Chinese Journal of Physiology 42(1): 33-39, 1999

A Further Investigation of ATP-Induced Calcium Mobilization in MDCK Cells

Chung-Ren Jan[1,2], Sheng-Nan Wu[1,2] and Ching-Jiunn Tseng[1]

[1]*Department of Medical Education and Research*
Veterans General Hospital-Kaohsiung
Kaohsiung 813
and
[2]*Department of Biology and Institute of Life Sciences*
National Sun Yat-Sen University
Kaohsiung 804, Taiwan, ROC

Abstract

We have previously reported that La^{3+} inhibited the ATP-induced rise in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) measured by fura-2 fluorimetry in Madin Darby canine kidney (MDCK) cells. Here we further investigated the ATP-induced Ca^{2+} signal. ATP caused a rise in $[Ca^{2+}]_i$ dose-dependently between 1 μM-1 mM. The rises induced by 10 μM-1 mM ATP were inhibited by Ca^{2+} removal. The pleateau phase of the ATP response was primarily maintained by Ca^{2+} influx because it was reduced or eliminated by Ca^{2+} removal. ATP failed to elevate $[Ca^{2+}]_i$ after the endoplasmic reticulum Ca^{2+} store had been depleted by 2,5-di-tert-butylhydroquinone or cyclopiazonic acid, suggesting that the ATP-induced Ca^{2+} influx was capacitative Ca^{2+} entry. Capacitative Ca^{2+} entry was directly measured by addition of 5 mM $CaCl_2$ to cells pretreated with ATP (0.1 mM) in Ca^{2+}-free medium. This capacitative Ca^{2+} entry was inhibited by econazole (25 μM) or SKF96365 (50 μM). The ATP response was significantly enhanced by extracellular alkalization to pH 8 or pretreatment with gly-phe-β-naphthylamide. Pretreatment with carbonylcyanide m-chlorophenylhydrazone (CCCP) or extracellular Na^+ removal had no enhancement, implicating that efflux via plasmalemmal Ca^{2+} pumps (but not Na^+/Ca^{2+} exchange) and buffering by lysosomes (but not mitochondria) might be involved in the decay of the ATP response.

Key Words: ATP, MDCK cells, P2 receptors, fura-2, calcium signaling

Introduction

Extracellular ATP acting on P2 receptors triggers a number of physiological responses in many tissues. P2 receptors have been subdivided into P_{2x}, P_{2y}, P_{2z}, P_{2u} and P_{2t} receptors based on the potency order of several nucleotides (2, 6). In a newer nomenclature, P2 receptors are separated into two large catogories: P_{2x} and P_{2y}. P_{2x} receptors are of ionotropic type, while P_{2y} receptors are coupled to specific G proteins. So far, seven subtypes have been named for both P_{2x} and P_{2y} receptors based on differences in molecular structure and pharmacology (1, 6, 8, 29).

In Madin Darby canine kidney (MDCK) cells, extracellular ATP has been shown to activate a short-circuit current (28), induce arachidonic acid release (7), and elevate intracellular levels of cAMP and several phospholipases (26). ATP also elevated $[Ca^{2+}]_i$ (12, 23) which subsequently activates Ca^{2+}-dependent K^+ currents leading to cell hyperpolarization (23) and chloride secretion (28). We have recently demonstrated that ATP increases $[Ca^{2+}]_i$ by releasing the endoplasmic reticulum (ER) Ca^{2+} followed by a La^{3+}-sensitive capacitative Ca^{2+} entry, and that La^{3+} appears to directly inhibit the ATP receptors in a competitive manner (12). Additionally, we have also reported evidence showing that $P2_{y2}$ and $P2_{y1}$ receptors coexist in MDCK cells based on the rank order of potency of ATP and several ATP analogues on $[Ca^{2+}]_i$ measured by fura-2 fluorimetry (13). Here we further investigated the ATP-induced Ca^{2+} signal. The

Corresponding author: Dr. C. R. Jan, Ph.D., Dept. Medical Education and Research, Veterans General Hospital-Kaohsiung, 386 Ta Chung 1st Rd, Kaohsiung 813, Taiwan, ROC. Fax: 886-7-3468056, E-mail: crjan@isca.vghks.gov.tw
Received January 30, 1999; Revised February 19, 1999; Accepted February 20, 1999.

mechanisms underlying the decay of the ATP response was also investigated.

Materials and Methods

Cell Culture

MDCK cells obtained from American Type Culture Collection (CRL-6253, MD, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2-containing humidified air.

