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Article

Mechanisms of Rise and Decay of ADP-Evoked Calcium Signal in MDCK Cells

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Abstract

We have studied the effects of ADP on intracellular calcium levels ($[Ca^{2+}]_i$) measured by fura-2 fluorimetry in Madin Darby canine kidney (MDCK) cells. ADP evoked $[Ca^{2+}]_i$ rises dose-dependently by releasing Ca^{2+} from the inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} pool followed by capacitative Ca^{2+} entry. The Ca^{2+} signal consisted of a peak and a gradual decline which reached a plateau in the case of 0.1-1 mM ADP; a plateau was not seen in the response to 10 mM ADP. ADP acted by stimulating P_{2u} and P_{2y} receptors based on rank order of agonist potency: ATP = UTP > ADP > 2-methylthio-ATP > 2-methylthio-ADP > adenosine > α,β-methylene ATP. Buffering by lysosomes and efflux via plasmalemmal Ca^{2+} pumps might play important roles in the decay of the Ca^{2+} signal. The Ca^{2+} signal was dramatically inhibited by 100 μM $LaCl_3$.

Key Words: ADP, MDCK cells, Ca^{2+} signaling, cytosolic Ca^{2+}, fura-2

Introduction

It has been well established that intracellular nucleotides play essential roles in nucleic acid synthesis, enzyme regulation and energy metabolism. It is widely appreciated now that extracellular nucleotides also exert important physiological actions. Extracellular adenosine diphosphate (ADP) is a ubiquitous nucleotide which could activate a great number of cell types and tissues. Of particular importance, in platelets ADP, via activating the P_{2t} purinoceptors, induces aggregation and transformation and the release of their granule contents, leading to plug formation (1). $[Ca^{2+}]_i$ rises have been found to be a key signal in these activation phenomena and considerable interest has been focused on its source and regulatory mechanisms (2, 3). ADP also induces $[Ca^{2+}]_i$ rises in many other cell types such as hepatocytes (4), glial cells (5), and epithelial cells (6). Physiologically, extracellular ADP could be formed by pathways including direct release from cells such as platelets and chromaffin cells (7), or conversion from extracellular ATP, which is released by nerve terminals and many secretory cells, via ectonucleotidases (8), or from damaged cells.

Madin-Darby canine kidney (MDCK) cell line has been used as a model for epithelial cells (9). Ca^{2+} homeostasis is important in many aspects of MDCK cell function. It has been reported that ATP could elevate $[Ca^{2+}]_i$ by activating phospholipase C (PLC)-specific inositol 1,4,5-trisphosphate (IP_3) formation and mobilizing stored Ca^{2+} (10). Other hormones of similar effects include bradykinin (11, 12), carbachol (12) and prostaglandins (13). The Ca^{2+} signal could activate Ca^{2+}-dependent K^+ currents (10) leading to cell hyperpolarization (14) and chloride secretion (15). ATP and ADP as well as other nucleotides such as UTP, ITP, GTP, UDP and GDP have been shown to hyperpolarize the cell membrane by increase of the cell membrane potassium conductance (14, 16). However, the effect of ADP on $[Ca^{2+}]_i$ in MDCK cells has not been studied previously.

Recently we observed that ADP evoked robust $[Ca^{2+}]_i$ transients in MDCK cells. In this study, we address several important questions related to the ADP-evoked $[Ca^{2+}]_i$ rises including characterization of the Ca^{2+} signal, the receptor type acted by ADP, and the mechanisms of rise and decay of the signal.

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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. Only cells from passages 70-80 were used.

Solutions

Normal buffer (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 1.8; Hepes 10; glucose 5. Ca^{2+}-free buffer contained no added Ca^{2+} plus 0.1 mM EGTA. In Na^+-free buffer, NaCl was isomolarly replaced with choline chloride. The experimental solution contained less than 0.1% of solvent (DMSO or ethanol) which did not affect $[Ca^{2+}]_i$ (n=3).

