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The $[Ca^{2+}]_i$ increase induced in murine thymocytes by extracellular ATP does not involve ATP hydrolysis and is not related to phosphoinositide metabolism

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We have previously demonstrated that exogenous ATP can give medullary thymocytes the calcium message required for the induction of their blastogenesis. In the present study, using the highly sensitive calcium indicator Indo-1, we have measured the effect of exogenous nucleotides on the cytosolic-free calcium concentration $[Ca^{2+}]_i$ of thymocytes, and determined inositol phosphate (IP) formation in the same cells, in parallel assays. The results were compared to those obtained with the mitogenic lectin concanavalin A (ConA) in similar experiments. They show that ATP does not mobilize calcium from its internal stores but stimulates its influx from the extracellular medium. Nevertheless, these data do not rule out the possibility that the nucleotide acts through specific P2 purinergic sites.

ATP, extracellular; Ca^{2+} concentration, cytosolic free; ATP analog; Inositol phosphate; (Mouse thymocyte)

1. INTRODUCTION

It has been shown that, in a large range of concentrations, extracellular ATP has pharmacological effects on many tissues (review [1]) and influences cell functions by changing membrane permeability, ionic fluxes and/or intracellular free calcium concentration $[Ca^{2+}]_i$. In some cells, this effect appears to be mediated by P2 purinergic sites linked to the phosphoinositide metabolism [2–8]. A few years ago, we have demonstrated the existence of A2 purinergic receptors on lymphocyte membranes [9] and shown that they are involved in a negative control of the function of these cells. More recently [10] we found that, in the presence of extracellular calcium, ATP increases $[Ca^{2+}]_i$ of both cortical and medullary thymocytes and induces the blastogenesis of the mature (medullary) population in the presence of PMA, a protein kinase C activator. Our data suggested that this effect might be mediated through P2 purinergic sites. The present study was performed in order to determine which form of ATP was active, and how it modified thymocyte $[Ca^{2+}]_i$ to give a positive signal. Cells were loaded with the highly sensitive calcium indicator Indo-1 [11] and the effects of ATP on $[Ca^{2+}]_i$ were measured; in parallel experiments, inositol phosphate (IP) formation was determined. These effects were compared to those triggered by the mitogenic lectin Con A.

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Abbreviations: $[Ca^{2+}]_i$, cytosolic-free calcium concentration; ConA, concanavalin A; ADP-β-S, adenosine 5'-*O*-(2-thiodiphosphate); p[NH]ppA, 5'-adenylimidodiphosphate; pp$[CH_2]$pA, α,β-methylene adenosine triphosphate; p$[CH_2]$ppA, β,γ-methylene adenosine triphosphate; ATP-γ-S, adenosine 5'-*O*-(3-thio-triphosphate); 2-Me-thio ATP, 2-methyl-thio-adenosine triphosphate; PMA, phorbol-12-myristate-13-acetate

2. MATERIALS AND METHODS

2.1. *Materials*

ConA was purchased from IBF (France), ATP, ADP, ADP-β-S, AMP, adenosine, pp$[CH_2]$pA, p[NH]ppA, p$[CH_2]$ppA, 2'-deoxyATP, GTP, ITP, UTP were from Sigma. ATP-γ-S

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was from Boehringer, 2-methylthio-ATP from RBI, Indo-1-AM from Calbiochem, ionomycin from Fluka, *myo*-[2-^{3}H]inositol from NEN, Dowex 1X-8 anion-exchange columns from Bio-Rad and inositol-free RPMI 1640 from Gibco.

2.2. *Cells*

Thymocytes were prepared from 3-week-old male Swiss mice as in [10].

2.3. *$[Ca^{2+}]_i$ determination*

This was performed as described by Gelfand et al. [12]. Cells were incubated in Hank's solution (3×10^7/ml) with 1 μM Indo-1-AM for 30 min at 37°C, then washed twice and resuspended in pH 7.3 buffer, 140 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes. When indicated $MgCl_2$ was added. In Ca-free medium, $CaCl_2$ was replaced by 0.2 mM EGTA. Measurements were performed at 6×10^6 cells per ml at 37°C with a Perkin-Elmer-Hitachi spectrofluorimeter (λ_{ex} 340 nm, λ_{em} 400 nm). Calibration was performed with ionomycin and Mn^{2+} and $[Ca^{2+}]_i$ were calculated as described [13].