Solutions

Ca^{2+} medium (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; Hepes 10; glucose 5. Ca^{2+}-free medium contained no Ca^{2+} plus 1 mM EGTA. The experimental solution contained 0-1% of solvent (DMSO or ethanol) which did not affect $[Ca^{2+}]_i$ (n=3). In Na^+-free medium, NaCl was repalced with choline chloride without changing osmolarity.

Optical Measurements of $[Ca^{2+}]_i$

Trypsinized cells (10^6/ml) were loaded with 2 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N,N-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM) for 30 min at 25°C in DMEM. Cells were washed and resuspended in Ca^{2+} medium and were washed every hour during experiments to minimize extracellular dye. Fura-2 fluorecence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette normally contained 1 ml of medium and 0.5 million of cells unless otherwise stated. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Japan) by continuously recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-s intervals. Maximal and minimal fluorescence values were obtained by adding TX-100 (0.1%) and EGTA (20 mM) sequentially at the end of an experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ as described previously (9). Mn^{2+} quench experiments were performed in Ca^{2+} medium containing $MnCl_2$ (50 μM) by recording excitation signals which alternated at 340, 360, and 380 nm and emission signal at 510 nm in 1-s intervals. Our previous studies have shown that trypsinized cells prepared by our protocol responded to stimulation of ATP (12), ADP (13), UTP (14), bradykinin (15) or thapsigargin (17) similarly to cells attached to coverslips. We decided to use trypsinized cells because this procedure is easier and less time-consuming. All experiments were performed at room temperature (25°C).

Chemical Reagents

The reagents for cell culture were from Gibco (NY, USA). Fura-2/AM was from Molecular Probes (OR, USA). 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SKF96365) and 2,5-di-tert-butylhydroquinone (BHQ) were from Biomol (Plymouth Meeting, PA, USA). The other reagents were from Sigma (MO, USA).

Statistical Analysis

All values are reported as means±S.E. of 3-4 experiments. Statistical comparisons were determined by using the Student's t test, and significance was accepted when $P < 0.05$.

Results

Effects of ATP on $[Ca^{2+}]_i$

We have previously reported that at concentrations of 1 μM-1 mM ATP increased $[Ca^{2+}]_i$ in a dose-dependent manner (12). In the present study we explored the effect of Ca^{2+} removal on the dose-response curve. Figure 1A shows that in Ca^{2+} medium the $[Ca^{2+}]_i$ rises induced by 0.1-1 mM ATP (*traces a, b*) consisted of a rapid peak, a gradual decay and a sustained plateau; while that by 1-10 μM ATP (*traces c, d*) lacked a plateau. Effect of 0.1 μM ATP was negligible. Thapsigargin is a drug which inhibits the ER Ca^{2+} pump allowing Ca^{2+} to leak from the ER store (30). *Trace e* is the thapsigargin-induced $[Ca^{2+}]_i$ rise without ATP prestimulation. The decay of 1 mM ATP-induced $[Ca^{2+}]_i$ rise was significantly faster than that by 0.1 mM ATP. Addition of ATP (1 μM-1mM; *traces a, b, c, d*) dose-dependently inhibited the thapsigargin-induced $[Ca^{2+}]_i$ rises (*trace e*) suggesting that ATP mobilized the thapsigargin-sensitive Ca^{2+} store. Figure 1B shows that Ca^{2+} removal (no added Ca^{2+} + 1 mM EGTA) reduced the peak $[Ca^{2+}]_i$ induced by 1 mM ATP by ~20% (495±20 nM vs. 620±12 nM; n=3; $P < 0.05$), significantly reduced the area under the curve, and substantially inhibited the plateau of the $[Ca^{2+}]_i$ rise, suggesting that Ca^{2+} influx occurred which contributed to both the rising phase and the plateau phase of the ATP response. Qualitatively similar results were found for 0.1 mM ATP except that the plateau phase was abolished by Ca^{2+} removal. The response induced by 10 μM ATP, which lacked a

