ELSEVIER

European Journal of Pharmacology 336 (1997) 89–97

ejp

Na^+-dependent release of intracellular Ca^{2+} induced by purinoceptors in parotid acinar cells of the rat

Yasue Fukushi [a,*], Terutaka Ozawa [a], Takahiro Kanno [b], Makoto Wakui [b]

[a] *Department of Physiology, Tohoku University School of Medicine, Seiryo-cho, Sendai 980-77, Japan*
[b] *Department of Physiology, Hirosaki University School of Medicine, Hirosaki 036, Japan*

Received 16 January 1997; revised 28 July 1997; accepted 1 August 1997

Abstract

In rat parotid acinar cells, ATP caused a transient increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the absence of external Ca^{2+}. The ATP-induced Ca^{2+} response was strongly suppressed by removal of external Na^+. The sequence of potency in increasing $[Ca^{2+}]_i$ was 3′-*o*-(4-benzoyl) benzoyl-ATP > ATP > uridine 5′-triphosphate (UTP). Adenosine, AMP, ADP or α,β-metylene ATP did not cause an increase in $[Ca^{2+}]_i$. The 3′-*o*-(4-benzoyl) benzoyl-ATP-induced increase in $[Ca^{2+}]_i$ was abolished by removal of external Na^+, but the UTP-induced response was not. The threshold external Na^+ concentration required for ATP- or 3′-*o*-(4-benzoyl) benzoyl-ATP-induced Ca^{2+} release was 10–20 mM. ATP but not UTP caused a rise in the intracellular Na^+ concentration ($[Na^+]_i$). Ca^{2+} release stimulated by caffeine or treatment with ryanodine reduced the Ca^{2+} release evoked by ATP. These results suggest that ATP, acting through P_{2Z} purinoceptors, causes Na^+ entry by opening cation-permeable channels, and thereafter the increase in $[Na^+]_i$ triggers Ca^{2+} release from ryanodine-sensitive stores. UTP, acting through P_{2U} purinoceptors, causes Ca^{2+} release independent of external Na^+. © 1997 Elsevier Science B.V.

Keywords: Ca^{2+} release; Na^+, external; P_2 purinoceptor; Parotid acinar cell

1. Introduction

Evidence suggests a role for ATP as a major cotransmitter in regulating the autonomic nervous system (Burnstock, 1972). Extracellular ATP elevates $[Ca^{2+}]_i$ in many cell types: (1) by activating phospholipase C in some cell types (Charest et al., 1985; Barry and Cheek, 1994) and (2) by directly opening Ca^{2+}-permeable ion channels in other cell types (Sasaki and Gallacher, 1990; Soltoff et al., 1992).

In the parotid gland, in which there is a rich innervation of autonomic nerve fibers, extracellular ATP causes a great rise in $[Ca^{2+}]_i$ in two distinct ways: (1) via activation of Ca^{2+} entry from the external fluid (McMillian et al., 1988; Soltoff et al., 1990) and (2) through an induction of Ca^{2+} release from the intracellular stores (McMillian et al., 1988; Soltoff et al., 1990). There is evidence that P_{2Z}-subtype purinoceptors are involved in the mechanism of ATP-induced Ca^{2+} entry (McMillian et al., 1988; Soltoff et al., 1990). P_{2Z} as well as P_{2X} purinoceptors, subtypes of the P_2 purinoceptor, are suggested to be coupled to ligand-gated ion channels or pores (Nuttle and Dubyak, 1994). It has been established that P_{2X} purinoceptors are present predominantly in excitable cells and may act in neuronal transmission (Bean, 1992), whereas P_{2Z} purinoceptors are present in nonexcitable cells. However, P_{2Z} purinoceptors recently have been cloned and identified as a member of the P_{2X} family (Surprenant et al., 1996). P_{2Z} purinoceptors activate the formation of nonselective pores, permeable to molecules with $Mr \leq 900$ in some cell lines (Cockroft and Gomperts, 1979; Greenberg et al., 1988; Tatham et al., 1988). In parotid acinar cells, P_{2Z} purinoceptors are coupled to the rapidly gated cation channel (Soltoff et al., 1992; McMillian et al., 1993).

The mechanism by which ATP releases Ca^{2+} from the intracellular stores in parotid acinar cells is still poorly understood. P_{2Y} as well as P_{2U} purinoceptors, subtypes of the P_2 purinoceptor, are suggested to be coupled to the mobilization of intracellular Ca^{2+} stores, inducing rapid activation of inositol hydrolysis and accumulation of inositol 1,4,5-trisphosphate ($InsP_3$) (Dubyak and El-Moatassim, 1993). However, the mechanism in this cell type appears to differ from that linked to phospholipase C because (1) ATP does not produce large changes in inositol 1,4,5-trisphosphate production, and electrophysiological

* Corresponding author. Tel.: (81-22) 717-8070; Fax: (81-22) 717-8098.

effects of ATP do not require guanine nucleotide binding protein (McMillian et al., 1988; Soltoff et al., 1990); and (2) effects of ATP on $[Ca^{2+}]_i$ in parotid acinar cells are additive to the effects of other Ca^{2+}-mobilizing agonists (McMillian et al., 1988; Soltoff et al., 1990).

Recently, evidence of Ca^{2+} release due to Na^+ entry from the extracellular fluid has been obtained in an insulin-secreting cell line (Leech et al., 1995; Holz et al., 1995). Peptides coupled to the generation of cAMP, such as pituitary adenylate cyclase-activating polypeptide (Leech et al., 1995), or glucagon-like peptide-1 (Holz et al., 1995), caused an elevation of $[Ca^{2+}]_i$ initiated by Na^+ entry through nonselective cation channels opened by a cAMP-dependent mechanism. In B cells, elevation of $[Na^+]_i$ also triggered Ca^{2+} release (Herchuelz and Malaisse, 1980). In Sertoli cells, activation of the P_2 purinoceptor subtype coupled to the Na^+ entry pathway caused a release of Ca^{2+} from intracellular stores (Foresta et al., 1995). Therefore, in the present study we aimed to examine (1) the purinoceptors responsible for Ca^{2+} release in this cell, and (2) the relationships between Ca^{2+} release, activation of purinoceptors and external Na^+. Here, we provide evidence that ATP, by activation of P_{2Z} purinoceptors, induces Na^+ influx through cation-permeable channels. It is further suggested that the increase in intracellular Na^+ concentration triggers Ca^{2+} release from caffeine- and ryanodine-sensitive Ca^{2+} stores. In contrast, UTP, by acting through P_{2U} purinoceptors, of which there are few in this cell type, induces Ca^{2+} release independently of external Na^+.

