-stimulated hormone secretion. Moreover, because glucose metabolism involves glycolysis, whereas that of α-ketoisocaproic acid does not, both secretagogues were used in parallel experiments.

MATERIALS AND METHODS

Islet isolation and perifusion

Fed male Wistar rats (200–250 g; Hilltop Laboratory Animals, Scottdale, PA) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Pancreatic islets were isolated using collagenase (Serva, New York, NY) [8] and separated from the digest on Ficoll gradients (Sigma, St. Louis, MO) [9].

Perfusion was carried out using a modification of the method of Weaver *et al.* [10] as described previously [11]. The apparatus was modified to allow rapid removal of islets and minimize the chamber dead space. The upper portion of the Swinnex 13-mm filter holder (Millipore, Bedford, MA) used to secure a Nitex filter (10 μm mesh; Tetko, Elmsfold, NY) was replaced with a retaining ring (o.d. 15 × 3 mm, i.d. 10 × 3 mm; nominal upstream dead volume 0.24 mL), which was secured to the lower portion of the filter holder by a single threaded turn so that it could be removed rapidly by a 360° rotation. The lower portion of the filter holder was unmodified, giving a nominal downstream space of 2.35 mL.

Perifusion experiments

Protocol 1. *Perifusion with bicarbonate-containing media (bicarbonate/CO_2).* Isolated islets were

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cultured for 1 hr in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin in an atmosphere of humidified 95% air and 5% CO_2 at 37°. Batches of 100 islets were loaded into paired chambers. One of the chambers was maintained at pH 7.4 with Krebs bicarbonate buffer (mM: 115 NaCl, 5 KCl, 2.2 $CaCl_2$, 1 $MgCl_2$, 1 Na_2HPO_4, 24 $NaHCO_3$ equilibrated with 95% O_2/5% CO_2 at 37°), supplemented with 0.25% bovine serum albumin, as a control, while the other was perifused with a medium adjusted to a desired pH by manipulating the concentration of bicarbonate. Sodium chloride was altered appropriately to maintain constant osmolarity. The concentrations of bicarbonate were as follows: pH 6.5, 3.04–4.25 mM; pH 6.8, 6.06–6.75 mM; pH 7.1, 13.80 mM; pH 7.7, 66.87 mM. Concentration of H^+ in the media was monitored continuously with a pH electrode. After 40 min of perifusion with a buffer containing 5 mM glucose, stock solutions of glucose (2 M) and α-ketoisocaproic acid (2 M) were added to the medium to attain a final concentration of 16 and 10 mM of each secretagogue, respectively.

Protocol 2. Perifusion with bicarbonate-free media (Hepes/O_2). After 1 hr of culture as described above, each chamber containing 100 islets was perifused with a bicarbonate-free medium equilibrated with 100% O_2 at 37° and containing sodium-Hepes adjusted to the desired pH. The concentration of sodium-Hepes was either 2 or 10 mM, and the results of insulin secretion were the same at both concentrations. The concentration of H^+ was monitored with a pH electrode. The same secretagogues were examined as in Expt. 1.

Measurements of adenine and nucleotides

Two hundred islets were used and perifused as described above. The preparations were quenched after either 45 min of perifusion (control, non-stimulated) or 5 min of stimulation (experimental) using a slight modification of the previous method [11]. Islets were snap-frozen on dry ice and transfered into vials containing 250 μL of 2 M $HClO_4$ at −10°. They were then sonicated with two bursts of 11 pulses and extracted for 10 min in the same solution. The power setting and duty cycle of the sonicator (Heat Systems Sonicator model W-385, Plainview, NY) were 3 and 50%, respectively. The $HClO_4$ extracts were centrifuged at 9000 *g* for 5 min at 4° and the supernatant was neutralized by the addition of 2.5 M K_2CO_3. The potassium perchlorate precipitate was removed by centrifugation in a Beckman Microfuge B for 2 min at 4°. Supernatants were stored at −70°.

Analytic methods

After perifusion, the filters containing adhering islets were removed from the chambers and stored at −70° for determining DNA. These islets were extracted at room temperature in a saline solution (2 M NaCl, 50 mM sodium-phosphate, 1.8 mM EDTA, pH 7.4) by sonicating with 80 pulses from the sonicator with a power setting of 3 and a 50% duty cycle. DNA was measured fluorometrically with Hoechst 33258 dye and calf thymus DNA (Sigma) as standard [12].

