Caffeine causes glycerophosphorylcholine accumulation through ryanodine-inhibitable increase of cellular calcium and activation of phospholipase A₂ in cultured MDCK cells

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Abbreviations: MDCK, Mardin-Darby canine kidney; GPC, glycerophosphorylcholine; GPC:choline PDE, glycerophosphorylcholine: choline phosphodiesterase; $[Ca^{2+}]_i$, intracellular free calcium; PLA₂, phospholipase A₂; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid; PBS, phosphate buffered saline; PCA, perchloric acid; TLC, thin layer chromatography

Abstract

Glycerophosphorylcholine (GPC) is a renal medullary compatible organic osmolyte that is derived from choline via phosphatidylcholine, which is catalyzed in part by phospholipase A₂ (PLA₂) and its degradation by GPC:choline phosphodiesterase (GPC:choline PDE). We found that caffeine elevated intracellular free calcium ($[Ca^{2+}]_i$) and GPC level in cultured MDCK cells, canine kidney epithelial cells, and propose a possible biochemical mechanism. When MDCK cells were incubated for 3 h with 1 to 10 mM caffeine, cellular GPC was elevated in a dose-dependent manner, and this occurred independently of the extracellular osmolality. Caffeine stimulated the rate of [¹⁴C]choline incorporation into [¹⁴C]GPC and PLA₂ activity. Whereas, GPC:choline PDE activity was accompanied by less of increase. These enzyme changes demonstrate the increased net synthesis of MDCK GPC. In order to identify what triggers the PLA₂ activation, $[Ca^{2+}]_i$ was measured by using a fluorescence dye, Fura-2. Caffeine (10 mM) resulted in a typical transient increase in MDCK $[Ca^{2+}]_i$ concentration, and this increase was greatly inhibited by pretreatment of MDCK cells with 10 mM ryanodine for 5 min. Ryanodine (10 mM) also inhibited the caffeine-induced stimulation of PLA₂ activity. These findings provide the first evidence that caffeine in MDCK cells causes a ryanodine-inhibitable increase of $[Ca^{2+}]_i$ and PLA₂ activity, resulting in cellular GPC accumulation.

Key words: caffeine, $[Ca^{2+}]_i$, glycerophosphorylcholine,

phospholipase A₂, MDCK

Introduction

In bacteria, plant, and mammalian cells, glycerophosphorylcholine (GPC) is generally considered as a "compatible" solute (Yancey *et al.*, 1982; Bagnasco *et al.*, 1986), which enables cells to osmotically adapt and protects denaturing of cellular macromolecules under the extracellular hypertonic stress. As initially demonstrated in hepatocytes (Dawson, 1955), GPC is generated from choline via phosphatidylcholine which is a predominant phospholipid in mammalian renal cells (Morgan *et al.*, 1963) and synthesized through the Kennedy pathway (Wilgram and Kennedy, 1963). GPC synthesis entails the removal of fatty acid in part by the phospholipase A₂ (PLA₂) activity (Zablocki *et al.*, 1991). The GPC produced by enzymatic deacylations is degraded by hydrolysis to glycerol 3-phosphate and choline in a reaction catalyzed by GPC:choline phosphodiesterase (GPC:choline PDE).

It is well known that in a variety of cell types such as muscular cells (Fleischer *et al.*, 1985; Campbell *et al.*, 1987; Iino *et al.*, 1988), neurons (Lipscombe *et al.*, 1988; Hernandez-Cruz *et al.*, 1990; Zacchetti *et al.*, 1991), endocrine cells (Burgoyne *et al.*, 1989; Malgaroli *et al.*, 1990) and the pancreatic acinar cells (Wakui *et al.*, 1990), caffeine increases the intracellular free calcium ($[Ca^{2+}]_i$) level through ryanodine, a neutral plant alkaloid, -sensitive or -insensitive Ca²⁺ channel located in intracellular calcium stores. However, there is no information about the action of caffeine and ryanodine on the cellular calcium change in renal epithelial cells. Based on the biochemical and physiological regards, $[Ca^{2+}]_i$ level serves as a critical transducer in a variety of cellular responses. For example, PLA₂ activity is dependent on the $[Ca^{2+}]_i$ concentration in rat kidney (Gronich *et al.*, 1990; Nakamura *et al.*, 1991) and cultured MDCK cells (Slivka *et al.*, 1987; Weiss and Insel, 1991).

