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# P2-purinoceptor-stimulated phosphoinositide turnover in chick myotubes

## Calcium mobilization and the role of guanyl nucleotide-binding proteins

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ATP, a trigger of P2-purinoceptor-mediated polyphosphoinositide (PI) turnover in cultured myotubes, increased cytosolic calcium levels in a time- and dose-dependent manner (quin2 fluorescence). The calcium was released from intracellular stores, as acute addition of 5 mM EGTA was without significant effect. Adenosine 5'-(3-thiotriphosphate) and 5'-adenylyl imidodiphosphate also increased intracellular levels of inositol phosphates (InsP) and cytosolic calcium levels. Treatment with cholera or pertussis toxin of myotube cultures did not affect the P2-purinoceptor-mediated InsP increase although PI turnover in permeabilized myotubes was stimulated by guanosine 5'-(3-thiotriphosphate). The results suggest that myotube P2-purinoceptors trigger PI turnover and increase intracellular free calcium levels, via a mechanism insensitive to ADP-ribosylation, by cholera or pertussis toxin of guanyl nucleotide-binding (G) proteins. However, the presence of a phospholipase C-coupled G-protein was otherwise demonstrated.

P2-purinoceptor; ATP; Inositol phosphate; cytosolic $Ca^{2+}$; Quin-2; Myotube

## 1. INTRODUCTION

Signal transmission at the neuromuscular synapse involves the release of not only the neurotransmitter acetylcholine (ACh) from the presynapse but also that of adenosine 5'-triphosphate (ATP). ATP release may occur from the nerve terminal [1,2] and from depolarized skeletal muscle [3]. We have recently shown [4,5] that ATP, when extracellularly applied to chick myotubes in culture, induces a rapid decrease in polyphosphoinositides (PI) and, in chick and mouse myotubes, an accumulation of inositol phosphates (InsP). This observation points to a regulatory role for extracellular ATP in skeletal muscle calcium homeostasis through P2-purinoceptors. This paper presents further evidence in support of our previous findings and describes the action of extracellular ATP on cytoplasmic calcium levels in cultured chick myotubes as well as the involvement of guanyl nucleotide-binding proteins (G-proteins) in this process.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

The chemicals used were of the highest quality available and were purchased from Sigma, Boehringer, Merck and Serva. Cholera toxin was purchased from Calbiochem. Pertussis toxin was a gift from Dr P. Askelöf (SBL, Stockholm) and arylazidoaminoproprionyl-ATP from Dr B. Fredholm (Karolinska Institutet, Stockholm). Material for cell cultures was obtained from Flow and Nordvacc, Stockholm.

### 2.2. *Culture of cells and PI-turnover experiments*

Chick myotubes were prepared and maintained in culture as in [6]. They were labelled with *myo*-[2-$^3$H]inositol (5 $\mu$Ci/2-3 ml inositol-free Ham's F10 medium containing 2% horse serum) for 18-24 h at 37°C. Labelled cells were thoroughly washed at room temperature and, prior to ex-

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periments, incubated with 10 mM LiCl, in physiological buffer (composition in mM: 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 5.5 d-glucose, 1.0 $NaH_2PO_4$, 20.0 Hepes; pH 7.4) for 20 min at 37°C. LiCl was added during InsP-accumulation experiments. Experiments on permeabilized cells were performed in a cytosol-like buffer of the following composition [in mM: 110 KCl, 1.0 $KH_2PO_4$, 5.0 $MgSO_4$, 0.202 $CaCl_2$ (calculated free Ca: 50 nM), 1.0 EGTA, 20.0 Hepes; pH 7.2; 2 g/l bovine serum albumin]. Permeabilization was achieved by addition of 100 $\mu$g/ml of saponin [7]. In experiments employing cholera and pertussis toxin, cultures were incubated with toxins for 3–4 h before labelling with *myo*-[2-$^3$H]inositol was stopped. Incubation with compounds triggering InsP accumulation was stopped by addition of 3% (final concentration) perchloric acid. The samples were neutralized with 1.5 M KOH/75 mM Hepes and InsPs were separated by ion-exchange chromatography [8]. Radioactivity was counted in an LKB Rackbeta scintillation counter using Optiphase MP (LKB, Bromma, Sweden) as scintillation cocktail.