2. Materials and methods

2.1. Solutions and materials

The standard extracellular solution contained 140 mM NaCl, 4.7 mM KCl, 1.13 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES-NaOH (pH 7.2). To make a Ca^{2+}-free solution, $CaCl_2$ was removed from and 0.5 mM EGTA was added to the standard solution, in which the free Ca^{2+} concentration was estimated to be 1 nM (Fabiato and Fabiato, 1979). Na^+-free solutions were prepared by replacing NaCl with *N*-metyl-D-glucamine (NMDG) or LiCl, and the pH was adjusted to 7.2 with HCl. Reagents were obtained from Sigma (St. Louis, MO, USA) except the following drugs: fura-2/AM, sodium benzofuran isophthalate (SBFI) (Dojin Chemical Institute, Fukuoka, Japan), and collagenase (Wako, Osaka, Japan). Fura-2/AM was dissolved in dimethyl sulfoxide (DMSO) (final [DMSO] = 0.01%).

2.2. Preparation of parotid acinar cells

Parotid glands from male Wistar rats were isolated, and blood vessels passing through the tissue and the surrounding connective tissue were removed. The glands were minced and chemically digested for 10 min in a standard solution containing collagenase (200 U/ml). The suspension was continuously fully oxygenated at 37°C and agitated in a shaking water bath (at 100 cycles/min). The suspension was transferred to a Ca^{2+}-free solution containing 2 mM EDTA (pH 7.2) for 10 min. At the end of the EDTA treatment, the cells were washed and resuspended for an additional 50 min in a solution containing collagenase. At 25 min intervals, the suspension was gently pipetted up and down five times in a pipette tip (1–1.5 mm wide) polished by heat. At the end of the digestion period, cells were mechanically dispersed by pipetting (about 100 times) to obtain further separation. The suspension was washed twice and resuspended in an oxygenated bovine serum albumin-(0.2%) containing solution. Cells were fully oxygenated, agitated in a shaking water bath (at 60 cycles/min) for an additional 2 h, and then the experiments were performed.

2.3. Fluorescence measurements

In the experiments for $[Ca^{2+}]_i$ measurements, cells were loaded with fura-2 by incubation with 1–1.5 μM fura-2/AM at 37°C for 40 min, and washed with the external solution by centrifugation, as previously described (Fukushi et al., 1995). The cells were used within 10–60 min after fura-2 loading. In the experiments for $[Na^+]_i$ measurements, cells were loaded with the Na^+-sensitive fluorescent indicator, sodium benzofuran isophthalate, by incubation with 10 μM sodium benzofuran isophthalate/AM in the presence of 0.1% pluronic acid at 37°C for 1.5–2 h (Harootunian et al., 1989). The cover slip with the cells was mounted in a chamber on the stage of an inverted microscope (TMD-EFQ; Nikon, Tokyo, Japan), and the cells were perifused continuously (bath volume = 400 μl; flow rate = 0.5 ml/min) with a stream of external solution at 23.5°C. The arrangement of the superfusion system permitted rapid exchange of the bath solution in the vicinity of the cell. Fura-2 imaging, construction of ratio images and quantification were the same as previously described (Fukushi et al., 1995). The change in $[Ca^{2+}]_i$ was estimated from the ratio of fluorescence traces at the two excitation wavelengths (340 nm/380 nm). $[Ca^{2+}]_i$ was calculated using the equation given by Grynkiewicz et al. (1985).

$$[Ca^{2+}]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \frac{S_{f2}}{S_{b2}},$$

where K_d is the dissociation constant for fura-2/Ca^{2+} (224 nM), R is the intensity ratio for fluorescence at the two wavelengths, R_{max} and R_{min} are ratios at zero and saturation, respectively, and S_{f2}/S_{b2} is the ratio of excitation efficiencies for free and bound fura-2 at the higher of the wavelengths. The calibration solution contained 50 μM fura-2, 100 mM KCl, 25 mM 3-[*N*-morpholino]pro-

panesulfonic acid (MOPS), 1 mM $MgCl_2$, 10 mM EGTA and 0–1 mM free Ca^{2+} (pH 7.20). We used the values of R_{max}, R_{min} and S_{f2}/S_{b2} as 10.4, 0.24 and 8.45.

2.4. Calibration of $[Na^+]_i$

Calibration of $[Na^+]_i$ was accomplished by application of gramicidin (4 μM) or nigericin (3.3 μM). Cells were perifused with different Na^+ concentrations made from appropriate mixtures of high Na^+ and Na^+-free solutions containing *N*-methyl-D-glucamine for 10 min. Gramicidin or nigericin was introduced for 20 min to allow complete equilibration. The sodium indicator sodium benzofuran isophthalate permits excitation ratio measurements and can be used with the same equipment as fura-2. The recorded 340/380 nm excitation fluorescence ratio (emission 510 nm) was converted to mM (Harootunian et al., 1989).

2.5. Statistics

Statistical significance was analyzed with the Mann-Whitney *U* test. Data were expressed as means ± S.E.M.

3. Results

3.1. Effects of ATP on $[Ca^{2+}]_i$

When exposed to 2 mM ATP (maximally effective concentration), the rat parotid acinar cells showed a rapid increase in $[Ca^{2+}]_i$ in an external solution containing 1 mM Ca^{2+} (a typical example of 10 experiments). The change in $[Ca^{2+}]_i$ in response to ATP reached a peak within 10 s from the start of the stimulation and thereafter $[Ca^{2+}]_i$ remained at a high level (Fig. 1a). The increase in $[Ca^{2+}]_i$ induced by ATP was decreased by washing the cells with the external solution. The second application of ATP did not evoke a further response. When the external Ca^{2+} concentration was reduced to 1 nM, ATP caused a transient increase in $[Ca^{2+}]_i$ (Fig. 1b), which was due to Ca^{2+} released from intracellular stores. The time to the peak of the initial phase was similar to that observed in the normal external solution. When the external Ca^{2+} concentration was increased to 1 mM in the continuing presence of ATP, an agonist-dependent Ca^{2+} influx resulted (Fig. 1b). When exposed to thapsigargin, a Ca^{2+}-ATPase inhibitor, cells produced a large increase in $[Ca^{2+}]_i$ due to Ca^{2+} released from stores. Sequential application of ATP did not elicit a response (Fig. 1c).