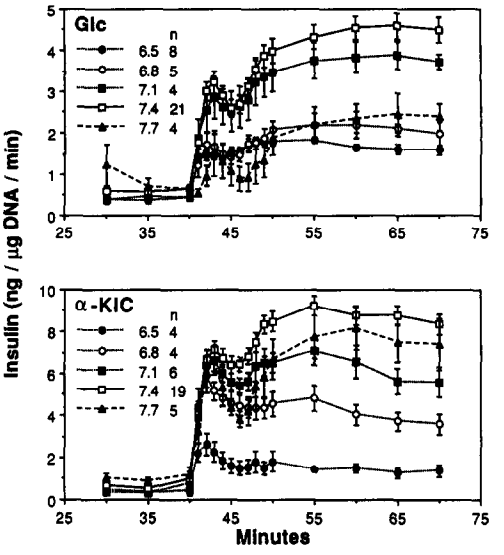

Fig. 1. Secretion profiles for insulin in pancreatic islets perifused with buffers adjusted to indicated pH values with bicarbonate. Islets were isolated and perifused as described in Materials and Methods. Results are means ± SEM for the number of experiments indicated (n) in the figure.

Nucleotides were analyzed by HPLC using an Alltech Associates C18 Adsorbosphere HS column (100 × 4 mm, 3 μm particle size; Deerfield, IL). The pairing reagent was 20 mM tetrabutyl ammonium phosphate in 90 mM $NH_4H_2PO_4$, pH 5.05. Elution was accomplished with a 5 to 36% methanol gradient at a flow rate of 1 mL/min. Nucleotides were detected spectrophotometrically at 260 nm and quantitated by area-to-response factors calculated from standards. Linearity and dynamic response ranges have been described previously together with control experiments for recovery and hydrolysis of the nucleotides [11]. Nucleotide concentrations were calculated using DNA content and the conversion factor of 90 pL internal islet cell water per ng of DNA [11]. In these experiments DNA was measured in two batches of at least 20 islets selected at random from each preparation.

Insulin was measured using a single antibody radioimmunoassay with separation of bound and free ligand by charcoal-dextran precipitation [13]. The standard was rat insulin (Eli Lilly & Co., Indianapolis, IN).

Integration of perifusion data to determine total insulin release was performed using the trapezoid rule. Statistical analysis was performed by *t*-test or multifactor ANOVA using Macintosh Statistical System software (Statsoft, Tulsa, OK).

RESULTS

Insulin secretion in bicarbonate-containing media at various pH *values*

Insulin release was measured at five different pH

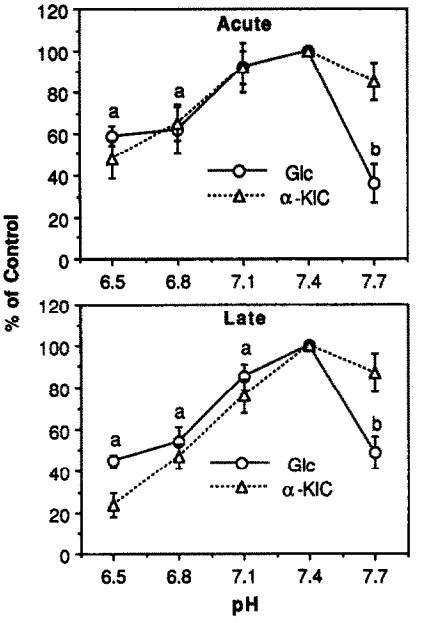

Fig. 2. Effect of $[H^+]$, expressed as a percentage of values at pH 7.4 (100%), on insulin release by islets perifused with bicarbonate buffer and stimulated with either 16 mM glucose or 10 mM α-ketoisocaproic acid. Conditions of perifusion are described in detail in Materials and Methods. The integrated values were used to calculate percentages of paired controls. The numbers of observations per data point are indicated in Fig. 1. Values are means ± SEM. Acute release represents that between 40 and 45 min of perifusion and late that between 45 and 70 min. Control values for the acute and late phases of high glucose-stimulated secretion at pH 7.4 are given in Table 1. The values for secretion when α-ketoisocaproic acid was used for stimulation at pH 7.4 were 142 ± 10 and 359 ± 17 ng/μg DNA for the acute and late phase, respectively. Statistical significance: $P < 0.05$ or better when compared to control (pH 7.4), (a) both secretagogues, and (b) glucose only.