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

Loading 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) was performed in trypsinized cells (10^6/ml) for 30 min at 25°C in DMEM. Cells were washed and resuspended in normal buffer and were washed again before each experiment to minimize extracellular dye. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring, which normally contained 1 ml of buffer and 0.5 million of cells unless otherwise stated. Fluorescence was monitored with a Hitachi F-4500 spectrofluorophotometer (Japan) by continuously collecting excitation signals at 340 nm and 380 nm and emission signal at 510 nm in 1-s intervals. Maximal and minimal fluorescences were obtained by adding TX-100 (0.1%) and EGTA (20 mM) sequentially at the end of the experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ as described previously (17) assuming a K_d of 155 nM. Mn^{2+} entry experiments were performed in normal buffer containing 50 µM $MnCl_2$ by collecting the excitation signals at 340, 360, and 380 nm and emission signal at 510 nm which were continuously alternated in 1-s intervals. Preliminary experiments showed that using trypsinized cells yielded qualitatively similar resluts as using cells attached on coverslips. We decided to use trypsinized cells because this procedure is easier and more time-saving.

Chemical Reagents

The reagents for cell culture were from Gibco (Grand Island, NY, USA). Fura-2/AM was from Molecular Probes (Eugene, OR., USA). Bradykinin, 2-methylthio-ATP, 2-methylthio-ADP, α,β-methylene ATP were from RBI (Natick, MA, USA). SK&F96365 was from Biomol (Plymouth Meeting, PA, USA). All other reagents were from Sigma (St. Loius, MO, USA).

Statistical Analysis

All values were reported as mean±S.E.. Statistical comparisons were determined utilizing the Student's t test, and significance was accepted when $P < 0.05$.

Results

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

ADP at a concentration range between 10 µM and 1mM evoked a rise in $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 1A). The $[Ca^{2+}]_i$ rise evoked by 0.1-1 mM ADP (traces *a* and *b*) consisted of a rapid peak, a gradual decay and a sustained plateau; while that by 10 µM ADP (trace *c*) did not have a plateau. Thapsigargin (TG) is a drug which inhibits the endoplasmic reticulum (ER) Ca^{2+} pump allowing Ca^{2+} to leak from the ER store (18). Addition of ADP (0.1-1 mM; traces *a* and *b*) significantly inhibited the TG-evoked $[Ca^{2+}]_i$ rises as shown in trace *d* suggesting that ADP substantially mobilized TG-sensitive Ca^{2+} pools. Figure 1B shows that removal of extracellular Ca^{2+} (no added Ca^{2+} + 0.1 mM EGTA) reduced the $[Ca^{2+}]_i$ peak evoked by 1 mM ADP by ~19% (589±20 nM vs. 700±12 nM; n=5; $P < 0.05$), significantly reduced the area under the curve, and completely abolished the plateau of the $[Ca^{2+}]_i$ rises, suggesting that the plateau resulted from Ca^{2+} influx. Qualitatively similar results were found for 0.1 mM ADP, while the effects of Ca^{2+} removal on $[Ca^{2+}]_i$ rises evoked by 10 µM ADP was insignificant suggesting that Ca^{2+} influx only occurred on stimulation of high doses of ADP. Additionally, in Ca^{2+}-free buffer, the TG-evoked $[Ca^{2+}]_i$ peak was reduced by ~ 50% (trace *d*; 201±20 nM vs. 405±23 nM; n = 5; $P < 0.05$) suggesting that TG induced significant Ca^{2+} influx. Pretreatment of 0.1-1 mM ADP abolished the TG-evoked response in Ca^{2+}-free buffer (traces *a* and *b*) implicating that ADP and TG mobilized the same Ca^{2+} pools. In normal buffer, ADP failed to evoke $[Ca^{2+}]_i$ rises after pretreatment of either 10 µM U73122, a phospholipase C inhibitor (19), or 0.1 µM TG for 15 min (Fig. 2A, left panel). U73343, an inactive structural analogue