2.4. *Phosphoinositide breakdown*

Thymocytes prepared in an inositol-free culture medium were labelled for 18–20 h with 5 μCi/ml *myo*-[2-^{3}H]inositol. They were washed twice and resuspended in the medium used for $[Ca^{2+}]_i$ determination, then incubated for 20 min at 37°C. Aliquots (15×10^6 cells in 0.5 ml) were treated by ATP or ConA (10 μg/ml). The reaction was stopped 20 min later with perchloric acid and IP levels were measured after chromatography on anion exchange Dowex 1X-8, as described [14].

3. RESULTS

Experiments were performed on unseparated thymocytes as we have shown that ATP triggered the same $[Ca^{2+}]_i$ rise in mature and immature cells and that calcium could be mobilized by ConA in both subpopulations [10], a result confirmed by others [15].

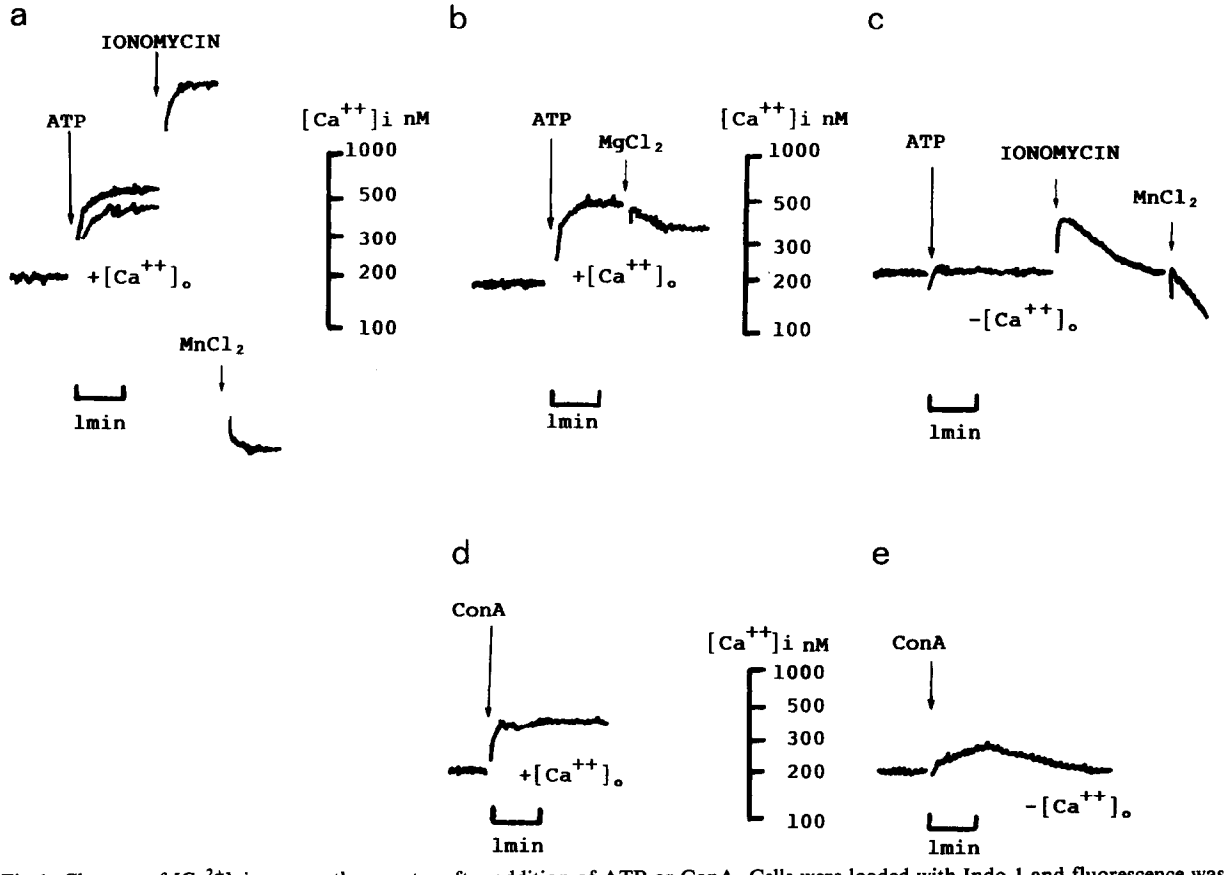

Fig.1. Changes of $[Ca^{2+}]_i$ in mouse thymocytes after addition of ATP or ConA. Cells were loaded with Indo-1 and fluorescence was determined as described in section 2 (adduct concentrations: 0.3 mM ATP, 10 μg/ml ConA, 0.3 μM ionomycin, 0.3 mM $MnCl_2$, 5 mM $MgCl_2$). This experiment is representative of many others.

