Effects of Caffeine on Gluconeogenesis and Urea Synthesis Induced by α -Adrenergic Stimulation in Suspensions of Rat Hepatocytes

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Abstract Effects of caffeine on gluconeogenesis and urea synthesis of rat isolated hepatocytes were investigated in the presence of hormonal agonists. Phenylephrine at $10\ \mu\text{M}$ stimulated 1.7-fold gluconeogenesis and 1.9-fold (compared to control) urea synthesis from 4 mM glutamine. Stimulative effects of caffeine in the range from 0.1 to 10 mM were biphasic depending on its concentration, and it showed maxima at about 1 mM. Caffeine at 1 mM stimulated 2.1-fold gluconeogenesis and 2.4-fold urea synthesis. Caffeine without phenylephrine did not stimulate both syntheses. These effects of caffeine and phenylephrine diminished in the absence of extracellular Ca^{2+} . Results on uptake of $^{45}\text{Ca}^{2+}$ into hepatocytes and change in quin-2 fluorescence indicated that phenylephrine induced Ca^{2+} influx into the cell and consequently increased the intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]$, and that the addition of caffeine did not further stimulate the effect of phenylephrine on $[\text{Ca}^{2+}]$. Therefore, we suggest that stimulation of gluconeogenesis and urea synthesis by phenylephrine is due to increase in $[\text{Ca}^{2+}]$. Since caffeine is known to inhibit phosphodiesterase, the additional stimulation of both syntheses by caffeine plus phenylephrine may be due to the synergistic effect of increases in cAMP and $[\text{Ca}^{2+}]$. The increase in the rates of gluconeogenesis and urea synthesis similarly depended on the caffeine concentration. Furthermore, the ratio of [acetoacetate]/[3-OH-butyrate] which shows intramitochondrial redox state, also depended on the caffeine concentration, indicating a possible coupling of the redox function of mitochondria with $[\text{Ca}^{2+}]$.

Key words: caffeine, gluconeogenesis, urea synthesis, cellular energy metabolism, isolated hepatocytes.

In hepatocytes, hormones such as α -adrenergic agonists, vasopressin, and angiotensin II cause an increase in cytosolic $[Ca^{2+}]$, inducing various cellular functions (WILLIAMSON *et al.*, 1981; EXTON, 1985). Two different sources for the increase have been proposed: Ca^{2+} release from intracellular pools (CHEN *et al.*, 1978; EXTON, 1981; WILLIAMSON *et al.*, 1981), and Ca^{2+} influx from the extracellular medium (ASSIMACOPOULOS-JEANNET *et al.*, 1977; GARRISON and BORLAND, 1979; MAUGER *et al.*, 1984). Methylxanthine derivatives such as theophylline and 3-isobutyl-1-methylxanthine were reported to enhance the α -adrenergic stimulation of glucose output (CHAN and EXTON, 1978; FORSTER and BLAIR, 1978).

Caffeine is also a methylxanthine derivative and an important ingredient of tea and coffee. Effects of caffeine on gluconeogenesis and urea synthesis in hepatocytes have not been reported, although several studies showed that caffeine strongly potentiated the glucose stimulation of glycogen synthase phosphatase reaction (GILBOE, 1986) and that it did not affect the ATP dependent Ca^{2+} uptake into mitochondrial and endoplasmic reticulum pools (BURGESS *et al.*, 1983).

Here, we studied effects of caffeine on both gluconeogenesis and urea synthesis from glutamine in rat isolated hepatocytes. Glutamine uptake into hepatocytes has been suggested not to be rate-limiting for these syntheses (JOSEPH and MCGIVAN, 1978; OCHS and LARDY, 1983; KASHIWAGURA *et al.*, 1985). Furthermore, we also studied effects of caffeine on cellular energy metabolism, that is, respiratory rate, mitochondrial $[NAD^+]/[NADH]$ and phosphorylation potential.

MATERIALS AND METHODS

Isolation of liver cells. Liver cells were prepared by the method of BERRY and FRIEND (1969) with modifications (CORNELL *et al.*, 1973) from 48-h starved male rats of the Wistar strain, weighing 170–220 g. Rats were anesthetized with pentobarbital (5 mg/100 g). Collagenase (0.4 mg/ml) was dissolved in Krebs-Henseleit solution (KREBS and HENSELEIT, 1932). The flow rate of oxygenated perfusion solution during collagenase infusion was 40 ml/min at 37°C. Isolated liver cells were suspended in Krebs-Henseleit saline supplemented with 2% bovine serum albumin. The suspension of cells was maintained at room temperature by gentle shaking under a 95% O_2 : 5% CO_2 atmosphere.

