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Xanthine derivatives inhibit the increase in intracellular Ca^{2+} concentration induced by acetylcholine in nasal gland acinar cells of the guinea-pig

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Xanthine derivatives inhibit the increase in intracellular Ca^{2+} concentration induced by acetylcholine in nasal gland acinar cells of the guinea-pig. K. Ishitani, K. Ikeda, H. Sunose, D. Wu, H. Honda, T. Takasaka. ©ERS Journals Ltd 1995.

ABSTRACT: Intracellular calcium is considered to play a major role in secretory responses of various exocrine cell types. We examined whether xanthine derivatives can inhibit Ca^{2+} mobilization and entry in secretory cells in the airways. Therefore, the inhibitory effect of xanthines in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in the isolated submucosal acinar cells of the guinea-pig nasal septum was investigated by means of fluorescence ratio microscopy.

The inhibitory effects on Ca^{2+} release from stores was examined in Ca^{2+}-free conditions. Effects on Ca^{2+} entry were estimated by two different protocols; 1) the sustained phase in a long-term application of acetylcholine (ACh) and 2) the $[Ca^{2+}]i$ overshoot following removal of ACh in Ca^{2+}-free conditions.

Xanthine derivatives, 3-isobutyl-1-methyl-xanthine (IBMX), caffeine, and theophylline, significantly inhibited the increase in $[Ca^{2+}]i$ evoked by ACh; both mobilization from internal Ca^{2+} stores and Ca^{2+} entry from the external space. The rank order of potency of these xanthine derivatives was IBMX>theophylline> caffeine. The addition of dibutyryl-cyclic adenosine monophosphate (cAMP) and forskolin to nasal gland acinar cells failed to inhibit the ACh-evoked increase in $[Ca^{2+}]i$. Furthermore, a protein kinase A inhibitor, H-89, did not affect the inhibitory effect of the xanthine derivatives. The action of xanthines on the present acinar cells did not involve Ca^{2+}-induced Ca^{2+} release (CICR) or an interaction with purinergic receptors.

Thus, xanthines have a direct inhibitory effect both on Ca^{2+} release and entry in nasal gland acinar cells, and might thereby have antisecretory activity within the airways.

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It is well-known that Ca^{2+} is a very important factor for secretory responses in various types of exocrine cells. In pancreatic and parotid acinar cells, Ca^{2+} has been thought of as a trigger for the secretion of digestive enzymes and salivary fluids, respectively [1, 2]. In general, the stimulation of exocrine cells by appropriate neurotransmitters and hormones is known to cause an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), which is a biphasic response resulting from Ca^{2+} release from intracellular stores (a transient phase) followed by Ca^{2+} entry from the extracellular space (a sustained phase). Inositol-1, 4, 5-trisphosphate (IP_3) is the most probable candidate to release Ca^{2+} from internal stores by acting on the membrane receptor [3, 4]. However, the underlying mechanism remains to be defined, although the increased entry of Ca^{2+} from the extracellular fluid is obviously observed [5].

So far, several drugs or chemicals are known to inhibit the IP_3-mediated Ca^{2+} release and Ca^{2+} entry [5–7]. Xanthine derivatives are also known to have inhibitory effects [8–12]. In the present paper, we provide confirmatory evidence that xanthines directly inhibit both Ca^{2+} mobilization and entry induced by acetylcholine (ACh) in the nasal gland acinar cells.