2.3. *Estimation of cytosolic levels of calcium*

Cytosolic calcium levels in monolayers of cultured myotubes were determined by measuring quin2 fluorescence in a custom-built fluorimeter (Lindmark Innovation, Stockholm), equipped with a deuterium lamp and a photon counter. Excitation (340 nm) and emission (510 nm) wavelengths were restricted by filter units (bandwidth 10 nm). Emitted light was monitored at 450 nm relative to the excitation light beam. Culture dishes were placed directly in the light path. Myotubes were incubated with 10 $\mu$M quin2/AM in physiological buffer (see above) for 30 min at 37°C. After washing, myotubes were further incubated for 30 min at 37°C to allow for formation of quin2. Fluorescence was recorded on a chart recorder after digital/analog conversion. Maximum and minimum fluorescence was determined after addition of 0.1% Triton X-100 and 5 mM EGTA, respectively.

## 3. RESULTS

Stimulation of cultured chick myotubes with ATP causes a time- and concentration-dependent accumulation of InsPs [4]. Further, in cultured rat and mouse [5] myotubes, ATP and slowly hydrolyzed derivatives of ATP enhanced InsP accumulation while in a rat diaphragm preparation only the derivatives were effective (not shown). When applied to cultured myotubes ATP induced an increase in cytosolic levels of calcium as determined in the quin2 experiments (fig.1A). This increase was not due to influx of calcium over the plasma membrane as short-term treatment with 5 mM EGTA neither inhibited the ATP-induced increase in cytosolic calcium nor the accumulation of InsPs (fig.1B,D). The time courses of InsP and cytosolic calcium increases were closely parallel and had reached maximal levels after 10 s incubation (fig.1A,C). Also, the dose-effect curves for the increase in cytosolic calcium and InsPs overlapped (fig.2A). Both effects were significant at 5–10 $\mu$M ATP. We have previously shown [4] that within the series of purines ATP, ADP, AMP and adenosine, ATP was the most effective in inducing PI turnover while adenosine was ineffective. This suggests a P2-purinoceptor-mediated mechanism. Here, adenosine had no effect on quin2 fluorescence in chick myotubes. Besides ATP and adenosine we also investigated two slow-

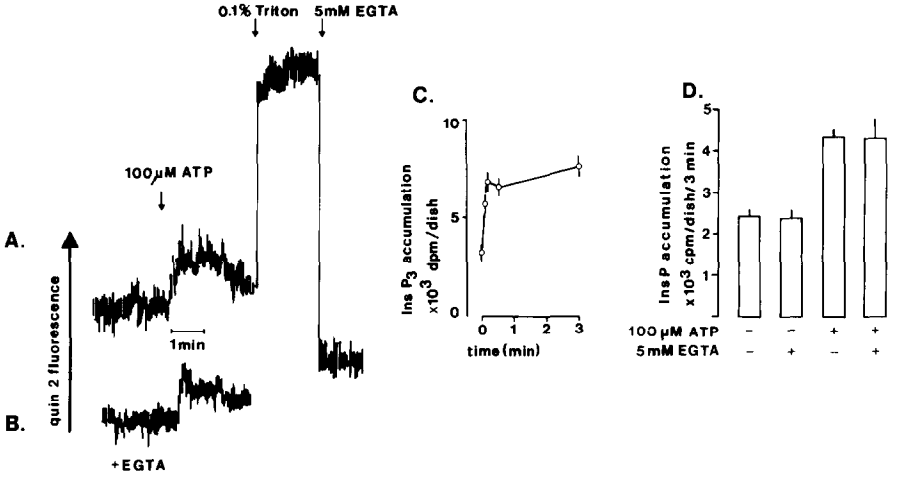


Fig.1. Effect of ATP on levels of inositol phosphates and cytoplasmic free calcium in cultured chick myotubes. (A,B) Quin2 fluorescence after treatment of myotubes with ATP in the absence (A) and presence (B) of EGTA. (C) Time course of ATP (100 $\mu$M)-induced inositol triphosphate accumulation (mean ± SE, $n = 5$). (D) Effect of EGTA on basal and ATP-induced inositol phosphate accumulation. ATP and EGTA added simultaneously (mean ± SE, $n = 5$).