Incubation of liver cells. A 2 ml cell suspension (about 40 mg wet weight/ml of cells) was mixed with 2 ml of Krebs-Henseleit solution containing 8 mM glutamine (final 4 mM) and 2% albumin. When caffeine (≤ 10 mM) was added, the isotonicity of the cell suspension mixture was not readjusted. However, it was confirmed separately that the osmotic effect was negligibly small, in which experiment hypertonicity was generated by choline Cl. The mixture was incubated in a 50 ml Erlenmeyer flask with shaking (100–110 cycle/min) for 45 min at 37°C. The pH of the cell suspension was adjusted to 7.4 or an indicated value with CO_2 -bicarbonate or 2-[N-morpholino]ethanesulfonic acid (MES)- CO_2 -bicarbonate buffer. The gas phase was 95% O_2 : 5% CO_2 . In some experiments, the pH of the reaction medium

was quickly measured before quenching.

Reactions in cells were quenched by injecting 0.4 ml of 40% perchloric acid at the end of incubation. The flask containing samples was placed on ice. Then samples were centrifuged to remove insoluble material. The supernatant was neutralized with 2 M K_2CO_3 -0.58 M triethanolamine and used for assay of metabolites. The dry weight of the cell suspension was determined for each preparation. A factor of 3.5 was used to convert dry weight into wet weight of cells (KASHIWAGURA *et al.*, 1985).

Measurements of metabolites. We measured ATP enzymatically by the method of LAMPRECHT and TRAUTSCHOLD (1974), ADP by that of JAWOREK *et al.* (1974), glucose by that of BERGMAYER *et al.* (1974), urea by that of GUTMANN and BERGMAYER (1974), and acetoacetate and 3-OH-butyrate by that of WILLIAMSON *et al.* (1962).

Measurements of cellular respiration. The rate of oxygen consumption by cells was measured at 37°C with a Clark-type oxygen electrode (Rank Brothers).

Determination of inorganic phosphate. Cells were rapidly passed by centrifugation through a silicone oil layer (specific gravity 1.05, Shin-etsu Silicone K-45) into a quenching solution containing 4% HClO_4 and 10% NaCl. Phosphate in the quenching solution was measured by the method of YODA and HOKIN (1970).

Measurements of $^{45}\text{Ca}^{2+}$ influx. The influx rate of Ca^{2+} into hepatocytes was measured by the method of MAUGER *et al.* (1984). Briefly, the cells were preincubated for 5 min at 37°C in Krebs-Henseleit solution containing 1.3 mM Ca^{2+} and 2% bovine serum albumin. A 250 μl volume of the cell suspension was mixed with the same volume of Krebs-Henseleit solution containing 1.3 mM Ca^{2+} , 2% bovine serum albumin, 8 mM glutamine, and 2 $\mu\text{Ci/ml}$ of $^{45}\text{CaCl}_2$ with a constant gas phase (95% O_2 , 5% CO_2). The amount of $^{45}\text{Ca}^{2+}$ taken in cells was determined at 15, 45, 75, and 105 s. Each 100 μl of sample was rapidly filtered through a Whatman GF/C glass filter. Then the filter was washed 3 times with 4 ml of the ice-cold washing solution containing 144 mM NaCl, 5 mM CaCl_2 , and 5 mM Tris-HCl (pH 7.4). Care was taken that the filter did not contact with air before the termination of the washing procedure. Then the radioactivity in the filter was counted.

