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Original

Involvement of phosphodiesterase 4 in β-adrenoceptor agonist-induced amylase release in parotid acinar cells

Keitaro Satoh[1)], Ming-Yu Guo[1)] and Nakayasu Sairenji[2)]

[1)]Department of Physiology, Nihon University School of Dentistry at Matsudo, Matsudo, Japan
[2)]Department of Anesthesiology, Nihon University School of Dentistry at Matsudo, Matsudo, Japan

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Abstract: β-Adrenoceptor activation increases intracellular cAMP levels and consequently induces exocytotic amylase release in parotid acinar cells. Phosphodiesterase (PDE) catalyses the hydrolysis of cAMP, which terminates the downstream signaling of this second messenger. We investigated the involvement of PDE4, a cAMP-PDE, in β-adrenoceptor agonist-induced amylase release in mouse, rat and rabbit parotid acinar cells by using the specific PDE4 inhibitor rolipram. cAMP-PDE activity was detected in mouse, rat and rabbit parotid acinar cells. In the presence of rolipram, cAMP-PDE activity was reduced by about 31%, 38% and 33% in mouse, rat and rabbit parotid acinar cells, respectively. The increase in cAMP levels induced by the β-adrenoceptor agonist isoproterenol was enhanced in the presence of rolipram in mouse, rat and rabbit parotid acinar cells. Isoproterenol-induced amylase release, but not constitutive amylase release, was also enhanced in the presence of rolipram in mouse, rat and rabbit parotid acinar cells. These results suggest that the rolipram-sensitive cAMP-PDE, PDE4, is involved in β-adrenoceptor agonist-induced amylase release in parotid acinar cells. (J Oral Sci 51, 173-179, 2009)

Keywords: phosphodiesterase; rolipram; amylase release; cAMP; parotid acinar cells.

Correspondence to Dr. Keitaro Satoh, Department of Physiology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-nishi, Matsudo, Chiba 271-8587, Japan
Tel: +81-47-360-9326
Fax: +81-47-360-9327
E-mail: satoh.keitaro@nihon-u.ac.jp

Introduction

Cyclic adenosine-3',5'-monophosphate (cAMP) is an intracellular signal molecule involved in a wide variety of cellular functions (1). The intracellular cAMP level is regulated by adenylate cyclase and cyclic nucleotide phosphodiesterase (PDE), which are the enzymes responsible for the synthesis and breakdown of cAMP, respectively (2).

PDE catalyses the hydrolysis of cAMP, which terminates the downstream signaling of this second messenger. PDE has been classified into 11 families on the basis of substrate and inhibitor specificities, allosteric properties and amino acid sequences (3-5). Among the PDE families, 8 have been reported to hydrolyze cAMP: Ca^{2+}/calmodulin-dependent PDE (PDE1), cGMP-stimulated PDE (PDE2), cGMP-inhibited PDE (PDE3), cGMP-insensitive PDE (PDE4), rolipram-insensitive PDE (PDE7), rolipram and 3-isobutyl-1-methylxanthine-insensitive PDE (PDE8), and the recently recognized PDE10 and PDE11 (3-5).

Exocytosis is the process by which cells release the contents of their secretory granules. This process is continuous in most cells (constitutive exocytosis), but it can be greatly accelerated by appropriate cellular signals such as neural stimulation (regulatory exocytosis). In parotid acinar cells, stimulation of β-adrenoceptors increases the intracellular cAMP level, and consequently induces exocytotic release of amylase (6,7).

Rolipram has been shown to be a potent cAMP-PDE inhibitor, and was originally developed as a possible antidepressant agent (3,4,8,9). PDE4, the rolipram-sensitive cAMP-PDE, is known to be present in rat parotid gland (10), but its cellular function is still unclear. Here we investigated the role of PDE4 in amylase release from

isolated parotid acinar cells.

Materials and Methods

Materials

Collagenase A, bovine serum albumin (BSA) and Protease Inhibitor Cocktail (Complete™) were purchased from Roche (Basal, Switzerland). Trypsin (type III), trypsin inhibitor (type I-S), isoproterenol, rolipram, and *Crotalus atrox* snake venom were obtained from Sigma (St. Louis, MO, USA). Calmodulin and AG 50W-X4 resin (200 – 400 mesh; hydrogen form) were obtained from Wako (Osaka, Japan) and Bio-Rad (Hercules, CA, USA), respectively. [8-^{3}H]cAMP (1187.7 GBq/mmol) was obtained from Perkin-Elmer (Wellesley, MA, USA). An cAMP enzyme immunoassay kit was purchased from Amersham (Piscataway, NJ, USA).

