Effect of adenine nucleotides on *myo*-inositol-1,4,5-trisphosphate-induced calcium release

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The effects of a whole series of adenine nucleotides on Ins(1,4,5) P_3 -induced Ca^{2+} release were characterized in permeabilized A7r5 smooth-muscle cells. Several adenine nucleotides activated the Ins(1,4,5) P_3 receptor. It was observed that 3'-phosphoadenosine 5'-phosphosulphate, CoA, di(adenosine-5')tetraphosphate (Ap₄A) and di(adenosine-5')pentaphosphate (Ap₅A) were more effective than ATP. Ap₄A and Ap₅A also interacted with a lower EC₅₀ than ATP. In order to find out how these adenine nucleotides affected Ins(1,4,5) P_3 -induced Ca^{2+} release, we have measured their effect on the response of permeabilized A7r5 cells to a progressively increasing Ins(1,4,5) P_3 concentration. Stimulatory ATP and Ap₅A concentrations had no effect on the threshold Ins(1,4,5) P_3 concentration for initiating Ca^{2+} release, but they stimulated Ca^{2+} release in the presence of

supra-threshold Ins(1,4,5) P_3 concentrations by increasing the co-operativity of the release process. Inhibition of the Ins(1,4,5) P_3 -induced Ca^{2+} release at higher ATP concentrations was associated with a further increase in co-operativity and also with a shift in threshold towards higher Ins(1,4,5) P_3 concentrations. ATP had no effect on the non-specific Ca^{2+} leak in the absence of Ins(1,4,5) P_3 . We conclude that the adenine-nucleotide-binding site can be activated by many different adenine nucleotides. Binding of these compounds to the transducing domain of the Ins(1,4,5) P_3 receptor increases the efficiency of transmitting Ins(1,4,5) P_3 binding to channel opening. The inhibition by high ATP concentrations is exerted at a different site, related to Ins(1,4,5) P_3 binding.

INTRODUCTION

Ins(1,4,5) P_3 is a second messenger used by many cell types to release Ca^{2+} from internal stores [1]. Ins(1,4,5) P_3 binds at the N-terminus of the Ins(1,4,5) P_3 receptor (InsP₃R), whereas the channel region is located at the C-terminus. There is a large transducing domain between the Ins(1,4,5) P_3 binding region and the channel region [2]. A number of regulatory sites are proposed to reside in this transducing region, including adenine-nucleotide-binding sites. In the type I InsP₃R, two or three putative adenine-nucleotide-binding sites have been proposed, depending on the splice isoform [2–5].

ATP in the millimolar range can displace Ins(1,4,5) P_3 from its binding site and thereby inhibit Ins(1,4,5) P_3 -induced Ca^{2+} release [6]. The [ATP] needed to inhibit the InsP₃R seems to vary [6–10]. These divergent values may be related to differences in the [Ins(1,4,5) P_3] used, to differences in assay conditions, or to differences in InsP₃Rs expressed in the various cell types.

Adenine nucleotides can also interact with the InsP₃R with high affinity and this results in a more pronounced Ca^{2+} release in the presence of Ins(1,4,5) P_3 . The nature of the stimulation is, however, unclear. Kaplin et al. [8] reported that stimulatory ATP concentrations did not affect the B_{max} and K_d of [³H]Ins(1,4,5) P_3 binding, whereas Spät et al. [11] observed an increase in specific [³H]Ins(1,4,5) P_3 binding by ATP. It is unclear whether the latter effect represented an effect on the B_{max} or on the K_d and whether the increased [³H]Ins(1,4,5) P_3 binding represented binding to the active or the inactive conformation of the InsP₃R [12,13]. Although stimulatory effects of ATP on Ca^{2+} release at various Ins(1,4,5) P_3 concentrations have been described [7,10,14], determination of the Ca^{2+} release at near-threshold concentrations

of Ins(1,4,5) P_3 is usually not accurate. In addition, EC₅₀ values were not very reliable, since, usually, insufficient Ins(1,4,5) P_3 concentrations at the upper end of the dose–response curve are tested to accurately determine the maximal release.