3.2. Effects of ATP analogues

In order to study which purinoceptor subtypes are involved in Ca^{2+} release from intracellular stores in this cell type, the effects of various ATP analogues on $[Ca^{2+}]_i$ were examined in presence of 1 nM external Ca^{2+} to eliminate

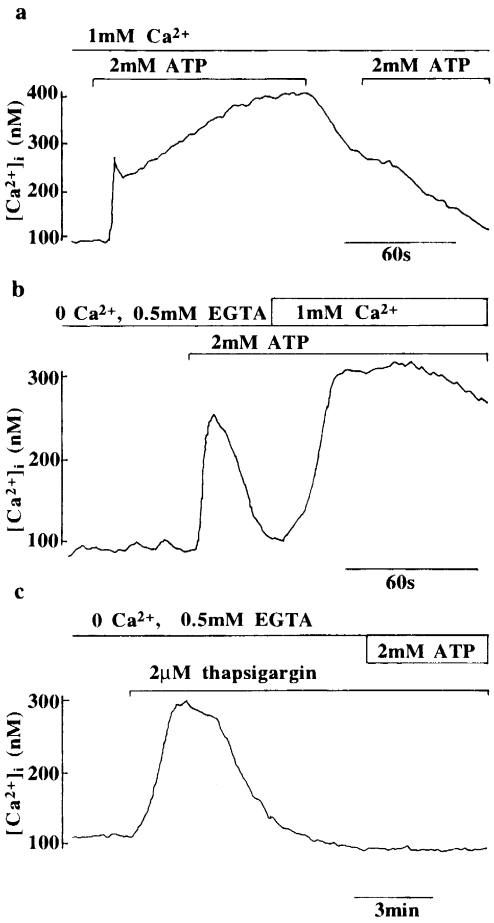

Fig. 1. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) evoked by extracellular ATP in the presence of 1 mM or 1 nM external Ca^{2+}. (a) ATP-induced Ca^{2+} response in the presence of external Ca^{2+} (1 mM). ATP (2 mM) was applied repeatedly at the times indicated. (b) Increase in $[Ca^{2+}]_i$ in cells due to mobilization of Ca^{2+} stores by 2 mM ATP. Stimulation was in the presence of an external Ca^{2+} concentration ($[Ca^{2+}]_o$) of 1 nM. At 30 s after the addition of ATP, the external Ca^{2+} was increased to 1 mM. The subsequent increase in $[Ca^{2+}]_i$ is due to an influx of Ca^{2+}. (c) Changes in $[Ca^{2+}]_i$ after exposure of cells to 2 μM thapsigargin and then to ATP. $[Ca^{2+}]_o$ was 1 nM throughout.

any Ca^{2+} influx. Adenosine, AMP and ADP were ineffective. α,β-metyleneadenosine 5′-triphosphate, a selective agonist of the P_{2X} purinoceptor, was also ineffective. Uridine 5′-triphosphate (UTP), a selective agonist of the P_{2U} purinoceptor, was less potent than ATP. In contrast, 3′-*o*-(4-benzoyl) benzoyl-ATP, the putative agonist of the P_{2Z} purinoceptor, was the most potent and effective among the ATP analogues tested. The $\Delta[Ca^{2+}]_i$ of the initial response after exposure to various concentrations of ATP analogues is shown in Fig. 2. These results demonstrate the possible existence of P_{2Z} (major) and P_{2U} purinoceptors (minor) in this cell.

3.3. Cross-desensitization of Ca^{2+} mobilizing responses by ATP and UTP

It was examined whether the UTP-induced increase in $[Ca^{2+}]_i$ in this cell type was mediated through pyrim-

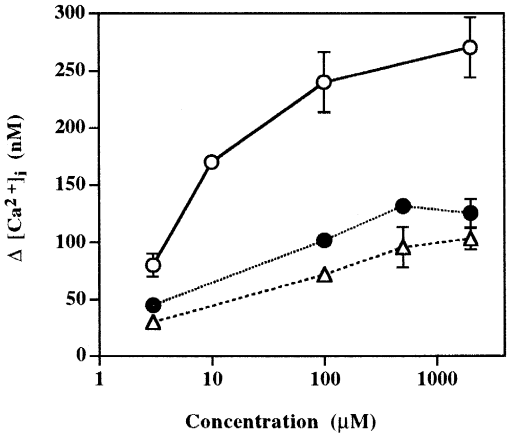

Fig. 2. Dose–response curves for the change in $[Ca^{2+}]_i$ evoked by 3′-*o*-(4-benzoyl) benzoyl-ATP (open circles), ATP (filled circles), and UTP (open triangles) in the presence of 1 nM external $[Ca^{2+}]_o$. $\Delta[Ca^{2+}]_i$ was measured by subtracting the prestimulated level of $[Ca^{2+}]_i$ from the peak response. Each point represents means ± S.E.M. (n = 5–40/nucleotides).

idinoceptors or P_{2U} purinoceptors. Fig. 3a shows that pre-exposure to ATP at 2 mM for 82 s blocked the UTP-induced increase in $[Ca^{2+}]_i$ in the continuing pres-

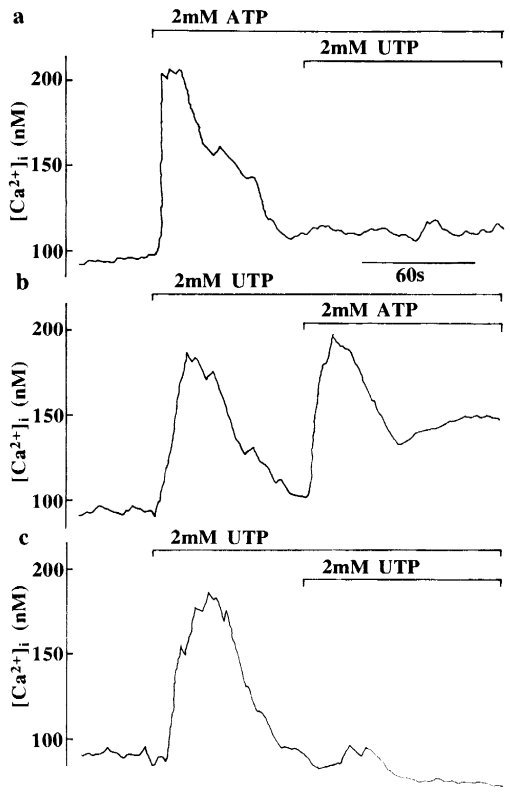

Fig. 3. Cross-desensitization of Ca^{2+}-mobilizing response of UTP and ATP. External $[Ca^{2+}]_o$ was 1 nM. (a) After pre-exposure of cells to ATP at 2 mM for 60 s, UTP at 2 mM was added sequentially. Resting $[Ca^{2+}]_i$ is approximately 70 nM in parotid acinar cells. (b) After pre-exposure of cells to UTP at 2 mM for 60 s, ATP at 2 mM was added. (c) After pre-exposure of cells to UTP at 2 mM for 60 s, the concentration of UTP was increased to 4 mM. Tracings are representative of three experiments using 30–50 cells.