Preparation of parotid acinar cells

Acinar cells from the parotid glands of male ddy mice, Sprague-Dawley rats (200 – 250 g) and Japanese white rabbits (2 – 2.5 kg) were prepared as described previously (11). The parotid glands were removed and placed in a small volume with Krebs-Ringer-bicarbonate (KRB) solution of the following composition (in mM): NaCl, 116; KCl, 5.4; $MgSO_4$, 0.8; $CaCl_2$, 1.8; NaH_2PO_4, 0.96; $NaHCO_3$, 25; HEPES (pH 7.4), 5; and glucose 11.1. The KRB solution was equilibrated in an atmosphere of 95% O_2/5% CO_2. After mincing with a razor, the glands were treated with KRB buffer with 0.5% BSA in the presence or absence of enzyme. First, the glands were incubated with trypsin (0.5 mg/ml) at 37°C for 10 min. After incubation, the trypsin-treated glands were removed by centrifugation at 200 ×*g* for 1 min. The glands were subsequently incubated in Ca^{2+}-Mg^{2+}-free KRB solution containing 1 mM EGTA and trypsin inhibitor (0.5 mg/ml) at 37°C for 5 min. After the solution had been removed by centrifugation (200 ×*g* for 1 min), the glands were incubated in Ca^{2+}-Mg^{2+}-free KRB solution without trypsin inhibitor at 37°C for 5 min. After removal of the solution by centrifugation (200 ×*g* for 1 min), the glands were incubated in KRB solution with collagenase (1.5 mg/ml) at 37°C for 20 min. The suspension was passed through eight layers of nylon mesh to separate the dispersed cells from undigested connective tissue, and was gently put on KRB solution containing 4% BSA. After centrifugation (50 ×*g* for 5 min), the cells were suspended in appropriate amounts of KRB solution containing 0.5% BSA and 0.02% trypsin inhibitor. All experimental protocols were approved by the Laboratory Animal Committee of the Nihon University School of Dentistry at Matsudo.

Amylase release

The dispersed acinar cells from a rabbit, a rat or three mice were suspended in 28 ml of KRB solution containing 0.5% BSA and 0.02% trypsin inhibitor and incubated at 37°C with the indicated agents. The cell suspension was separated into 4 parts (7 ml each), and incubated with vehicle, rolipram (50 μM), isoproterenol (1 μM), or rolipram/isoproterenol. Isoproterenol was added after preincubation with vehicle or rolipram for 10 min. After incubation times of 0, 5, 10 and 15 min, 1 ml of cell suspension was removed and passed through filter paper. Amylase activity in the filtrates was measured according to Bernfeld (12). Total amylase activities were measured in acinar cells homogenized in 20 mM phosphate buffer (pH 6.9) containing 0.01% Triton X-100.

Measurement of the intracellular cAMP level

The dispersed acinar cells from a rabbit, a rat or three mice were suspended in 8 ml of KRB solution containing 0.5% BSA and 0.02% trypsin inhibitor and incubated at 37°C with the indicated agents. The cell suspension was separated into 4 parts (2 ml each), and incubated with vehicle, rolipram (50 μM), isoproterenol (1 μM), or rolipram/isoproterenol. Isoproterenol was added after preincubation with vehicle or rolipram for 10 min. After incubation times of 0, 0.5, 1, 2, 3, 5 and 10 min, 200 μl of cell suspension was taken, mixed with 30 μl of 35% perchloric acid, and put on ice for 30 min. After addition of 60 μl of 17.5% potassium hydroxide to the mixture for neutralization, the mixture was centrifuged at 10,000 ×*g* for 5 min, and the supernatant was isolated. The cAMP concentration in the supernatant was measured using an enzyme immunoassay kit.

Assay of PDE activity

Parotid acinar cells were homogenized with 10 ml of 20 mM Tris-HCl (pH 7.4) containing 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1.3 mM benzamidine, and Protease Inhibitor Cocktail (Complete™) and centrifuged at 1,000 ×*g* for 10 min at 4°C. The supernatant was used for assay of PDE activity. cAMP-PDE activity was determined by the two-step method (13). The enzymatic reaction was performed in a total volume of 0.1 ml. The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.4 μM calmodulin, and the substrate (0.1 μM [^{3}H] cAMP; 3.7 kBq) and enzyme. The mixture was incubated at 30°C for 10 min, and then the reaction was terminated by heating at 90°C for 5 min. [^{3}H]5'-AMP formed by PDE was converted to [^{3}H]adenosine by the action of nucleotidase (25 μl of 1 mg/ml snake venom at 30°C for 10 min). The

reaction was terminated by adding 0.5 ml of water and the denatured protein was removed by centrifugation (10,000 ×*g*, 3 min). The clear supernatant fluid (0.5 ml) was applied to a 0.5-ml column of AG 50W-X4 resin (200-400 mesh; hydrogen form). The reaction product, [^{3}H]adenosine, was eluted with 1.5 ml of 3 N ammonium hydroxide after washing the column with 15 ml of water, and radioactivity was measured using a liquid scintillation counter.