We have now characterized the effects of adenine nucleotides on the InsP₃R in permeabilized A7r5 smooth-muscle cells. Many adenine nucleotides activated the InsP₃R and some of them were even more effective than ATP. In order to find out how these nucleotides modulated Ins(1,4,5) P_3 -induced Ca^{2+} release, we have accurately measured the response of permeabilized A7r5 cells to a progressively increasing [Ins(1,4,5) P_3]. Stimulatory ATP and di(adenosine-5')pentaphosphate (Ap₅A) concentrations had no effect on the threshold [Ins(1,4,5) P_3] for initiating Ca^{2+} release. In contrast, Ca^{2+} release at each supra-threshold [Ins(1,4,5) P_3] was enhanced by ATP and Ap₅A as a result of an increase in co-operativity. The inhibitory effect of ATP was associated with a shift in threshold towards higher Ins(1,4,5) P_3 concentrations. We conclude that the adenine-nucleotide-binding site can be activated by many different adenine nucleotides, including (3'-phospho)adenosine 5'-phosphosulphate (PAPS), CoA, di(adenosine-5')tetraphosphate (Ap₄A) and Ap₅A. They increase the efficiency of transmitting Ins(1,4,5) P_3 binding to channel opening, apparently by increasing the co-operativity of the release process.

MATERIALS AND METHODS

A7r5 cells from embryonic rat aorta were used between the 7th and the 19th passage after receipt from the American Type Culture Collection (Bethesda, MD, U.S.A.) and subcultured weekly by trypsin treatment. The cells were cultured at 37 °C

Abbreviations used: InsP₃R, *myo*-inositol 1,4,5-trisphosphate receptor; Ap₂A, Ap₃A, Ap₄A, Ap₅A, Ap₆A, di(adenosine-5')pyro-, tri-, tetra-, penta-, hexaphosphate; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; ara-ATP, 9-β-D-arabinofuranosyl adenine 5'-triphosphate; p[NH]ppA, adenosine 5'-[β,γ-imido]triphosphate; ATP[S], adenosine 5'-[γ-thio]triphosphate; APS, adenosine 5'-phosphosulphate.

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in a 9% CO₂ incubator in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal-calf serum, 3.8 mM L-glutamine, 0.9% (v/v) non-essential amino acids, 85 units/ml penicillin and 85 µg/ml streptomycin. The cells were seeded in 12-well dishes (4 cm²; Costar Europe, Badhoevedorp, The Netherlands) at a density of approx. 10⁴ cells/cm².

⁴⁵Ca²⁺ fluxes on permeabilized cells were studied on a thermostatically controlled plate at 25 °C. The culture medium was aspirated and replaced by 1 ml of permeabilization medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM MgCl₂, 1 mM ATP, 1 mM EGTA and 20 µg/ml saponin. The saponin-containing solution was removed after 10 min and the cells were washed once with a similar saponin-free solution. ⁴⁵Ca²⁺ uptake into the non-mitochondrial Ca²⁺ stores was accomplished by incubation for 60 min in 2 ml of loading medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 5 mM MgCl₂, 5 mM ATP, 0.44 mM EGTA, 10 mM NaN₃ and 100 nM free ⁴⁵Ca²⁺. After this phase of ⁴⁵Ca²⁺ accumulation, the monolayers were incubated in 1 ml of efflux medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8) and 1 mM EGTA. Additions of Ins(1,4,5)P₃ or adenine nucleotides are indicated in the Figures and Tables below. This efflux medium was replaced each 2 min or each 6 s. The latter was done by automatically transferring the efflux medium from the cells into scintillation vials using a high-speed P-50 Pump (Pharmacia Biotech, Sweden) connected to a Pharmacia LKB SuperFrac fraction collector. At the end of the experiment the ⁴⁵Ca²⁺ remaining in the stores was released by incubation in 1 ml of 2% (w/v) SDS for 30 min.

RESULTS

Effect of adenine nucleotides on Ins(1,4,5)P₃-induced Ca²⁺ release

Permeabilized A7r5 cells loaded to equilibrium with ⁴⁵Ca²⁺ slowly lost their accumulated ⁴⁵Ca²⁺ during incubation in efflux medium. A short exposure to 1 µM Ins(1,4,5)P₃ accelerated the rate of Ca²⁺ loss (Figure 1, ○). The release was enhanced if 1 mM ATP was added at the time of Ins(1,4,5)P₃ addition (Figure 1, ●). Although ATP is likely to power the Ca²⁺ pumps, it is unlikely that the stimulatory effect of ATP represented an effect on the Ca²⁺ pumps, since the free [Ca²⁺] in the efflux medium was too low to stimulate the pumps. In addition, a