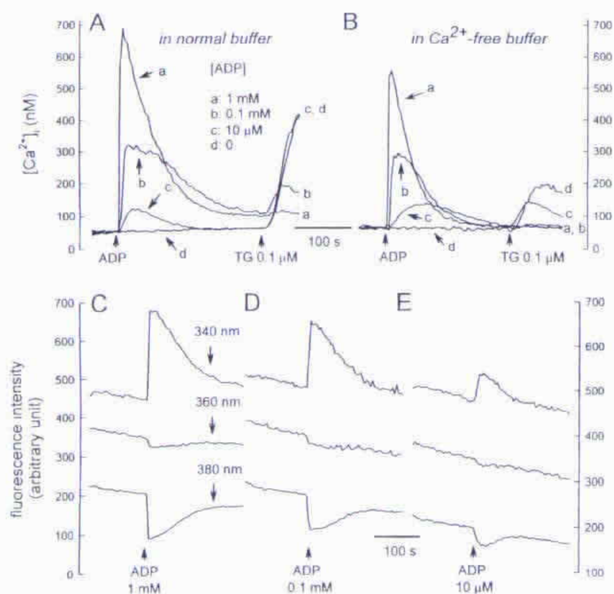

Fig. 1. Effects of ADP on $[Ca^{2+}]_i$ in fura-2 loaded MDCK cells. ***A***. Dose-response relationship determined in normal buffer. ADP was added at the indicated time at 1 mM (trace *a*), 0.1 mM (trace *b*) or 10 µM (trace *c*). Trace *d* was control without addition of ADP. TG (0.1 µM) was added at the indicated time. ***B***. Similar to ***A*** except that extracellular Ca^{2+} was removed (no added Ca^{2+} + 0.1 mM EGTA). ***C-E***. ADP-evoked Ca^{2+} influx detected by Mn^{2+} entry measurements. $MnCl_2$ (50 µM) was added to cells 90 s prior to ADP stimulation. Concentration of ADP was 1 mM in ***C***, 0.1 mM in ***D***, and 10 µM in ***E***. Excitation signals which alternated at 340, 360, and 380 nm and emission signal at 510 nm were continuously collected in 1-s intervals. The *bars* represent time scales of 100 s. The traces were representative of five experiments.

of U73122, was tested as a control for the U73122 effect and was found not to affect the ADP response (Fig. 2A, right panel).

We applied another maneuver to prove that Ca^{2+} influx occurred during ADP 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 (20). Thus, Mn^{2+} can aid in detecting 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 normal buffer containing $MnCl_2$ (50 µM). Figure 1C shows that ADP (1 mM) induced a rise in 340 nm signal accompanied by a corresponding drop in 380 nm signal. Concomitantly there was a slight but significant drop in 360 nm signal which occurred early upon ADP addition and gradually recovered to prestimulatory baseline. Similar, but smaller, effects were observed with 0.1 mM ADP. Mn^{2+} influx induced by 10 µM ADP was negligible. Thus it is clear that 0.1-1 mM ADP evoked Ca^{2+} influx.

ADP raises $[Ca^{2+}]_i$ by Activating P_{2u} and P_{2y} Receptors

At present, P_2 nucleotide receptors have been subdivided pharmacologically into at least P_{2u}, P_{2t}, P_{2x}, P_{2y} and P_{2z} (7). To determine which receptors mediated the ADP-evoked $[Ca^{2+}]_i$ rises in MDCK cells, the effects of several nucleotides on $[Ca^{2+}]_i$ were determined and compared. At 0.1 mM, the order of potency of these nucleotides in raising $[Ca^{2+}]_i$ (peak height) was: ATP = UTP > ADP > 2-methylthio-ATP > 2-methylthio-ADP > adenosine > α,β-methylene ATP (Fig. 2B); AMP being ineffective.