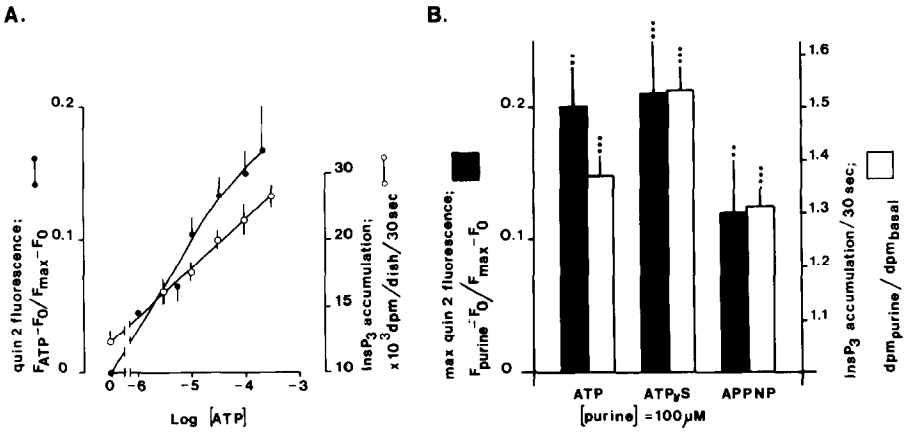


Fig.2. (A) Concentration dependence for ATP-induced inositol triphosphate accumulation and quin2 fluorescence. $F_{ATP}$, fluorescence in the presence of ATP; $F_{max}$, in the presence of 0.1% Triton X-100; $F_o$, in the presence of 0.1% Triton X-100 and 5 mM EGTA (mean ± SE, $n=4$). (B) Comparison of the effects of ATP, ATPγS and APPNP on quin2 fluorescence and inositol triphosphate accumulation in chick myotubes. $^{**}p<0.01$; $^{***}p<0.001$ vs basal levels in each case.

ly hydrolyzed derivatives of ATP for effects on cytosolic calcium levels. Both adenosine 5′-(3-thiotriphosphate) (ATPγS) and 5′-adenylyl imidodiphosphate (APPNP), at 100 μM, increased quin2 fluorescence and PI turnover, suggesting that ATP need not be hydrolyzed in order to elicit its effect. Further evidence for a P2-purinoceptor-mediated mechanism was obtained from experiments using the putative P2-purinoceptor antagonist, aryl-

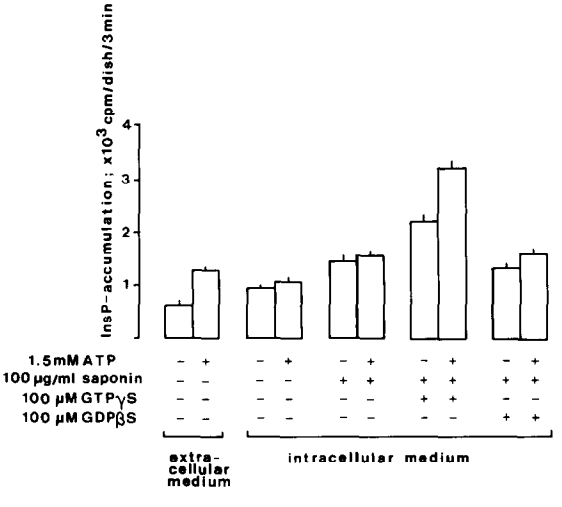


Fig.3. Action of guanyl nucleotides on inositol phosphate accumulation in permeabilized chick myotubes. Results shown are from one representative experiment (mean ± SE, $n=5$). For media compositions see section 2. The ATP concentration was chosen to be high in order to mimic intracellular conditions.