Figure 1 Effect of ATP on Ins(1,4,5)P₃-induced Ca²⁺ release

Non-mitochondrial Ca²⁺ stores, loaded to steady state with ⁴⁵Ca²⁺, were incubated in efflux medium for 10 min, at which time 1 µM Ins(1,4,5)P₃ with (●) or without (○) 1 mM ATP was added for 2 min, as indicated by the horizontal bar. Ca²⁺ release was plotted as fractional loss, i.e. the amount of Ca²⁺ leaving the stores in 2 min divided by the total store Ca²⁺ content at that time. Typical for 74 independent experiments each performed in duplicate.

Table 1 Effect of adenine-containing compounds on Ins(1,4,5)P₃-induced Ca²⁺ release

Stimulation of Ins(1,4,5)P₃ (1 µM)-induced Ca²⁺ release by 1 mM of the indicated compounds, added at the time of Ins(1,4,5)P₃ addition, was determined as in Figure 1. The stimulation was calculated as the increase in fractional loss in the presence of 1 µM Ins(1,4,5)P₃ plus the indicated adenine nucleotide relative to the increase in fractional loss in the presence of 1 µM Ins(1,4,5)P₃ in the absence of the adenine nucleotide. Values are means ± S.E.M. for the number of observations given in parentheses.

Compound	Stimulation (%)	Compound	Stimulation (%)
Adenine	13 ± 3 (2)	2-Methylthio-ATP	130 ± 5 (2)
Adenosine	36 ± 4 (4)	1, N ⁶ -etheno-ATP	19 ± 6 (5)
2'-O-methyladenosine	82 ± 4 (3)	8-Bromo-ATP	149 ± 5 (3)
3'-O-methyladenosine	68 ± 8 (3)	dATP	144 ± 13 (3)
1-Methyladenosine	0 ± 3 (2)	Ara-ATP	155 ± 3 (2)
N ⁶ -methyladenosine	0 ± 4 (2)	p[NH]ppA	130 ± 7 (3)
S-adenosyl-L-methionine	1 ± 3 (2)	ATP[S]	135 ± 8 (3)
Adenosine 5'-sulphate	73 ± 8 (3)	Adenosine 5'-tetraphosphate	58 ± 4 (2)
AMP	74 ± 2 (6)	CoA	140 ± 4 (9)
1, N ⁶ -etheno-AMP	8 ± 4 (2)	1, N ⁶ -etheno-CoA	31 ± 2 (5)
N ⁶ -methyl-AMP	1 ± 5 (3)	Desulpho-CoA	125 ± 9 (3)
3':5'-cAMP	72 ± 3 (7)	Acetyl-CoA	117 ± 9 (3)
8-Amino-3':5'-cAMP	67 ± 2 (3)	Malonyl-CoA	102 ± 6 (2)
2':3'-cAMP	93 ± 6 (3)	Ap ₂ A	98 ± 5 (3)
2':3'-cAMP-5'P	143 ± 5 (2)	Ap ₃ A	118 ± 6 (2)
APS	85 ± 8 (3)	Ap ₄ A	144 ± 7 (3)
PAPS	152 ± 4 (5)	Ap ₅ A	141 ± 7 (3)
2':3'-cPAPS	146 ± 8 (2)	Ap ₆ A	90 ± 4 (2)
ADP	129 ± 5 (6)	NAD	50 ± 4 (6)
1, N ⁶ -etheno-ADP	38 ± 3 (2)	1, N ⁶ -etheno-NAD	0 ± 2 (2)
ADP-glucose	99 ± 4 (2)	NADH	89 ± 3 (5)
ADP-ribose	74 ± 9 (5)	NADP	43 ± 3 (9)
ADP-ribose-2'P	81 ± 10 (3)	NADPH	71 ± 3 (6)
ATP	128 ± 6 (74)	FAD	39 ± 2 (2)
N ¹ -oxide-ATP	48 ± 2 (4)		

similar stimulation by ATP was observed in the presence of 2 µM thapsigargin (results not shown). These findings suggest that ATP stimulated Ins(1,4,5)P₃-induced Ca²⁺ release.