Fig.1a shows that under physiological conditions, 0.3 mM ATP caused a sustained enhancement of the fluorescence of Indo-1-loaded thymocytes which reached a plateau in less than 2 min. Thymocyte $[Ca^{2+}]_i$ increased from 197 to 494 nM, a result slightly different from that which we obtained with Quin-2-loaded cells [10] (increase from 105 to 199 nM under the same conditions). When the same experiments were performed in the absence of external Mg^{2+}, the fluorescence of Indo-1-labelled thymocytes increased more rapidly and the plateau was higher (557 nM). When Mg^{2+} was added to ATP-activated thymocytes, it partially reversed the ATP response within a few minutes (from 514 to 389 nM with 5 mM Mg^{2+}, fig.1b). In agreement with these data, fig.2B shows that Mg^{2+} added before the stimulus dose dependently inhibited the ATP-triggered $[Ca^{2+}]_i$ rise. In both cases the ATP effect was dose dependent; in the absence of Mg^{2+} its effect occurred at a nucleotide concentration lower than in the presence of Mg^{2+}: the half-maximal concentration of ATP shifted from 0.25 to 0.16 mM with 5 mM $MgCl_2$ (fig.2A). These data establish that $MgATP^{2-}$ is not the entity involved in $[Ca^{2+}]_i$ enhancement and consequently demonstrate that ATP hydrolysis is not required, this complex being the preferential substrate of ATPases and kinases [16].

This result explains that the non-hydrolysable ATP analogs ATP-γ-S and ADP-β-S caused a marked $[Ca^{2+}]_i$ rise in thymocytes: 71.5 and 57%, respectively, of that observed with ATP (table 1), an effect also shown by p[NH]ppA, p[CH_2]ppA and pp[CH_2]pA in the absence of Mg^{2+} but not in its presence [10]. We measured the effects of other nucleotides and nucleosides in Mg-free buffer (table 1). 2-Methyl-thio-ATP, an agonist of P2 purinergic receptors, and ADP led to a $[Ca^{2+}]_i$ increase but to a lesser extent than did ATP; GTP, ITP and UTP exert only a small positive effect, while AMP and adenosine had no significant effect.

In some cell types [2–8], ATP directly mobilizes calcium from its intracellular stores. Since the experimental conditions (use of Quin-2, presence of Mg^{2+}) of our previous work [10] were not optimal for observing such an effect, we repeated the same experiments (absence of external calcium) under the present conditions (use of Indo-1, absence of Mg^{2+}). Fig.1c shows that ATP was unable to promote the transient increase of $[Ca^{2+}]_i$ corresponding to an internal calcium mobilization. Moreover, when the calcium ionophore ionomycin was added to the cells after ATP, it induced the brief $[Ca^{2+}]_i$ rise followed by the decrease to its basal level, which shows that calcium was still pre-

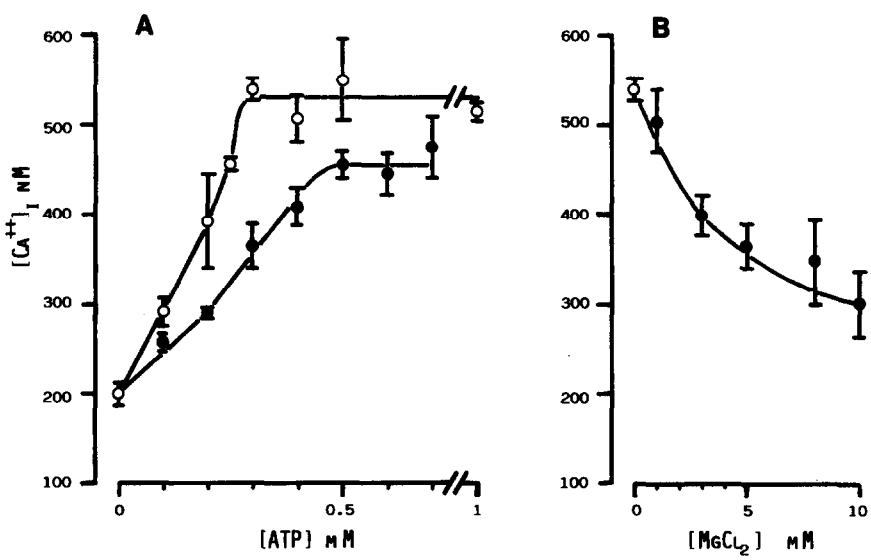

Fig.2. Dose-dependent increase of $[Ca^{2+}]_i$ by ATP, in mouse thymocytes and inhibition by Mg^{2+}. Cells were loaded with Indo-1 and fluorescence was determined as described in section 2. (A) Effect of ATP in the absence (○) or presence (●) of 5 mM $MgCl_2$; (B) inhibition by Mg^{2+} of the effect of 0.3 mM ATP.