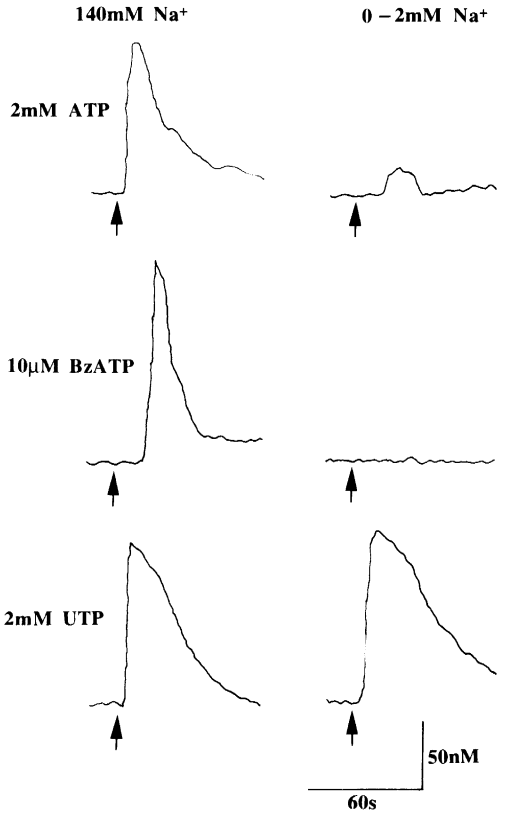

Fig. 4. Effects of Na^+ removal from the external solution on the increases in $[Ca^{2+}]_i$ induced by ATP, 3′-*o*-(4-benzoyl) benzoyl-ATP or UTP. $[Ca^{2+}]_o$ was 1 nM throughout. Tracings in the left panel were obtained for 140 mM Na^+ external solution and those in the right panel for the Na^+ free external solution (although 2 mM in the case of UTP). Concentrations of nucleotides (ATP and UTP, 2 mM: 3′-*o*-(4-benzoyl) benzoyl-ATP, 10 μM) were adjusted to be equipotent (although 3′-*o*-(4-benzoyl) benzoyl-ATP was 1.3 times more potent than the other nucleotides). Na^+ was iso-osmotically replaced by *N*-methyl-D-glucamine (NMDG). Note that increases in $[Ca^{2+}]_i$ induced by ATP, or 3′-*o*-(4-benzoyl) benzoyl-ATP, but not UTP, were markedly reduced by omitting external Na^+. The record shown is typical of three independent experiments.

ence of ATP. However, UTP did not inhibit the ATP-induced increase in $[Ca^{2+}]_i$ (Fig. 2b), although UTP inhibited the UTP-induced Ca^{2+} release (Fig. 3c).

3.4. Effects of Na^+ removal from the external solution on increases in $[Ca^{2+}]_i$ induced by ATP, 3′-o-(4-benzoyl) benzoyl-ATP and UTP

In the following experiments we assessed the previously mentioned hypothesis that Ca^{2+} release from intracellular stores induced by ATP is possibly coupled to Na^+ entry through cation-permeable channels. Tracings in the left panel in Fig. 4 show the Ca^{2+} mobilization from the stores induced by ATP (upper), 3′-*o*-(4-benzoyl) benzoyl-ATP (middle) and UTP (lower) in Na^+-rich external solution. The concentration used (2 mM) was maximally effective in the case of ATP and UTP, and at the concentration used

Table 1
Increases in $[Ca^{2+}]_i$ induced by ATP, UTP and 3′-*o*-(4-benzoyl) benzoyl-ATP (BzATP) in the presence of 140 mM or 0–2 mM external Na^+ solution

	$[Ca^{2+}]_i$ (nM)		
	prestimulated level	net change $\Delta[Ca^{2+}]_i$	*n*
2 mM ATP			
1 mM Ca^{2+}, 140 mM Na^+	103.5 ± 3.0	616.5 ± 119.9	50
0 Ca^{2+}, 140 mM Na^+	61.7 ± 4.6	125.5 ± 12.2	29
0 Ca^{2+}, 0 Na^+ (NMDG)	51.5 ± 1.5	46.6 ± 6.7 [a]	49
0 Ca^{2+}, 0 Na^+ (Li^+)	70.0 ± 2.8	26.0 ± 5.8 [a]	41
2 mM UTP			
0 Ca^{2+}, 140 mM Na^+	75.5 ± 0.8	103.0 ± 9.4	38
0 Ca^{2+}, 2 mM Na^+ (NMDG)	55.5 ± 0.8	110.9 ± 9.6	35
0 Ca^{2+}, 2 mM Na^+ (Li^+)	66.5 ± 4.5	102.3 ± 1.1	28
10 μM BzATP			
0 Ca^{2+}, 140 mM Na^+	70.5 ± 2.0	169.9 ± 7.4	31
0 Ca^{2+}, 0 Na^+ (NMDG)	56.5 ± 1.2	0	27
0 Ca^{2+}, 0 Na^+ (Li^+)	70.8 ± 2.2	0	40

0 Ca^{2+} means that $[Ca^{2+}]_o$ was 1 nM. $\Delta[Ca^{2+}]_i$ was measured by subtracting the prestimulated level of $[Ca^{2+}]_i$ from the peak response.
[a] $P < 0.001$. Control means values after stimulation with nucleotides in 0 Ca^{2+} and 140 mM Na^+ external solution.

(10 μM), 3′-*o*-(4-benzoyl) benzoyl-ATP was 1.3 times more potent than 2 mM ATP or 2 mM UTP. As shown, the nucleotides caused a large transient increase in $[Ca^{2+}]_i$ under Na^+-rich conditions. In detail, the 3′-*o*-(4-benzoyl) benzoyl-ATP-induced rise in $[Ca^{2+}]_i$ was characterized by a slower onset and shorter duration than the onset and duration of the increase in $[Ca^{2+}]_i$ induced by the other nucleotides.