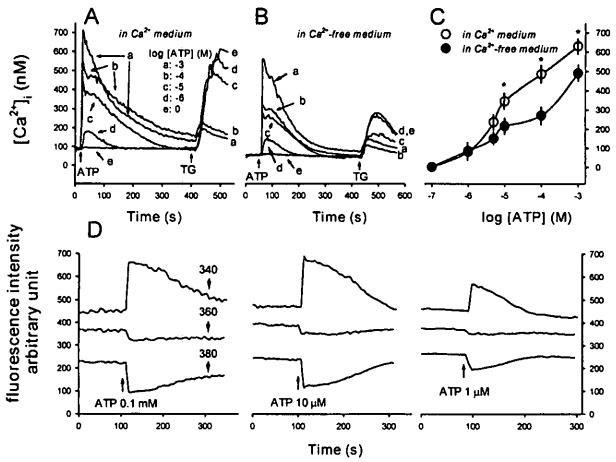

Fig. 1. Effect of ATP on $[Ca^{2+}]_i$ in fura-2-loaded MDCK cells. A, Dose-response relationship determined in Ca^{2+} medium. ATP was added at concentrations of 10^{-3} M (*trace a*), 10^{-4} M (*trace b*), 10^{-5} M (*trace c*), 10^{-6} M (*trace d*) or zero (*trace e*). Thapsigargin (TG; 0.1 μM) was added subsequently. ***B***, Similar to ***A*** except that extracellular Ca^{2+} was removed (no added Ca^{2+} plus 1 mM EGTA). ***C***, Dose-response curve plotted as peak $[Ca^{2+}]_i$ (nM) vs. concentration of ATP both in Ca^{2+} medium and in Ca^{2+}-free medium. Data are mean±S.E. of 3-4 experiments. * $P < 0.05$. ***D***, ATP-evoked Ca^{2+} influx detected by Mn^{2+} entry measurements. $MnCl_2$ (50 μM) was added 90 s prior to stimulation with three different concentrations of ATP as indicated. Excitation signals which alternated at 340, 360, and 380 nm and emission signal at 510 nm were continuously collected in 1-s intervals. The traces are typical of 3-4 experiments.

plateau, was also reduced by Ca^{2+} removal (*traces c*). The effect of Ca^{2+} removal on 1 μM ATP-induced $[Ca^{2+}]_i$ rise was insignificant. Additionally, in Ca^{2+}-free medium, the thapsigargin-induced $[Ca^{2+}]_i$ rises were reduced by 58% (traces e; 251±20 nM vs. 600±23 nM; n=3; $P < 0.05$) suggesting that thapsigargin induced Ca^{2+} influx. Pretreatment with 10 μM-1 mM ATP greatly reduced the thapsigargin-induced response in Ca^{2+}-free medium (*traces a, b, c vs. traces d, e*), again implicating that ATP mobilized the thapsigargin-sensitive Ca^{2+} store. Figure 1C compares the dose-response relationship of ATP-induced $[Ca^{2+}]_i$ rises in Ca^{2+} medium and in Ca^{2+}-free medium.

We applied another maneuver to prove that Ca^{2+} influx really occurred during ATP stimulation by using Mn^{2+} as a surrogate for Ca^{2+}. Mn^{2+} enters cells through similar pathways as Ca^{2+}, but quenches fura-2 fluorescence at all excitation wavelengths (24), thus providing a method to measure Ca^{2+} influx. Fluorescence intensity was monitored at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the Ca^{2+}-sensitive wavelengths of 340 nm and 380 nm alternatively in Ca^{2+} medium containing $MnCl_2$ (50 μM). Figure 1D shows that 0.1 mM ATP induced an increase in 340 nm signal accompanied by a corresponding decrease in 380 nm signal (*left traces*). Concomitantly there was a significant decrease in

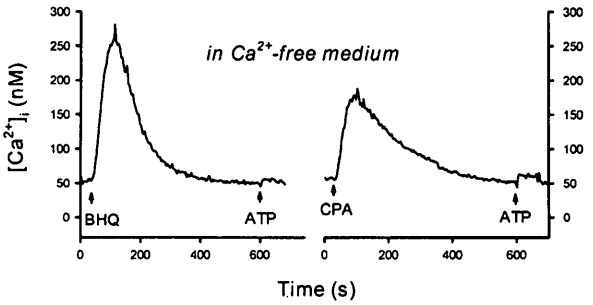

Fig. 2. Effects of BHQ or CPA pretreatment in the absence of extracellular Ca^{2+} on ATP-induced $[Ca^{2+}]_i$ rises. The experiment was performed in Ca^{2+}-free medium. BHQ (50 μM; *left*) or CPA (100 μM; *right*) was added followed by ATP (0.1 mM) approximately 10 min afterward. The traces are typical of 3-4 experiments.

360 nm signal which occurred early upon ATP addition and did not recover to prestimulatory baseline within 250 s. With 10 μM ATP, a decrease in 360 nm signal smaller than that stimulated by 0.1 mM ATP was also observed which recovered to prestimulatory baseline in 150 s. Mn^{2+} influx induced by 1 μM ATP was negligible. Thus, it is clear that 10 μM-0.1 mM ATP induced Ca^{2+} influx.