Measurements of intracellular Ca^{2+} by quin-2/AM. Hepatocytes (45–50 mg wet weight/ml) was suspended in Krebs-Henseleit bicarbonate buffer containing 2% bovine serum albumin and 1.3 mM Ca^{2+} . They were preincubated at 37°C for 15 min with constant gassing (95% O_2 , 5% CO_2). Then they were incubated with 100 μM quin-2-tetra(acetoxymethyl) ester (quin-2/AM) for 15 min at 37°C. They were washed three times by centrifugation at $50 \times g$ for 2 min and diluted to 12–15 mg wet weight/ml in the fresh Krebs-Henseleit bicarbonate buffer. The resuspended cells were incubated for 10–15 min at 37°C prior to use in fluorescence measurements. Control hepatocytes were similarly treated without quin-2/AM. Change in the intensity of the free tetraanion (quin-2) of quin-2/AM fluorescence was measured at an excitation wavelength of 340 nm (1 nm slit) and an emission wavelength of 492 nm (5 nm slit) using a Hitachi Model 650-10 spectrofluorometer fitted with a magnetic stirrer and a thermostatted cell holder. The results were expressed as the

change in fluorescence (mV) from a base line in an arbitrary unit. The cell viability, as assessed by trypan blue exclusion test, was more than 90%.

Reagents. Collagenase was obtained from Wako Pure Chem. Ind. Co., enzymes (hexokinase Type IV, glucose-6-phosphate dehydrogenase, urease, and 3-OH-butyrate dehydrogenase) from Sigma, enzymes (glutamate dehydrogenase, lactate dehydrogenase, and pyruvate kinase) from Boehringer Mannheim, quin-2/AM from Dojindo Laboratories, and $^{45}\text{CaCl}_2$ from Amersham.

RESULTS

The effect of caffeine on gluconeogenesis and urea synthesis in the presence of phenylephrine

Gluconeogenesis and urea synthesis from glutamine in hepatocytes have been found to be very sensitive to action of various effectors (JOSEPH and MCGIVAN, 1978; KASHIWAGURA *et al.*, 1985). Therefore, we used 4 mM glutamine as a substrate in all experiments. Figure 1A and B show effects of caffeine on glucose and urea syntheses in the presence of 10 μM phenylephrine at pH 7.4. In both syntheses, stimulative effects of caffeine were biphasic depending on its concentration, and it showed maxima at about 1 mM. For studying the mechanism of stimulating effects of caffeine, we chose 1 mM caffeine in subsequent experiments. It was also found that caffeine per se did not disturb the enzymatic assay systems of various metabolites.

Effect of extracellular Ca^{2+} on the effect of caffeine

In the presence of 1.3 mM Ca^{2+} , 10 μM phenylephrine increased 1.7-fold the rate of gluconeogenesis and 1.9-fold that of ureagenesis compared to the control (Fig. 2). Caffeine alone did not stimulate both syntheses; however, caffeine (1 mM) plus phenylephrine (10 μM) further stimulated them. The effects of phenylephrine and phenylephrine plus caffeine were abolished by 0.3 μM prazosin, α -antagonist (data not shown), indicating that the effect of caffeine was manifested through α -agonistic effect of phenylephrine. Dibutyryl cAMP (Bt_2cAMP) alone stimulated 1.4-fold both production; however, Bt_2cAMP plus caffeine induced no further stimulation.

In the absence of Ca^{2+} , the stimulating effects of phenylephrine and phenylephrine plus caffeine diminished completely (Fig. 2). The control rates of gluconeogenesis and ureagenesis were 0.31 ± 0.01 and $0.52 \pm 0.03 \mu\text{mol}/(\text{min} \cdot \text{g wet weight})$, respectively.

Ca^{2+} influx in the presence and absence of caffeine

Uptake of $^{45}\text{Ca}^{2+}$ into hepatocytes was measured in the presence of phenylephrine, caffeine, or both (Fig. 3A). Caffeine alone was not effective. Phenylephrine greatly stimulated $^{45}\text{Ca}^{2+}$ uptake. No further stimulation was induced by phenylephrine plus caffeine. The rate of $^{45}\text{Ca}^{2+}$ uptake after the initial lag time of about 45 s was 12 pmol/(min $\cdot$ g wet weight) for control, 17 for 1 mM caffeine, 60 for 10 μM

phenylephrine, and 62 for $10\ \mu\text{M}$ phenylephrine plus $1\ \text{mM}$ caffeine.