Material and methods

Preparation of isolaled acini from submucosal nasal gland

Healthy albino guinea-pigs weighing 200–300 g were anaesthetized by inhalation of diethyl ether. After decapitation, the nasal septum was quickly removed and placed in an oxygenated cell-storage solution. The cell-storage solution was prepared by the addition of 0.2% bovine serum albumin (BSA) to a standard solution composed of (in mM) 139.2 Na^+, 4.69 K^+, 2.56 Ca^{2+}, 1.13 Mg^{2+},

136.1 Cl^-, 4.91 pyruvate$^-$, 5.38 fumarate$^-$, 4.92 glutamate$^-$, 2.8 glucose, and 5.0 N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)/tris- (hydroxymethyl)-aminomethane (Tris) mixture (pH 7.4 at 37°C). We developed an isolation procedure to prevent any contamination by the surface epithelium [13]. Briefly, the septal mucosa was separated from the cartilage beneath the cartilaginous membrane and inverted in a Petri dish containing the cell-storage solution. Following removal of the cartilaginous membrane, the area containing a large amount of nasal glands was carefully dissected without injuring the epithelial lining, and the tissue was minced into cubes varying in size from 0.2–0.5 mm. The pooled fragments were suspended in an isolation solution and incubated for 20–30 min at 37°C in a shaking water bath. The isolation solution was prepared by removing Ca^{2+} from the standard solution and adding 1 mM ethylene glycol-bis(β-amino ethyl ether)-N, N, N', N'-tetra-acetic acid (EGTA), 0.2% BSA, and 100 U·mL^{-1} collagenase type IV. The digested tissue was dissociated by pipetting it 10–20 times using a siliconized pipette tip, and the clusters of cells were washed and centrifuged (1,000 rpm for 1 min) three times. The resulting sediment was resuspended in a fresh cell-storage solution.

Micro/fluorimetric ratio imaging

The isolated acinar cells were incubated at 37°C for 50–60 min in an oxygenated solution containing 2–3 μM fura-2 acetoxymethyl ester (fura-2 AM). After dye loading, the acinar cells were washed once with the cell-storage solution, and stored under light-free conditions until use. Before use, 30–40 μL of the acinar cell suspension were placed on a coverslip coated with a natural cell adhesive, Cell Tak, in a Petri dish containing enough humidity for 20 min to allow the immobilization of the cells. The coverslip was then placed in a superfusion chamber with a bath capacity of 0.2 mL, which was mounted on the stage of an inverted epifluorescence microscope. During the experiments, acinar cells were continuously superfused with the desired solutions that were oxygenated and warmed to 37°C at a rate of 1.6 mL·min^{-1}, ensuring the exchange of the bathing solutions within several seconds. To observe the effects of reagents on a transient phase of ACh-induced $[Ca^{2+}]i$ increase, the acinar cells were pretreated with appropriate reagents 3 min prior to, and during, the application of 10^{-6} M ACh. In our previous paper [14], removal of external Ca^{2+} by adding EGTA resumed the sustained phase in $[Ca^{2+}]i$ to the basal level, indicating that the sustained phase is totally dependent on the influx of external Ca^{2+} across the membrane. Therefore, to observe the effects of reagents on Ca^{2+} entry induced by ACh, the acinar cells were treated with appropriate reagents for 3 min after the $[Ca^{2+}]i$ increase reached the sustained phase.

The instrumentation used for fluorimetric ratio imaging microscopy has been reported previously [15]. Briefly, the cells mounted on the epifluorescence microscope were alternately illuminated at 340 and 380 nm excitation from a xenon lamp through neutral density filters, 10 nm bandpass filters, a 440 nm dichroic mirror, and an objective lens (DPlan Apo 10×UV, N.A.: 0.40; Olympus, Tokyo, Japan). The emitted light path included the objective, dichroic mirror and a 510 nm bandpass filter. Images were focused on a silicone intensifier target camera (SIT camera C-2400-8, Hamamatsu Photonics, Japan) and analysed using a digital image processor (Argus 50, Hamamatsu Photonics). The viewfield of the SIT camera consisted of 512×483 picture elements (pixels). One pixel corresponded to a 1.0×1.0 μm^2 objective area. All the images were obtained for a pair of excitation radiations by averaging 16 frames·s^{-1} for each excitation wavelength, usually at 10 s intervals. The excitation radiation was shut off except for the data-collection cycle. After background subtraction, the images of the fluorescence ratio at 340 and 380 nm excitations (F_{340} and F_{380}, respectively) were calculated frame-by-frame for each pixel. To minimize the photochemical effect due to excitation, neutral density filters (×128) were incorporated in the excitation light path. The fluorescence signal was still within the dynamic range of the TV camera.