In a previous report, we showed that thapsigargin and ATP shared the same ER Ca^{2+} store (12). We next examined whether 2,5-di-tert-butylhydroquinone (BHQ) and cyclopiazonic acid (CPA), two drugs that were thought similar to thapsigargin in inhibiting the ER Ca^{2+} pump and depleting the ER Ca^{2+} store (4, 18), act similarly to thapsigargin. Figure 2 shows that in Ca^{2+}-free medium (to prevent Ca^{2+} influx-induced refilling of Ca^{2+} stores), BHQ and CPA caused significant $[Ca^{2+}]_i$ rises with a peak value of 200-250 nM which decayed to baseline in ~ 400 s. ATP (0.1 mM) added afterward failed to induce significant $[Ca^{2+}]_i$ rises in either case, suggesting that BHQ and CPA acted similarly to thapsigargin in depleting the ATP-sensitive ER Ca^{2+} store.

ATP-Induced Capacitive Ca^{2+} Entry was Inhibited by Econazole and SKF96365

We have previously shown that ATP (0.1 mM) induced capacitative Ca^{2+} entry which was abolished by La^{3+} (0.1 mM) (12). Because we have recently shown that econazole inhibited CPA-induced capacitative Ca^{2+} entry (20), and SKF96365 inhibited thapsigargin- and UTP-induced capacitative Ca^{2+} entry (19), we went on to investigate whether these two drugs could inhibit ATP-induced capacitative Ca^{2+} entry. Figure 3A shows that in Ca^{2+}-free medium, after prior stimulation with 0.1 mM ATP for 300 s, $CaCl_2$ (5 mM) induced a rise in $[Ca^{2+}]_i$ (200±14 nM; n=3) which was significantly greater than control (without ATP prestimulation; 81±5 nM; n=4;

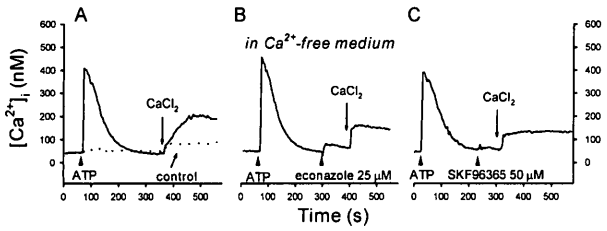

Fig. 3. ATP-induced capacitative Ca^{2+} entry was inhibited by econazole and SKF96365. The experiments were performed in Ca^{2+}-free medium. ***A***, *solid trace*: ATP (0.1 mM) was added as indicated. 5 mM $CaCl_2$ was added at 360 s. *Dashed line*: control without ATP prestimulation. ***B***, after ATP (0.1 mM) prestimulation, econazole (25 μM) was added at 300 s for 100 s followed by $CaCl_2$. C, Similarly to ***B***, SKF96365 was added at 240 s for 100 s followed by $CaCl_2$. The traces are typical of 3-4 experiments.

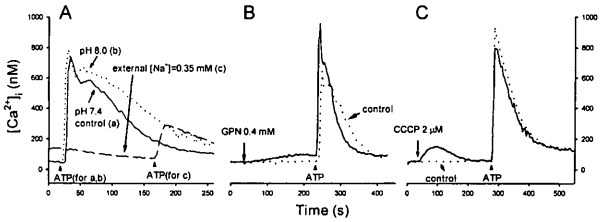

Fig. 4. Mechanisms of decay of the ATP response. ***A***, ATP-induced $[Ca^{2+}]_i$ rises were measured in a medium of pH 7.4 (control; *trace a*), pH 8.0 (*trace b*), and low $[Na^+]$ medium (trace c), and the results were compared. Extracellular $[Na^+]$ was lowered to 0.35 mM by replacing NaCl with choline chloride (see Results for detail). 0.1 mM ATP was added at 25 s (*traces a, b*) or 165 s (*trace c*). ***B***, *Solid trace*: in Ca^{2+} medium, GPN (0.4 mM) was added for 200 s before addition of ATP (0.1 mM). *Dashed trace* (control): without GPN pretreatment. ***C***, Effect of CCCP pretreatment on ATP-induced $[Ca^{2+}]_i$ rise. The experiment was performed in Ca^{2+} medium. *Solid trace*: CCCP (2 μM) was added for 240 s before addition of 0.1 mM ATP. *Dashed trace* (control): without CCCP pretreatment. The traces are typical of 3-4 experiments.

