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Plasma membrane Ca^{2+} pumping plays a prominent role in adenosine A_1 receptor mediated changes in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells

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Abstract

Adenosine A_1 receptor mediated formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and accumulation of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) were investigated in DDT_1 MF-2 smooth muscle cells. A strong reduction of the adenosine and N^6-cyclopentyladenosine (CPA) induced rise in $[Ca^{2+}]_i$ was observed after blocking Ca^{2+} entry across the plasma membrane with $LaCl_3$. This effect of $LaCl_3$ was not observed in the absence of extracellular Ca^{2+}; it was not caused by reduced $Ins(1,4,5)P_3$ formation or changed $Ins(1,4,5)P_3$ induced Ca^{2+} release, or influenced by temperature. The inhibition of the CPA induced increase in $[Ca^{2+}]_i$ by $LaCl_3$ was strongly counteracted in the presence of ortho-vanadate, an inhibitor of plasma membrane Ca^{2+} ATPase. Ortho-vanadate might also reduce protein tyrosine-phosphate phosphatase activity involved in tyrosine kinase mediated phospholipase C (PLC) activation. However, ortho-vanadate and tyrphostin 25, a tyrosine kinase inhibitor, did not affect the CPA induced formation of $Ins(1,4,5)P_3$. Taken together, these results show a strong contribution of Ca^{2+} pumping across the plasma membrane to the regulation of $[Ca^{2+}]_i$ mediated by adenosine A_1 receptors. Na^+/Ca^{2+} exchange only played a minor role in the initial phase of CPA induced Ca^{2+} metabolism as measured in low Na^+ containing solution. The mechanism by which adenosine A_1 receptors activate plasma membrane Ca^{2+} ATPase pumps does not include direct stimulation of pumps, but most likely involves an indirect pathway activated by a rapid increase in $[Ca^{2+}]_i$.

Keywords: Adenosine A_1 receptor; Ca^{2+}; Ca^{2+} pumping; DDT_1 MF-2 cell; (Temperature)

1. Introduction

Adenosine receptors are classified into different subtypes, $A_{1,2A,B,3}$, based on agonist and antagonist properties and are coupled via GTP binding proteins to a wide variety of effectors (reviewed by Fredholm et al., 1994).

The adenosine receptor signal transduction system has been well characterized in DDT_1 MF-2 smooth muscle cells. Adenosine A_1 receptors that inhibit adenylyl cyclase and adenosine A_2 receptors that stimulate adenylyl cyclase have been identified on these cells (Gerwins et al., 1990; Gerwins and Fredholm, 1991; Ramkumar et al., 1990; Shryock et al., 1993). Moreover, stimulation of adenosine A_1 receptors caused a pronounced activation of phospholipase C leading to a formation of inositol phosphates, Ca^{2+} release from internal $Ins(1,4,5)P_3$ sensitive stores and Ca^{2+} entry across the plasma membrane. It was reported that the adenosine A_1 receptor mediated rise in $[Ca^{2+}]_i$ was not reduced in the absence of extracellular Ca^{2+} (Gerwins and Fredholm, 1992a,b; White et al., 1992; Dickenson and Hill, 1993). In contrast, Schachter et al. (1992) observed only a minor activation of phospholipase C on adenosine A_1 receptor stimulation which was abolished after the removal of extracellular Ca^{2+}.

In a preliminary study we observed a strong inhibition of the adenosine A_1 receptor mediated rise in $[Ca^{2+}]_i$ when Ca^{2+} entry was blocked with $LaCl_3$ but not in the absence of extracellular Ca^{2+}. We investigated the mechanism which caused this difference by measuring $Ins(1,4,5)P_3$ formation and rises in $[Ca^{2+}]_i$ at different temperatures, and determined the involvement of plasma membrane Ca^{2+} pumping and Na^+/Ca^{2+} exchange in the adenosine A_1 receptor mediated Ca^{2+} response.

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2. Materials and methods

2.1. Cell culture

DDT_1 MF-2 cells, derived from a Syrian hamster vas deferens (Norris et al., 1974) were cultured in Dulbecco's modified essential medium supplemented with 7 mM $NaHCO_3$, 10 mM Hepes at pH 7.2 (DMEM) and 10% fetal calf serum at 37°C in 5% CO_2 (Hoiting et al., 1990).

2.2. Measurement of Ins(1,4,5)P_3

DDT_1 MF-2 cells were grown in monolayers in 9.6 cm^2 plastic wells as described earlier (Sipma et al., 1995). The medium was replaced by 2 ml DMEM at 20°C, 30 min before starting the experiment by adding agonists. After removing the medium, reactions were stopped with 400 μM 5% trichloroacetic acid (TCA) and placed on ice for at least 45 min. Samples were washed 3 times with 800 μl water saturated diethylether and neutralised with KOH (25 μl, 0.2 M).

Mass measurements of Ins(1,4,5)P_3 were performed as described earlier, using a standard curve of Ins(1,4,5)P_3 in ether extracted TCA solution (Molleman et al., 1991). In short, samples were assayed in 25 mM Tris/HCl (pH 9.0), 1 mM EDTA, 1 mg bovine serum albumin, [^{3}H]Ins(1,4,5)P_3 (3.3 Ci/mmol, 2000 cpm/assay) and about 1 mg binding protein for 15 min. The binding protein was isolated from fresh beef liver (Chilvers et al., 1989). Bound and free radioactivities were separated by centrifugation. The radioactivity in the pellet was determined by liquid scintillation counting.

