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# Inhibitory effects of extracellular ATP on the $Ca^{2+}$ mobilization evoked by muscarinic stimulation in rat parotid acinar cells

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## Abstract

In rat parotid acinar cells, extracellular ATP reduced the increase in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) induced by the muscarinic receptor agonist carbachol (CCh) dose-dependently. This inhibitory effect was also observed in the absence of extracellular $Ca^{2+}$, indicating that the $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores was prevented by ATP. Formation of inositol trisphosphate induced by CCh stimulation was strongly attenuated in the presence of extracellular ATP, suggesting that the effect of ATP on the CCh-induced $[Ca^{2+}]_i$ response was the result of a decreased phosphoinositide hydrolysis. Extracellular ATP had no effect on the CCh-induced increase in $[Ca^{2+}]_i$ in rat lacrimal acinar cells that lack the purinergic receptors. This result supports the view that the inhibitory effect of ATP on the CCh-induced $[Ca^{2+}]_i$ response in parotid acinar cells is mediated through activation of the purinergic receptros. In rat parotid acinar cells, the purinergic receptors may play a role in modulating the activity of muscarinic receptors.

**Key words** : Parotid acinar cells, ATP, Purinergic receptors, Intracellular $Ca^{2+}$, Phosphoinositide hydrolysis

## Introduction

In rat salivary acinar cells, extracellular ATP causes a rapid increase in cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) without significant activation of phosphoinositide hydrolysis[1,2]. The ATP-induced increase in $[Ca^{2+}]_i$ is mainly due to the entry of $Ca^{2+}$ from extracellular medium,

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since a removal of extracellular $Ca^{2+}$ markedly diminishes this respose[1,2]. Based on the pharmacological potencies of various nucleotides, it has been believed that the effects of ATP observed in salivary acinar cells is mediated through its action on $P_{2Z}$, a subtype of $P_2$-purinergic receptors, which is highly sensitive to the ionized form of ATP ($ATP^{4-}$)[3]. Although previous work suggests that extracellular ATP may play an important role in regulating salivary functions, its precise role is not fully understood.

Activation of muscarinic-cholinergic receptors stimulates phosphoinositide hydrolysis leading to production of inositol phosphates and causes an increase in $[Ca^{2+}]_i$ due to a release of $Ca^{2+}$ from intracellular stores and a sustained entry of $Ca^{2+}$ across the plasma membrane. This $Ca^{2+}$ response is an essential intracellular signal for fluid secretion in salivary glands[4]. It was recently reported that extracellular ATP suppresses the $Ca^{2+}$ response induced by stimulation of muscarinic receptors in rat submandibular and parotid acinar cells[5,6]. This suggests that there is an interaction between the ATP-activated intracellular signals and those activated through stimulation of muscarinic receptors. To address this, a further examination of the effects of extracellular ATP on $Ca^{2+}$ mobilization and inositol trisphosphate ($IP_3$) induced by muscarinic stimulation in rat parotid acinar cells was made.

## Materials and Methods

### Materials

Carbachol (CCh), collagenase, trypsin (type III), trypsin inhibitor (typeII-S), bovine serum albumin (BSA), ATP(disodium salt) were obtained from Sigma (St Louis, MO, USA). Fura-2/acetoxymetyl ester (fura-2/AM), EGTA and HEPES were from Dojin Laboratories (Kumamoto, Japan). The *myo*-[2-$^3$H]-inositol was from American Radiolabeled Chemicals, Inc (St Louis, MO, USA). All other regents were Wako Pure Chemicals (Osaka, Japan).

### Preparation of parotid and lacrimal acinar cells

Male Wistar-strain rats, weighing about 300 g, were anesthetized with diethyl ether and killed by cardiac puncture. Dispersed acinar cells were prepared by enzyme digestion with trypsin and collagenase as previously described elsewhere[7]. After dispersion, the cells were washed and resuspended in a Hanks' balanced salt solution buffered with 20mM HEPES (pH 7.4) (HBSS-H) containing 0.2% BSA.

### Measurement of $[Ca^{2+}]_i$

Acinar cells were incubated for 40min with 2$\mu$M fura-2/AM in HBSS-H containing 0.2% BSA at 37°C. The fura-2-loaded cells were washed twice, resuspended in fresh HBSS-H containing 0.2% BSA and stored at room temperature until use. Fura-2 fluorescence was measured at 37°C in a magnetically stirred cuvette in a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan) with excitation at 340 and 380nm and emission at 510nm. The $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence, as described by Grinkiewicz et al.[8].