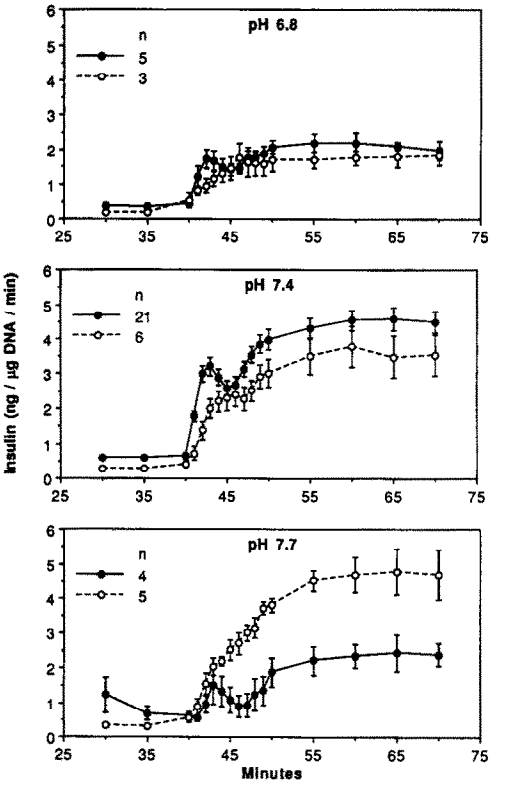

Fig. 3. Profiles of glucose-induced insulin secretion in pancreatic islets perifused with various pH value buffers containing either Hepes (○) or bicarbonate (●). Procedures for perifusion and stimulation are given in Materials and Methods. Secretion profiles designated with closed circles are the same as those in Fig. 1 (Glc). Values are means ± SEM for the number of experiments indicated (n) in the figure.

values: 6.5, 6.8, 7.1, 7.4 and 7.7, using two metabolic secretagogues, glucose (Glc) and α-ketoisocaproic acid. The combined secretion profiles displayed in Fig. 1 demonstrate that release was dependent on external pH in both cases, although there were differences between the two seretagogues tested.

To evaluate the differences more accurately, the two phases of stimulated secretion, the early (acute, 40–45 min), and the late (45–70 min), were quantified separately (Fig. 2). Moreover, to minimize errors arising from daily variations, only paired experiments (i.e. carried out simultaneously) were used for analysis and the results were expressed as percentages of controls, i.e. islets perifused with the medium of pH 7.4. Figure 2 shows that release with high glucose exhibited a bell-shaped dependence on pH, with decreases at both the acid and alkaline sides of pH 7.1–7.4. The largest reduction concerned the acute phase at pH 7.7 which declined to less than 40% of the value at pH 7.4.

Secretion of insulin elicited by α-ketoisocaproic acid had a similar dependence on $[H^+]$ in both the acute and late phases; the amount released was smaller at pH values less than 7.1 but remained constant in the range 7.1–7.7.

Insulin secretion at various pH *values in the absence of bicarbonate*

All experiments described above were carried out in media containing bicarbonate/CO_2 as a buffer. To determine whether or not the nature of the buffer had any effect on secretion, parallel experiments were carried out at the same pH (6.8, 7.4 and 7.7) either with bicarbonate/CO_2 or with Hepes/O_2 in the media. There were no statistically significant buffer-dependent differences in insulin release induced by α-ketoisocaproic acid at the three pH values investigated (data not shown). However, with glucose as the secretagogue the decrease seen at pH 7.7 in the presence of bicarbonate was not observed with Hepes (Fig. 3). Consequently in the latter condition, pH-dependent secretion profile for glucose resembled that obtained with α-keto-isocaproic acid.