In the particular experiment of Figure 1, 1 µM Ins(1,4,5)P₃ increased the rate of release from 8 to 46% per 2 min in the absence of ATP and from 8 to 88% per 2 min in the presence of 1 mM ATP. The increase in rate of Ca²⁺ release in the presence of ATP was therefore 80% per 2 min, whereas the increase observed in the absence of ATP was 38% per 2 min. The additional stimulation induced by 1 mM ATP (i.e. 80% - 38% = 42%) over and above that given by 1 µM Ins(1,4,5)P₃ alone (i.e. 38%) was therefore 111% in this particular experiment. A similar protocol was used to test the percentage stimulation by a whole series of adenine nucleotides (Table 1). Most adenine nucleotides (1 mM) activated the InsP₃R. Some of them, like the sulphate donor PAPS, CoA, Ap₄A and Ap₅A exerted a larger effect than ATP. Inactive compounds were 1-methyladenosine, S-adenosyl-L-methionine and a number of adenine compounds substituted at the N⁶ position.

CoA has a free thiol group. Since thiol reagents can activate the InsP₃R [15,16], we had to investigate whether the free SH group on CoA was needed for the potentiation of Ins(1,4,5)P₃-induced Ca²⁺ release. Desulpho-CoA, which lacks this terminal thiol group, was equally effective as CoA in stimulating the release (Table 1). Acetyl- and malonyl-CoA, where the SH group is occupied, also stimulated the release. These findings indicate that CoA was not acting as a thiol reagent. The presence of an etheno group at position N⁶ of the adenine dramatically reduced

Table 2 Modulation of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release by phosphorylation of the ribose

Stimulation of $\text{Ins}(1,4,5)\text{P}_3$ ($1\ \mu\text{M}$)-induced Ca^{2+} release by $1\ \text{mM}$ of the indicated compounds and of their phosphorylated derivatives (either in the 2'- or 3'-position) was determined using the same experimental protocol as in Figure 1. The stimulations were calculated as in Table 1. The values are expressed as means $\pm$ S.E.M. for the number of observations given in parentheses. n.d.: not determined because the compound was not available.

Compound	Stimulation (%)	
	+2'-P	+3'-P
Dephospho-CoA	21 ± 6 (5)	n.d.
NAD	50 ± 4 (6)	43 ± 3 (9)
NADH	89 ± 3 (5)	71 ± 3 (6)
APS	85 ± 8 (3)	52 ± 8 (2)
AMP	74 ± 2 (6)	70 ± 7 (4)
Adenosine	36 ± 4 (4)	82 ± 5 (4)

Table 3 Modulation of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release by changing the base in the nucleotide

The experimental protocol was the same as in Figure 1. Stimulation of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release by the indicated concentrations of nucleoside tri-, di- and monophosphates (NTP, NDP, NMP) and NDP-glucose of the indicated bases. Stimulations were calculated as in Table 1 and are the means $\pm$ S.E.M. for the number of observations given in parentheses. n.d.: not determined because the compound was not available.

	Stimulation (%)				
	Adenine	Guanine	Cytosine	Uracil	Hypoxanthine
NTP $1\ \text{mM}$	128 ± 6 (74)	38 ± 9 (3)	50 ± 5 (3)	38 ± 7 (3)	35 ± 6 (2)
$32\ \mu\text{M}$	68 ± 3 (5)	6 ± 3 (4)	6 ± 2 (5)	8 ± 4 (4)	7 ± 3 (6)
NDP $1\ \text{mM}$	129 ± 5 (6)	34 ± 3 (2)	42 ± 9 (2)	45 ± 9 (2)	36 ± 1 (2)
$32\ \mu\text{M}$	65 ± 3 (4)	7 ± 2 (4)	9 ± 1 (4)	11 ± 2 (5)	5 ± 3 (6)
NMP $1\ \text{mM}$	74 ± 2 (6)	9 ± 6 (3)	0 ± 5 (3)	0 ± 2 (3)	3 ± 5 (3)
NDP-glucose $1\ \text{mM}$	99 ± 5 (2)	13 ± 5 (3)	0 ± 8 (3)	0 ± 5 (3)	n.d.

the efficiency of CoA, similarly to that observed for AMP, ADP, ATP and NAD (Table 1). These findings suggest that CoA stimulated the Ca^{2+} release through its ADP moiety.