### Measurement of inositol triphosphate

Parotid acinar cells were prelabelled with *myo*-[2-$^3$H] -inositol, as described by Tanimura et al.[9]. The labelled cells were preincubated for 5min in the presence of 10mM LiCl and stimulated with ATP, CCh or a combination of ATP and CCh for a further 5min. The reactions were stopped by the addition of $HClO_4$ (final concentration 4.5%). A portion of the supernatant was then neutralized with 0.5M KOH/9mM $Na_2B_4O_7$. Labelled inositol trisphosphate ($IP_3$) was collected using a Bio-Rad AG 1-X8 column according to the method of Berridge et al.[10].

## Results

Fura-2-loaded parotid acinar cells were stimulated with various concentrations (0.1, 0.25 and 0.5mM) of ATP in the presence of extracellular $Ca^{2+}$. As shown in Fig. 1, the addition of ATP increased $[Ca^{2+}]_i$ in a dose-dependent manner. When 10 $\mu$M CCh was added to the cells at the plateau phase of the ATP-induced increase in $[Ca^{2+}]_i$, further increases in $[Ca^{2+}]_i$ in response to CCh were reduced with increasing the concentration of added ATP. Prestimulation with 0.5mM ATP completely blocked the $[Ca^{2+}]_i$ response induced by CCh.

To show whether ATP affects the $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores, the effect of ATP on the CCh-induced $Ca^{2+}$ response was examined in a $Ca^{2+}$-free medium containing 1mM EGTA (Fig. 2). Addition of ATP up to 0.5mM had little or no effect on $[Ca^{2+}]_i$, indicating that ATP itself did not mobilize $Ca^{2+}$ from intracellular $Ca^{2+}$ stores. In the control cells, subsequent addition of 10$\mu$M CCh evoked a rapid and large increase in $[Ca^{2+}]_i$ due to a $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores, but in the presence of extracellular ATP the CCh-induced $Ca^{2+}$ mobilization was markedly reduced with increasing concentrations of ATP.

To obtain direct evidence that ATP inhibits phosphoinositide hydrolysis activated by muscarinic stimulation, the levels of $IP_3$ produced by CCh were measured in the presence and absence of ATP (Fig. 3). Incubation for 5min with 10$\mu$M CCh alone caused a marked increase in $IP_3$ levels, but the combined addition of 10$\mu$M CCh and 1mM ATP had only a littel effect on

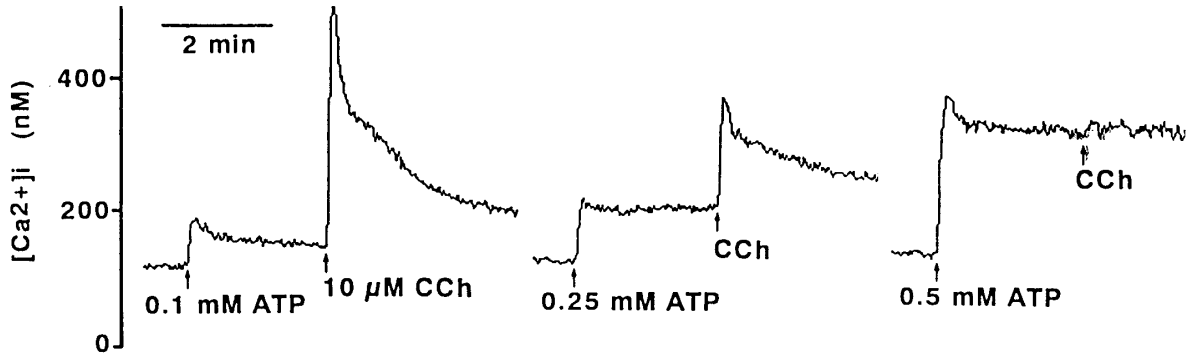


Fig. 1 Effect of ATP on the increase in $[Ca^{2+}]_i$ induced by CCh in the presence of extracellular $Ca^{2+}$. Fura-2-loaded parotid acinar cells were stimulated with 0.1, 0.25, or 0.5 mM ATP, and then 10$\mu$M CCh was added at the plateau phase of the ATP-induced increase in $[Ca^{2+}]_i$.