Table 1

Effects of ATP and other nucleotides in the absence of magnesium, and of ConA (± $MgCl_2$) on $[Ca^{2+}]_i$

Addition	n	$[Ca^{2+}]_i$	%
None	58	199 ± 14	0
0.3 mM ATP	14	540 ± 17	100
0.4 mM ADP	4	442 ± 38	71
0.3 mM AMP	3	220 ± 8	6[a]
0.3 mM adenosine	5	176 ± 12	−7[a]
0.3 mM ATP-γ-S	4	443 ± 4	71.5
0.3 mM 2-Me-Thio-ATP	3	437 ± 22	67.9
0.3 mM ADP-β-S	3	395 ± 1	57.3
0.3 mM p[NH]ppA	3	316 ± 2	34.1
0.3 mM 2'-deoxy-ATP	3	299 ± 5	29.1
0.3 mM pp[CH_2]pA	3	293 ± 6	27.4
0.3 mM p[CH_2]ppA	4	287 ± 9	25.6
0.3 mM GTP	4	290 ± 9	26
0.3 mM ITP	3	270 ± 1	20.6
0.3 mM UTP	3	246 ± 1	13.5
10 μg/ml ConA	8	379 ± 20	52.6
10 μg/ml ConA + 0.3 mM ATP	5	759 ± 17	164.4
10 μg/ml ConA + 5 mM $MgCl_2$	5	368 ± 15	49.4

[a] Difference relative to control not significant as determined with Student's t-test ($P > 0.05$)

Indo-1-loaded cells (12 × 10^6 cells/2 ml) were suspended in medium containing 1 mM $CaCl_2$; $[Ca^{2+}]_i$ was measured as indicated in section 2, at optimal effector concentrations. Results are the mean ± SE of n measurements

sent in its internal stores. Similar results were obtained with ATP concentrations ranging from 0.05 to 0.5 mM (not shown).

In the presence of extracellular calcium, mitogenic concentrations of ConA induced $[Ca^{2+}]_i$ rises (from 200 to 395 nM) lower than those induced by ATP (from 197 to 494 nM); however, as reported by Hesketh et al. [17], we found that in calcium-free medium ConA mobilized intracellular calcium (fig.1e), $[Ca^{2+}]_i$ increasing from 200 to 300 nM. When Mg^{2+} was omitted from the medium, the ConA-promoted $[Ca^{2+}]_i$ rise was unchanged, whatever the buffer calcium concentration was. Moreover, when ATP and ConA were added together, their effects were found to be additive (table 1).

The ConA-induced $[Ca^{2+}]_i$ rise is due to phospholipase C activation and to inositol triphosphate formation [18]. IP levels were measured in thymocytes incubated with either ConA or different ATP concentrations, for various periods of time, in the presence of lithium. Whereas ConA induced a significant increase (300–400%) of the IP level within 20 min, ATP had no effect even at concentrations up to 1.0 mM (table 2). Similar results were observed with or without Mg^{2+} and for different incubation times ranging from 0.5 to 10 min (not shown). Altogether these results show that the changes of $[Ca^{2+}]_i$ induced by ConA or by ATP do not involve the same mechanism.

4. DISCUSSION

The purpose of this work was to determine the

Table 2

Effects of ConA and ATP on IP levels

Effector	IP-associated radioactivity (dpm/15 × 10^6 cells)			
	A	B		
	Total IPs	IP_1	IP_2	IP_3
Control	525 ± 11	259 ± 76	181 ± 23	107 ± 6
10 μg/ml ConA	1586 ± 108	724 ± 174	727 ± 90	262 ± 70
0.1 mM ATP	510 ± 64[a]			
0.25 mM ATP	595 ± 66[a]	227 ± 33[a]	221 ± 3[a]	112 ± 5[a]
0.5 mM ATP	572 ± 24[a]	228 ± 23[a]	215 ± 20[a]	119 ± 6[a]
1.0 mM ATP	604 ± 65[a]			