For the *in vitro* calibration of the $[Ca^{2+}]i$ measurements, Ca^{2+}/EGTA buffer solution containing 20 μM fura-2 were placed between glass coverslips spaced 20 μM apart and the fluorescence intensity ratio, R (F_{340}/F_{380}), was determined. $[Ca^{2+}]i$ was calculated from the following equation:

$$[Ca^{2+}]i = K(R-R_{min})/(R_{max}-R)$$

where R_{max} and R_{min} are the maximum and minimum F_{340}/F_{380} obtained at saturating and zero Ca^{2+} concentrations, respectively. The parameter K is the product of $K_D(F_O/F_S)$, where K_D is the effective dissociation constant of the indicator at 37°C in an ionic milieu simulating vertebrate cytosol, and F_O/F_S is the ratio of excitation efficiencies of free-indicator to Ca^{2+}-bound indicator at 380 nm. R_{min} with 2.5 mM EGTA and 0 Ca^{2+} was 0.7, while R_{max} with 1.0 mM Ca^{2+} was 20.2 in the representative experiment. R_{max} and R_{min} were calibrated each time after renewal of the lamp. K_D and F_O/F_S were taken as 224 nM and 6.7, respectively, the latter being experimentally determined in a fura-2 solution with and without 1 mM Ca^{2+}.

Chemicals

Fura-2 and fura-2 AM solutions were purchased from Dojindo (Kumamoto, Japan), Cell Tak from Collaborative Research Inc. (Bedford, Ma,USA), and H-89 from Seikagaku Kogyo (Tokyo, Japan). Collagenase type IV, ACh, db-cAMP, 3-isobutyl-l-methylxanthine (IBMX) and caffeine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were of the special reagent grade supplied from Wako Pure Chemical Industries Ltd (Tokyo, Japan).

Statistics

The data are expressed as the mean±SE. Statistical significance was analysed by paired t-test; a p-value of less than 0.05 was accepted as significant.

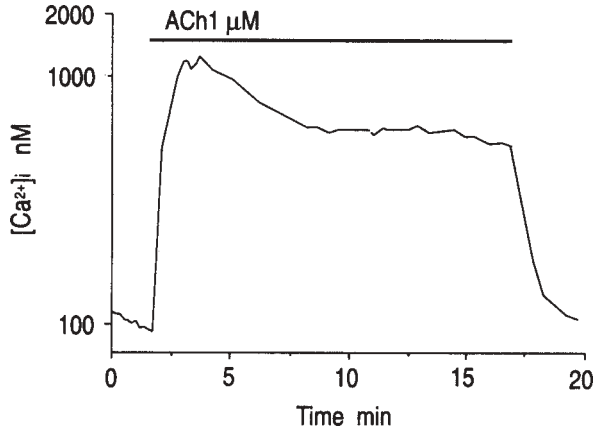

Fig. 1. – Acetylcholine (ACh)-induced responses in intracellular Ca^{2+} concentration $[Ca^{2+}]i$. ACh was applied to the isolated nasal gland acinar cells, resulting in the initial rapid increase followed by a sustained plateau.

Results

Effects of ACh on $[Ca^{2+}]i$

A typical trace of the ACh-induced $[Ca^{2+}]i$ increase is shown in figure 1. The application of 10^{-6} M ACh exhibited a rapid increase in $[Ca^{2+}]i$ (a transient phase), which was followed by a partial decline to a sustained $[Ca^{2+}]i$ plateau (a sustained phase). The average $[Ca^{2+}]i$ under resting conditions, and a transient peak and sustained phase after 5 min induced by ACh were 113±12 nM (n=44), 1,289±221 nM (n=21), and 693±147 nM (n=21), respectively. After discontinuing the application of ACh, the $[Ca^{2+}]i$ promptly returned to the basal level.