The increase in $[Ca^{2+}]_i$ induced by ATP or 3′-*o*-(4-benzoyl) benzoyl-ATP was strongly reduced when external Na^+ was reduced to 0–2 mM (tracings in the right panel in Fig. 4: ATP, upper: 3′-*o*-(4-benzoyl) benzoyl-ATP, middle). In the case of ATP, a small increase in $[Ca^{2+}]_i$ remained after Na^+ removal, and the second challenge to ATP failed to effect a further response (data not shown). In the case of 3′-*o*-(4-benzoyl) benzoyl-ATP, the increase in $[Ca^{2+}]_i$ was abolished by Na^+ removal. Similar results were obtained when external Na^+ was replaced with LiCl. In contrast, UTP caused a transient increase in $[Ca^{2+}]_i$ even when the external Na^+ was reduced to 2 mM (tracing in the right panel in Fig. 4, lower). The comparative results of the changes in $[Ca^{2+}]_i$ induced by nucleotides in the presence and absence of external Na^+ are summarized in Table 1.

3.5. Threshold external Na^+ concentrations required for the increase in $[Ca^{2+}]_i$

The increase in $[Ca^{2+}]_i$ induced by ATP or 3′-*o*-(4-benzoyl) benzoyl-ATP varied depending upon the external Na^+ (Fig. 5). Different Na^+ concentrations were made from appropriate mixtures of high Na^+ and Na^+-free solutions containing *N*-methyl-D-glucamine. In the absence of Na^+, the rise in $[Ca^{2+}]_i$ was completely suppressed in the case of 3′-*o*-(4-benzoyl) benzoyl-ATP or greatly suppressed in the case of ATP. In the presence of 10–20 mM Na^+ (10 mM in 3′-*o*-(4-benzoyl) benzoyl-ATP; 20 mM in ATP), an increase in $[Ca^{2+}]_i$ was observed.

3.6. Changes of intracellular Na^+ concentration ($[Na^+]_i$) by application of ATP or UTP

Fig. 6a shows the change in $[Na^+]_i$ elicited by ATP, detected by using a Na^+-sensitive fluorescent dye, sodium benzofuran isophthalate. The basal $[Na^+]_i$ was 7.1 ± 0.4 mM ($n = 48$), and ATP caused an immediate and rapid increase in $[Na^+]_i$, which reached a maximum 36 s after the addition of ATP. The net change in $[Na^+]_i$ was 55.3 ± 1.6 mM ($n = 48$). When external Na^+ was replaced with *N*-methyl-D-glucamine, ATP caused a small decrease in $[Na^+]_i$ (data not shown). In contrast, UTP at 2 mM did not cause any increase in $[Na^+]_i$, even in the Na^+-rich external solution (Fig. 6b).

3.7. ATP releases Ca^{2+} from caffeine and ryanodine-sensitive Ca^{2+} stores

In order to determine whether the Ca^{2+} release from intracellular stores is mediated by ryanodine receptors, the

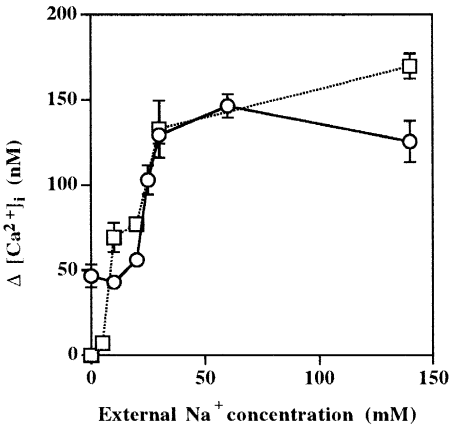

Fig. 5. Relationship between external Na^+ concentration and change in $[Ca^{2+}]_i$ in response to ATP, 3′-*o*-(4-benzoyl) benzoyl-ATP. Changes in $[Ca^{2+}]_i$ caused by 2 mM ATP (open circles) and 10 μM 3′-*o*-(4-benzoyl) benzoyl-ATP (open squares) in different external Na^+ concentrations were plotted. Solutions of different Na^+ concentrations were made from appropriate mixtures of high Na^+ and Na^+-free solutions containing *N*-methyl-D-glucamine. Cells were perifused with a solution of appropriate concentrations of Na^+ for 10 min and recording was started. External $[Ca^{2+}]_o$ was 1 nM. $\Delta[Ca^{2+}]_i$ was measured by subtracting the prestimulated level of $[Ca^{2+}]_i$ from the peak response. Each point represents means ± S.E.M. of three experiments using 17–24 cells (3′-*o*-(4-benzoyl) benzoyl-ATP) and 12–49 cells (ATP).

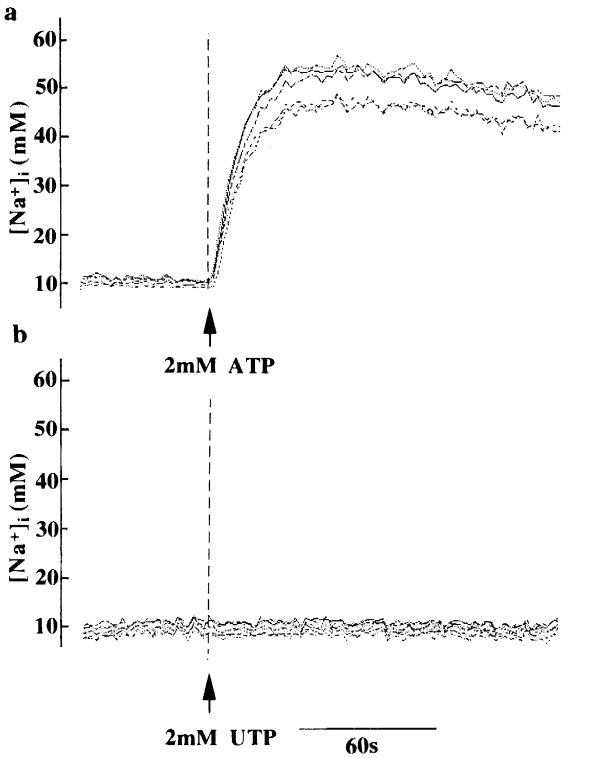

Fig. 6. Changes in intracellular Na^+ concentration ($[Na^+]_i$) evoked by extracellular ATP or UTP. $[Na^+]_i$ was measured with the Na^+-sensitive fluorescent indicator benzofuran isophthalate. Stimulation at the arrow and at the broken line in each trace was in the presence of Ca^{2+} at 1 nM. The record shown is typical of three independent experiments using 48–50 cells.