All xanthines including caffeine produce a mild diuresis by increasing glomerular filtration and blocking tubular reabsorption of sodium ion in the kidney. This effect of caffeine on renal function is physiologically related with a mechanism of urine concentration which contributes to built-up an osmotic gradient in the kidney. Recently, we have found that caffeine increases cellular GPC content in cultured MDCK cells, Mardin-Darby canine kidney epithelial cells. In biochemical and pharmacological terms, we have a hypothesis to clarify a postulated mechanism for caffeine-induced MDCK GPC accumulation as following: although there is no available results about caffeine-induced $[Ca^{2+}]_i$ mobilization in MDCK cells, if caffeine increases MDCK $[Ca^{2+}]_i$ level, the elevated

$[Ca^{2+}]_i$ concentration may be involved in the stimulation of MDCK PLA₂ activity, followed by MDCK GPC accumulation. To test this hypothesis, we investigated what extent an induction of GPC synthesis and its degradation accounts for the elevation of GPC caused by caffeine, and effects of caffeine and ryanodine on $[Ca^{2+}]_i$ concentration in cultured MDCK cells.

Materials and Methods

Chemicals

L- α -glycerophosphorylcholine, L- α -glycerophosphorylcholine phosphodiesterase, L- α -phosphatidylcholine dipalmitoyl, deoxycholic acid, caffeine, 4-aminoantipyrine, bovine serum albumin (type V), choline oxidase and $CaCl_2$ were purchased from the Sigma Chemical company. Peroxidase was from the Boehringer Mannheim Biochemicals. [methyl- ^{14}C]choline chloride and L-3-phosphatidylcholine, 1-palmitoyl-2-[1- ^{14}C]palmitoyl were from the Amersham. [methylcholine- ^{14}C]GPC was prepared from L- α -dipalmitoyl-[choline-methyl- ^{14}C]phosphatidylcholine (NEN Research Production) by saponification with LiOH (Spanner and Ansell, 1987). Ryanodine and fura-2/AM were obtained from the Calbiochem (San Diego, CA) and Molecular Probes (Eugene, OR), respectively. Microcrystalline cellulose and silica gel chromatogram sheets for TLC were from the Eastman Kodak Company (Rochester, NY). Media for cell culture were purchased from the Irvine Scientific (Santa Ana, CA). All other chemicals were the highest grade available.

Cell culture

MDCK cells (American Type Culture Collection, Rockville, MD) were used in a passage 63-68. They were grown in a defined medium (315 mosmol/Kg H₂O) containing 50% Dulbecco's modified Eagle's medium and 50% Coon's improved medium mF-12, plus 10 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 5 mg/ml transferrin, 5 mg/ml insulin, 5 pM triiodothyronine (T₃), 50 nM hydrocortisone, 10 nM Na₂SeO₃ · 5H₂O, 25 ng/ml prostaglandin E1 and 2 mM L-glutamine (Taub *et al.*, 1979). For hypertonic medium (510 mosmol/Kg H₂O), NaCl was additionally added into the medium. All cultures were maintained at 37°C in 5% CO₂/95% air.

Cell GPC assay

Control and caffeine-treated MDCK cells were washed 3 times with Dulbecco's phosphate buffered saline (PBS), then extracted with perchloric acid (PCA). GPC was assayed by the combined chemical and enzymatic method previously described (Zablocki *et al.*, 1991) that uses GPC:choline PDE to catalyze production of choline from GPC, then measures the choline in a

mixture of choline oxidase, peroxidase, phenol, and 4-aminoantipyrine that produces a "red dye". The absorbance was read at 500 nm wavelength in a spectrophotometer (Lambda 3B, Perkin-Elmer) against color reagent blank.