Effects of xanthine derivatives on ACh-induced $[Ca^{2+}]i$ increase

Three types of xanthine derivatives, IBMX, theophylline and caffeine, were examined with respect to their effects on the increase in $[Ca^{2+}]i$ induced by ACh. A typical trace of the effects of IBMX on the transient phase of the ACh-induced $[Ca^{2+}]i$ increase is shown in figure 2. At a concentration of 10^{-4} M IBMX, the transient phase of $[Ca^{2+}]i$ increase induced by ACh was inhibited by 73.6±7.1% (n=5), whereas theophylline and caffeine were inactive. All the xanthine derivatives significantly inhibited the transient increase in $[Ca^{2+}]i$ at a concentration of 3×10^{-3} M. The rank order of potency of these xanthine derivatives was IBMX>theophylline>caffeine (table 1). No significant change in $[Ca^{2+}]i$ was observed during application of each xanthine derivative itself, even when 10^{-6} M thapsigargin was simultaneously applied in the Ca^{2+}-free conditions with 0.2 mM EGTA. In experiments designed to investigate the effects of these xanthines on the sustained phase of $[Ca^{2+}]i$, results similar to those obtained with the transient phase were observed. IBMX inhibited the sustained phase in a concentration-dependent manner in the range of 10^{-4} M to 10^{-3} M (fig. 3).

Similarly the rank order of potency was IBMX>theophylline>caffeine at 10^{-3} M (table 1). One of the actions of xanthines is to inhibit the binding of adenosine to cell

Table 1. – Inhibitory effects of xanthine derivatives on acetylcholine-induced intracellular calcium increase in nasal gland acinar cells isolated from guinea-pig

	Inhibition %			
Molar	Transient phase		Sustained phase	
IBMX				
1×10^{-4}	ND		10.9±5.1*	(n=5)
3×10^{-4}	ND		31.9±7.5**	(n=5)
1×10^{-3}	73.6±7.1**	(n=5)	77.8±4.1**	(n=5)
Theophylline				
1×10^{-3}	7.0±9.0	(n=6)	40.8±3.8**	(n=8)
3×10^{-3}	47.6±2.9**	(n=4)	ND	
Caffeine				
1×10^{-3}	6.6±6.1	(n=5)	33.4±5.8**	(n=6)
3×10^{-3}	25.6±3.0**	(n=4)	ND	

IBMX: isobutyl-methyl-xanthine; ND; not determined. Each value is expressed as mean±SEM. *: P<0.05; **: P<0.01.

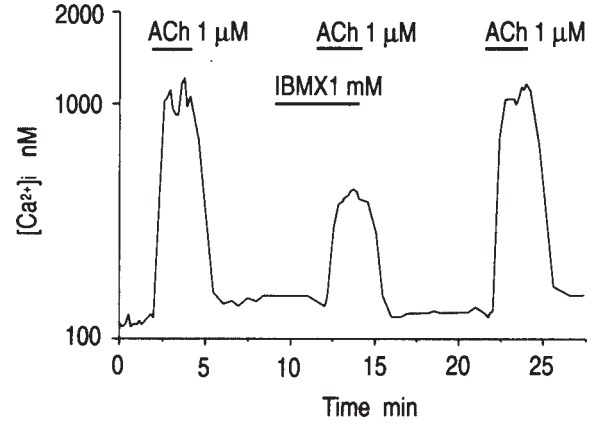

Fig. 2. – Effect of 3-isobutyl-1-methyl-xanthine (IBMX) on the acetylcholine (ACh)-induced transient increase in intracellular Ca^{2+} concentration $[Ca^{2+}]i$. Addition of 1 mM IBMX prior to and during ACh inhibited the transient increase in $[Ca^{2+}]i$ induced by application of 1 μM ACh. Following wash-out of IBMX for a sufficient time, the $[Ca^{2+}]i$ response induced by ACh was almost completely recovered to the initial response.