Mechanisms Underlying the Decay of the ADP-evoked Ca^{2+} Signal

In most cells, many mechanisms could underlie the decay of $[Ca^{2+}]_i$ transients evoked by an agonist. For example, desensitization of receptors or channels,

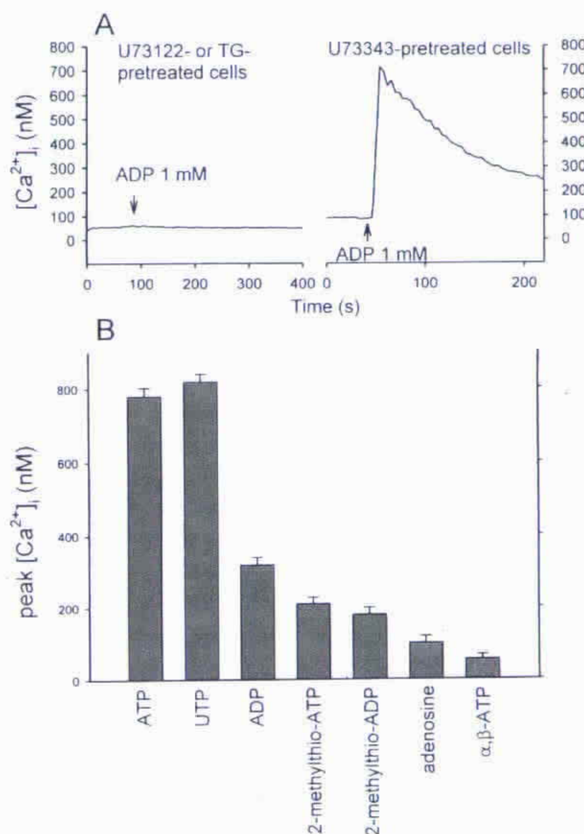

Fig. 2. ***A***. left panel: cells were pretreated with U73122 (10 µM) or thapsigargin (TG; 0.1 µM) for 15 min before adding ADP (1 mM). Right panel: cells were pretreated with U73343 (10 µM) for 15 min before adding ADP (1 mM). Traces were representative of four experiments. ***B***. Effects of several nucleotides on $[Ca^{2+}]_i$. The concentration was 0.1 mM for all nucleotides. Data were mean±S.E. (n = 4) of the peak height (nM) of the $[Ca^{2+}]_i$ rises evoked by the nucleotides. Student's *t* test was performed to test the statistical difference between any two bars. Except between ATP and UTP, all other pairs were significantly different. All experiments in this figure were performed in normal buffer.

buffering by Ca^{2+} binding proteins, efflux via plasmalemmal Ca^{2+} pumps and Na^+/Ca^{2+} exchange and sequestration by internal stores (21). How the Ca^{2+} signal evoked by an agonist decays in MDCK cells has not been investigated previously. We determined whether the following mechanisms were responsible for the decay of the ADP-evoked Ca^{2+} signal: Ca^{2+} efflux via the Ca^{2+} pump or Na^+/Ca^{2+} exchange and sequestration by the ER, mitochondria and lysosomes.

We first examined the role of the Ca^{2+} pump. For the plasmalemmal Ca^{2+} pump, a selective inhibitor is not available. We tried three manipulations which could depress the pump: extracellular alkalization (22), adding eosin (23) or La^{3+} (22). Extracellular alkalization to pH 8.0 significantly slowed the decay of the ADP-evoked $[Ca^{2+}]_i$ transient (Fig. 3A) without altering the resting $[Ca^{2+}]_i$ or the evoked $[Ca^{2+}]_i$ peak height, although the rise of the transient was slightly slower than control. The data suggest that the Ca^{2+} pump might play a significant role in the decay of the ADP-evoked Ca^{2+} signal. Eosin (1-10 μM) interfered with fura-2 fluorescence and could not be tested. Because La^{3+} potently inhibited the Ca^{2+} signal (see below), its inhibition on the Ca^{2+} pump was difficult