azidoaminoproprionyl ATP (ANAPP3). This photoaffinity ligand irreversibly blocks P2-purinoceptors in the vas deferens [9]. At 10 μM, ANAPP3 reduced the ATP-induced (100 μM) PI turnover by 50% ($p<0.05$, $n=5$) under the appropriate conditions, i.e. after light. Cultures treated, in total darkness, with ANAPP3 and then ATP responded to ATP similarly to ANAPP3-untreated cultures (not shown).

Receptor-mediated PI turnover is known to occur via G-proteins (e.g. [10]). In a series of experiments we studied the influence of G-proteins on P2-purinoceptor-mediated PI turnover. The action of cholera or pertussis toxin (concentrations up to $10^{-8}$ M) was investigated. No significant effect on ATP-stimulated PI turnover was, however, observed (not shown). On the other hand, the presence of a G-protein type of transducer coupled to phospholipase C was observed, as indicated by the effect of 100 μM guanosine 5′-(3-thiotriphosphate) (GTPγS) in saponin-treated myotubes where the amount of accumulated InsPs increased by approx. 300% (fig.3). Guanosine 5′-(2-thiodiphosphate) (GDPβS) was without effect.

## 4. DISCUSSION

Based on the observed similarities in time courses and dose-effect relationships, and on the observed insensitivity of calcium release to acute exposure of myotubes to EGTA, this study allows

us to draw the conclusion that ATP-induced inositol triphosphate ($InsP_3$) accumulation and the increase in cytosolic calcium are coupled (probably consecutive) phenomena. Based on current knowledge that $IP_3$ is a trigger of calcium mobilization, we suggest that the P2-purinoceptor-stimulated increase in $InsP_3$ gives rise to the release of calcium from intracellular stores into the cytosol. This mechanism seems to be insensitive to cholera and pertussis toxin (e.g. [11]). Nevertheless, transducer proteins probably play an important role in ATP-induced calcium release since, in permeabilized cells, GTP$\gamma$S increased basal InsP levels as compared to controls. Further, GTP also increased InsP levels but to a lesser extent (not shown). However, it is difficult to decide whether ATP enhances GTP$\gamma$S-induced InsP accumulation through a receptor-mediated mechanism as the ATP added to permeabilized cells also maintains cellular levels of the rapidly turning over receptor-sensitive pool of PIs. Nevertheless, there is a significant increase in effect between the presence and absence of ATP when comparing the conditions with and without GTP$\gamma$S (fig.3).

The action of ATP on chick myotube PI turnover is most probably due to the activation of P2-purinoceptors. This suggestion is strengthened by our finding that ANAPP3, a putative P2-purinoceptor antagonist, reduces ATP-stimulated PI turnover. The physiological role of P2-purinoceptors present on the myotube in culture and on the skeletal muscle fiber membrane is not known. One obvious function would be for the receptors to participate in or to modulate the muscular contraction mechanism which is strictly calcium-dependent. The action of ATP described by us in this and previous papers has also been reported for other cell types such as hepatocytes [12], aortic myocytes [13], medullary endothelial cells [14], Ehrlich ascites cells [15], glioma C6 and neuroblastoma N1E 115 cells (unpublished). This suggests that the stimulation of PI turnover and increase in cytosolic calcium in response to extracellular ATP is not muscle-specific but rather a more general mechanism, pointing to a ('primitive'?) cell hormone action. Hypothetically, it may be the specialization of a particular cell type that determines the physiological target of the P2-purinoceptor-mediated increase in cytosolic calcium, such that in skeletal muscle cells contraction may be modulated while in, for example hepatocytes, glycogenolysis [12] is triggered.

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