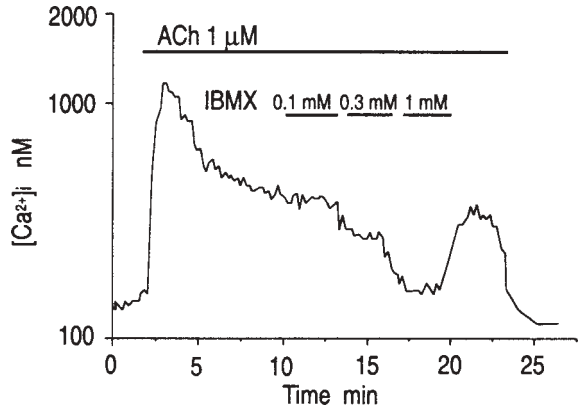

Fig. 3. – Concentration-dependent inhibition of 3-isobutyl-1-methyl-xanthine (IBMX) on the sustained phase of acetylcholine (ACh)-induced increase in intracellular Ca^{2+} concentration $[Ca^{2+}]i$. After the $[Ca^{2+}]i$ response induced by 1 μM ACh reached the sustained phase, 0.1, 0.3, 1 mM of IBMX was subsequently applied to the cell. Wash-out of IBMX returned the $[Ca^{2+}]i$ response to the value before addition of IBMX.

Table 2. – Effects of forskolin and db-cAMP on ACh-induced intracellular calcium increase in nasal gland acinar cells isolated from guinea pig

		Inhibition %	
	[M]	Transient phase	Sustained phase
Forskolin	1×10^{-4}	-3.9±4.4 (n=4)	-2.8±0.9 (n=3)
db-cAMP	1×10^{-3}	-7.1±3.8 (n=6)	-2.2±1.7 (n=10)

Each value is expressed as mean±SEM. db-cAMP: dibutyrylcyclic adenosine monophosphate; ACh: acetylcholine.

surface purinergic receptors [16]. However, the addition of adenosine (10^{-4} M) did not affect the basal [Ca^{2+}]i (98.4±1.0% of the control; n=4) or the the ACh-induced [Ca^{2+}]i (106.6±4.2% of the control; n=4). Thus, the inhibitory effect of xanthines on ACh-induced [Ca^{2+}]i increase did not apparently involve the inhibition of purinergic receptor activation by endogenous adenosine.

Effects of cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) system

Phosphodiesterase inhibitors are known to increase cAMP and to activate PKA, leading to a variety of cellular responses [17]. Thus, studies were performed to determine whether the inhibitory effects of IBMX on the ACh-induced increase in [Ca^{2+}]i were due to activation of the cAMP/PKA system. Neither forskolin, an activator of adenylylcyclase, nor db-cAMP, a membrane-permeable analogue of cAMP, had an effect on the ACh-induced [Ca^{2+}]i increase (table 2).

H-89 is a relatively selective inhibitor of PKA [18]. As shown in figure 4, the inhibitory effect of IBMX on both the transient and sustained phases of ACh-induced increase in [Ca^{2+}]i was unaffected by preincubation of acinar cells with 10^{-5} M H-89. No effect of H-89 on the resting [Ca^{2+}]i was seen at this concentration.