To allow for a more accurate comparison of differences obtained in the two buffers with glucose, the results were quantified by integration, and are

Table 1. Effect of buffer on insulin release in islets stimulated with 16 mM glucose at various pH values

pH	Buffer	Insulin release (ng/μg DNA) Basal	Acute	Late	Total
6.8	HCO_3^-/CO_2	4.67 ± 0.60 (14)	6.98 ± 1.14* (5)	49.71 ± 4.81* (5)	60.45 ± 5.19* (5)
	Hepes	3.80 ± 0.61 (11)	5.16 ± 1.00 (3)	42.96 ± 7.09* (3)	50.77 ± 8.71* (3)
7.4	HCO_3^-/CO_2	6.15 ± 0.40 (57)	12.51 ± 0.94 (21)	104.88 ± 6.95 (21)	123.31 ± 8.06 (21)
	Hepes	4.96 ± 0.82 (17)	7.65 ± 1.08† (6)	83.08 ± 12.20 (6)	93.77 ± 13.29 (6)
7.7	HCO_3^-/CO_2	8.78 ± 12.5* (14)	5.18 ± 1.36* (4)	51.40 ± 9.41* (4)	64.67 ± 12.43* (4)
	Hepes	5.62 ± 0.94† (16)	8.16 ± 0.96 (5)	106.86 ± 10.23† (5)	118.83 ± 10.78† (5)

Conditions of perifusion are described in detail in Materials and Methods. Values are means ± SEM for the number of experiments in parentheses. The time frames for the secretion ranges are defined as follows: basal, 30–40 min; acute, 40–45 min; late, 45–70 min and total, 30–70 min.

*,† Statistical analysis, $P < 0.05$ or better: *same buffer with respect to the value at 7.4; and †buffer at the same pH.

displayed in Table 1. The table also shows that basal (i.e. non-stimulated) release in the Hepes-buffered media had very little dependence on external pH. Non-stimulated secretion in a bicarbonate-containing solution was not significantly different at pH 6.8 and 7.4 but increased at pH 7.7.

Effect of pH *on energy level of pancreatic islets*

The levels of ATP and ADP were measured in islets perifused at various pH values either with or without bicarbonate, and before and after stimulation with glucose or α-ketoisocaproic acid. These results, together with the calculated [ATP]/[ADP], are presented in Table 2 and Fig. 4. It can be seen that the concentrations of ATP and ADP, and consequently their sums, were considerably larger in islets perifused with buffers containing no bicarbonate, at all pH values tested (Table 2). Addition of 16 mM glucose caused a rise in [ATP] and a fall in [ADP]. Although changes in the concentrations of individual nucleotides were relatively small, they were quite consistent. The increases in [ATP]/[ADP] upon addition of secretagogues were substantial and statistically significant. The absolute values of [ATP]/[ADP] ratios, both before and after stimulation, were somewhat higher in islets perifused with Hepes buffer than in those perifused with bicarbonate. The sums of ATP and ADP were not significantly different at various pH values tested when media contained the same buffer. By contrast the [ATP]/[ADP] progressively declined as the $[H^+]$ was decreased in both buffers used. This reduction was observed in non-stimulated as well as stimulated islets and irrespective of the secretagogues used. A notable observation was that addition of 16 mM glucose to islets perifused with the bicarbonate buffer at pH 7.7 resulted in only a small rise in [ATP]/[ADP]: from 4.08 to 5.74.

DISCUSSION

The results presented in this paper lead to the following conclusions: (1) The content of adenine nucleotides in perifused islets depends on the buffer used for perifusion; it is lower in a bicarbonate-containing than in a Hepes-buffered medium. (2) The [ATP]/[ADP] in perifused islets is markedly dependent on external pH in the range 6.5–7.7 and changes irrespective of the buffer used. (3) Insulin release obtained with 5 mM glucose (basal) correlates neither with [ATP] nor with the absolute value of [ATP]/[ADP]. (4) Stimulation of insulin secretion by metabolic secretagogues is always accompanied by an elevation in [ATP]/[ADP]. However a rise in [ATP]/[ADP], albeit necessary, might not always be the limiting event for the stimulated response.

Before discussing our results in some detail we wish to point out that although all events studied in this work could be influenced predominantly, if not exclusively, by internal $[H^+]$, the variable that was monitored and modulated is external pH. The internal $[H^+]$ was measured under very similar conditions by other authors and the results obtained are directly relevant to the current study. They can be briefly summarized as follows: (i) pH_i in bicarbonate-containing buffers is higher than in the absence of bicarbonate although the difference seems to be less than 0.1 pH unit [14]. (ii) pH_i changes as a function of external pH, and in the same direction albeit by a lesser amount [15]. This is consistent with studies on other cells (see, for example, Ref. 6). (iii) Addition of 16 mM glucose or 10 mM α-ketoisocaproate causes a slow and very small internal alkalinization [16]. In the absence of bicarbonate no change in internal pH is observed upon infusion of high glucose [14].