NAD and NADH also stimulated $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release (Table 1). Kaplin et al. [8] reported previously a stimulation of Ca^{2+} release by NAD and NADH, while in their study NADP and NADPH were totally ineffective in stimulating the cerebellar InsP_3R . We, however, observed a pronounced stimulation of the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release by both NADP and NADPH in permeabilized A7r5 cells (Table 1). Since NADP(H) differs from NAD(H) by the presence of one phosphate in the 2'-position of the ribose, we investigated the functional consequence of this phosphate in a series of adenine nucleotides (Table 2). All compounds tested remained active when phosphorylated in the 2'-position. It therefore remains unclear why NADP and NADPH were totally ineffective in stimulating the cerebellar InsP_3R [8]. One possible explanation is the fact that cerebellum and A7r5 cells express different isoforms of the InsP_3R [17], which differ in adenine-nucleotide-binding sites [5,18]. Table 2 also shows that a phosphate in the 3'-position of the ribose potentiated the stimulatory effect of the adenine nucleotides on the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release. This effect was especially striking for CoA, because dephospho-CoA, which lacks the 3'-phosphate, was almost ineffective at stimulating the InsP_3R .

Figure 2 Effect of various ATP and Ap_5A concentrations on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release

Non-mitochondrial Ca^{2+} stores, loaded to steady state with $^{45}\text{Ca}^{2+}$, were incubated in efflux medium for 10 min, at which time $1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ plus the indicated ATP (A) or Ap_5A (B) concentrations were added for 2 min. The stimulation of the Ca^{2+} release by ATP or Ap_5A is expressed as the percentage increase in the Ca^{2+} release above the control value, obtained in the absence of these compounds. Data are means of four independent experiments. The S.E.M. was less than 10%.

We have measured the EC_{50} for activating $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release for some of the most active compounds. Ap_5A and Ap_4A , the concentration of which may rise above $100\ \mu\text{M}$ during heat shock and oxidative stress [19], interacted with lower EC_{50} (16 and $23\ \mu\text{M}$) than ATP ($29\ \mu\text{M}$), ADP ($30\ \mu\text{M}$), NADH ($32\ \mu\text{M}$), CoA ($117\ \mu\text{M}$) or PAPS ($130\ \mu\text{M}$).

The stimulation strictly required an adenine group, since nucleotides derived from guanine, cytosine, uracil or hypoxanthine were much less effective (Table 3). Di- and tri-phosphates were, however, equally effective (Table 3).

Bell-shaped stimulation of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release by ATP and Ap_5A

Since the concentrations of ATP needed to stimulate or inhibit $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release differ in the various reports [6–11,14], we had to determine the effective concentrations in permeabilized A7r5 cells. We therefore measured the Ca^{2+} release in the presence of $1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ and a series of ATP concentrations. The stimulation of the Ca^{2+} release by ATP is shown in Figure 2(A). The stimulation reached a maximum at approx. $1\ \text{mM}$ ATP and at higher concentrations, the Ca^{2+} release by $1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ again declined.

Figure 2(B) shows the effect of various concentrations of Ap_5A on the Ca^{2+} release in the presence of $1\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$. The

Figure 3 Lack of effect of a stimulatory [ATP] on the threshold for Ins(1,4,5) P_3 -induced Ca^{2+} release

(A) The non-mitochondrial Ca^{2+} stores were loaded to steady state with $^{45}Ca^{2+}$. After 7 min incubation in efflux medium with (●) or without (○) 0.3 mM ATP, 3.2 nM Ins(1,4,5) P_3 was added. The [Ins(1,4,5) P_3] was then increased from 3.2 nM to 3.2 μ M in 60 steps, each lasting 6 s. (B) shows the fitting of the data points at the lower Ins(1,4,5) P_3 concentrations with the Hill equation. Individual data points were not plotted. The broken line in (B) extrapolates the basal rate of Ca^{2+} release at sub-threshold concentrations of Ins(1,4,5) P_3 , in order to better determine the threshold for Ins(1,4,5) P_3 -induced Ca^{2+} release. Both plots deviate from the broken line at the same [Ins(1,4,5) P_3]. Means for five experiments are shown. The S.E.M. was always less than 3%.

effect was again bell-shaped, with again a maximum at 1 mM Ap_5A .