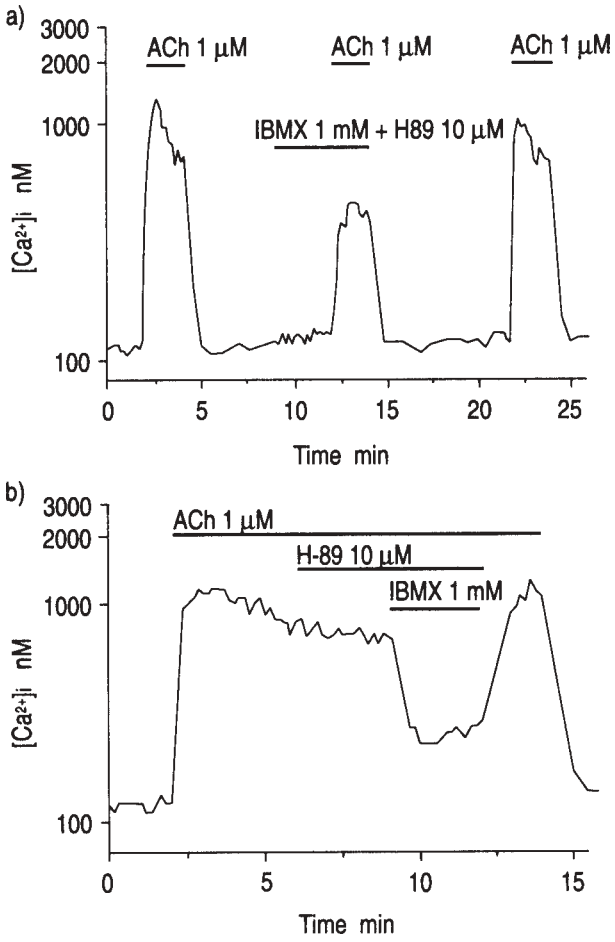

Fig. 4. – Effect of a protein kinase A inhibitor, H-89, on 3-isobutyl-1-methyl-xanthine (IBMX)-sensitive inhibition of acetylcholine (ACh)-induced intracellular Ca^{2+} concentration [Ca^{2+}]i increase. The experimental protocol in (a) and (b) was similar to that described in figure 2 and 3, respectively, except for additional application of 10 µM H-89 with IBMX.

Effect of IBMX on Ca^{2+} release and entry induced by ACh

To better clarify the site(s) responsible for the inhibition of the ACh-induced [Ca^{2+}]i increase by IBMX, we examined the release of Ca^{2+} from the cytosolic pool. Under Ca^{2+}-free conditions (+0.2 mM EGTA), a transient increase in [Ca^{2+}]i evoked by the addition of ACh originates from the intracellular Ca^{2+} pool in nasal gland acinar cells [14]. Exposure of these cells to 10^{-3} M IBMX inhibited the transient [Ca^{2+}]i increase induced by ACh in the absence of external Ca^{2+} by 37.6±3.7% (n=5) (fig. 5).

The sustained phase in [Ca^{2+}]i induced by ACh involves a variety of Ca^{2+} transport processes, such as Ca^{2+} influx and efflux across the plasma membrane and the release and refilling of Ca^{2+} in the intracellular stores. According to the capacitative model of Ca^{2+} entry [19], depleting the intracellular Ca^{2+} pool activates the Ca^{2+} entry mechanism even in the absence of receptor activation or increase in inositol polyphosphates. Fura-2 loaded acinar cells were incubated in Ca^{2+}-free solution containing 0.2 mM EGTA. Following release of the intracellular Ca^{2+} pool, ACh stimulation was eliminated by wash-out of Ca^{2+}-free standard solution. Control cells were identically treated, except that the intracellular Ca^{2+} pool was not released by addition of ACh. Replacement with Ca^{2+}-containing standard solution caused a rapid and substantial increase in [Ca^{2+}]i compared to controls. The Ca^{2+}