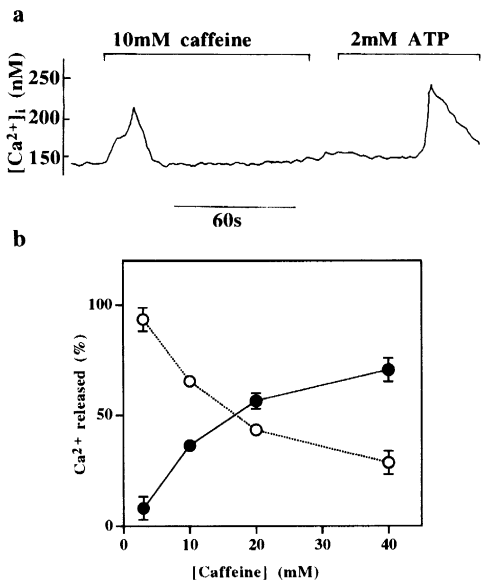

Fig. 7. Concentration-dependent inhibition by caffeine of the ATP-induced rise in $[Ca^{2+}]_i$. Cells were stimulated with caffeine, and then within 98 s further stimulated with 2 mM ATP, in the presence of Ca^{2+} at 1 nM. (a) $[Ca^{2+}]_i$ changes evoked by 10 mM caffeine and 2 mM ATP. (b) Plot of the complete experiment of $\Delta[Ca^{2+}]_i$ of the initial caffeine-induced response (filled circles) and the sequential ATP-induced response (open circles). The data are means ± S.E.M., $n = 5$.

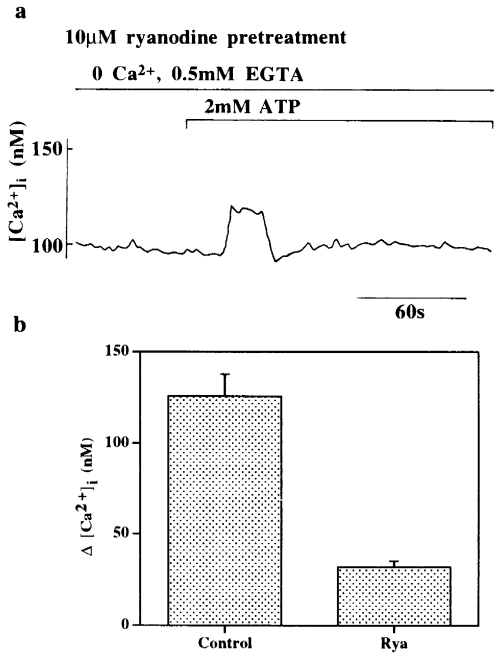

Fig. 8. Ryanodine inhibition of the rise of $[Ca^{2+}]_i$ evoked by extracellular ATP. External $[Ca^{2+}]_o$ was 1 nM. (a) Cells were pretreated with 10 μM ryanodine for 15 min. ATP (2 mM) was added at the time indicated. (b) $\Delta[Ca^{2+}]_i$ in cells treated (Rya) and not treated (Control) with ryanodine. Data were obtained from three experiments using 15 cells.

effect of caffeine and ATP on $[Ca^{2+}]_i$ was examined. Caffeine at 3 mM showed little effect on $[Ca^{2+}]_i$, and the sequential application of ATP at 2 mM caused an increase in $[Ca^{2+}]_i$. When applied at 10 mM, caffeine caused a transient increase in $[Ca^{2+}]_i$, and the sequential stimulation with ATP also caused an elevation of $[Ca^{2+}]_i$, although its onset was delayed (Fig. 7a). Caffeine at 40 mM caused a large transient increase in Ca^{2+} release, and the subsequent ATP stimulation caused another increase in $[Ca^{2+}]_i$. Fig. 7b shows the $\Delta[Ca^{2+}]_i$ evoked by caffeine and 2 mM ATP. The larger the amount of Ca^{2+} released by caffeine, the smaller the amount of Ca^{2+} released by ATP. Fig. 8a shows the effect of ryanodine, a ligand for Ca^{2+}-induced Ca^{2+} release channels, on the release of Ca^{2+} induced by ATP. The cells treated with ryanodine (10 μM) showed an increase in $[Ca^{2+}]_i$ in response to 2 mM ATP. However, the magnitude of the $\Delta[Ca^{2+}]_i$ in the cells treated with ryanodine was much smaller than that obtained in the control group (Fig. 7b).

4. Discussion

4.1. External ATP causes Ca^{2+} release from intracellular stores by activation of two distinct P_2 purinoceptors

In rat parotid cells, external ATP is known to activate P_{2Z} purinoceptors coupled to the cation-permeable chan-

nels permeable to Na^+ and Ca^{2+}, resulting in Ca^{2+} entry into the cell from the external solution (Soltoff et al., 1992; McMillian et al., 1993). In the present study, the presence of the P_{2Z} purinoceptor was confirmed (Fig. 2). However, the activation of the P_{2Z} purinoceptor was coupled firstly to Ca^{2+} mobilization from intracellular stores and secondly to Ca^{2+} entry. This conclusion was based on the following findings: (1) in the presence of Na^+ and Ca^{2+} in the external solution, ATP caused an increase in $[Ca^{2+}]_i$ consisting of the initial transient peak followed by a large sustained phase (Fig. 1a); (2) in the absence of external Ca^{2+}, ATP caused only the initial response of $[Ca^{2+}]_i$ (Fig. 1b); and (3) 3′-*o*-(4-benzoyl) benzoyl-ATP was the most potent and effective among the ATP analogues tested in elevating $[Ca^{2+}]_i$ in the absence of external Ca^{2+} (Fig. 2). In addition, UTP induced Ca^{2+} mobilization in this cell type (Fig. 2). P_{2U} purinoceptors are believed to be expressed in many cell types (Conigrave and Jiang, 1995), although there has been controversy about whether ATP and UTP interact with the same receptor or with distinct purinoceptors and pyrimidinoceptors (Seifert and Schulz, 1989). The finding that pre-exposure of cells to ATP desensitized UTP-induced Ca^{2+} release (Fig. 3) suggests that ATP shares a common P_{2U} purinoceptor. In addition, UTP did not desensitize the ATP response (Fig. 3b), suggesting that it could be a partial agonist. The idea that UTP may be a partial agonist is supported by the finding that UTP produced a lower peak response than ATP (Fig. 2).