Effect of ATP and Ap_5A on the threshold for Ins(1,4,5) P_3 -induced Ca^{2+} release

The effect of ATP and Ap_5A on the threshold for Ins(1,4,5) P_3 action was determined as described previously [20]. The [Ins(1,4,5) P_3] was gradually increased from 3.2 nM to 3.2 μ M in the presence and absence of 0.3 mM ATP (Figure 3A). Ca^{2+} release was plotted as fractional loss, i.e. the amount of Ca^{2+} leaving the stores in 6 s divided by the total store Ca^{2+} content at that time. The lowest [Ins(1,4,5) P_3], at which the fractional loss significantly increased above baseline, was taken as the threshold. Figure 3(B) shows the fitting of the data points at the lower Ins(1,4,5) P_3 concentrations with the Hill equation; individual data points were not plotted, in order to better visualize the threshold for Ins(1,4,5) P_3 action. ATP (0.3 mM) stimulated Ca^{2+} release induced by supra-threshold Ins(1,4,5) P_3 concentrations, but did not change the threshold for Ins(1,4,5) P_3 action. ATP (0.3 mM) decreased the EC_{50} for Ca^{2+} release from 541 to 232 nM Ins(1,4,5) P_3 (Table 4). ATP also increased the co-

Table 4 Effects of ATP and Ap_5A on the Hill coefficient and EC_{50} of Ins(1,4,5) P_3 -induced Ca^{2+} release

Values were calculated from the fitted curves shown in Figures 3(B), 4(B) and 5(B).

Condition	Hill coefficient	EC_{50} (nM)
Control	1.9	541
0.3 mM ATP	2.4	232
5.0 mM ATP	3.1	446
0.3 mM Ap_5A	2.6	197

Figure 4 Lack of effect of 0.3 mM Ap_5A on the threshold for Ins(1,4,5) P_3 -induced Ca^{2+} release

(A) The [Ins(1,4,5) P_3] was increased from 3.2 nM to 3.2 μ M in efflux medium containing 0.3 mM Ap_5A (●) or in control medium without Ap_5A (○). (B) shows the fitting of the data at the lower Ins(1,4,5) P_3 concentrations with the Hill equation. The broken line extrapolates the basal rate of Ca^{2+} release. Means for three experiments are shown. The S.E.M. was always less than 5%.

operativity of the release process, since the Hill coefficient increased from 1.9 to 2.4 (Table 4).

The effect of 0.3 mM Ap_5A on the threshold for Ins(1,4,5) P_3 action is shown in Figure 4. Ap_5A only stimulated Ca^{2+} release at supra-threshold concentrations of Ins(1,4,5) P_3 . The threshold for initiating Ca^{2+} release was again not affected. This concentration of Ap_5A decreased the EC_{50} for Ca^{2+} release from 541 to 197 nM Ins(1,4,5) P_3 and increased the Hill coefficient from 1.9 to 2.6 (Table 4). Ap_5A was therefore more effective than ATP.

Figure 5 Effect of an inhibitory [ATP] on the threshold for $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release

(A) The $[\text{Ins}(1,4,5)\text{P}_3]$ was gradually increased from 3.2 nM to 3.2 μM in an efflux medium containing 0.3 mM (○) or 5.0 mM (●) ATP. (B) shows the fitting of the data at the lower $\text{Ins}(1,4,5)\text{P}_3$ concentrations with the Hill equation. The broken line extrapolates the basal rate of Ca^{2+} release. Means for three experiments are shown. The S.E.M. was always less than 5%.

Figure 5 shows the effect of 5.0 mM ATP, i.e. a concentration that induced a less pronounced stimulation of the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release than 0.3 mM ATP (Figure 2A). The co-operativity of the release was higher at 5.0 mM ATP than at 0.3 mM ATP (Hill coefficient of 3.1 and 2.4 respectively, Table 4). However, the EC_{50} for Ca^{2+} release increased from 232 nM to 446 nM $\text{Ins}(1,4,5)\text{P}_3$ upon increasing the [ATP] from 0.3 mM to 5.0 mM (Table 4). This effect was due to a shift in threshold for $\text{Ins}(1,4,5)\text{P}_3$ action towards higher $\text{Ins}(1,4,5)\text{P}_3$ concentrations (Figure 5). $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ -binding experiments revealed a decrease in specific $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ -binding to A7r5 microsomes under similar conditions (results not shown).