[^{14}C]Choline incorporation into GPC

Incorporation of [^{14}C]choline into GPC was measured to estimate the rate of synthesis of GPC from choline, as previously described (Zablocki *et al.*, 1991). In brief, confluent MDCK monolayers were first incubated for 3 h with or without 10 mM caffeine in the regular medium that contains 65 mM choline, were washed 4 times with choline-free medium, then were incubated with or without 10 mM caffeine in medium containing [^{14}C]choline (65 mM, 3.6 mCi/ml). After 30, 60 or 90 min, the radioactivity in medium was decanted and the monolayer was rinsed 4 times with ice-cold PBS. Cellular [^{14}C]GPC was extracted with 1 ml of 7% PCA (vol/vol), and neutralized by adding 2 M K₂CO₃. [^{14}C]GPC was separated by thin-layer chromatography (TLC) on microcrystalline cellulose sheets, with the solvent system ethanol/2.7 M ammonium acetate, pH 5.0, 7:3 (vol/vol). GPC spots were visualized with iodine vapor and identified by comparison to standards. radioactivity was measured by liquid scintillation counter (LS 9800, Beckman).

Phospholipase A₂ assay

PLA₂ was assayed as previously described (Zablocki *et al.*, 1991). Briefly, confluent MDCK cells treated with caffeine (10 mM) and/or ryanodine (10 mM) were rinsed 3 times with ice-cold PBS, then scraped into 50 mM Tris/HCl (pH 8.6) containing 0.38% sodium deoxycholate. A homogenate was prepared in a Potter-Elvehjem apparatus with motor-driven Teflon pestle and centrifuged at 600 *g* for 10 min to remove nuclei and unbroken cells. The assay mixture contained 0.3 ml of cell homogenate, 10 mM CaCl₂, 7.2 mM 1-palmitoyl-2-[1- ^{14}C]palmitoyl phosphatidylcholine (0.1 mCi) in a total volume of 505 μ l. After incubation at 37°C for 2 h, the reaction was terminated by adding chloroform/methanol/H₂O, 2:2:1.8 (vol/vol/vol). The organic phase was evaporated under N₂ gas and taken up in 1 ml of chloroform/methanol, 2:1 (vol/vol). The radioactivity of 50 μ l samples was measured to determine extraction efficiency. The remaining samples were concentrated under N₂ gas and chromatographed on Silica Gel 60 plates in chloroform/methanol/H₂O, 65:25:4 (vol/vol/vol). The lipids were visualized with iodine vapor and identified by comparison to standards (palmitic acid, phosphatidylcholine and lysophosphatidylcholine). Radioactivity of scraped free fat acid spots was measured by liquid scintillation counter.

GPC:choline phosphodiesterase

GPC:choline PDE activity was measured as described

previously (Zablocki *et al.*, 1991). In brief, confluent MDCK cells were rinsed 3 times with ice-cold PBS, scraped into 0.25 M sucrose solution containing 0.38% deoxycholic acid, then homogenized as described above. 40 ml of supernatant were incubated for 2 h in 50 mM glycine/KOH buffer containing 10 mM $[^{14}C]$ GPC at pH 9.0 (total volume 80 ml). $[^{14}C]$ choline was separated by TLC using ethanol/2.7 M ammonium acetate, pH 5.0, 7:3 (vol/vol). $[^{14}C]$ choline spots were visualized with iodine vapor, identified by comparison to standards (choline, GPC and phosphocholine), and scraped into scintillation vials for liquid scintillation counting.

Cellular calcium measurement

Measurement of $[Ca^{2+}]_i$ was done by using fluorescence indicator, fura-2 (Grynkiewicz *et al.*, 1985). MDCK cells grown to confluence on cover slips were washed with 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) buffer containing (in mM) 127 NaCl, 2.0 Na_2HPO_4 , 4.0 KCl, 1.2 $MgSO_4$, 2.0 $CaCl_2$, 10 mM glucose and 2 mM glutamine, pH 7.3. Then, dye was loaded into the cells by incubation at 37°C for 30 min in the same buffer containing 10 mM fura-2/AM, followed by rinsing to remove fura-2 ester. The preparations were placed into a chamber on a microscope stage, and washed with dye-free buffer for approximately 15 min at a flow rate of 2.3 ml/min before adding experimental agents dissolved in the buffer. Solution change in the assay chamber was completed within 15 sec. New solution was prewarmed to 37°C before use.