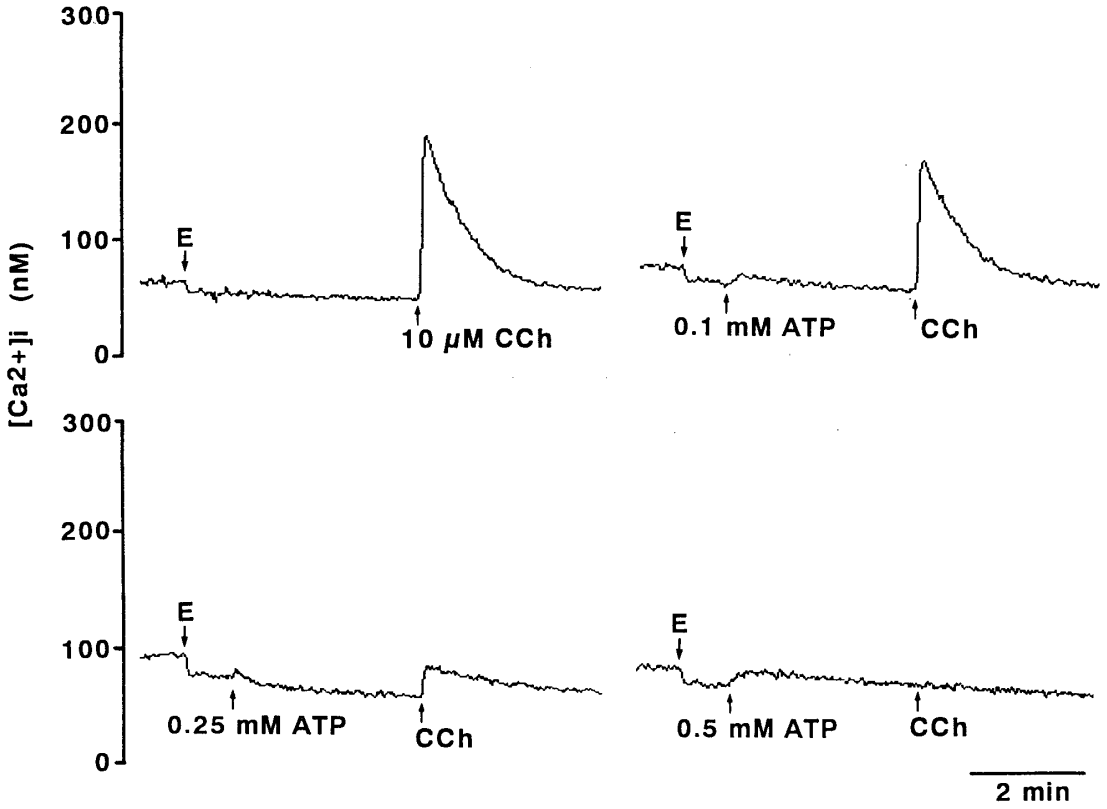


Fig. 2 Effect of ATP on $Ca^{2+}$ release induced by CCh in the absence of extracellular $Ca^{2+}$. Fura-2-loaded parotid acinar cells were exposed to various concentrations of ATP in a $Ca^{2+}$-free medium containing 1mM EGTA (E) and then stimulated with 10$\mu$M CCh.

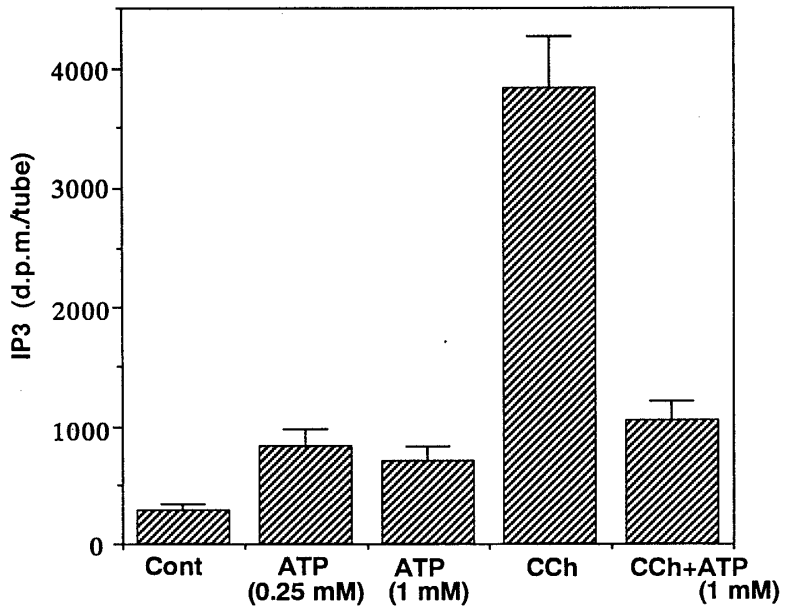


Fig. 3 Inositol trisphosphate ($IP_3$) formation induced by ATP and CCh. [$^3$H] Inositol-labeled parotid acinar cells were incubated with ATP, CCh, or a combination of ATP and CCh for 5min. Values are means ± S.E. of 5 experiments.