2.3. Measurements of intracellular Ca^{2+}

Suspension: intracellular Ca^{2+} concentrations were measured as described earlier (Hoiting et al., 1990). Cells (10^6 cells/ml) suspended in DMEM containing 10% foetal calf serum were loaded with Indo-1/AM (1.5 μM) for 45 min at 37°C. The cells were collected by centrifugation (5 min, 1000 × g) and washed two times before the fluorescence measurement with a buffer solution containing: NaCl (145 mM), KCl (5 mM), $MgSO_4$ (0.5 mM), $CaCl_2$ (1 mM), D-glucose (10 mM), Hepes 10 mM, (pH 7.4) (Hesketh et al., 1983). Low Na^+ buffer contained 10 mM NaCl and 135 mM glucamide-chloride. Ca^{2+}-free solution contained Mg^{2+} (6.2 mM) to prevent membrane leakage and EGTA (0.1 mM) to remove extracellular Ca^{2+} (Den Hertog, 1981). Indo-1 fluorescence of the cells (excitation: 325 nm; emission 400 nm and 480 nm) was measured at 22°C or 37°C. The cell suspension was continuously magnetically stirred. The internal calcium concentration was calculated (Hesketh et al., 1983) using 0.015% of Triton X-100 as permeabilizing agent. $LaCl_3$ did not interfere with the Indo-1 fluorescence or calibration of the measurements.

Single cells: cells were plated at a density by 1.5–2.0 10^5 in six well plates for 48–72 h. On the day of experiment the culture medium was replaced with a solution of the following composition: 120 mM NaCl, 5 mM KCl, 1.6 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 20 mM Hepes, 10 mM D-glucose and 0.1% bovine serum albumin (pH adjusted at 7.4 with NaOH). Fura-2 was loaded in cytosol by an incubation with 2 μM Fura-2/AM, during 30–40 min, in the dark, at room temperature. Under these conditions the compartmentalization of the dye is minimal, as judged by the remaining fluorescence (4.8 ± 1.1%, n = 4) after selective permeabilization of plasmalemma with 10–15 μg/ml digitonin. At the end of the incubation period cells were washed several times with Fura-free solution and incubated for at least 15 min to allow a complete de-esterification of the dye.

The coverslip was then mounted in a specially designed chamber (capacity 0.150 ml) in a stage of inverted fluorescence microscope (Zeiss, Axiovert 35) equipped for Fura-2 microfluoremetry. The temperature of the chamber was maintained at 37°C and the cells were perfused at a rate by 0.9 ml/min. The entire bath solution was changed in less than 6 s. The Zeiss MSP system switches between the two excitation wavelengths (340 nm and 380 nm) and ratios of emitted light at 510 nm was acquired every 2.5 s. These ratios were converted to calcium levels using the classical equation described by Grynkiewicz et al. (1985). The R_{min} value for minimal fluorescence was measured after perfusion with solution without calcium and with 20 mM EGTA, 5 mM Tris-HCl, and 10 μM ionomycin (pH 8.2, no bovine serum albumin added) and the R_{max} of the equation was measured in the presence of 10 mM $CaCl_2$ and 10 μM ionomycin. The autofluorescence of the cell was determined as that remaining in the presence of 5 mM $MnCl_2$ and 10 μM ionomycin.

In each experiment, fluorescence measurements were limited to one cell, by means of an adjustable external diaphragm. The fluorescence signal collected from one cell was stable for more than 20 min, and the bleaching was minimal.

2.4. $^{45}Ca^{2+}$ efflux measurements

The cells were plated on poly-L-lysine (0.01 mg/ml) coated wells (10^6 cells/well) 15 h before the start of the experiment. The experiments were carried out at 22°C, following the same procedure as described before (Van der Zee et al., 1995). In brief, cells were equilibrated for 1 h with a modified Krebs solution containing (in mM): 135 NaCl, 5.9 KCl, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 11.6 Hepes and 11.5 glucose. The cells were then permeabilized by an incubation for 10 min with saponin (40 μg/ml) in a solution containing (in mM): 100 KCl, 30 imidazole, 2 $MgCl_2$, 1 ATP, and 1 EGTA (pH 7.0) and subsequently loaded for 5 min with $^{45}Ca^{2+}$ by exposure to a solution containing 10.5 μCi/ml $^{45}CaCl_2$ (specific activity: 19.3

Ci/g) with a final composition of (in mM): 100 KCl, 30 imidazole, 5 $MgCl_2$, 5 ATP, 0.44 EGTA, 5 NaN_3 and 0.12 $CaCl_2$ (pH 7.0); the free Ca^{2+} concentration of this solution was 0.15 μM. The efflux was performed by adding 1 ml of a solution containing (in mM) 100 KCl, 30 imidazole, 2 $MgCl_2$, 1 ATP, 1 EGTA and 5 NaN_3 (pH 7.0) to the cells and replacing it every 2 min during 30 min. The $^{45}Ca^{2+}$ present in each of the efflux samples and the remaining $^{45}Ca^{2+}$ in the cells at the end of the efflux procedure was measured by liquid scintillation counting. The time course of the tracer wash-out was calculated by summing in retrograde order the amount of tracer remaining in the cells at the end of the efflux and the amount of tracer collected during the successive time intervals. This time course became mono-exponential after 8–10 min. The $^{45}Ca^{2+}$ release was represented as the fractional loss of $^{45}Ca^{2+}$ per minute, representing the amount of $^{45}Ca^{2+}$ leaving the cell, normalized to the amount of labelled $^{45}Ca^{2+}$ present in the cell at that time.