Measurements of quin-2 fluorescence gave similar results (Fig. 3B). Phenylephrine at $10\ \mu\text{M}$ induced a large fluorescence change. The change was mainly

Fig. 1. The effect of caffeine in the presence of $10^{-5}\ \text{M}$ phenylephrine on glucose (A) and urea (B) production from $4\ \text{mM}$ glutamine. Values are expressed as percentages of controls. Hepatocytes were incubated for 45 min at 37°C , at $18\text{--}25\ \text{mg}$ wet weight/ml in Krebs-Henseleit buffer containing $4\ \text{mM}$ glutamine and 2% bovine serum albumin. Aliquots of the cell suspension were quenched by addition of cold HClO_4 , rapidly cooled, and then further treated as described under MATERIALS AND METHODS. Each point is the mean of duplicate determinations in one typical experiment. Dotted lines show the levels in the absence of caffeine.

Fig. 2. The effect of Ca^{2+} on glucose (A) and urea (B) synthesis in the presence of various effectors. The cells were suspended as described in the legend of Fig. 1. $\text{Ca}(+)$ denotes medium containing 1.3 mM extracellular calcium and $\text{Ca}(-)$ denotes medium without Ca^{2+} . PE, phenylephrine; CAF, caffeine; and Bt_2cAMP , dibutyryl cAMP. The results are represented as mean (% of control) values $\pm$ S.E. for five separate determinations. * $p < 0.05$ with respect to the result of PE (t statistic for two means).

due to Ca^{2+} influx from the extracellular medium (1.3 mM Ca^{2+}), since corresponding change was very small in the absence of extracellular Ca^{2+} (data not shown). Caffeine in the absence of phenylephrine did not increase the intensity of fluorescence. Furthermore, caffeine did not stimulate the effect of phenylephrine.

Fig. 3. A: the time course for uptake of $^{45}\text{Ca}^{2+}$ into rat hepatocytes. $^{45}\text{Ca}^{2+}$ ($2 \mu\text{Ci}/\text{ml}$) were added into the suspension of hepatocytes ($20 \text{ mg wet weight}/\text{ml}$) at zero time. Samples were taken at the indicated time, washed and counted for radioactivity as described in the MATERIALS AND METHODS. Effectors such as phenylephrine (PE) and caffeine were added together with $^{45}\text{Ca}^{2+}$. Each point is the mean values $\pm$ S.E. B: the effect of phenylephrine and caffeine on fluorescence of quin-2. Quin-2-loaded hepatocytes were prepared as described under MATERIALS AND METHODS. The results are presented as change in the intensity of fluorescence in an arbitrary unit against time. At arrows, agents were added.

Fig. 4. Effects of phenylephrine and caffeine on glucose (A) and urea (B) production from 4 mM glutamine at various extracellular pH. Hepatocytes were incubated for 45 min with and without added agonists. The pH of the medium was adjusted by varying $[\text{HCO}_3^-]$ or, at more acid pH values, by using a MES- CO_2 -bicarbonate buffer.

Effects of extracellular pH on the effect of caffeine

Results described above were obtained at pH 7.4. Figure 4A and B show effects of pH on glucose and urea syntheses from 4 mM glutamine in the presence of phenylephrine, caffeine, or both. Glucose production showed a maximum at pH 7.5–7.6 and urea synthesis at pH 7.3–7.6. The former declined by 50% at pH 6.7–6.8 on the acid side of the maximum and at pH 8.0 on the alkaline side. The 50% decreases for ureagenesis occurred at pH 6.3 on the acid side and pH 7.9 on the alkaline side.

The effect of caffeine on the phosphorylation potential

Table 1 shows [ATP]/[ADP] ratios, concentrations of intracellular inorganic phosphate, and phosphorylation potentials in various conditions. The [ATP]/[ADP] ratio in the presence of 10 μ M phenylephrine plus 1 mM caffeine was not significantly different from the control. The ratios in the various conditions listed in the table ranged from 4.5 to 6.0. The concentration of inorganic phosphate (2.2–2.9 mM) was the same as the control. Therefore, the phosphorylation potential ($[ATP]/[ADP][P_i]$) was nearly constant and was in the range from 1.7×10^{-3} to $2.4 \times 10^{-3} \text{ M}^{-1}$. These results show that the phosphorylation potentials in the various conditions were almost constant.

The effect of caffeine on the respiratory rate

The control levels of the respiratory rate in the presence and absence of Ca^{2+} were 5.07 ± 1.32 and $4.96 \pm 0.94 \mu\text{mol}/(\text{min} \cdot \text{g wet weight})$, respectively. Caffeine

Table 1. The phosphorylation values in the presence of various effectors.