[a] Difference relative to control not significant as determined with Student's t-test ($P > 0.05$)

Separation and extraction of total IPs (experiment A) and those of inositol mono-, di- and triphosphate (experiment B) were performed as described in section 2. Results are the mean ± SE of triplicate determinations in one representative experiment out of three

mechanism whereby extracellular ATP can deliver the calcium message required for lymphocyte activation. Our previous data suggested the existence of P2 purinergic receptors on the membrane of these cells [10]. Since in many cell types [2–8] such sites are correlated to phospholipase C activation, we studied the effect of ATP on IP formation. Under conditions allowing the induction by ATP of $[Ca^{2+}]_i$ rise in thymocytes, the nucleotide had no effect on IP accumulation, even at high concentrations consistent with P2z sites [1]. This negative result was not due to a failure of the assay or to a peculiarity of the cells, since we observed, under the same conditions, that ConA induced IP formation in these cells, as reported by others [18].

The variations of $[Ca^{2+}]_i$ we observed in response to either ATP or ConA are consistent with these results. Experiments were performed using Indo-1, a fluorescent probe more sensitive than Quin-2, which allows much lower probe concentrations, thus decreasing the Ca^{2+}-buffering power and potential side effects [12]. In ConA-stimulated cells, the IP_3-mediated calcium release was rapid, transient, independent of extracellular calcium (fig.1e) and correlated with IP formation [18]. The ConA-induced $[Ca^{2+}]_i$ rise was higher in the presence of extracellular calcium than in its absence, which is consistent since it resulted from both mobilization from internal stores and influx from external calcium [17]. In contrast, the ATP-induced $[Ca^{2+}]_i$ rise was rapid, lasted longer and was strictly dependent upon external calcium, which confirms that the phosphoinositide cycle is not involved in this response. This result agrees with the absence of ATP-induced IP formation reported above. It also explains the additive effects of ConA and ATP on the $[Ca^{2+}]_i$ rise, these two effectors acting through different pathways. As in macrophages [19], parotid acinar cells [20] and pancreatic islets [21], the biological effect of ATP might be mediated by an increase in $[Ca^{2+}]_i$ resulting from a net influx of extracellular calcium.

We have no direct evidence that the observed calcium influx is related to a specific receptor as in the case of cholera toxin B subunit [22], EGF [17], or FGF [23]. Nevertheless, our data show that: (i) the effect of ATP is specific: other nucleotides are less efficient; (ii) the efficiency order of the adenine derivative is ATP > ADP ⩾ AMP > adenosine, a characteristic of P2 purinergic sites in Burnstock's classification [24]; (iii) an agonist of P2 sites, 2-methyl-thio-ATP, is active; (iv) the non-hydrolysable analogs of ATP are efficient, demonstrating that phosphate group transfer is not involved in the response; (v) the ATP-induced $[Ca^{2+}]_i$ increase is much higher in the absence of Mg^{2+}, showing that $MgATP^{2-}$ complex is not involved. This last point is consistent with the inefficiency of p[NH]ppA, p[CH_2]ppA and pp[CH_2]pA, in the presence of Mg^{2+} [10], since these analogs display affinity constants for divalent cations 2.5-fold higher than ATP [25], and with the non-involvement of phosphate group transfer in the ATP effect.

It has been demonstrated that, in many tissues, ATP^{4-} is the nucleotide form interacting with P2 sites [25–27]. Our data suggest that such receptors linked to increased calcium influx might mediate the $[Ca^{2+}]_i$ rise in thymocytes, maybe by pore formation as suggested by Tathan et al. [26] in the case of rat mast cells, which might explain that ATP in the presence of EDTA has been used to permeabilize human T lymphocyte membrane to impermeable compounds [29]. The high ATP concentration (in the range of 100 μM) needed to promote the calcium response might be explained by the presence of ecto-ATPase on the cell membrane [10], the Ca-ATP complex being a substrate of this enzyme; such concentrations have been reported in other tissues for P2 receptors linked to the activation of the phosphoinositide pathway [4–6,8].

Our data and our conclusions completely disagree with those of Lin et al. [28], as discussed in [10]. New data support our hypothesis: we recently showed that ATP-γ-S, like ATP, induces the blastogenesis of PMA-treated medullary thymocytes (not shown). Further investigations will be necessary to firmly establish that, besides A2 receptors, P2 receptors exist on thymocytes.

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