Stimulatory ATP concentrations (0.3 mM) had no effect on the threshold for $\text{Ins}(1,4,5)\text{P}_3$ action (Figure 3). Since high ATP concentrations shifted the threshold towards higher $\text{Ins}(1,4,5)\text{P}_3$ concentrations (Figure 5), we were concerned that the lack of effect on the threshold by 0.3 mM ATP could already have been due to some inhibitory effect of ATP. We therefore also tested the effect of a much lower [ATP] (50 μM) on the threshold for $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release. ATP at a concentration of 50 μM again had no effect on the threshold for $\text{Ins}(1,4,5)\text{P}_3$ action (results not shown).

Effect of adenine nucleotides on the non-specific Ca^{2+} leak

The Ca^{2+} leak in the presence of sub-threshold concentrations of $\text{Ins}(1,4,5)\text{P}_3$ was affected neither by 0.3 mM (Figure 3) nor by 5.0 mM ATP (Figure 5). This finding indicates that ATP did not

Figure 6 Effect of adenosine 5'-tetrphosphate on the non-specific Ca^{2+} leak

The non-mitochondrial Ca^{2+} stores were loaded to steady state with $^{45}\text{Ca}^{2+}$ and then incubated in efflux medium without ATP and without $\text{Ins}(1,4,5)\text{P}_3$. The efflux medium was supplemented with 1 mM adenosine 5'-tetrphosphate with (●) or without (○) 1 mM ATP during the time period indicated by the horizontal bar. Typical for three experiments.

affect the non-specific Ca^{2+} leak from the stores in saponin-permeabilized A7r5 cells. We also tested the effect of the other adenine nucleotides on the rate of passive Ca^{2+} release from the stores. None of the following compounds had an effect on the non-specific Ca^{2+} leak at a concentration of 1 mM: adenine, adenosine, AMP, ADP, ATP, dATP, 9- β -D-arabinofuranosyl adenine 5'-triphosphate (ara-ATP), adenosine 5'-[β , γ -imido]-triphosphate (p[NH]ppA), adenosine 5'-[γ -thio]triphosphate (ATP[S]), S-adenosyl-L-methionine, adenosine 5'-phosphosulphate (APS), PAPS, cAMP, CoA, desulpho-CoA, dephospho-CoA, ADP-glucose, ADP-ribose, ATP-ribose, NAD, NADH, NADP, NADPH, FAD, di(adenosine-5')pyrophosphate (Ap_2A), di(adenosine-5')triphosphate (Ap_3A), Ap_4A , Ap_5A and di(adenosine-5')hexaphosphate (Ap_6A) (results not shown). Adenosine 5'-tetrphosphate was the only compound that affected the non-specific Ca^{2+} leak (Figure 6). This effect was not prevented by the simultaneous presence of 1 mM ATP.

DISCUSSION

We have found that ATP is only one of the many compounds that activate the stimulatory adenine-nucleotide-binding site(s) on the InsP_3R . ATP was not the most effective compound with respect to maximal stimulation or to affinity. This finding evokes the question of whether changes in [ATP] would eventually affect $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release in an intact cell, where other compounds are also present.

We have further investigated how adenine nucleotides affected $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release. Our results indicate that stimulatory ATP and Ap_5A concentrations had no effect on the threshold for $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release, but only stimulated Ca^{2+} flux at supra-threshold $\text{Ins}(1,4,5)\text{P}_3$ concentrations. This finding suggests that ATP enhanced the efficiency of coupling $\text{Ins}(1,4,5)\text{P}_3$ binding to channel opening. This more efficient coupling was associated with an increase in co-operativity of the $\text{Ins}(1,4,5)\text{P}_3$ dependence of the release process. The Hill coefficient further increased when the [ATP] was raised to inhibitory levels. This finding indicates that the InsP_3R became

inhibited despite the achievement of an optimal coupling via the activatory ATP-binding site. This confirms the hypothesis that the inhibition occurred at a site different from that eliciting the stimulation. It is still debated whether the inhibition by ATP was exerted at the level of the $\text{Ins}(1,4,5)\text{P}_3$ -binding domain [6,7,9,10,21] or at a low-affinity ATP-binding site distinct from this $\text{Ins}(1,4,5)\text{P}_3$ -binding site [14]. Our data, showing that the inhibition was associated with a shift in threshold towards higher $\text{Ins}(1,4,5)\text{P}_3$ concentrations and with an inhibition of $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding, would favour an interaction with the $\text{Ins}(1,4,5)\text{P}_3$ -binding domain, although an allosteric interaction cannot be excluded. The physiological relevance of this inhibition by ATP is probably that a 'sick cell' containing 1 mM ATP would release its Ca^{2+} stores at lower $\text{Ins}(1,4,5)\text{P}_3$ levels than a healthy cell containing 5 mM ATP.