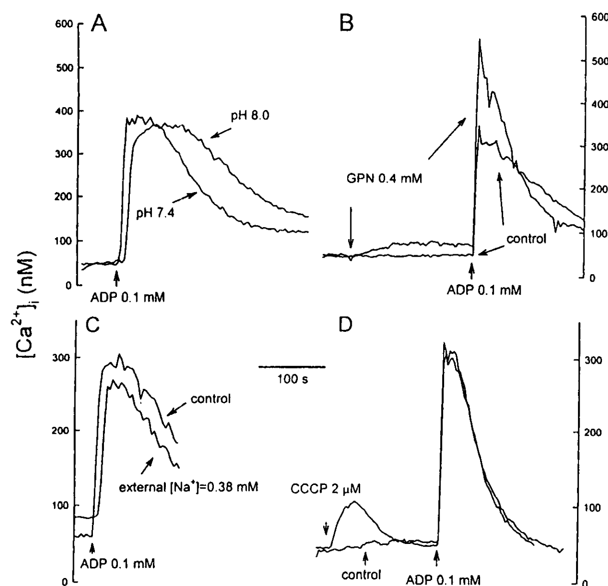

Fig. 3. Mechanisms of the decay of the ADP-evoked $[Ca^{2+}]_i$ rises. ***A***, 0.1 mM ADP-evoked $[Ca^{2+}]_i$ rises were measured in buffer of pH 7.4 (normal buffer) and pH 8.0, and the results were compared. ***B***, In normal buffer, GPN (0.4 mM) was added for 160 s before addition of ADP (0.1 mM). Control: without GPN pretreatment. ***C***, Effects of 0.1 mM ADP on $[Ca^{2+}]_i$ in low $[Na^+]$ buffer. External $[Na^+]$ was lowered to 0.38 mM by replacing NaCl with choline chloride (see ***Results*** for detail). Control: ADP effects in normal buffer. ***D***, Effects of CCCP pretreatment on the ADP-evoked $[Ca^{2+}]_i$ rises. The experiments were performed in Ca^{2+}-free buffer. Control: without CCCP pretreatment. The *bar* represents a time scale of 100 s. The traces were representative of five experiments.

to determine. Lysosomes have been implicated in IP_3-mediated Ca^{2+} release in MDCK cells and could be depleted of stored Ca^{2+} by Gly-Phe-β-naphthylamide (GPN) via permeabilization of the lysosomal membrane (24). We next examined whether lysosomes contributed to buffering the ADP-evoked Ca^{2+} signal. Figure 3B shows that GPN (0.4 mM) caused a tiny (~20 nM) and gradual rise in $[Ca^{2+}]_i$ for 160 s. ADP added subsequently evoked a $[Ca^{2+}]_i$ transient which had a peak ~ 70% higher (586±13 vs. 350±5 nM; n = 5; $P < 0.05$) than control with a faster decay. Thus lysosomes may contribute to buffering the Ca^{2+} signal; however, this contribution may be of limited importance because GPN pretreatment appeared to facilitate the decay of the Ca^{2+} signal via some unknown mechanism.

We next examined the contribution of Na^+/Ca^{2+} exchange which has been found to be active in MDCK cells (25). If external Na^+-dependent Ca^{2+} efflux occurs during the Ca^{2+} signal, lowering external $[Na^+]$ to less than 1 mM should be expected to block the Ca^{2+} efflux and result in a potentiation of the signal. To inhibit Na^+-dependent Ca^{2+} efflux, we lowered extracellular $[Na^+]$ by adding 5 μl of cell suspension in normal buffer to 2 ml of Na^+-free buffer (Na^+ replaced with choline) in a cuvette. This gave an extracellular $[Na^+]$ of 0.38 mM. Immediately after cells being added to Na^+-free buffer, the basal $[Ca^{2+}]_i$ was 83±10 nM (Fig. 3C; n = 5) which was significantly higher than that measured in normal buffer (control; 61±6 nM; n = 5; $P < 0.05$). Stimulation of ADP (0.1mM) elicited a $[Ca^{2+}]_i$ transient indistinguishable from control in kinetics, and the peak height of the transient was even reduced instead of increased compared with control. Thus it is unlikely that Na^+-dependent Ca^{2+} efflux contributed to the decay of the Ca^{2+} signal. The reduced $[Ca^{2+}]_i$ rise seen in low $[Na^+]$ buffer was probably due to sensitivity of ADP receptors to the Na^+ substitute, choline, because the $[Ca^{2+}]_i$ rises were even much more reduced when N-methyl-glucamine (NMG) was the Na^+ substitute (not shown). In contrast, bradykinin-evoked $[Ca^{2+}]_i$ rises in MDCK cells were not altered by Na^+ substitution with choline or NMG (not shown).