2.5. Data analysis

Data are represented as means ± S.E.M. Data were considered significantly different from control values when $P < 0.05$ using Student's unpaired t-test. A sigma plot logistic curve fit program (Jandel Scientific, USA) was used to determine EC_{50} values and to analyze binding parameters obtained from the Ins(1,4,5)P_3 radioligand binding assay.

2.6. Chemicals

Inositol 1,4,5-trisphosphate sodium salt and Fura-2/AM were obtained from Boehringer (Germany). Indo-1/AM was from Molecular Probes (USA). Thapsigargin and N^6-cyclopentyladenosine (CPA) were purchased from Sigma (USA). Tyrphostin 25 was from Biomol (USA). Sodium ortho-vanadate and adenosine were from Janssen Chimica (Belgium). Saponin was from ICN Biochemicals (USA). $^{45}CaCl_2$ was obtained from Amersham International (UK) and D-[2-^{3}H]inositol 1,4,5-trisphosphate from Du Pont-New England Nuclear (USA). Hepes, imidazole and $LaCl_3$ and all other chemicals were from Merck (Germany).

3. Results

3.1. Adenosine A_1 receptor mediated increase in $[Ca^{2+}]_i$ at 22°C and 37°C

The rise in $[Ca^{2+}]_i$ upon exposure of DDT_1 MF-2 cells to a maximal effective concentration of the adenosine A_1 receptor agonist, CPA (100 nM), was reported to be similar in magnitude in the presence or absence of extracellular Ca^{2+} at 37°C (Gerwins and Fredholm, 1992a; White et al., 1992). We observed a similar result at 22°C

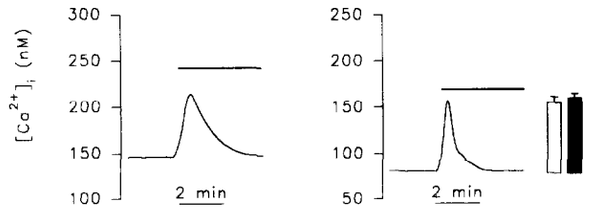

Fig. 1. The adenosine A_1 receptor mediated change in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. The CPA (100 nM, horizontal bar) induced response was measured at 22°C in the presence (left panel) or absence (right panel) of extracellular Ca^{2+}. Maximum amplitudes are presented (means ± S.E.M.) by the bars on the right-hand side. Each tracing is representative of 8 experiments.

(Fig. 1, Table 1), even though basal $[Ca^{2+}]_i$ was reduced when extracellular Ca^{2+} was removed. In contrast, we found that inhibition of Ca^{2+} entry by pretreatment of cells with $LaCl_3$ (50 μM, 2 min) strongly diminished the CPA induced rise in $[Ca^{2+}]_i$ (Fig. 2A, Table 1). The CPA induced increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} was not affected by $LaCl_3$ even at a four-times higher concentration (Table 1). In order to investigate whether the difference between these two strategies of measuring Ca^{2+} release from internal stores was dependent on temperature, increases in $[Ca^{2+}]_i$ were also measured at room temperature (22°C). At this temperature the rise in $[Ca^{2+}]_i$ after adenosine A_1 receptor stimulation reached a similar maximal value in the absence and presence of $LaCl_3$ as observed at 37°C (Fig. 2B, Table 1). The time to reach the maximum increase in $[Ca^{2+}]_i$ at 22°C ($t = 38 \pm 6$ s, $n = 12$) was about twice as long as that observed at 37°C (18 ± 3 s, $n = 5$). Furthermore, a prolonged elevation in $[Ca^{2+}]_i$ was observed at the lower temperature. These effects were examined on multiple cells and it is therefore possible that the apparently prolonged responses were due to heterogeneity within the cell population rather than to changes in individual cells. We

Table 1
Adenosine A_1 receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells

Treatment	Basal $[Ca^{2+}]_i$	Increase $[Ca^{2+}]_i$	
		CPA	adenosine
37°C			
Control	149 ± 4	76 ± 10	80 ± 12
$LaCl_3$ (50 μM)	157 ± 6	23 ± 8 [a]	30 ± 4 [a]
22°C			
Control	152 ± 4	77 ± 8	74 ± 5
$LaCl_3$ (50 μM)	160 ± 4	22 ± 5 [a]	23 ± 5 [a]
Ca^{2+}-free	77 ± 4 [b]	86 ± 7	88 ± 5
Ca^{2+}-free + $LaCl_3$ (200 μM)	81 ± 5	79 ± 6	

The CPA (100 nM) and adenosine (10 μM) induced increases in $[Ca^{2+}]_i$ were measured in non-pretreated cells, in cells pretreated with $LaCl_3$ (2 min) and in the absence of extracellular Ca^{2+}. Different from control stimulation, [a] $P < 0.01$. Different from control unstimulated level, [b] $P < 0.01$. Data are expressed as means ± S.E.M. of at least 4 experiments.