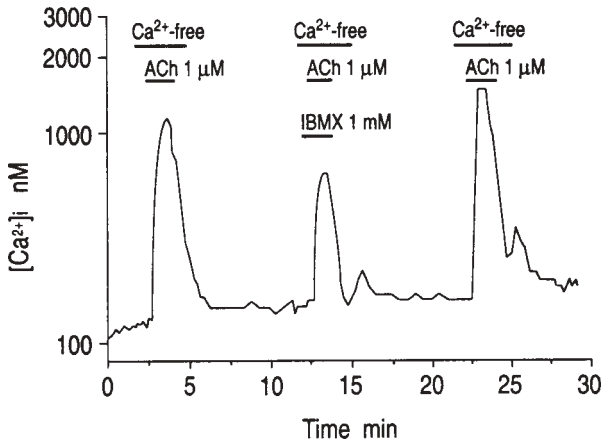

Fig. 5. – Effect of 3-isobutyl-1-methyl-xanthine (IBMX) on the Ca^{2+} release from intracellular stores induced by acetylcholine (ACh). The Ca^{2+} release induced by ACh was isolated under external Ca^{2+}-free conditions.

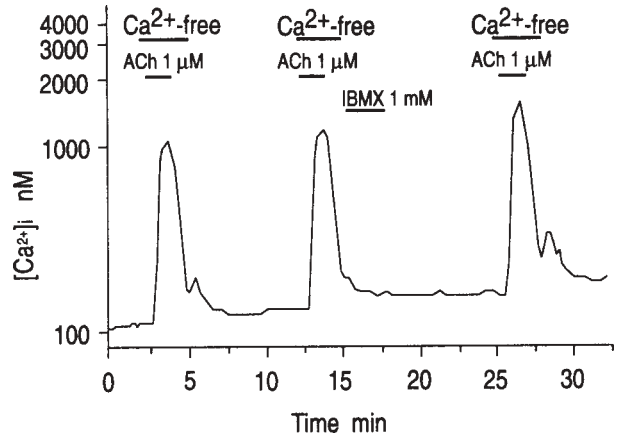

Fig. 6. – Effect of 3-isobutyl-1-methyl-xanthine (IBMX) on the Ca^{2+} entry following acetylcholine (ACh) stimulation. The Ca^{2+} overshoot after wash-out of ACh in the absence of external Ca^{2+} implied Ca^{2+} entry.

overshoot implies the increased $[Ca^{2+}]i$ purely *via* the Ca^{2+} entry mechanism [20]. The addition of 10^{-3} M IBMX inhibited the Ca^{2+} overshoot observed in the protocol by 97.6±2.5% (n=6) (fig. 6). Thus, both the Ca^{2+} release from intracellular stores and the Ca^{2+} entry from the extracellular space were directly inhibited by xanthine derivatives.

Discussion

The present study demonstrated that xanthine derivatives apparently inhibited the increase in $[Ca^{2+}]i$ evoked by ACh in the submucosal nasal gland acinar cells of the guinea-pig. Both mobilization from internal Ca^{2+} stores and Ca^{2+} entry from the external space were significantly suppressed by these drugs.

Purified IP_3 receptors can be phosphorylated by PKA [6, 21]. Xanthine derivatives are known to act by inhibiting phosphodiesterase enzymes [17], leading to the elevation of intracellular cAMP. There are some reports that have investigated the effects of phosphodiesterase inhibitors on agonist-induced changes in $[Ca^{2+}]i$. AKAIKE *et al.* [22] reported that phosphodiesterase inhibitors accumulating intracellular cAMP suppressed the $[Ca^{2+}]i$ increase induced by adenosine diphosphate (ADP) or IP_3 in rat megakaryocyte. In contrast, GRUNE *et al.* [23] reported that cAMP increased the $[Ca^{2+}]i$ in rat hepatocyte. However, it seems that the effect of xanthine derivatives on the ACh-evoked increase in $[Ca^{2+}]i$, in the nasal acinar gland cells cannot arise through changes in cyclic nucleotide metabolism, since application of the adenylyl cyclase activator, forskolin, or the cell-permeable cAMP analogue, db-cAMP, did not inhibit the ACh-induced $[Ca^{2+}]i$ increase, and PKA inhibitor H-89 did not affect the inhibitory effect of xanthine derivatives.