As previously described (Grynkiewicz *et al.*, 1985), fluorescence was recorded under computer control at excitation wavelength of 340 nm and 380 nm and an

emission wavelength of 510 nm. $[Ca^{2+}]_i$ concentration was calculated from the ratio of fluorescence intensity at the two excitation wavelength (340/380).

Protein assay

Protein in extracted cells and homogenates was dissolved in 0.5 N NaOH and was measured with a protein assay kit (Bio-Rad laboratories, Richmond, CA).

Data analysis

All experiments were done in duplicate or triplicate. Results are presented as mean $\pm$ S.E. Statistical significance was analyzed by using Student's t-test for two groups and one way analysis of variance for multi-group comparisons. $P < 0.05$ is considered statistically significant.

Results

Caffeine increases MDCK cell GPC

When MDCK cells were incubated for 3 h with 1 to 10 mM of caffeine, their GPC level increased in a dose-dependent fashion (Figure 1). This effect occurred independently of whether the cells were in isotonic or hypertonic medium. The remaining studies were aimed at investigating the mechanism of this effect and were carried out using 10 mM caffeine under isotonic condition.

Caffeine increases the rate of incorporation of $[^{14}C]$ choline into GPC

In MDCK cells, GPC is synthesized from choline with phosphatidylcholine presumed to be an intermediate (Zablocki *et al.*, 1991). In order to estimate the rate of

Figure 1. Effect of caffeine on GPC content in MDCK cells. Cells grown in isotonic medium (310 mOsm/kg H₂O) were either kept in the same medium or exposed to hypertonic medium (510 mOsm/kg H₂O) for 5 min. Then, 0 to 10 mM caffeine were added for 3 h. Control (no caffeine) cell GPC was 25.8 ± 3.2 in cells cultured in isotonic medium and 31.7 ± 3.1 μ mol/mg protein in cells cultured hypertonic medium (n=5)

Figure 2. Effect of caffeine on incorporation of $[^{14}C]$ choline into GPC. MDCK cells were grown and maintained in isotonic medium (310 mOsm/Kg H₂O), then were exposed to 10 mM caffeine (or none) for 3 h, followed by incubation for 0 to 90 min under the same conditions, but with 65 μ M $[^{14}C]$ choline added.

Figure 3. Effect of caffeine on MDCK cellular phospholipase A₂ activity. Cells were grown and maintained in isotonic medium, then 10 mM caffeine was added. In the absence of caffeine, phospholipase A₂ activity was 0.37 ± 0.03 pmol of free fat acid per mg protein per minute (n=5).

Figure 4. Effect of caffeine on GPC:choline phosphodiesterase activity. MDCK cells were grown and maintained in isotonic medium, then 10 mM caffeine was added. In the absence of caffeine, GPC:choline phosphodiesterase activity was 0.39 ± 0.037 nmol/mg protein/min (n=5).

GPC synthesis from choline, we measured the incorporation of [¹⁴C]choline into GPC. The assumptions and limitations of this method were discussed previously (Zablocki *et al.*, 1991). The MDCK cells were incubated with or without caffeine for 3 h, followed by additional incubation for 30, 60 and 90 min in the presence of [¹⁴C]choline. As shown in Figure 2, the incorporation of [¹⁴C]choline into [¹⁴C]GPC was linear with incubation time, as previously observed (Zablocki *et al.*, 1991), and was increased approximately 4-folds by caffeine treatment. This result suggests that caffeine raises MDCK cell GPC by increasing its synthesis.

Caffeine increases phospholipase A₂ activity, but has relatively little effect on GPC:choline phosphodiesterase activity

Synthesis of GPC from phosphatidylcholine is catalyzed in part by PLA₂, and degradation of GPC is catalyzed by GPC:choline PDE (Zablocki *et al.*, 1991). Activity of these enzymes was measured to further characterize the effect of caffeine on MDCK cell GPC change.