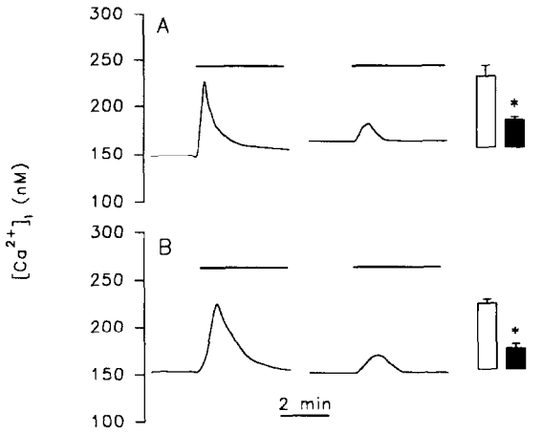

Fig. 2. The effect of $LaCl_3$ on adenosine A_1 receptor mediated increases in cytoplasmic Ca^{2+}. The CPA (100 nM, horizontal bar) induced rise in $[Ca^{2+}]_i$ was obtained at 37°C (A) and 22°C (B) in non-pretreated DDT_1 MF-2 cells (left panel) and in cells pretreated with $LaCl_3$ (50 μM, 2 min, right panel). Maximum amplitudes are presented (means ± S.E.M.) by the bars on the right-hand side. Different from stimulation without $LaCl_3$, * $P < 0.01$. Each tracing is representative of at least 6 experiments.

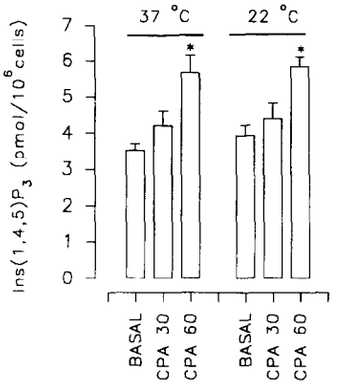

Fig. 4. The time course of adenosine A_1 receptor mediated $Ins(1,4,5)P_3$ formation at different temperatures. The basal level of $Ins(1,4,5)P_3$ (BASAL) and the accumulation of $Ins(1,4,5)P_3$ after exposure to CPA (100 nM) for 30 s (CPA 30) and 60 s (CPA 60) are shown at 37°C and 22°C. Different from unstimulated level, * $P < 0.01$. Data are expressed as means ± S.E.M. of 6 experiments.

therefore examined $[Ca^{2+}]_i$ changes in single DDT_1 MF-2 cells at different temperatures. Typically, the magnitude of the changes were similar at both temperatures, but the responses were more prolonged at 22°C than at 37°C (Fig. 3). Occasionally cells at 22°C showed clearcut calcium oscillations (not shown).

Exposure of DDT_1 MF-2 cells to the physiological adenosine receptor agonist, adenosine (10 μM), elicited changes in $[Ca^{2+}]_i$ which showed the same characteristics as observed with CPA at different temperatures in the presence and absence of $LaCl_3$ (50 μM) and also in the absence of extracellular Ca^{2+} (Table 1).

3.2. The CPA induced $Ins(1,4,5)P_3$ formation

The measurements at different temperatures were extended to the formation of $Ins(1,4,5)P_3$. The CPA (100 nM) induced rises in the $Ins(1,4,5)P_3$ level at 22°C and 37°C were of similar magnitude and followed the same time-course in attached cells. $Ins(1,4,5)P_3$ formation was maximal at 60 s at both temperatures (Fig. 4).

Besides a direct action on Ca^{2+} entry, $LaCl_3$ and the removal of extracellular Ca^{2+} may interfere indirectly with $[Ca^{2+}]_i$ via modulation of CPA induced $Ins(1,4,5)P_3$ formation. Pretreatment of attached DDT_1 MF-2 cells with $LaCl_3$ (50 μM, 2 min) did not affect the CPA (100 nM) induced formation of $Ins(1,4,5)P_3$ (Fig. 5). Moreover, the ability of a submaximal concentration of $Ins(1,4,5)P_3$ (3 μM, Van der Zee et al., 1995) to release preloaded $^{45}Ca^{2+}$ from permeabilized DDT_1 MF-2 cells was not changed by $LaCl_3$ (50 μM, 101 ± 6% vs. control-$Ins(1,4,5)P_3$, $n = 4$). In the absence of extracellular Ca^{2+}, a reduced basal level of $Ins(1,4,5)P_3$ and reduced CPA evoked $Ins(1,4,5)P_3$ formation were observed (Fig. 5).

3.3. Ca^{2+} extrusion and internal $[Ca^{2+}]$

The level of $[Ca^{2+}]_i$ is maintained by Na^+/Ca^{2+} exchange, Ca^{2+} pumping across the plasma membrane and