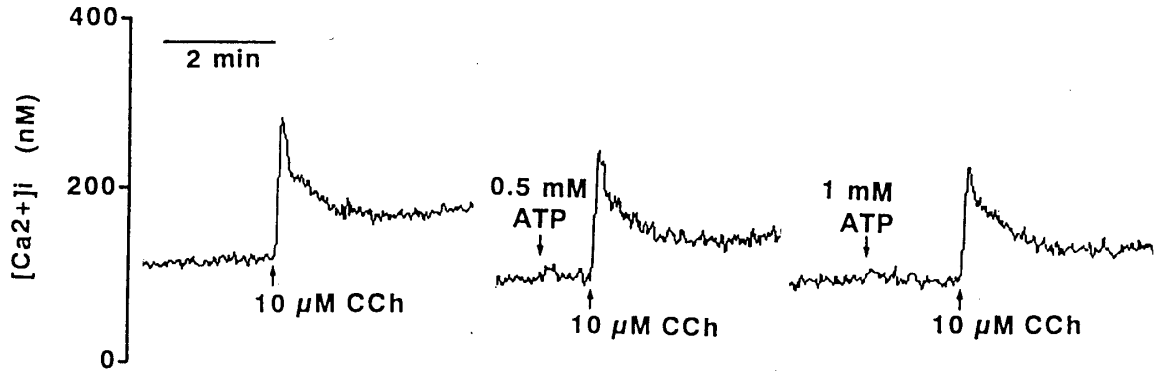


Fig. 4 Effect of ATP on the increase in $[Ca^{2+}]_i$ induced by CCh in rat lacrimal acinar cells. In the presence of extracellular $Ca^{2+}$, fura-2-loaded cells were exposed to 0.5 or 1mM ATP and then stimulated with 10$\mu$M CCh. The ATP did not attenuate the increase in $[Ca^{2+}]_i$ induced by CCh.

$IP_3$ formation. The $IP_3$ level was similar to that in the cells incubated with ATP alone.

Further, to show whether the inhibition by ATP of the $Ca^{2+}$ mobilization takes place in cell types that lack purinergic receptors, the effect of ATP on the CCh-induced increase in $[Ca^{2+}]_i$ was examined in rat lacrimal acinar cells. As shown in Fig. 4, lacrimal acinar cells did not respond to 0.5 and 1mM ATP in the presence of extracellular $Ca^{2+}$, indicating that $P_2$-purinergic receptors do not exist in rat lacrimal acinar cells. In this cell type, extracellular ATP did not inhibit the increase in $[Ca^{2+}]_i$ induced by a subsequent addition to 10$\mu$M CCh.

## Discussion

This study supports the findings that extracellular ATP suppresses the $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores induced by muscarinic receptor agonists in rat submandibular gland and parotid acini[5,6]. There are several possible explanations for this inhibitory effect of ATP. As ATP itself causes a marked increase in $[Ca^{2+}]_i$ due to $Ca^{2+}$ entry from extracellular medium, the inhibition by ATP may be secondary to the increase in $[Ca^{2+}]_i$. However, this cannot be the explanation that ATP inhibited the CCh-induced $[Ca^{2+}]_i$ response similarly in the presence and absence of extracellular $Ca^{2+}$. Another possibility is that ATP inhibits the CCh-induced $[Ca^{2+}]_i$ response by directly interfering with the specific binding of CCh to muscarinic receptors. If so, ATP would be expected to inhibit activation of muscarinic receptors in any cell type. The present study, however, shows that ATP had no effect on the CCh-induced increase in $[Ca^{2+}]_i$ in rat lacrimal acinar cells which lack purinergic receptors. Hurley et al.[5] reported that ATP did not affect the binding of $[^3H]$ acetylcoline to the plasma membrane of submandibular gland acini, also indicating that ATP does not directly interfere with the binding of receptor agonists to the receptors.

Thus, it is possible to conclude that the inhibitory effects of ATP on the muscarinic responses are mediated through activation of $P_{2Z}$ purinergic receptors. The mechanism by which the acitivation of $P_{2Z}$ receptors inhibit the intracellular signal pathway activated by muscarinic

stimulation is not clear, but in the present study extracellular ATP also strongly reduced the production of $IP_3$ induced by CCh, indicating that the activation of $P_{2Z}$ receptors suppressed the $[Ca^{2+}]_i$ response through inhibition of phosphoinositide hydrolysis. The inhibitory effect of ATP on $IP_3$ formation may be the result of a reduced phospholipase C activity.

The physiological role of purinergic receptors in salivary glands is not established. As ATP has little or no effect on amylase release in rat parotid acinar cells[1,2], it is unlikely that ATP is a neurotransmitter inducing amylase release. Since ATP causes a release of $K^+$ from rat parotid acinar cells[3], it may be possible that ATP is involved in fluid secretion. However, unlike CCh, ATP induces $K^+$ release even in the absence of extracellular $Ca^{2+}$[2], and the mechanism of the ATP-induced $K^+$ release appears to be different from that for $K^+$ release induced by muscarinic stimulation. This result raises doubts about ATP as a physiological stimulus in fluid secretion from salivary glands. Several investigations[5,6] have proposed that ATP may play a role as a negative modulator in regulating the activity of muscarinic and other autonomic receptors. Further study is necessary to determine this possibility.

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