PLA₂ activity increased more than 4-folds within 5 min after 10 mM caffeine was added to MDCK cells, then remained with sustained phase through at least 3 h (Figure 3). This result is consistent with caffeine-increasing GPC synthesis, as also suggested by the experiments in Figure 2. GPC:choline PDE activity was also elevated by caffeine (Figure 4), but the effect was slower and relatively smaller than that on PLA₂ activity (Figure 3). Increased GPC:choline PDE activity should lower cellular GPC, rather than elevate it, as is observed (Figure 1). Perhaps, the rise in GPC:choline PDE activity is a secondary effect, and rapid incorporation of choline into GPC depletes cellular choline or phosphatidylcholine store.

Taken together, these results suggest that caffeine elevates MDCK cellular GPC by increasing activity of a rate limiting enzyme that catalyzes its synthesis. The following studies were aimed at defining which of the many pathways of caffeine action is involved.

Caffeine increases MDCK Ca²⁺ concentration and ryanodine inhibits this effect

PLA₂ activation may depend on the [Ca²⁺]_i concentration (Slivka and Insel, 1987; Gronich *et al.*, 1990; Nakamura *et al.*, 1991; Weiss and Insel, 1991), and millimolar concentrations of caffeine may increase [Ca²⁺]_i concentration both in muscular (Fleischer *et al.*, 1985; Campbell *et al.*, 1987; Iino *et al.*, 1988) and non-muscular cells (Lipscombe *et al.*, 1988; Burgoyne *et al.*, 1989; Hernandez-Cruz *et al.*, 1990; Malgaroli *et al.*, 1990; Zacchetti *et al.*, 1991). Caffeine mobilizes cellular Ca²⁺ from intracellular stores by activating Ca²⁺ channels that are sensitive to ryanodine. With this in mind, we tested the effect of caffeine and ryanodine on the MDCK [Ca²⁺]_i level.

[Ca²⁺]_i concentration was measured by using the indicator dye fura-2. As shown in Figures 5 and 6, caffeine increased [Ca²⁺]_i approximately 2-folds within 2 min. This effect was greatly reduced by ryanodine added 5 min before the caffeine. Ryanodine, by itself, did not change significantly the basal [Ca²⁺]_i level.

The most likely explanation of this result is that caffeine mobilizes cellular Ca²⁺ concentration in MDCK cells through activation of ryanodine-sensitive Ca²⁺ channels.

Ryanodine inhibits the increases in phospholipase A₂ caused by caffeine

If activation of PLA₂ by caffeine resulted from the increased [Ca²⁺]_i concentration, it should also be inhibited by ryanodine. As shown in Figure 7, ryanodine,

Figure 5. Representative experiments showing the effects of caffeine and ryanodine on $[Ca^{2+}]_i$ in MDCK cells. The cells were grown under isotonic condition (310 mOsm/Kg H₂O) in cover slip. Changes in the $[Ca^{2+}]_i$ concentration were measured using the fluorescence indicator, fura-2. Dye-loaded cells were treated with 10 mM caffeine (A) or 10 μ M ryanodine plus 10 mM caffeine (B).

by itself, had no effect on MDCK cell PLA₂ activity, yet it inhibited by 76% the caffeine-induced increase in activity. Thus, the caffeine-induced increase in MDCK cell PLA₂ activity apparently is caused by the associated increase in cellular Ca^{2+} concentration.

Discussion

In cultured MDCK cells, Zablocki *et al.* (1991) have reported the metabolic pathway for cellular GPC synthesis and degradation as follows: choline → phosphocholine → cytidine phosphocholine → phosphatidylcholine → GPC. Phosphatidylcholine is found almost exclusively in the membrane of almost all mammalian cells including kidney cells, and constitutes approximately 50% of the phospholipid species (Morgan *et al.*, 1963). Additionally, numerous reports demonstrated that the metabolic characteristics of phosphatidylcholine turnover in 3T3-L1 (Besterman *et al.*, 1986), in hepatocyte (Sanghera and Vance, 1989), in C6 rat glioma cells (George *et al.*, 1991; George *et*

al., 1991) and in HeLa cells (Utal *et al.*, 1991) using several different experimental conditions. However, there is no available informations about the effect of adenosine analogues, such as caffeine, on the phosphatidylcholine and/or GPC turnover. In the present study, we demonstrated that milimolar concentration of caffeine increased the cellular Ca^{2+} concentration and PLA₂ activity in cultured MDCK cells whose activities were dramatically inhibited by the pretreatment of MDCK cells with micromolar concentration of ryanodine. These results, suggest that caffeine-induced increase of MDCK $[Ca^{2+}]_i$ concentration apparently serves as a transducer for stimulation of MDCK PLA₂ activity, followed by MDCK GPC accumulation (Figure 8). Importantly, we demonstrated for the first time caffeine-induced $[Ca^{2+}]_i$ mobilization, which is sensitive to the ryanodine, in cultured MDCK cells.