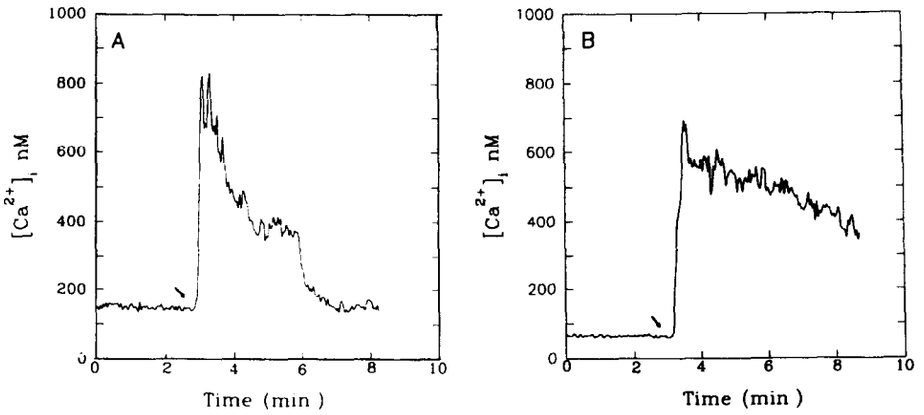

Fig. 3. Adenosine A_1 receptor mediated changes in $[Ca^{2+}]_i$ in single DDT_1 MF-2 cells. CPA (100 nM, arrow) induced changes in $[Ca^{2+}]_i$ were measured at 37°C (A) and 22°C (B). Each tracing is representative of at least 4 experiments.

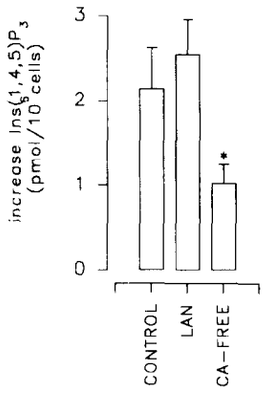

Fig. 5. Adenosine A_1 receptor mediated Ins(1,4,5)P_3 formation in the presence of $LaCl_3$ and after the removal of extracellular Ca^{2+}. The CPA (100 nM) induced formation of Ins(1,4,5)P_3 was measured at 22°C after blocking Ca^{2+} entry with $LaCl_3$ (50 μM, LAN) or in the absence of extracellular Ca^{2+} (CA-FREE). Unstimulated Ins(1,4,5)P_3 levels: control: 3.53 ± 0.19 pmol/10^6cells; LAN: 4.18 ± 0.39 pmol/10^6cells; CA-FREE: 3.03 ± 0.20 pmol/10^6cells #. Different from control unstimulated level, # $P < 0.05$. Different from CPA induced stimulation in the presence of extracellular Ca^{2+}, * $P < 0.05$. Data are expressed as means ± S.E.M. of 6 experiments.

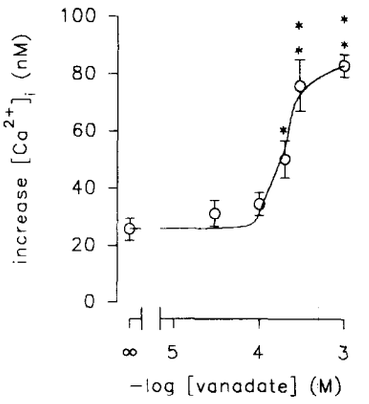

Fig. 7. The concentration dependency of the effect of ortho-vanadate. CPA (100 nM) induced increases in $[Ca^{2+}]_i$ were measured in DDT_1 MF-2 cells pretreated with $LaCl_3$ (50 μM, 2 min) and different concentrations of ortho-vanadate (2 min). Different from the increase in $[Ca^{2+}]_i$ in the absence of ortho-vanadate, * $P < 0.05$, ** $P < 0.01$. Data are expressed as means ± S.E.M. of at least 4 experiments.

active re-uptake into the Ca^{2+} stores. The Na^+/Ca^{2+} exchange process is inhibited by decreasing the extracellular Na^+ concentration, Ca^{2+} pumping can be inhibited by ortho-vanadate (Niggli et al., 1981) and re-uptake into the stores by thapsigargin (Thastrup et al., 1990).

Reduction of extracellular $[Na^+]$ to 10 mM affected neither basal $[Ca^{2+}]_i$ nor the shape or the maximal increase in $[Ca^{2+}]_i$ induced by 100 nM CPA (control increase: 78 ± 6 nM; low Na^+: 80 ± 7 nM, $n = 4$). In the presence of $LaCl_3$ (50 μM), CPA induced a slightly higher increase in $[Ca^{2+}]_i$ when low Na^+ conditions were applied (control increase: 28 ± 4 nM; low Na^+ 47 ± 6 nM, $P < 0.05$, $n = 4$).

Ortho-vanadate (300 μM) did not change basal $[Ca^{2+}]_i$ in DDT_1 MF-2 cells (not shown). After pretreatment of cells with ortho-vanadate for 2 min, the CPA (100 nM) induced increase in $[Ca^{2+}]_i$ was more pronounced than in the absence of the inhibitor (Fig. 6A, control increase: 75 ± 4 nM, with ortho-vanadate: 103 ± 5 nM, $P < 0.05$, $n = 4$). In the presence of $LaCl_3$, ortho-vanadate was even more effective in augmenting the CPA evoked rise in $[Ca^{2+}]_i$ (Fig. 6B, control increase: 21 ± 4 nM, with ortho-vanadate: 76 ± 7 nM, $P < 0.01$, $n = 8$). Ortho-vanadate counteracted the effect of $LaCl_3$ on the CPA evoked rise in $[Ca^{2+}]_i$ concentration dependently (Fig. 7, EC_{50}: 209 μM ± 16 μM). In contrast to the response in the presence of $LaCl_3$, the CPA induced increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} was not enhanced by ortho-vanadate (control increase: 83 ± 5 mM, with ortho-vanadate: 93 ± 8 nM, $n = 4$).