An unexpected finding of the present study was that [ATP] and [ADP] were considerably larger in islets perifused without bicarbonate and attained values close to those measured in fresh non-perifused islets [11]. The 2-fold differences seen at all pH values tested were statistically significant (Table 2) and were unlikely to arise from experimental inadequacies. The reduction in [ATP] and [ADP] required the presence of bicarbonate but was not dependent on its concentration in the range 3 to 67 mM. This is evidenced by the constancy of the sum of the two nucleotides at the pH values inspected (Table 2). This phenomenon is especially surprising because bicarbonate/carbon dioxide is the physiologic buffer and one would expect that it better

Table 2. Effect of pH on nucleotide levels and [ATP]/[ADP] in islets stimulated with 16 mM glucose (Glc)

pH	Treatment		N	ATP (mM)	ADP (mM)	ATP + ADP (mM)	[ATP]/[ADP]
6.8	Control	(A)	6	1.45 ± 0.07	0.19 ± 0.01	1.64 ± 0.08	7.59 ± 0.11*
		(B)	3	3.14 ± 0.17†	0.39 ± 0.04	3.53 ± 0.20†	8.15 ± 0.43
	Glc	(A)	5	1.65 ± 0.05‡	0.16 ± 0.01	1.81 ± 0.05	10.28 ± 0.55*‡
		(B)	3	3.25 ± 0.20†	0.25 ± 0.01	3.50 ± 0.20†	12.98 ± 1.00†‡
7.4	Control	(A)	5	1.53 ± 0.07	0.27 ± 0.01	1.80 ± 0.07	5.73 ± 0.13
		(B)	3	2.68 ± 0.26†	0.40 ± 0.03	3.07 ± 0.28†	6.71 ± 0.34†
	Glc	(A)	5	1.67 ± 0.10	0.22 ± 0.02	1.89 ± 0.12	7.70 ± 0.14‡
		(B)	3	3.36 ± 0.42†	0.33 ± 0.05	3.69 ± 0.47†	10.45 ± 0.49†‡
7.7	Control	(A)	3	1.24 ± 0.03*	0.31 ± 0.03	1.55 ± 0.03*	4.08 ± 0.44*
		(B)	3	2.03 ± 0.14†	0.36 ± 0.02	2.39 ± 0.17†	5.58 ± 0.03*†
	Glc	(A)	6	1.47 ± 0.08	0.26 ± 0.01	1.73 ± 0.09	5.74 ± 0.24*‡
		(B)	3	3.34 ± 0.09†‡	0.42 ± 0.03	3.76 ± 0.12†‡	8.01 ± 0.32*†‡

The pH of (A) was adjusted with $NaHCO_3$, while the pH of (B) was adjusted with Hepes-sodium (without $NaHCO_3$). Conditions of perifusion and analytical techniques for measurement of nucleotides are described in detail in Materials and Methods. Values are means ± SEM for the number (N) of experiments indicated.

*–‡ Statistical analysis, $P < 0.05$ or better: *same buffer with respect to the value at 7.4, †buffer at the same pH, and ‡high glucose at the same pH.

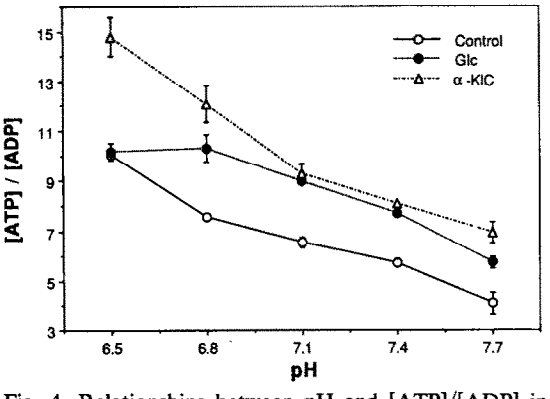

Fig. 4. Relationships between pH and [ATP]/[ADP] in islets perifused with bicarbonate/CO_2 buffer. Nucleotides were measured as described in Materials and Methods. Values are means ± SEM of results from at least three experiments. Values for controls and high glucose at pH 6.8, 7.4 and 7.7 are the same as in Table 2. In most cases the SEM bars were smaller than the symbols and, therefore, do not appear on the graph.

preserves the internal environment. At present we have no explanation for the finding. We wish, however, to point out that because nucleotide tri- and diphosphates do not "leak" across the intact plasma membrane, our observation seems to suggest that bicarbonate stimulates nucleotide catabolism and/or facilitates adenine/adenosine loss from islets.