Treatment	$\frac{[ATP]}{[ADP]}$	$[P_i]$ (mM)	$\frac{[ATP]}{[ADP][P_i]}$ (M^{-1})
Control (+)	4.64 ± 0.17 (11)	2.49 ± 0.16 (4)	1.86×10^3
Control (–)	4.53 ± 0.32 (5)	2.40 ± 0.07 (4)	1.89×10^3
10 μ M PE (+)	4.79 ± 0.20 (8)	2.16 ± 0.06 (4)	2.22×10^3
10 μ M PE (–)	4.65 ± 0.18 (3)	2.24 ± 0.14 (4)	2.08×10^3
10 μ M PE + 1 mM CAF (+)	4.96 ± 0.15 (8)	2.94 ± 0.12^a (4)	1.69×10^3
10 μ M PE + 1 mM CAF (–)	4.69 ± 0.24 (3)	2.63 ± 0.33 (4)	1.78×10^3
50 μ M Bt ₂ cAMP (+)	5.91 ± 0.26^a (3)	2.70 ± 0.10 (4)	2.19×10^3
50 μ M Bt ₂ cAMP (–)	5.39 ± 0.24 (3)	2.27 ± 0.16 (4)	2.37×10^3
50 μ M Bt ₂ cAMP + 1 mM CAF (+)	4.86 ± 0.27 (3)	2.55 ± 0.09 (4)	1.91×10^3
50 μ M Bt ₂ cAMP + 1 mM CAF (–)	4.75 ± 0.41 (3)	2.20 ± 0.10 (4)	2.16×10^3
1 mM CAF (+)	4.57 ± 0.74 (3)	2.39 ± 0.24 (4)	1.91×10^3
1 mM CAF (–)	4.63 ± 0.55 (3)	2.31 ± 0.26 (4)	2.00×10^3

^a $p < 0.05$ with respect to control (t statistics for two means). (+), with extracellular 1.3 mM Ca^{2+} ; (–), without extracellular Ca^{2+} ; PE, phenylephrine; CAF, caffeine; Bt₂cAMP, dibutyryl cAMP. Data are the mean $\pm$ S.E. (number of experiments).

Table 2. The effect of caffeine on oxygen consumption in hepatocytes incubated with 4 mM glutamine.

Treatment	Respiratory rate (%)
Control (+)	100
Control (−)	100
10 μ M PE (+)	118 $\pm$ 2 ^a (3)
10 μ M PE (−)	100 $\pm$ 4 (3)
10 μ M PE + 1 mM CAF (+)	131 $\pm$ 2 ^{a,b} (3)
10 μ M PE + 1 mM CAF (−)	108 $\pm$ 2 (3)
1 mM CAF (+)	107 $\pm$ 2 ^a (3)
1 mM CAF (−)	105 $\pm$ 1 (3)

Hepatocytes (18–25 mg wet weight/ml) were preincubated for 10 min with each of the indicated effectors at 37°C. Aliquots were then transferred to a sealed thermostated cuvette and the rate of oxygen uptake was monitored with a Clark-type oxygen electrode for the next 10–15 min. ^a $p < 0.05$ with respect to control (t statistics for two means). ^b $p < 0.05$ with respect to 10 μ M PE. (+), with extracellular 1.3 mM Ca^{2+} ; (−), without extracellular Ca^{2+} ; PE, phenylephrine; CAF, caffeine. Data are the mean $\pm$ S.E. (number of experiments).

Table 3. The effect of caffeine on redox states of intramitochondria in the presence of various effectors.

Treatment	$\frac{[\text{acetoacetate}]}{[\text{3-OH-butyrate}]}$
Control (+)	5.58 $\pm$ 0.42 (6)
Control (−)	5.29 $\pm$ 0.36 (4)
10 μ M PE (+)	3.96 $\pm$ 0.44 ^a (5)
10 μ M PE (−)	4.91 $\pm$ 0.33 (4)
10 μ M PE + 1 mM CAF (+)	2.24 $\pm$ 0.31 ^{a,b} (5)
10 μ M PE + 1 mM CAF (−)	5.35 $\pm$ 0.37 (4)
50 μ M Bt_2cAMP (+)	2.13 $\pm$ 0.45 ^a (3)
50 μ M Bt_2cAMP (−)	4.27 $\pm$ 0.07 ^a (3)
50 μ M Bt_2cAMP + 1 mM CAF (+)	2.22 $\pm$ 0.51 ^a (3)
50 μ M Bt_2cAMP + 1 mM CAF (−)	4.82 $\pm$ 0.88 (3)
1 mM CAF (+)	6.10 $\pm$ 0.88 (3)
1 mM CAF (−)	6.06 $\pm$ 0.35 (3)