High extracellular osmolality dies to NaCl and urea

Figure 6. Effect of caffeine and ryanodine on the MDCK $[Ca^{2+}]_i$ concentration. Mean results from the peak level of $[Ca^{2+}]_i$ in 5 experiments, including that in Figure 5

Figure 7. Effect of caffeine and ryanodine on MDCK cell phospholipase A₂ activity. MDCK cells were grown and maintained in isotonic medium (310 mOsm/kg H₂O), then were treated for 5 min with or without 10 μ M ryanodine, followed by incubation for 30 min in the absence or presence of 10 mM caffeine. Mean results of 5 experiments.

increases the MDCK GPC level by inhibiting its enzymatic degradation (Zablocki *et al.*, 1991). However, the present study showed that MDCK GPC level by caffeine increased independently to the extracellular osmolality (Figure 1). This result suggests that GPC accumulation by caffeine may occur through different mechanism from that by hypertonic stress. In addition, we can consider five hypo-theses for the role of caffeine in the increase of GPC in MDCK cells: 1) stimulation of GPC synthesis, 2) inhibition of GPC degradation, 3) increased net GPC synthesis compared to its degradation, 4) decreased net GPC degradation compared to the inhibition of its synthesis as under a hypertonic condition (Zablocki *et al.*, 1991), and 5) combination of both increased GPC synthesis and decreased degradation. To elucidate these hypotheses, two metabolic pathways (synthesis and degradation) for MDCK GPC were separately investigated. Caffeine increased both the rate of [^{14}C]choline incorporation into GPC and PLA₂ activity within relatively short time (Figures 2 and 3), demonstrating that caffeine stimulates the GPC synthesis. However, GPC:choline PDE activity was stimulated by caffeine with slower rate and lower effect than PLA₂ activity (Figure 4). In the biological system, we concentration that caffeine-mediated GPC:choline PDE activation may be involved in the secondary action to maintain the cellular phosphatidylcholine concentration, because an increase of the GPC level means the depletion of cellular phosphatidylcholine, and choline produced by GPC:choline PDE activation will be recycled as cellular phosphatidylcholine (Dawson, 1955; Pelech and Vance, 1989; Zablocki *et al.*, 1991). However, there is no evidence to support or refute this hypothesis based on the present results.

The potential alternative pathways of phospholipid

Figure 8. Possible mechanism for caffeine-induced increase of MDCK glycerophosphorylcholine. Mobilization of [Ca^{2+}]_i by caffeine apparently serves as a transducer for stimulation of phospholipase A₂ activity, followed by MDCK GPC accumulation. PC, phosphatidylcholine; PLA₂, phospholipase A₂; GPC, glycerophosphorylcholine; G3P, glycerol 3-phosphate.