$P < 0.05$). This is consistent with our previous findings (12). Figures 3B and 3C show that econazole (25 μM) and SKF96365 (50 μM) partly inhibited (~40-50%) the capacitative Ca^{2+} entry. Higher doses were not tested to avoid nonspecific effects.

In Ca^{2+} medium, pretreatment with verapamil (10 μM), nifedipine (10 μM), diltiazem (10 μM) and Ni^+ (1 mM) for 3 min did not affect the ATP-induced Ca^{2+} signal (not shown).

Mechanisms Underlying the Decay of the ATP-induced $[Ca^{2+}]_i$ rise

The decay of a $[Ca^{2+}]_i$ signal in most cells involves buffering by Ca^{2+} binding proteins, efflux via plasmalemmal Ca^{2+} pumps and Na^+/Ca^{2+} exchange, and buffering by internal stores (3). Here we examined whether the following mechanisms were involved in the decay of the ATP response: Ca^{2+} efflux via Ca^{2+} pumps or Na^+/Ca^{2+} exchange, and buffering by the ER, mitochondria and lysosomes.

For plasmalemmal Ca^{2+} pumps, a selective inhibitor is not available. We tried two manipulations which were shown to depress the pump: extracellular alkalization and addition of La^{3+} (25). Extracellular alkalization to pH 8.0 significantly slowed the decay of 0.1 mM ATP-induced $[Ca^{2+}]_i$ rise (Figure 4A, *traces a, b*) without altering the resting $[Ca^{2+}]_i$ or the induced peak $[Ca^{2+}]_i$. The under-curve area of *trace b* was 35±5% larger than that of *trace a* (n=4, $P < 0.05$). Because we found that La^{3+} abolished the ATP-induced capacitative Ca^{2+} entry and might interfere with ATP binding to its receptor (12), the effect of La^{3+} on the Ca^{2+} pump was difficult to determine.

We next examined the contribution of Na^+/Ca^{2+} exchange which was found to be active in MDCK cells (15, 16). If Na^+-dependent Ca^{2+} efflux occurs during the ATP response, lowering extracellular $[Na^+]$ to less than 1 mM should block Ca^{2+} efflux leading to a potentiation of the ATP response. To inhibit Na^+-dependent Ca^{2+} efflux, we lowered extracellular $[Na^+]$ by adding 5 μl of cell suspension in Ca^{2+} medium to 2 ml of Na^+-free buffer (Na^+ replaced with choline) in a cuvette. This gave an extracellular $[Na^+]$ of 0.35 mM. Measurements were started after incubating cells in low Na^+ medium for 15 min. The resting $[Ca^{2+}]_i$ was 120±10 nM (Figure 4A; *trace c*; n=3) which was significantly higher than that measured in Ca^{2+} medium (*trace a*; 51+6 nM; n=3; $P < 0.05$). The elevated resting $[Ca^{2+}]_i$ gradually declined to ~80 nM in 100 s. Subsequent stimulation with ATP (0.1mM) elicited a $[Ca^{2+}]_i$ rise which was similar to control in kinetics but the peak value was reduced by more than 50%.

Lysosomes were implicated in IP_3-mediated Ca^{2+} release in MDCK cells and were thought to be depleted of stored Ca^{2+} by gly-phe-β-naphthylamide (GPN) via permeabilization of the lysosomal membrane (10). We examined whether lysosomes could sequester the Ca^{2+} mobilized by ATP. Figure 4B shows that GPN (0.4 mM) caused a tiny (< 20 nM), gradual rise in $[Ca^{2+}]_i$ lasting for ~200 s. ATP (0.1 mM) added subsequently induced a $[Ca^{2+}]_i$ rise with a peak value 59% higher (basal subtracted: 890±23 vs. 560±15 nM; n=3; $P < 0.05$) than control and a faster decay.

Mitochondria play a role in sequestering Ca^{2+} in MDCK cells (16) and other cells such as chromaffin cells (11) and neuronal cells (21). If mitochondria contribute to buffering the ATP-induced $[Ca^{2+}]_i$ rise, inhibition of mitochondria would slow the decay of the Ca^{2+} signal. Figure 4C shows that the mitochondrial uncoupler CCCP (2 μM) induced a small $[Ca^{2+}]_i$ transient (peak value=150±25 nM; n=4), reflecting a release of Ca^{2+} from, and an inhibition of, the resting mitochondria. However, CCCP pretreatment did not alter the ATP-induced $[Ca^{2+}]_i$ rise. Similar data were

obtained from using another mitochondrial inhibitor, oligomycin (not shown).