4.2. P_{2Z} purinoceptor- but not P_{2U} purinoceptor-mediated Ca^{2+} release is Na^+-dependent

The present finding that ATP- and 3′-*o*-(4-benzoyl) benzoyl-ATP-induced Ca^{2+} release was greatly reduced by the removal of external Na^+ (Fig. 4) and that this effect varied depending upon external Na^+ (Fig. 5) clearly shows that there is a close relationship between Ca^{2+} release from intracellular stores and external Na^+. Furthermore, the results confirmed that Na^+ actually enters into the cell across the plasma membrane after stimulation with ATP (Fig. 6). There are two possible mechanisms to explain the link between the increase in $[Na^+]_i$ and the increase in $[Ca^{2+}]_i$: (1) Ca^{2+} extrusion from the cell is reduced, and (2) the rate of Ca^{2+} flux from the store to the cytosol is actually potentiated. With respect to the former possibility, elevation of $[Na^+]_i$ has been proposed to slow plasma membrane Na^+-Ca^{2+} exchange in pancreatic B cells (see Section 1). In the case of exocrine cells, however, Ca^{2+} extrusion from the cell is largely mediated by the Ca^{2+} pump (Tepikin et al., 1992). The latter possibility is supported by experimental evidence. In permeabilized rat submandibular acinar cells (Hurley and Ryan, 1988) and permeabilized rat pancreatic acinar cells (Bayerdörffer et al., 1984), it has been shown that Na^+ consistently reduces nonmitochondrial Ca^{2+} uptake by 20–30% as compared to the Ca^{2+} uptake in the presence of an equimolar concentration of K^+. Similar results have been obtained in experiments with microsomal preparations from pancreatic acinar cells (Thévenod and Schulz, 1988). The onset of Na^+ entry induced by ATP was very rapid (Fig. 6), whereas that of Ca^{2+} release induced by ATP or 3′-*o*-(4-benzoyl) benzoyl-ATP was roughly 10–15 s (Fig. 4), suggesting that Na^+ entry could indirectly cause Ca^{2+} release.

The mechanism of Ca^{2+} release through P_{2U} purinoceptor is not Na^+-dependent, which is supported by the finding that the removal of external Na^+ did not affect the UTP-induced increase in $[Ca^{2+}]_i$ (Fig. 4c) and that UTP did not evoke Na^+ entry (Fig. 6).

4.3. ATP-linked Na^+ entry releases Ca^{2+} from the caffeine- and ryanodine-sensitive stores

Experimental evidence has shown that nonmitochondrial Ca^{2+} stores other than inositol 1,4,5-triphosphate-sensitive stores are present in pancreatic acinar cells (Schmid et al., 1990). The presence of a Ca^{2+}-induced Ca^{2+} release mechanism has been proposed in salivary acinar cells (Foskett and Wong, 1991). In the present study, the presence of caffeine-sensitive stores was confirmed (Fig. 7a). ATP released Ca^{2+} from these stores, as shown in Fig. 7. This result was further confirmed by the findings with ryanodine. Ryanodine is a ligand for the Ca^{2+}-induced Ca^{2+} releasing channel, and is known to keep the channel in an open state (Imagawa et al., 1987). Therefore, it can empty Ca^{2+} stores possessing Ca^{2+}-induced Ca^{2+}-releasing channels (Fukushi et al., 1996). As shown in Fig. 8, in the cells pretreated with ryanodine, ATP caused only a small increase in $[Ca^{2+}]_i$. Recently, Ca^{2+} release by ATP from caffeine-sensitive stores in

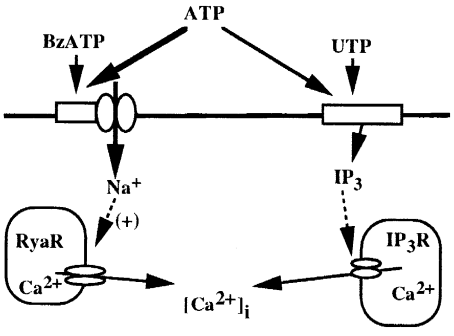

Fig. 9. Scheme showing receptor-mediated intracellular events that could account for ATP-induced Ca^{2+} signals in rat parotid acinar cells. Activation of a purinoceptor by UTP and ATP releases intracellularly stored Ca^{2+}. ATP releases Ca^{2+} from ryanodine-sensitive stores following Na^+ entry through channels opened by the activation of P_{2Z} purinoceptors. UTP releases Ca^{2+} possibly from inositol 1,4,5-triphosphate-sensitive stores by the activation of P_{2U} purinoceptors in a Na^+-independent manner.

PC12 cells was demonstrated (Barry and Cheek, 1994), whereas in submandibular acinar cells, activation of P_{2Z} purinoceptors inhibited acetylcholine-induced Ca^{2+} mobilization from inositol 1,4,5-triphosphate-sensitive stores, although activation of the receptor itself had little effect on Ca^{2+} mobilization (Hurley et al., 1993).

In conclusion, ATP triggers two distinct Ca^{2+} signaling pathways in parotid cells, as illustrated in Fig. 9: one through P_{2Z} purinoceptors and the other through P_{2U} purinoceptors. The Ca^{2+} signaling pathway of the former, which seems to be predominant in this cell type, is Na^+-dependent. Elevation of Na^+ in the cell can be achieved by opening of Na^+-permeable channels of the cell membrane by activation of P_{2Z} purinoceptors and elevation of $[Na^+]_i$, which, in turn, elevates $[Ca^{2+}]_i$ and triggers Ca^{2+} release from caffeine- and ryanodine-sensitive Ca^{2+} stores. In contrast, the Ca^{2+} signaling pathway of the latter, which seems to be minor in this cell type, is Na^+-independent. Further studies are required to explore the process(es) connecting intracellular Na^+ with Ca^{2+} release.

Acknowledgements

We would like to thank Professor Yoshio Maruyama and Professor John H. Exton for valuable comments on and discussion of this manuscript.

References

Barry, V.A., Cheek, T.R., 1994. Extracellular ATP triggers two functionally distinct calcium signalling pathways in PC12 cells. J. Cell. Sci. 107, 451–462.

Bayerdörffer, E., Streb, H., Eckhardt, L., Haase, W., Schulz, I., 1984. Characterization of calcium uptake into rough endoplasmic reticulum of rat pancreas. J. Membr. Biol. 81, 68–82.

Bean, B.P., 1992. Pharmacology and electrophysiology of ATP-activated ion channels. Trends Pharmacol. Sci. 13, 87–91.

Burnstock, G., 1972. Purinergic nerves. Pharmacol. Rev. 24, 509–581.