If mitochondria contribute to buffering the ADP-evoked Ca^{2+} signal, inhibition of mitochondria would slow the decay of the signal. Figure 3D shows that the mitochondrial uncoupler carbonylcyanide m-chlorophynylhydrozone (CCCP; 2 μM) evoked small $[Ca^{2+}]_i$ rises (peak = 100±25 nM; n = 5) in Ca^{2+}-free buffer, reflecting a release of Ca^{2+} from, and an inhibition of, the resting mitochondria, without affecting the ADP-evoked Ca^{2+} signal, suggesting that the decay of the Ca^{2+} signal was unlikely to be due to buffering by mitochondria. Our data also suggest that resting mitochondria in MDCK cells contain

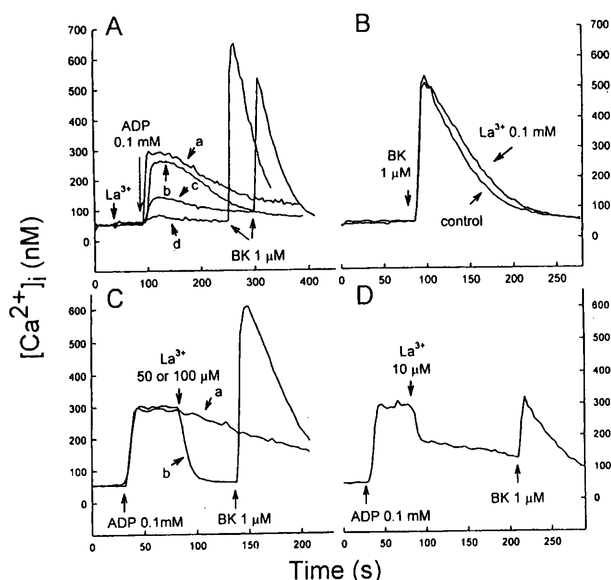

Fig. 4. Effects of La^{3+} on ADP-evoked $[Ca^{2+}]_i$ rises. ***A***, $LaCl_3$ was added for 30 s before addition of 0.1 mM ADP. Concentration of La^{3+} was zero in trace *a*, 10 μM in trace *b*, 50 μM in trace *c*, and 0.1 mM in trace *d*. In traces *c* and *d*, bradykinin (BK; 1 μM) was added at the indicated time. ***B***, Effects of La^{3+} (0.1 mM) on BK-evoked $[Ca^{2+}]_i$ rises. In La^{3+}-treated group, La^{3+} was added 30 s before addition of 1 μM BK. ***C***, La^{3+} was added during the decay of the 0.1 mM ADP-evoked $[Ca^{2+}]_i$ rises. Concentration of La^{3+} in trace *a* was zero, in trace *b* was 50 or 100 μM. In trace *b*, 1 μM BK was added at the indicated time. ***D***, Similar to ***C*** except that the concentration of La^{3+} was 10 μM. All experiments were performed in normal buffer. The traces were representative of five experiments.

significant amount of releasable Ca^{2+}.

La^{3+} Potently Inhibited the ADP-evoked $[Ca^{2+}]_i$ Rises

We investigated whether the ADP-evoked Ca^{2+} signal could be modulated pharmacologically. Among the several drugs we had examined, La^{3+} was found to have the most dramatic inhibition on the ADP-evoked Ca^{2+} signal. Figure 4A shows that La^{3+} (0.1mM) pretreatment for 30 s inhibited 90.8±1.3% of 0.1 mM ADP-evoked $[Ca^{2+}]_i$ peak (trace *d* vs. trace *a*; n = 5; $P < 0.05$). The inhibition of La^{3+} was dose-dependent with 50 μM La^{3+} inhibiting 55.1±3.9% of the signal (trace *c*; n = 5) and 10 μM La^{3+} inhibiting 10.8±2.5% (trace *b*; n = 5). Under the circumstances that the ADP-evoked Ca^{2+} signal was substantially blunted (Fig. 4A, traces *c*, *d*), bradykinin added subsequently could evoke robust $[Ca^{2+}]_i$ rises which were similar to (or even larger than) control without ADP pretreatment (Fig. 4B). The fact that the effect of bradykinin alone was slightly enhanced by La^{3+} pretreatment (Fig. 4B) could be because that La^{3+} inhibited the plasmalemmal Ca^{2+} pump and slowed the decay of the $[Ca^{2+}]_i$ rises. Interestingly, La^{3+} (50 or 100 μM) added during the decay of the ADP-evoked Ca^{2+} signal immediately and completely abolished the signal without affecting that evoked by subsequently added bradykinin (Fig. 4C; trace *b*), with 10 μM La^{3+} inhibiting the ADP-evoked $[Ca^{2+}]_i$ peak by ~ 40% (Fig. 4D). In Figure 4D, bradykinin-evoked $[Ca^{2+}]_i$ rises were much smaller than that seen in Figure 4C, probably because ADP and bradykinin shared similar Ca^{2+} pools which had been partially depleted by ADP pretreatment.