turnover in cell signal transduction have recently appreciated. For example, some agonists implicated in the coupling of cellular second messenger such as diacylglycerol and Ca^{2+} can stimulate the resynthesis of phosphatidylcholine via activation of CTP:phosphocholine cytidyltransferase (Besterman *et al.*, 1986; Sanghera and Vance, 1989; George *et al.*, 1991; George *et al.*, 1991; Utal *et al.*, 1991). In the rat hepatocyte, high concentration of calcium in medium stimulates phosphatidylcholine biosynthesis by causing a translocation of cytidyltransferase from the cytosol to membrane (Sanghera and Vance, 1989). A similar result has been observed by diacylglycerol in HeLa cells in culture (Utal *et al.*, 1991). Moreover, George *et al.* (1991) have suggested that the intermediates arising from CDP-choline pathway are not freely diffusible in electroporated rat glioma cells (C6), but are channeled toward synthesis of phosphatidylcholine. The intermediates are channeled most likely by [Ca^{2+}]_i levels that may coordinately regulate the conversion of choline to phosphatidylcholine. These informations suggest that cellular Ca^{2+} level is an important messenger to regulate the phospholipid turnover in a variety of cells. Although there are no available informations on the [Ca^{2+}]_i mobilization induced by caffeine in the renal epithelial cells including cultured MDCK cells, caffeine can be generally considered as a potentiator of Ca^{2+} -induced Ca^{2+} release (Schmid *et al.*, 1990; Stauderman and Murawsky, 1991; Pelech and Vance, 1989; Wakui *et al.*, 1990; Zacchetti *et al.*, 1991). Based on this assumption, we measured the changes of [Ca^{2+}]_i level by caffeine in MDCK cells and clarified the involvement of cellular Ca^{2+} in the caffeine-induced GPC accumulation. Caffeine significantly increased MDCK [Ca^{2+}]_i concentration and this was significantly inhibited by pretreatment of MDCK cells with ryanodine (Figures 5 and 6). Interestingly, ryanodine also inhibited the PLA₂ activity stimulated by caffeine (Figure 7). These results demonstrate that caffeine increases MDCK [Ca^{2+}]_i concentration and it is involved in the PLA₂ activity.

Caffeine and ryanodine are known to selectively influence the process of Ca^{2+} release in a variety of cells from intracellular calcium pool (Iino *et al.*, 1988; Malgaroli *et al.*, 1990; Schmid *et al.*, 1990; Stauderman and Murawsky, 1991; Wakui *et al.*, 1990; Zacchetti *et al.*, 1991). Biochemical, pharmacological and genetic studies have demonstrated the functional properties of Ca^{2+} channel modulated by these compounds. It is generally considered that depending on the experimental condition, "use-dependent" ryanodine either stimulates or inhibits the Ca^{2+} efflux from the cellular calcium pool (Meissner, 1986). For example, effect of ryanodine on muscle cells are complex and are dependent on the muscle type, the calcium activity, and the patterns of muscle stimulation as well as concentrations of ryanodine used. Recently, two different

types of full-length cDNA for ryanodine receptor have been cloned: skeletal muscle isoforms from rabbit (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990), human (Zorzato *et al.*, 1990) and porcine (Fujii *et al.*, 1991) as well as cardiac and brain isoforms from rabbit (Otsu *et al.*, 1990). In the skeletal muscle cells, ryanodine locks the) release of the "open state", then thus, Ca^{2+} is not reaccumulated Zorzato *et al.* (1990). The similar results were also demonstrated in the vascular smooth muscle of guinea-pig (Iino *et al.*, 1988). Zacchetti *et al.* (1991) have suggested that PC12 cells express two distinct intracellular Ca^{2+} channels, i. e. the receptor for caffeine-ryanodine and the inositol 1,4,5-triphosphate which appear to be colocalized. These suggestions are unlikely since caffeine-sensitive Ca^{2+} channel is not affected by ryanodine in pancreatic endo-plasmic reticulum vesicles (Schmid *et al.*, 1990). In the MDCK cells, caffeine-induced Ca^{2+} release was inhibited by ryanodine, demonstrating that Ca^{2+} channel modulated by caffeine are sensitive to the ryanodine. In summary, we suggest from the present study that although it is difficult to explain the detailed functional property of MDCK Ca^{2+} channel which responded sensitively to the caffeine, the biochemical effect of ryanodine are likely to be exerted the muscle types of ryanodine receptor than on the pancreatic types. Therefore, MDCK cells can be considered as an unique epithelial cells possessing the caffeine/ryanodine-sensitive Ca^{2+} channel.

Thus, this study underscores the role of intracellular Ca^{2+} in the regulation of MDCK GPC accumulation. Additionally, we show that MDCK cells have also the function of $[Ca^{2+}]_i$ mobilization by caffeine, which is sensitive to the ryanodine. Further biochemical studies on the caffeine-induced $[Ca^{2+}]_i$ increase, using intact MDCK cells or MDCK cell calcium stores such as endoplasmic reticulum, will contribute to the elucidation of detailed functional properties of ryanodine receptor located in renal epithelial cells.

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