Charest, R., Blackmore, P.F., Exton, J.H., 1985. Characterization of responses of isolated rat hepatocytes to ATP and ADP. J. Biol. Chem. 260, 15789–15794.

Cockroft, S., Gomperts, B.D., 1979. ATP induces nucleotide permeability in rat mast cells. Nature 279, 541–542.

Conigrave, A.D., Jiang, L., 1995. Review: Ca^{2+}-mobilizing receptors for ATP and UTP. Cell Calcium 17, 111–119.

Dubyak, G.R., El-Moatassium, C., 1993. Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. Am. J. Physiol. 265, C577–C606.

Fabiato, A., Fabiato, F., 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. J. Physiol. 75, 463–505.

Foresta, C., Rossato, M., Bordon, P., Virgilio, F.D., 1995. Extracellular ATP activates different signalling pathways in rat Sertoli cells. Biochem. J. 311, 269–274.

Foskett, J.K., Wong, D., 1991. Free cytoplasmic Ca^{2+} concentration oscillations in thapsigargin-treated parotid acinar cells are caffeine- and ryanodine-sensitive. J. Biol. Chem. 266, 14535–14538.

Fukushi, Y., Ozawa, T., Wakui, M., Nishiyama, A., 1995. Sr^{2+} can pass through Ca^{2+} entry pathway activated by Ca^{2+} depletion, but can be hardly taken up by the Ca^{2+} stores in the rat salivary acinar cells. Tohoku J. Exp. Med. 176, 83–97.

Fukushi, Y., Ozawa, T., Nishiyama, A., Kase, H., Wakui, M., 1996. Depletion of ryanodine-sensitive Ca^{2+} store activates Ca^{2+} entry in rat submandibular gland acinar cells. Tohoku J. Exp. Med. 178, 399–411.

Greenberg, S., Di Virgillio, F., Steinberg, T.H., Silverstein, S.C., 1988. Extracellular nucleotides mediate Ca^{2+} fluxes in J774 macrophages by two distinct mechanisms. J. Biol. Chem. 263, 10337–10343.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Harootunian, A.T., Kao, J.P.Y., Eckert, B.K., Tsien, R.Y., 1989. Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. J. Biol. Chem. 264, 19458–19467.

Herchuelz, A., Malaisse, W.J., 1980. Regulation of calcium fluxes in rat pancreatic islets: Dissimilar effects of glucose and sodium ion accumulation. J. Physiol. 302, 263–280.

Holz, G.G., Leech, C.A., Habener, J.F., 1995. Activation of a cAMP-regulated Ca^{2+}-signaling pathway in pancreatic β-cells by the insulinotropic hormone glucagon-like peptide-1. J. Biol. Chem. 270, 17749–17757.

Hurley, T.W., Ryan, M.P., 1988. The control of cytosolic Ca^{2+} concentration: studies of high affinity Ca^{2+} transport in permeabilized acini of rat submandibular glands. Arch. Oral Biol. 33, 793–800.

Hurley, T.W., Shoemaker, D.D., Ryan, M.P., 1993. Extracellular ATP prevents the release of stored Ca^{2+} by autonomic agonist in rat submandibular gland acini. Am. J. Physiol. 265, C1472–C1478.

Imagawa, T., Smith, J.S., Corinado, R., Campbell, K.P., 1987. Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+}-permeable pore of the calcium release channel. J. Biol. Chem. 262, 16636–16643.

Leech, C.A., Holz, G.G., Habener, J.F., 1995. Pituitary adenylate cyclase-activating polypeptide includes the voltage-independent activation of inward membrane currents and elevation of intracellular calcium in HIT-T15 insulinoma cells. Endocrinology 136, 1530–1536.

McMillian, M.K., Soltoff, S.P., Lechileiter, J.D., Cantley, L.C., Talamo, B.R., 1988. Extracellular ATP increases free cytosolic calcium in rat parotid acinar cells. Biochem. J. 255, 291–300.

McMillian, M.K., Soltoff, S.P., Cantley, L.C., Rudel, R.A., Talamo, B.R., 1993. Two distinct cytosolic calcium responses to extracellular ATP in rat parotid acinar cells. Br. J. Pharmacol. 108, 453–461.

Nuttle, L.C., Dubyak, G.R., 1994. Differential activation of cation channels and non-selective pores by macrophage P_{2Z} purinergic receptors expressed in Xenopus Oocytes. J. Biol. Chem. 269, 13988–13996.

Sasaki, T., Gallacher, D.V., 1990. Extracellular ATP activates receptor-operated cation channels in mouse lacrimal acinar cells to promote calcium influx in the absence of phosphoinositide metabolism. FEBS Lett. 264, 130–134.

Schmid, A., Dehlinger-Kremer, M., Schulz, I., Gögelein, H., 1990. Voltage-dependent $InsP_3$-insensitive calcium channels in membranes of pancreatic endoplasmic reticulum vesicles. Nature 346, 374–376.

Seifert, R., Schulz, G., 1989. Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and by uracil nucleotides. Trends Pharmacol. Sci. 10, 365–369.

Soltoff, S.P., McMillian, M.K., Cragoe, E.J., Cantley, L.C., Talamo, B.R., 1990. Effects of extracellular ATP on ion transport systems and $[Ca^{2+}]_i$ in rat parotid acinar cells. J. Gen. Physiol. 95, 319–346.

Soltoff, S.P., McMillian, M.K., Talamo, B.R., 1992. ATP activates a cation-permeable pathway in rat parotid acinar cells. Am. J. Physiol. 262, C934–C940.

Surprenant, A., Rassendren, F., Kawashima, E., North, R.A., Buell, G., 1996. The cytosolic P_{2Z} receptor for extracellular ATP identified as a P_{2X} receptor ($P2X_7$). Science 272, 735–738.

Tatham, P.E.R., Cusack, N.J., Gomperts, B.D., 1988. Characterization of

the ATP^{4-} receptor that mediates permeabilization of rat mast cells. Eur. J. Pharmacol. 147, 13–21.

Tepikin, A.V., Voronina, S.G., Gallacher, D.V., Petersen, O.H., 1992. Acetylcholine-evoked increase in the cytoplasmic Ca^{2+} concentration and Ca^{2+} extrusion measured simultaneously in single mouse pancreatic acinar cells. J. Biol. Chem. 267, 3569–3572.

Thévenod, F., Schulz, I., 1988. H^{+}-dependent calcium uptake into an IP_3-sensitive calcium pool from rat parotid gland. Am. J. Physiol. 255, G429–G440.