Another possible action of xanthine derivatives is related to Ca^{2+}-induced Ca^{2+} release (CICR). In exocrine cells, cytoplasmic Ca^{2+} oscillations are proposed to involve CICR, and caffeine is capable of causing the Ca^{2+} release from ryanodine-sensitive stores [8, 10, 24]. If xanthine derivatives were to liberate Ca^{2+} from intracellular stores, it might inhibit responses to ACh by depleting the amount of Ca^{2+} available for release induced by IP_3. Xanthine derivatives are well-recognized as causing slow Ca^{2+} release from internal Ca^{2+} stores [25]. The increased $[Ca^{2+}]i$ induced by the slow release of the stored Ca^{2+} in response to xanthine derivatives might be cancelled by Ca^{2+} extrusion mechanisms sufficiently to cause no measurable rise in $[Ca^{2+}]i$. However, xanthine derivatives did not themselves elicit any increase in $[Ca^{2+}]i$, when the Ca^{2+}-adenosine triphosphatase (ATPase) to elicit the active Ca^{2+} uptake process into internal Ca stores was inhibited by thapsigargin [26]. Therefore, it is unlikely that inhibition of an ACh-induced $[Ca^{2+}]i$ increase is brought about by CICR.

Caffeine inhibits the increase in $[Ca^{2+}]i$ evoked by ACh in mouse pancreatic acinar cells [8], and by vasopressin in a smooth muscle cell line [9]. The $[Ca^{2+}]i$ response induced by a direct application of IP_3 to the cytosol is inhibited by the addition of caffeine [10, 11]. PARKER and IVORRA [11] reported that the effect of caffeine was not mediated by changes in cyclic nucleotide metabolism. It has been shown that these inhibitory actions are brought about by a change in the binding of IP_3 to its receptor [11], and a reduction of agonist-evoked IP_3 generation [12]. Although the rank order of potency of xanthine derivatives in inhibiting the Ca^{2+} mobilization is different, the underlying mechanisms in the present study seem to be consistent with previous studies [11, 12] but remain to be confirmed.

The Ca^{2+} entry process plays a critical role in maintaining sustained fluid secretion in exocrine glands [27]. A variety of the mechanisms involved in activating this process have been proposed in nonexcitable cells [5]; 1) receptor operated Ca^{2+} channels directly activated by ligand binding to the receptor, 2) guanosine triphosphate (GTP)-binding protein activated Ca^{2+} channels; 3) second-messenger operated Ca^{2+} channels; 4) capacitative Ca^{2+} entry resulting from depletion of the intracellular stores of Ca^{2+} after IP_3 generation; and 5) inositol 1, 3, 4, 5-tetrakisphosphate-activated Ca^{2+} entry and non-selective cation channels. It has been suggested that the signal may be either a diffusible messenger like an inositol phosphate, or the IP_3 receptor itself, which links store release to Ca^{2+} entry [28, 29]. Our recent findings in the submucosal nasal gland acinar cells indicate that the Ca^{2+} entry is inhibited by Ni^{2+} but not by the organic Ca^{2+} antagonist, nifedipine, and is conductive in nature [14]. The molecular basis of a regulatory messenger has currently been advocated [30]. A small (molecular weight less than 500), anionic, phosphorylated compound that activates Ca^{2+} entry has been partially characterized. This Ca^{2+}-influx factor appears to have hydroxyls or a hydroxyl and amino group, on adjacent carbons. Furthermore, PAREKH *et al.* [31] found a Ca^{2+}-permeable current activated by store emptying using *Xenopus laevis* oocytes. The activation of this store depletion current involves both a phosphatase and an unidentified diffusible messenger.

Nevertheless, this is the first report demonstrating that xanthine derivatives have a direct inhibitory effect on the Ca^{2+} entry process in the exocrine cells. Further studies to reveal the precise and exact mechanisms of effects by xanthine derivatives for Ca^{2+} entry are clearly warranted.

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