Lack of Cross-Desensitization with ATP, UTP and Bradykinin

We next examined whether ADP cross-desensitized with ATP, UTP and bradykinin in evoking $[Ca^{2+}]_i$ rises. Figure 5 (A, B, C) shows that the ADP-evoked $[Ca^{2+}]_i$ rises did not cross-desensitize with that by ATP, UTP and bradykinin. In Figures 5A and 5B, when added after the ADP-evoked Ca^{2+} signal had decayed, maximal doses of ATP and UTP (0.1 mM) were used to overcome the ADP-induced partial depletion of the Ca^{2+} pools. In the same vein, when ATP and UTP were added prior to the ADP-evoked Ca^{2+} signal, a submaximal dose (1 μM) was used to avoid depletion of the Ca^{2+} pools so that the ADP-evoked Ca^{2+} signal could be observed. Depletion of the Ca^{2+} pools was observed in Figure 5C in which bradykinin-evoked $[Ca^{2+}]_i$ rises were substantially reduced by ADP pretreatment.

Discussion

In this study we have carefully examined the $[Ca^{2+}]_i$ rises evoked by external ADP in MDCK cells. Our results suggest that submaximal and maximal

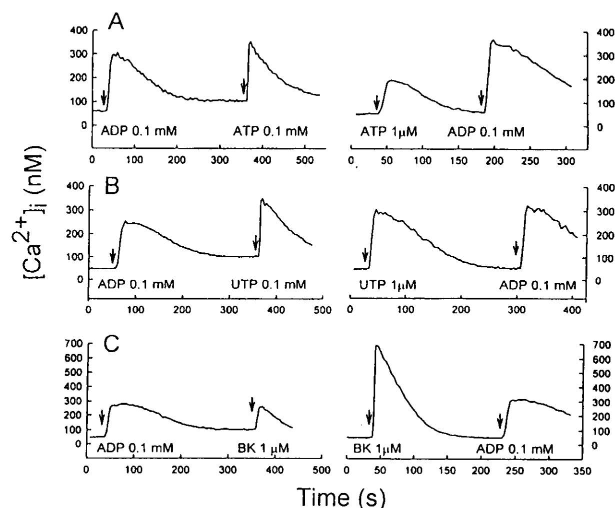

Fig. 5. Lack of cross-desensitization between ADP and ATP (or UTP and bradykinin). All experiments were performed in normal buffer. Drugs were added at the indicated time. The traces were representative of five experiments.

doses of ADP (0.1-1 mM) evoked $[Ca^{2+}]_i$ rises by activating PLC-specific IP_3 formation and mobilizing Ca^{2+} from the TG-sensitive pools. This Ca^{2+} signal was amplified by capacitative Ca^{2+} entry which contributed to part of the peak Ca^{2+} signal and was responsible for the plateau phase.