For measurements of [acetoacetate]/[3-OH-butyrate] ratio, hepatocytes were incubated as described in the legend of Fig. 1 and metabolites were assayed as described under MATERIALS AND METHODS. Values are mean $\pm$ S.E. (number of experiments). ^a $p < 0.05$ with respect to control (t statistics for two means) and ^b $p < 0.05$ with respect to PE in paired experiments. Notations and abbreviations are the same as in the legend of Table 1.

Fig. 5. Effects of caffeine on the ratio of [acetoacetate]/[3-OH-butyrate] in the presence of 10^{-5} M phenylephrine. The dotted line shows the control and the broken line shows the level at $10 \mu\text{M}$ phenylephrine.

slightly stimulated the respiratory rate (Table 2). Phenylephrine in the presence of Ca^{2+} stimulated 1.18-fold oxygen consumption compared to control. Phenylephrine plus caffeine stimulated 1.31-fold the rate. However, this effect of phenylephrine diminished in the absence of extracellular Ca^{2+} .

The effect of caffeine on intramitochondrial redox state

Measurements of 3-OH-butyrate and acetoacetate in hepatocytes showed that phenylephrine plus caffeine decreased the ratio of [acetoacetate]/[3-OH-butyrate] from 5.58 (control) to 2.24 (Table 3). This result indicated that the intramitochondrial redox state shifted to more reductive compared to control. However, the ratio did not change in the absence of Ca^{2+} . Caffeine alone shifted slightly the redox state to oxidative. Bt_2cAMP shifted the redox state to reductive in the presence of Ca^{2+} .

Figure 5 shows dose-dependent effects of caffeine on the ratio of [acetoacetate]/[3-OH-butyrate] in the presence of $10 \mu\text{M}$ phenylephrine. The change in the intramitochondrial redox state was a mirror image of the changes in gluconeogenesis and urea synthesis (Fig. 1A and B). At 1 mM caffeine, the rates of both syntheses showed maxima and the redox state shifted to the most reduced one.

DISCUSSION

Methylxanthines such as theophylline and 1-methyl-3-isobutylxanthine potentiate effects of α -adrenergic stimulation in liver. The effect has been attributed to change in $[\text{Ca}^{2+}]$ and not to change in $[\text{cAMP}]$ (CHERRINGTON *et al.*, 1976; CHAN and EXTON, 1978; FORSTER and BLAIR, 1978; GARRISON and BORLAND, 1979). We

found that stimulating effects of caffeine on both gluconeogenesis and urea production from glutamine completely depended on the action of phenylephrine (Figs. 1 and 2, and effects of prazosin). However, these effects of caffeine diminished in the absence of extracellular Ca^{2+} (Fig. 2). Phenylephrine induced influx of extracellular Ca^{2+} , inducing a rise in the intracellular Ca^{2+} concentration (Fig. 3). This effect of phenylephrine was observed when the extracellular Ca^{2+} concentration was higher than 0.1 mM (data not shown). This finding was consistent with previous results in which Ca^{2+} influx by norepinephrine was studied in liver (MAUGER *et al.*, 1984). Stimulative effects of caffeine on both syntheses were biphasic, i.e., increasing and decreasing with increasing the caffeine concentration (Fig. 1). Their maxima occurred at about 1 mM caffeine. For the decreasing effect of caffeine at higher concentrations, although we did not explore the actual reasons, nonspecific inhibitory effects on various enzymatic systems for both syntheses are considered.

Effects of phenylephrine and phenylephrine plus caffeine depended on the extracellular pH (Fig. 4). Both syntheses showed the highest rate at about pH 7.7, which was slightly more alkaline than physiological. These stimulating effects would be due to the elevated cytosolic Ca^{2+} concentration, which has been suggested to be maintained by assorted movements of Ca^{2+} via Ca^{2+} channel and $\text{Ca}^{2+}/\text{OH}^-$ symport or $\text{Ca}^{2+}/\text{H}^+$ antiport in the plasma membrane and also by initial rapid Ca^{2+} release from intracellular Ca^{2+} stores (BLACKMORE *et al.*, 1984; MAUGER *et al.*, 1984). The latter was triggered by increase in *myo*-inositol triphosphate induced by adrenergic agonists (JOSEPH *et al.*, 1984; CHAREST *et al.*, 1985).