The [ATP]/[ADP] ratios, but not the adenine nucleotide levels, were markedly dependent on external pH and changed in both buffers. The ratios were much higher at acid pH and declined when $[H^+]$ was lowered. Moreover, they were larger in islets perifused with solutions containing Hepes as the only buffer which is consistent with the lower internal pH measured under such conditions [14]. The decrease in [ATP]/[ADP] with increasing pH was perhaps not unexpected because H^+ is a product in the ATP hydrolysis reaction but it could also suggest that the free energy change for ATP hydrolysis, ΔG_{ATP}, decreases with a fall in $[H^+]$. However, ΔG_{ATP} is a sum of two terms, the standard free energy change, $\Delta G'^{\circ}_{ATP}$, and the concentration ratio, $RT\ln[ATP]/[ADP][P_i]$. $\Delta G'^{\circ}_{ATP}$ is also pH dependent and decreases when $[H^+]$ rises [17, 18]. Thus, the reduction in [ATP]/[ADP] with increasing pH may provide the means whereby the free energy available from ATP hydrolysis (ΔG_{ATP}) remains essentially constant. This behavior is very similar to that observed previously in isolated hepatocytes [6] and indicates that the same regulatory processes might be operating in the two types of cells. The occurrence of the same phenomenon in diverse systems may suggest that it represents an important principle of cellular homeostasis.

The postulate that a metabolically-evoked increase in [ATP] causes closure of the ATP-dependent K^+-channels [3, 4] involves the assumption that there is a "critical" concentration of the trinucleotide at which such transition occurs. We have shown previously [5], and confirmed it in this study (Table 2), that the experimentally measured increases in [ATP] which follow the addition of high glucose or α-ketoisocaproate are very small. This would suggest that the range of "critical" trinucleotide concentrations must be very narrow. Such a suggestion is difficult to reconcile with the finding that a 2-fold rise in [ATP], which was observed in islets perifused without bicarbonate (Table 2), did not augment basal insulin release, whereas with bicarbonate present, metabolic secretagogues induced massive hormone release at a substantially lower nucleotide level. The unavoidable conclusion is that there is no correlation between the absolute value of [ATP] and insulin release.

As a result of previous experiments carried out at various oxygen tensions [5], we have postulated that stimulation of insulin secretion by metabolic secretagogues is linked to a *rise* in islet [ATP]/[ADP]. Although in the current study we found little correlation between the *absolute* value of [ATP]/[ADP] and basal insulin secretion, nevertheless in all situations investigated an increase in the nucleotide ratio was necessary for stimulated hormone release. Moreover, at all pH values, augmentation in [ATP]/[ADP] was larger with high α-ketoisocaproate than with high glucose and was accompanied by larger secretion of insulin. Similarly a very small rise in the nucleotide ratio seen in islets perifused with a bicarbonate-containing buffer at pH 7.7 and stimulated with 16 mM glucose is coincident with an insignificant hormone release (Tables 1 and 2).

A careful analysis of our results leads to a somewhat unexpected conclusion. The relative constancy of basal insulin release in the pH range investigated (Table 1) correlates with an apparent constancy of ΔG_{ATP}, whereas stimulation of insulin release by high glucose or α-ketoisocaproate occurs simultaneously with a rise in ΔG_{ATP}. Although this may be a coincidence, it is worth pointing out that one of the unexplained observations in the area of islet physiology is a "phosphate flush," an efflux of inorganic phosphate into the external medium, which accompanies addition of metabolic secretagogues [19, 20]. Since a decrease in internal phosphate is synonymous with a rise in ΔG_{ATP}, the possibility that there is a step on the overall secretory pathway which is dependent on free energy of ATP hydrolysis deserves further study.

Finally, it has to be pointed out that if an increase in [ATP]/[ADP], or equivalent, is necessary for insulin release to occur, it is not always rate-limiting. An example of such a situation was stimulated secretion at pH below 7.0. The decline that was observed under such conditions must have been caused by other pH-dependent events that are part of the overall secretion pathway. It is possible that inhibition of movements of ions such as K^+ or Ca^{2+} by H^+, a well-known event [15, 16, 21–25], is responsible for curtailing secretion when $[H^+]$ is high.

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