Discussion

In this study we have further investigated several important questions related to the ATP-induced $[Ca^{2+}]_i$ rises in MDCK cells. The results present here together with our previous report (12) help to reveal how the ATP-induced $[Ca^{2+}]_i$ transient in MDCK cells rises and decays.

ATP induced a rise in $[Ca^{2+}]_i$ dose-dependently. This effect of ATP on $[Ca^{2+}]_i$ was not caused by the hydrolysis products of ATP, namely, ADP, AMP and adenosine, because these three compounds caused much smaller or no $[Ca^{2+}]_i$ rises; and that ATP-γ-S, a non-hydrolyzable ATP analogue, was of similar effect as ATP (13). At concentrations between 10 μM and 1 mM, ATP activated Ca^{2+} influx as demonstrated by extracellular Ca^{2+} removal and Mn^{2+} quench experiments; in contrast, at lower concentrations (1-5 μM) ATP only mobilized internal Ca^{2+} without activating Ca^{2+} influx. One important question is the identity of this Ca^{2+} influx pathway. We have demonstrated previously that this Ca^{2+} influx was solely mediated by capacitative Ca^{2+} entry (12). Consistently, nifedipine, verapamil and diltiazem could not inhibit the ATP response, which was not surprising because MDCK cells do not have voltage-gated Ca^{2+} channels (22). The finding of the dose-dependent activation of capacitative Ca^{2+} entry is interesting and implicate that the magnitude of capacitative Ca^{2+} entry might be tightly coupled to the extent of depletion of the ER Ca^{2+} store.

Econazole and SKF96365 partly inhibited ATP-induced capacitative Ca^{2+} entry (Figure 3). Because we have found that econazole inhibited the capacitative Ca^{2+} entry induced by U73122 (16) and CPA (20), and that SKF96365 inhibited the capacitative Ca^{2+} entry induced by U73122 (16), thapsigargin and UTP (19), it appears that the capacitative Ca^{2+} entry induced by these different agents is similar or identical. However, two different capacitative Ca^{2+} entry pathways were thought to exist in MDCK cells (5).

We found that the decay of the ATP response might involve efflux via plasmalemmal Ca^{2+} pumps based on that extracellular pH of 8.0 significantly slowed down the decay of the ATP response. However, because alkalization might interfere with many aspects of cell function, our data are only suggestive; conclusive results could not be obtained until a specific inhibitor of the plasmalemmal Ca^{2+} pump is available. We have also found that alkalization potentiated ADP-induced $[Ca^{2+}]_i$ rise (13), and that both alkalization and La^{3+} pretreatment potentiated thapsigargin- and BHQ-induced $[Ca^{2+}]_i$ rises (17, 18).

We also examined whether Na^+-dependent Ca^{2+} efflux had a contribution. The ATP-induced $[Ca^{2+}]_i$ peak was reduced by more than 50% in low $[Na^+]$ medium; however, the decay kinetics were largely similar to that of control, suggesting that Na^+/Ca^{2+} exchange is not important in mediating the decay of the ATP response. It is interesting that the ATP response was substantially blunted by substitution of Na^+ with choline and was even nearly abolished by substitution with another commonly used substitute, N-methylglucamine (not shown). We were not clear how this occurred but it appeared that P2 receptors in MDCK cells were sensitive to N-methylglucamine and choline because ADP- and UTP-induced $[Ca^{2+}]_i$ rises were also suppressed by substitution of Na^+ with N-methylglucamine or choline, while bradykinin-induced $[Ca^{2+}]_i$ rises were not affected at all (not shown). Similarly, Na^+/Ca^{2+} exchange did not contribute to the decay of the $[Ca^{2+}]_i$ rise induced by bradykinin (15) and U73122 (16). In contrast, the decay of the $[Ca^{2+}]_i$ rise induced by SKF96365 was significantly enhanced by Na^+ removal (19). Thus, it appears that Na^+/Ca^{2+} exchange is recruited as a pathway for Ca^{2+} efflux depending on the agonist used.