In order to investigate whether the release of Ca^{2+} from internal stores as such is sufficient to activate plasma

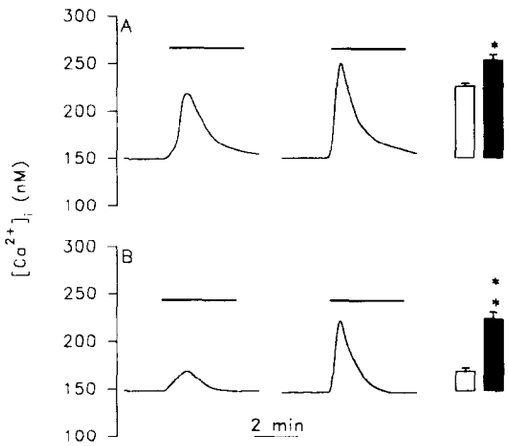

Fig. 6. The effect of ortho-vanadate on the adenosine A_1 receptor mediated change in cytoplasmic $[Ca^{2+}]$. CPA (100 nM, horizontal bar) induced increases in $[Ca^{2+}]_i$ were measured (A) in the absence of $LaCl_3$ and (B) in the presence of $LaCl_3$ (50 μM), without ortho-vanadate (left panel) and after pretreatment of cells with ortho-vanadate (300 μM, 2 min, right panel). Maximum amplitudes are presented (means ± S.E.M.) by the bars on the right-hand side. Different from response in the absence of ortho-vanadate, * $P < 0.05$. Different from stimulation in the presence of $LaCl_3$, ** $P < 0.01$. Data are presented as means ± S.E.M. of at least 4 experiments.

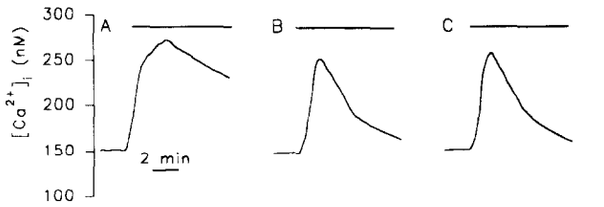

Fig. 8. The effect of $LaCl_3$ and ortho-vanadate on the thapsigargin induced increase in $[Ca^{2+}]_i$. The thapsigargin (1 μM, horizontal bar) induced increase in $[Ca^{2+}]_i$ was measured (A) in non-pretreated cells, (B) in cells pretreated with $LaCl_3$ (50 μM, 2 min) and (C) in cells pretreated with $LaCl_3$ and ortho-vanadate (300 μM, 2 min). Each tracing is representative for at least 4 experiments.

Table 2
The effect of ortho-vanadate and tyrphostin on the adenosine A_1 receptor induced formation of $Ins(1,4,5)P_3$ in DDT_1 MF-2 cells

Treatment	$Ins(1,4,5)P_3$ (pmol/10^6cells)	
	Basal level	CPA stimulated increase
Control	3.75 ± 0.12	2.50 ± 0.31 [b]
Ortho-vanadate	4.21 ± 0.31	2.22 ± 0.25 [b]
Tyrphostin 25	5.17 ± 0.40 [a]	2.13 ± 0.27 [b]

Attached DDT_1 MF-2 cells were stimulated with CPA (100 nM, 1 min) at 22°C after pretreatment with ortho-vanadate (300 μM, 2 min) or tyrphostin 25 (100 μM, 16 h) and the formation of $Ins(1,4,5)P_3$ was measured. Different from unstimulated level in non-pretreated cells, [a] $P < 0.05$. Different from respective unstimulated levels, [b] $P < 0.01$. Data are expressed as means ± S.E.M. of 6 experiments.

membrane Ca^{2+} pumping, the thapsigargin induced increase in $[Ca^{2+}]_i$ was measured in the presence and absence of ortho-vanadate. Thapsigargin (1 μM) caused an increase in $[Ca^{2+}]_i$, reaching a maximum after 4 min (121 ± 14 nM, Fig. 8A) and approaching its original value after about 15–20 min. $LaCl_3$ abolished the sustained phase of the thapsigargin induced increase in $[Ca^{2+}]_i$ (Fig. 8B), representing store-dependent Ca^{2+} entry (Putney, 1986). Ortho-vanadate did not change the shape of the response or the maximal increase in $[Ca^{2+}]_i$ induced by thapsigargin in the presence of $LaCl_3$ (Fig. 8B,C; thapsigargin induced increase: 106 ± 7 nM, with ortho-vanadate: 109 ± 12 nM, $n = 4$).

Stimulation of adenosine A_1 receptors might directly activate plasma membrane Ca^{2+}-ATPase, rather than indirectly by an increase in $[Ca^{2+}]_i$. To detect wether CPA can reduce previously enhanced $[Ca^{2+}]_i$, cells were challenged with CPA (100 nM), 10 min after emptying the internal stores with thapsigargin. Under these conditions, CPA did not cause an additional rise in $[Ca^{2+}]_i$ (not shown) or an increase in the rate of Ca^{2+} extrusion represented by the decline in $[Ca^{2+}]_i$ (control rate: 7.3 ± 1.8 nM/min; with CPA: 7.5 ± 1.6 nM/min, $n = 4$).