In MDCK cells, depletion of Ca^{2+} pools has been shown to trigger capacitative Ca^{2+} entry (26), a now widely appreciated Ca^{2+} influx process originally postulated by Putney (27). SK&F96365 and La^{3+} have been reported to inhibit capacitive Ca^{2+} entry in MDCK cells (26); however, we found that SK&F96365 (20-50 μM) itself induced robust $[Ca^{2+}]_i$ rises (not shown) rendering its effects on capacitative Ca^{2+} entry difficult to determine. Similarly, because La^{3+} (0.1 mM) inhibited as much as ~ 90% of the ADP-evoked $[Ca^{2+}]_i$ rises (Fig. 4), its inhibition on capacitative Ca^{2+} entry was difficult to assess.

To examine which P_2 receptor subtypes mediated the ADP response, we measured and compared the effects of several nucleotides on $[Ca^{2+}]_i$. The rank order of potency of these nucleotides strongly suggests that the P_{2u} subclass is the major P_2 nucleotide receptor in these cells. The P_{2y} receptor agonist 2-methylthio-ATP, induced robust $[Ca^{2+}]_i$ rises which were only slightly smaller than that by ADP, suggesting that P_{2y} receptors were also involved in mediating the ADP response. It has been reported recently that MDCK cells possess P_{2u} and P_{2y} receptors based on relative potencies of nucleotide analogues on release of arachidonic acid (28) and transepithelial ion transport (29). Our results demonstrate for the first time that P_{2u} and P_{2y} receptors coexist in MDCK cells based on $[Ca^{2+}]_i$ measurements, and that these two different subtypes of P_2 receptors might be responsible for the ADP-evoked $[Ca^{2+}]_i$ rises.

How Ca^{2+} signal decays has never been investigated in MDCK cells. Our data suggest that buffering by lysosomes (but not by mitochondria) and efflux via plasmalemmal Ca^{2+} pumps (but not via Na^+/Ca^{2+} exchange) might play important roles in the decay of the Ca^{2+} signal. Another mechanism that might underlie the decay of the ADP response is via buffering by the TG-sensitive ER Ca^{2+} pool. This possibility is unlikely because, as shown in Figs. 1A and 1B (traces *a*), TG added after the ADP-evoked Ca^{2+} signal had decayed to a plateau failed to induce any release of Ca^{2+} both in the presence and absence of external Ca^{2+}, demonstrating that the Ca^{2+} mobilized by ADP was not buffered by the TG-sensitive ER pool during the time period we observed (~ 4 min).

Another interesting finding in this study was that La^{3+} potently inhibited the ADP response. It has been well established that La^{3+} blocks many Ca^{2+} transport pathways in the plasma membrane in many cell types, including capacitative Ca^{2+} entry in MDCK cells (26). The dramatic inhibition of La^{3+} on the ADP-evoked Ca^{2+} signal would not be surprising if the Ca^{2+} signal was dominated by Ca^{2+} influx. However, comparing traces b in Figures 1A and 1B, it is obvious that Ca^{2+} influx only accounted for a small portion of the 0.1 mM ADP-evoked $[Ca^{2+}]_i$ rises, however, the ADP response was almost abolished by La^{3+} (Fig. 4A, trace *d*), rendering it impossible that La^{3+} exerted its inhibition solely via reducing Ca^{2+} influx. We have found similar potent inhibition of La^{3+} on the $[Ca^{2+}]_i$ rises induced by ATP and UTP in MDCK cells (30, 31). Further, taking into account of the fact that La^{3+} did not have any inhibition on the $[Ca^{2+}]_i$ rises induced by bradykinin which also mobilizes IP_3-dependent Ca^{2+} pools, the most likely explanation for the inhibition of La^{3+} on the $[Ca^{2+}]_i$ rises evoked by ADP would be that, in addition to block of capacitative Ca^{2+} entry, La^{3+} directly inhibits the P_2 receptors or interferes with nucleotide binding to the P_2 receptors. To our knowledge, dramatic inhibition of La^{3+} on nucleotide-evoked $[Ca^{2+}]_i$ rises has never been reported previously in any cell types.

In sum, we have investigated the mechanisms underlying the rise and decay, and also the modulation, of the Ca^{2+} signal induced by ADP. So far, most studies on ADP effects on Ca^{2+} homeostasis have been focused on platelets. It would be interesting to see how ADP affects $[Ca^{2+}]_i$ in other cell types.

Acknowledgments

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