If the decay of the ATP response is due to return of mobilized Ca^{2+} to the thapsigargin-sensitive store, it would be expected that thapsigargin added after the ATP response should induce $[Ca^{2+}]_i$ rises of identical magnitude as seen without prior ATP stimulation. The fact is that, as shown in Figures 1A and 1B, the thapsigargin-induced $[Ca^{2+}]_i$ rises were remarkably inhibited after prestimulation for 6-7 min with 0.1-1 mM ATP (*traces a, b*), suggesting that the majority of the Ca^{2+} mobilized by ATP did not return to the thapsigargin-sensitive store. Another possibility for the ATP response to decay is via buffering by mitochondria since we have previously shown that these stores play a dominant role in buffering the large loads of Ca^{2+} induced by U73122 in MDCK cells (16). If mitochondria have a contribution, inhibition of mitochondria would slow the decay of the Ca^{2+} signal. This possibility is ruled out because ATP induced a rise in $[Ca^{2+}]_i$ normally after pretreatment of cells with the mitochondrial inhibitors CCCP or oligomycin. CCCP evoked a small but significant rise in $[Ca^{2+}]_i$, reflecting a release of Ca^{2+} from, and an inhibition of, the resting mitochondria. Interestingly, in contrast to mitochondria, we found that lysosomes might play a role in buffering the ATP-mobilized Ca^{2+} because the peak ATP response was enhanced significantly (~59%) by prior permeabilization of lysosomes with GPN. However, this contribution of lysosomes may be of limited importance because GPN pretreatment appeared to facilitate the decay of the ATP-induced $[Ca^{2+}]_i$ rise via a unknown

mechanism. Similar faster decay of Ca^{2+} signal of higher peak amplitude was also observed in Figure 1A, *trace a vs. trace b*. The exact mechanism of this phenomenon is not clear. However, since our data suggest that plasmalemmal Ca^{2+} pumps might play a role in the decay of the ATP response, one possible interpretation for this phenomenon is that the facilitated decay might involve activation of plasmalemmal Ca^{2+} pumps by the higher levels of peak $[Ca^{2+}]_i$. Lysosomes were thought to participate in IP_3-mediated Ca^{2+} release in MDCK cells (10); we now provide evidence that lysosomes might also be involved in buffering agonist-induced $[Ca^{2+}]_i$ rises. Collectively, our data suggest that the decay of the ATP response involves efflux via plasmalemmal Ca^{2+} pumps and possibly buffering by lysosomes, and that efflux via Na^+/Ca^{2+} exchange and buffering by the thapsigargin-sensitive store and mitochondria have little contribution. To conclude, as a continuation of our previous work (12), here we have further examined the $[Ca^{2+}]_i$ rises induced by extracellular ATP in MDCK cells. Based on the combined results from this and our previous study, it is clear that in MDCK cells ATP induced a dose-dependent Ca^{2+} signal by triggering Ca^{2+} release from the ER Ca^{2+} store which is sensitive to depletion by thapsigargin, BHQ and CPA. The Ca^{2+} signal is amplified by capacitative Ca^{2+} entry which is inhibited by La^{3+}, econazole and SKF96365. ATP does not directly activate Ca^{2+} influx. The decay of the ATP response might involve Ca^{2+} efflux via the plasmalemmal Ca^{2+} pump and sequestration by lysosomes.

Acknowledgments

This work was supported by NSC88-2314-B-075B-003, VGHKS88-32 to CRJ. We thank CM Ho for culturing cells.