Ortho-vanadate may interfere with the activation of phospholipase C by inhibiting tyrosine-phosphate phosphatase, thereby indirectly affecting $[Ca^{2+}]_i$ (Grinstein et al., 1990; Heffetz et al., 1990). Basal and CPA induced $Ins(1,4,5)P_3$ production was not changed in the presence of ortho-vanadate (Table 2). Furthermore, pretreatment of cells with the tyrosine kinase inhibitor, tyrphostin 25 (100 μM) for 16 hrs (Lee et al., 1993) increased the basal level of $Ins(1,4,5)P_3$ but did not inhibit CPA induced $Ins(1,4,5)P_3$ formation (Table 2).

4. Discussion

The rise in $[Ca^{2+}]_i$ on adenosine A_1 receptor stimulation is due to mobilization of Ca^{2+} from internal stores by $Ins(1,4,5)P_3$ and Ca^{2+} entering the cytoplasm from the extracellular environment in DDT_1 MF-2 cells (Gerwins and Fredholm, 1992a,b; White et al., 1992; Schachter and Wolfe, 1992; Dickenson and Hill, 1993). Ca^{2+} entry is prevented by $LaCl_3$ or by removing Ca^{2+} from the extracellular solution (Den Hertog, 1992). Unexpectedly, we observed a marked reduction in the CPA induced rise in $[Ca^{2+}]_i$ in the presence of $LaCl_3$, which was not found in the absence of extracellular Ca^{2+}. Since $LaCl_3$ did not interfere with CPA induced $Ins(1,4,5)P_3$ formation in intact cells and with $Ins(1,4,5)P_3$ induced Ca^{2+} release in permeabilized cells, the effects of $LaCl_3$ are explained completely by the inhibition of Ca^{2+} entry. In agreement, $LaCl_3$ did not change the CPA induced increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, showing that the actions of $LaCl_3$ are related to Ca^{2+} entry. $LaCl_3$ is likewise known to inhibit plasma membrane Ca^{2+} ATPase (Carafoli, 1992). However, much higher concentrations are needed for such an inhibitory effect. Moreover, inhibition of plasma membrane Ca^{2+} pumps would increase $[Ca^{2+}]_i$, whereas we observed a reduction of CPA enhanced $[Ca^{2+}]_i$ in the presence of $LaCl_3$.

It was shown that temperature did not affect the maximal increase in $[Ca^{2+}]_i$ elicited by adenosine A_1 receptor stimulation with or without external $[Ca^{2+}]_i$ and in the presence of $LaCl_3$, respectively. In accord, the kinetics of CPA induced $Ins(1,4,5)P_3$ formation were similar at 22°C and 37°C. The faster onset of the rise in $[Ca^{2+}]_i$ observed at the higher temperature was observed previously in other intact cells (Alonso et al., 1991; Rojas et al., 1994) and might be explained by a faster $Ins(1,4,5)P_3$ induced Ca^{2+} release process (Champeil et al., 1989).

In contrast to blockade of Ca^{2+} entry with $LaCl_3$, the CPA induced $Ins(1,4,5)P_3$ formation was even reduced in the absence of extracellular Ca^{2+}, while the rise in $[Ca^{2+}]_i$ was not affected under these conditions. This indicates that $Ins(1,4,5)P_3$ formation cannot account for the discrepancy in Ca^{2+} homeostasis observed after blocking CPA induced Ca^{2+} entry with $LaCl_3$ or by the removal of extracellular Ca^{2+}.

Extrusion of Ca^{2+} across the plasma membrane by Na^+/Ca^{2+} exchange or Ca^{2+} ATPase activity may account for this discrepancy. Ortho-vanadate has been reported to preferentially inhibit plasma membrane Ca^{2+} ATPase (Carafoli, 1992), also in intact cells (Nelson and Hinkle, 1994). In contrast, thapsigargin, preferentially inhibits the Ca^{2+}-ATPase of internal stores (Thastrup et al., 1990; Lytton et al., 1991; Carafoli, 1992). Thapsigargin but not ortho-vanadate elicited an increase in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells, showing that ortho-vanadate did not inhibit intracellular Ca^{2+} pumps. The difference in the CPA induced maximal increase in $[Ca^{2+}]_i$ between the two strategies of inhibiting Ca^{2+} entry was abolished in the presence of ortho-vanadate. The ortho-vanadate concentration necessary to obtain a maximal effect was reached within one concentration-decade, which is in close agreement with its effect observed on purified Ca^{2+} ATPase (Niggli et al., 1981). The large effect of ortho-vanadate in