Although phenylephrine induced the increase in intracellular $[\text{Ca}^{2+}]$, further increase was not observed by addition of caffeine (Fig. 3A and B). CHAN and EXTON (1978) reported that epinephrine or 1-methyl-3-isobutylxanthine did not induce a rise in cAMP in hepatocytes, but epinephrine plus 1-methyl-3-isobutylxanthine induced a large rise in cAMP. Furthermore, it was reported that caffeine inhibited phosphodiesterase, for example, in guinea pig colon and rat erythrocyte (POCH and UMFÄHRER, 1976) and dog tracheal smooth muscle (POLSON *et al.*, 1978) and that caffeine did not change calcium accumulation by microsomes (BATRA and DANIEL, 1971). Based on these reports, although we did not measure the intracellular cAMP, phenylephrine plus caffeine may increase intracellular cAMP, which stimulates gluconeogenesis and urea synthesis in association with the increase in intracellular free $[\text{Ca}^{2+}]$ induced by phenylephrine. Another possibility was considered that trace amount of binding calcium within the cell was released by caffeine.

We could not find the stimulating effect of caffeine in the presence of angiotensin II or vasopressin (data not shown). This finding may reflect the recent finding that the pattern of change in aequorin bioluminescence induced by phenylephrine in hepatocytes was different from that induced by vasopressin or angiotensin II, suggesting that the mode of action of phenylephrine on cytoplasmic free calcium concentration differs from that of either vasopressin or angiotensin II (MINE *et al.*, 1986).

Phenylephrine-induced increase in syntheses of glucose and urea utilized more than $3.1 \mu\text{mol ATP}/(\text{min} \cdot \text{g wet weight})$. The phenylephrine-induced increase in oxygen uptake yields $5.5 \mu\text{mol ATP}/(\text{min} \cdot \text{g wet weight})$ under the assumption that one O_2 molecule yields 6 ATP. These results indicated that both synthetic pathways were supplied fully with ATP energy. Similarly, the additional increase caused by caffeine in production of glucose and urea (i.e., the difference of effects between phenylephrine plus caffeine and phenylephrine) utilized more $1.7 \mu\text{mol ATP}/(\text{min} \cdot \text{g wet weight})$. Corresponding increase in oxygen uptake yields $4.0 \mu\text{mol ATP}/(\text{min} \cdot \text{g wet weight})$. Effects of caffeine on hormonal responses were almost parallel in both glucose and urea productions (Fig. 1), indicating that the effects of caffeine had no selectivity for energy supply to both syntheses.

As shown in Table 1, phosphorylation potentials of hepatocytes were almost constant in various conditions, suggesting that respiratory rate increased to maintain a constant level for ATP supply even under acceleration of both synthetic pathways accompanied with higher ATP consumption. Consequently, phosphorylation potential may be the most important factor in the cellular energy metabolism (WILSON *et al.*, 1974; ERECINSKA and WILSON, 1982).

Phenylephrine and phenylephrine plus caffeine in the presence of extracellular Ca^{2+} decreased the intramitochondrial $[\text{NAD}^+]/[\text{NADH}]$ ratio, which is estimated from the change in the ratio of [acetoacetate]/[3-OH-butyrate] (WILLIAMSON *et al.*, 1967) (Fig. 5 and Table 3). The decrease in the ratio of $[\text{NAD}^+]/[\text{NADH}]$ may assist gluconeogenesis and urea synthesis. Recently, effectors that elevate the intracellular Ca^{2+} were shown to induce reduction of pyridine nucleotide in hepatocytes and gastric glands (CHAREST *et al.*, 1983; CHEW, 1986). These facts are similar to ours. Although it is not clear how calcium exerts the defined effect on the $[\text{NAD}^+]/[\text{NADH}]$ ratio in liver cells, the influence of calcium on the mitochondrial oxidation-reduction state may be an important factor in regulating cellular functions via several pyridine nucleotide-linked reactions and in maintaining the phosphorylation state of the adenine nucleotide system.

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