References

1. Abbracchio, M.P. and Burnstock. G. Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol. Therapeut.* 64: 445-475, 1994.
2. Boarder, M.R., Weisman, G.A., Turner, J.T. and Wilkinson. G.F. G protein-coupled P2 purinoceptors: from molecular biology to functional responses. *Trends Pharmacol. Sci.* 16: 133-139, 1995.
3. Clapman, D.E. Calcium signaling. *Cell* 80: 259-268, 1995.
4. Demaurex, N., Lew, D.P. and Krause. K.H. Cyclopiazonic acid depletes intracellular Ca^{2+} stores and activates an influx pathway for divalent cations in HL-60 cells. *J. Biol. Chem.* 267: 2318-2324, 1992.
5. Dietl, P., Haller, T., Wirleitner, B. and Friedrich. F. Two different store-operated Ca^{2+} entry pathways in MDCK cells. *Cell Calcium* 20: 11-19. 1996.
6. Dubyak, G.R., el-Moatassim, C. and el-Moatassim C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *C. Am. J. Physiol.* 265: C577-C606, 1993.
7. Firestein, L., Xing, M., Hughes, R.J., Corvera, C.U. and Insel. P.A. Heterogeneity of P2u- and P2y-purinergic receptor regulation of phospholipases in MDCK cells. *Am. J. Physiol.* 27: 1 F610-F618, 1996.
8. Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P. and Williams. M. Nomenclature and classification of purinoceptors. *Pharmacol. Rev.* 46: 143-156, 1994.
9. Grynkiewicz, G., Poenie, M. and Tsien. R.Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440-3450, 1985.
10. Haller, T., Dietl, P., Deetjen, P. and Volkl. H. The lysosomal compartment as intracellular calcium store in MDCK cells: a possible involvement in InsP3-mediated Ca^{2+} release. *Cell Calcium* 19: 157-165, 1996.
11. Herrington, J., Park, Y.B., Babcock, D.F. and Hille. B. Dominant role of mitochondria in clearance of large Ca^{2+} loads from rat adrenal chromaffin cells. *Neuron* 16: 219-228, 1996.
12. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng. C.J. Mechanism of lanthanum inhibition of extracellular ATP-evoked calcium mobilization in MDCK cells. *Life Sci.* 62: 533-540, 1998.
13. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng. C.J. ADP-evoked calcium signals in MDCK cells. *Chin. J. Physiol.* 41: 67-73, 1998.
14. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng. C.J. La^{3+} inhibits the UTP-induced Ca^{2+} mobilization in MDCK cells. *Chin. J. Physiol.* 41: 59-64, 1998.
15. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng. C.J. Bradykinin-evoked Ca mobilization in MDCK cells. *Eur. J. Pharmacol.* 355: 219-233, 1998.
16. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng. C.J. The phospholipase C inhibitor U73122 elevates cytoplasmic calcium levels in Madin Darby canine kidney cells by activating calcium influx and releasing stored calcium. *Life Sci.* 63: 895-908, 1998.
17. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng. C.J. Mechanism of rise and decay of thapsigargin-evoked calcium signals in MDCK cells. *Life Sci.* 64: 259-267, 1999.
18. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng. C.J. Mechanism of rise and decay of 2,5-di-tert-butylhydroquinone-induced Ca^{2+} signals in MDCK cells. *Eur. J. Pharmacol.* 365: 111-117, 1999.
19. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng. C.J. Multiple effects of 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole (SKF96365) on Ca^{2+} signaling in MDCK cells: depletion of thapsigargin-sensitive Ca^{2+} store followed by capacitaive Ca^{2+} entry, activation of a direct Ca^{2+} entry, and inhibition of thapsigargin-induced capacitative Ca^{2+} entry. *N-S Arch Pharmacol.* In press, 1999.
20. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng. C.J. Multiple effects of econazole on calcium signaling: depletion of thapsigargin-sensitive calcium store, activation of extracellular calcium influx, and inhibition of capacitative calcium entry. *Biochim. Biophys. Acta.* 1448: 533-542, 1999.
21. Kiedrowski, L. and Costa. E. Glutamate-induced destabilization of intracellular calcium concentration homeostasis in cultured cerebellar granule cells: role of mitochondria in calcium buffering. *Mol. Pharmacol.* 47: 140-147, 1995.
22. Lang, F. and Paulmichl. M. Properties and regulation of ion channels in MDCK cells. *Kidney Int.* 48: 1200-1205, 1995.
23. Lang, F., Plockinger, B., Haussinger, D. and Paulmich. M. Effects of extracellular nucleotides on electrical properties of subconfluent Madin Darby canine kidney cells. *Biochim. Biophys. Acta* 943: 471-476, 1988.
24. Merrit, J.E., Jocob, R. and Hallam. T.J. Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J. Biol. Chem.* 264: 1522-1527, 1989.
25. Milanick, M.A. Proton fluxes associated with the Ca pump in human red blood cells. *Am. J. Physiol.* 258: C552-C562, 1990.
26. Post, S.R., Jacobson, J.P. and Insel. P.A. P2 purinergic receptor

agonists enhance cAMP production in Madin-Darby canine kidney epithelial cells via an autocrine/paracrine mechanism. *J. Biol. Chem.* 271: 2029-2032, 1996.

27. Simmons, N.L. Identification of a purine (P2) receptor linked to ion transport in a cultured renal (MDCK) epithelium. *Br. J. Pharmacol.* 73: 379-384, 1981.
28. Simmons, N.L. Stimulation of Cl^- secretion by exogenous ATP in cultured MDCK epithelial monolayers. *Biochim. Biophys. Acta* 646: 231-242, 1981.
29. Surprenant, A., Rassendren, F., Kawashima, E., North, R.A. and G. Buell. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Purinoceptors: are there families of P2X and P2Y purinoceptors? *Science* 272: 735-738, 1996.
30. Thastrup, O., Cullen, P.T., Drobak, B.K., Hanley, M.R. and Dawson. A.P. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA* 87: 2466-2470, 1990.