the presence of $LaCl_3$ points to an important role of plasma membrane pumps in counteracting the CPA induced rise in $[Ca^{2+}]_i$. In contrast, ortho-vanadate did not augment the CPA induced increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. This discrepancy might be due to the low basal $[Ca^{2+}]_i$ in Ca^{2+}-free medium compared to that in the presence of $LaCl_3$. A threshold $[Ca^{2+}]_i$ of about 100 nM was reported for plasma membrane Ca^{2+}-ATPase activation (Niggli et al., 1981). The increase in $[Ca^{2+}]_i$ mediated by adenosine A_1 receptors in the absence of extracellular Ca^{2+} may be too small to activate plasma membrane Ca^{2+}-ATPase in a rapid fashion. Therefore, the pump is supposed to be working in the presence of $LaCl_3$, but not under Ca^{2+}-free conditions. It is noted that responses of larger magnitude, generated by histamine or ATP/UTP, are equally effected (40% inhibition of peak increase in $[Ca^{2+}]_i$) in the presence of $LaCl_3$ or in Ca^{2+}-free medium (Den Hertog, 1992). Apparently, low basal $[Ca^{2+}]_i$ under Ca^{2+}-free conditions does not restrict rapid activation of the pumps by stimuli provoking much Ca^{2+} release (in contrast to a CPA response). Plasma membrane Ca^{2+} pumps are most likely to be maximally activated by histamine and ATP/UTP both in the presence of $LaCl_3$ and after the removal of extracellular Ca^{2+}, and therefore, these responses are equally effected under these two conditions. In case of a small Ca^{2+} response, as observed after adenosine A_1 receptor stimulation, maximal activation of the pump at regular basal $[Ca^{2+}]_i$, has a relatively large effect on total internal Ca^{2+} accumulation. Since the plasma membrane Ca^{2+} ATPase has a low capacity for Ca^{2+} (Villa and Meldolesi, 1994), it is proposed that Ca^{2+} entry in the absence of $LaCl_3$ leads to a saturation of the plasma membrane Ca^{2+}-ATPase and therefore to an accumulation of cytoplasmic Ca^{2+}. It is concluded that the discrepancy between the CPA induced response in the presence of $LaCl_3$ and the response in Ca^{2+}-free medium can be explained by the selective activation of the plasma membrane pumps in the presence of $LaCl_3$. In agreement, inhibition of the pumps by ortho-vanadate fully resolved this discrepancy.

Ortho-vanadate has been shown to reduce protein-tyrosinephosphate phosphatase activity (Klarlund, 1985; Grinstein et al., 1990; Heffetz et al., 1990), which could give rise to an enhanced $Ins(1,4,5)P_3$ level, achieved by tyrosine kinase mediated phospholipase C stimulation (Atkinson et al., 1993; Kobayashi et al., 1994; Piiper et al., 1994). Ortho-vanadate however, did not enhance the CPA induced formation of $Ins(1,4,5)P_3$ in DDT_1 MF-2 cells. Moreover, the finding that a tyrosine kinase inhibitor, tyrphostin 25, did not reduce $Ins(1,4,5)P_3$ formation further supports the hypothesis that the CPA induced activation of phospholipase C is not mediated by tyrosine phosphorylation. Thus, the effects observed in the presence of ortho-vanadate can be explained by its action on the plasma membrane Ca^{2+}-ATPase.

The application of low extracellular $[Na^+]$ had only a small effect on the CPA evoked increase in $[Ca^{2+}]_i$. Therefore, it is concluded that compared to plasma membrane Ca^{2+} ATPase-pumps, Na^+/Ca^{2+} exchange does not play an important role in the CPA induced Ca^{2+} metabolism in DDT_1 MF-2 cells.

It has been suggested previously, that agonist induced activation of plasma membrane Ca^{2+} ATPase is mediated directly by heterotrimeric GTP binding proteins in hepatocytes and GH3 pituitary cells (Duddy et al., 1989; Nelson and Hinkle, 1994). Such a mechanism is not likely to be activated by adenosine A_1 receptors in DDT_1 MF-2 cells, since CPA did not enhance the rate of Ca^{2+} extrusion after treatment of cells with thapsigargin.

Measurements of monitoring changes in $[Ca^{2+}]_i$ in dye-loaded intracellular compartments, have revealed the presence of high $[Ca^{2+}]_i$-sequestering- and $Ins(1,4,5)P_3$-sensitive organelles near the plasma membrane and around the nucleus of DDT_1 MF-2 cells (Short et al., 1993). Adenosine A_1 receptor mediated $Ins(1,4,5)P_3$ formation and increases in $[Ca^{2+}]_i$ are quite modest compared to other receptors in DDT_1 MF-2 cells (Gerwins and Fredholm, 1992a,b; Sipma et al., 1995). Therefore it might be suggested that CPA induced $Ins(1,4,5)P_3$ almost exclusively reaches the peripheral Ca^{2+} stores, provoking a rapid increase in $[Ca^{2+}]_i$ near the plasma membrane. It likely that such a rapid rise in $[Ca^{2+}]_i$ is necessary to activate plasma membrane Ca^{2+} ATPase. The rise in $[Ca^{2+}]_i$ induced by thapsigargin, supposed to release all $Ins(1,4,5)P_3$ sensitive stores, was rather slow but large compared to the response evoked by adenosine A_1 receptors. Therefore, it is suggested that the major part of Ca^{2+} clearance after a thapsigargin challenge is not achieved by a high affinity and low capacity plasma membrane Ca^{2+} ATPase (Villa and Meldolesi, 1994), but by a high capacity Na^+/Ca^{2+} exchange or by an uptake in a high capacity and thapsigargin resistant intracellular store, such as mitochondria (Rizuto et al., 1993).

In conclusion, next to Ca^{2+} release from internal stores and Ca^{2+} entry from the extracellular environment, plasma membrane ATPase pumps play an prominent role in the regulation of adenosine A_1 receptor induced Ca^{2+} metabolism in DDT_1 MF-2 cells. Most likely, a rapid rise in $[Ca^{2+}]_i$ after receptor stimulation activates the ATPase mediated Ca^{2+} extrusion. This mechanism might be of particular importance for those cell systems in which a moderate agonist mediated Ca^{2+} response is present.

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