Stimulatory ATP concentrations have been reported to increase the $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding [11], but in other reports no effect could be observed [8]. Our results, indicating that the threshold for $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was not affected by stimulatory ATP concentrations, suggest that possible effects on $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding are of little importance in stimulating the release. Bezprozvanny and Ehrlich [7] came to a similar conclusion when comparing the 10-fold increase in channel activity with the small (30%) potentiation of $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding [11].

The present findings on the effects of stimulatory ATP concentrations are also of interest in explaining the partial or 'quantal' nature of the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release [22], whereby a submaximal $[\text{Ins}(1,4,5)\text{P}_3]$ is unable to empty the whole $\text{Ins}(1,4,5)\text{P}_3$ -sensitive store. The all-or-none model proposes that a low $[\text{Ins}(1,4,5)\text{P}_3]$ will completely empty a fraction of the stores while leaving the other fraction unaffected, because each individual store unit has its own threshold for $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release [22–25]. The percentage Ca^{2+} release at a particular $[\text{Ins}(1,4,5)\text{P}_3]$ is therefore proportional to the fraction of responsive versus the total number of store units. Stimulatory effects of modulators like ATP can in an all-or-none model only be explained if there is a transition from originally non-responding store units to responding units. Such transition implies that ATP should decrease the threshold $[\text{Ins}(1,4,5)\text{P}_3]$ in a large subfraction of the total store population. We, however, failed to demonstrate the predicted shift in threshold for $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release in A7r5 cells. This finding suggests that differences in threshold for $\text{Ins}(1,4,5)\text{P}_3$ action in individual stores units (as required by the all-or-none model) are unlikely to be the only mechanism for partial Ca^{2+} release in A7r5 cells. A steady-state model for quantal Ca^{2+} release [26,27], in contrast with the all-or-none hypothesis, can easily explain why ATP triggers further Ca^{2+} release: ATP allows each store unit to release more Ca^{2+} in the presence of a submaximal $[\text{Ins}(1,4,5)\text{P}_3]$.

ATP had no effect on the non-specific Ca^{2+} leak in saponin-permeabilized A7r5 cells (this work) and portal-vein smooth muscle [10]. In contrast, increasing the $[\text{ATP}]$ from 0.375 mM to 4 mM in streptolysin-O-permeabilized BHK-21 fibroblasts increased the non-specific Ca^{2+} leak 3.6-fold [28]. The reason for this discrepancy is unclear. The authors, however, pointed out that the effect of ATP in BHK-21 fibroblasts may have been an indirect one. It is known that some fatty acyl-CoA esters can increase the Ca^{2+} permeability of the stores [29] and that enough fatty acids and acyl-CoA synthetase is present in permeabilized cells to form these esters if CoA and ATP are added [29,30].

Interestingly, $\text{p}[\text{NH}]\text{ppA}$ and $\text{ATP}[\text{S}]$, which did not increase the non-specific Ca^{2+} leak [28], are ineffective substrates for the acyl-CoA synthetase [31]. It is therefore possible that differences in the endogenous $[\text{CoA}]$ can account for the different effects of ATP in saponin-permeabilized A7r5 cells or portal-vein smooth muscle and streptolysin-O-permeabilized BHK-21 cells.

We conclude that the adenine-nucleotide-binding site can be activated by many different adenine nucleotides. ATP binding, probably to one or more binding sites in the transducing domain of the InsP_3R , increases the efficiency of transmitting $\text{Ins}(1,4,5)\text{P}_3$ binding to channel opening, without affecting the $\text{Ins}(1,4,5)\text{P}_3$ binding itself. The inhibition by high ATP concentrations seems to be exerted at a different site, which apparently affects $\text{Ins}(1,4,5)\text{P}_3$ binding. ATP had no effect on the non-specific Ca^{2+} leak in the absence of $\text{Ins}(1,4,5)\text{P}_3$.

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