Protein quantification

Protein concentrations were determined by the method described previously (14) with a BSA standard

Statistical analysis

Significance of differences was determined using Student's *t*-test. The level of statistical significance was taken as $P < 0.05$.

Results

PDE4 activity in parotid acinar cells

We determined cAMP-PDE activity in mouse, rat and rabbit parotid acinar cells (Fig. 1). The specific activity of cAMP-PDE in mouse parotid acinar cells was lower than those in rat and rabbit cells. Next, we examined the effect of rolipram, a specific inhibitor of PDE4, on the cAMP-PDE activity. Rolipram (50 μM) inhibited cAMP-PDE activities to 30.6%, 37.9% and 32.6% of the control in mouse, rat and rabbit parotid acinar cells, respectively (Fig. 1). These results suggest that about 60 – 70% of cAMP-PDE in mouse, rat and rabbit parotid acinar cells is PDE4.

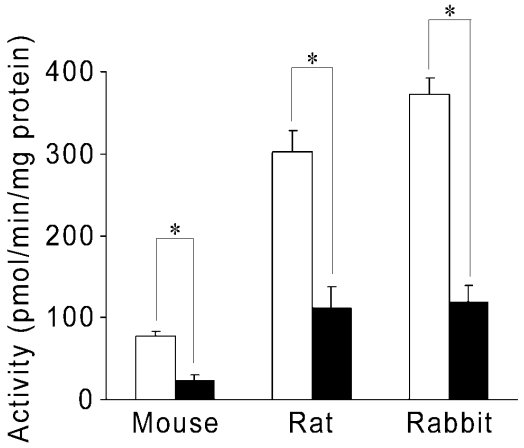

Fig. 1 Effect of rolipram on cAMP-PDE activity in acinar cells isolated from mouse, rat and rabbit parotid glands. cAMP-PDE activities in parotid acinar cells were assayed in the absence (clear column) or presence (solid column) of rolipram (50 μM). Results are means ± S.E.M. from three independent experiments. Statistical analysis: *, $P < 0.05$.

Effect of rolipram on isoproterenol-induced cAMP elevation in parotid acinar cells

We studied the effect of rolipram on cAMP elevation stimulated by β-adrenoceptor activation in mouse (Fig. 2A), rat (Fig. 2B) and rabbit (Fig. 2C) parotid acinar cells. The β-adrenoceptor agonist isoproterenol (1 μM) invariably increased cAMP levels in the absence of rolipram (Fig. 2, left column). The basal and isoproterenol-induced cAMP levels in rabbit parotid acinar cells were higher than those in mouse and rat. In the case of preincubation with rolipram (50 μM) for 10 min, isoproterenol-induced cAMP elevation was robustly enhanced (Fig. 2, right column). These results suggest that PDE4 is involved in regulation of the intracellular cAMP level induced by β-adrenoceptor activation in parotid acinar cells.

Effect of rolipram on isoproterenol-induced amylase release in parotid acinar cells

We next examined the effect of rolipram on amylase release induced by isoproterenol in mouse (Fig. 3A), rat (Fig. 3B) and rabbit (Fig. 3C) parotid acinar cells. Isoproterenol (1 μM) induced amylase release time-dependently, and isoproterenol-induced amylase release was enhanced by preincubation with rolipram (50 μM) for 10 min compared with controls. Rolipram had no effect on constitutive amylase release. These observations suggest that PDE4 is involved in isoproterenol-induced amylase release in parotid acinar cells.

Comparison of cAMP elevation and amylase release in parotid acinar cells

In parotid acinar cells, isoproterenol stimulates amylase release via an increase in the intracellular cAMP level (6,7). We compared the effects of rolipram on isoproterenol-induced cAMP elevation and amylase release in mouse, rat and rabbit parotid acinar cells (Fig. 4). Rolipram increased isoproterenol-induced cAMP levels about 9.9-, 2.7- and 7.2-fold (Fig. 4A), whereas this inhibitor enhanced isoproterenol-induced amylase release about 1.6-, 1.5- and 1.8-fold in mouse, rat and rabbit parotid acinar cells, respectively (Fig. 4B), indicating that the increase in cAMP levels is not linearly correlated with the enhancement of amylase release.

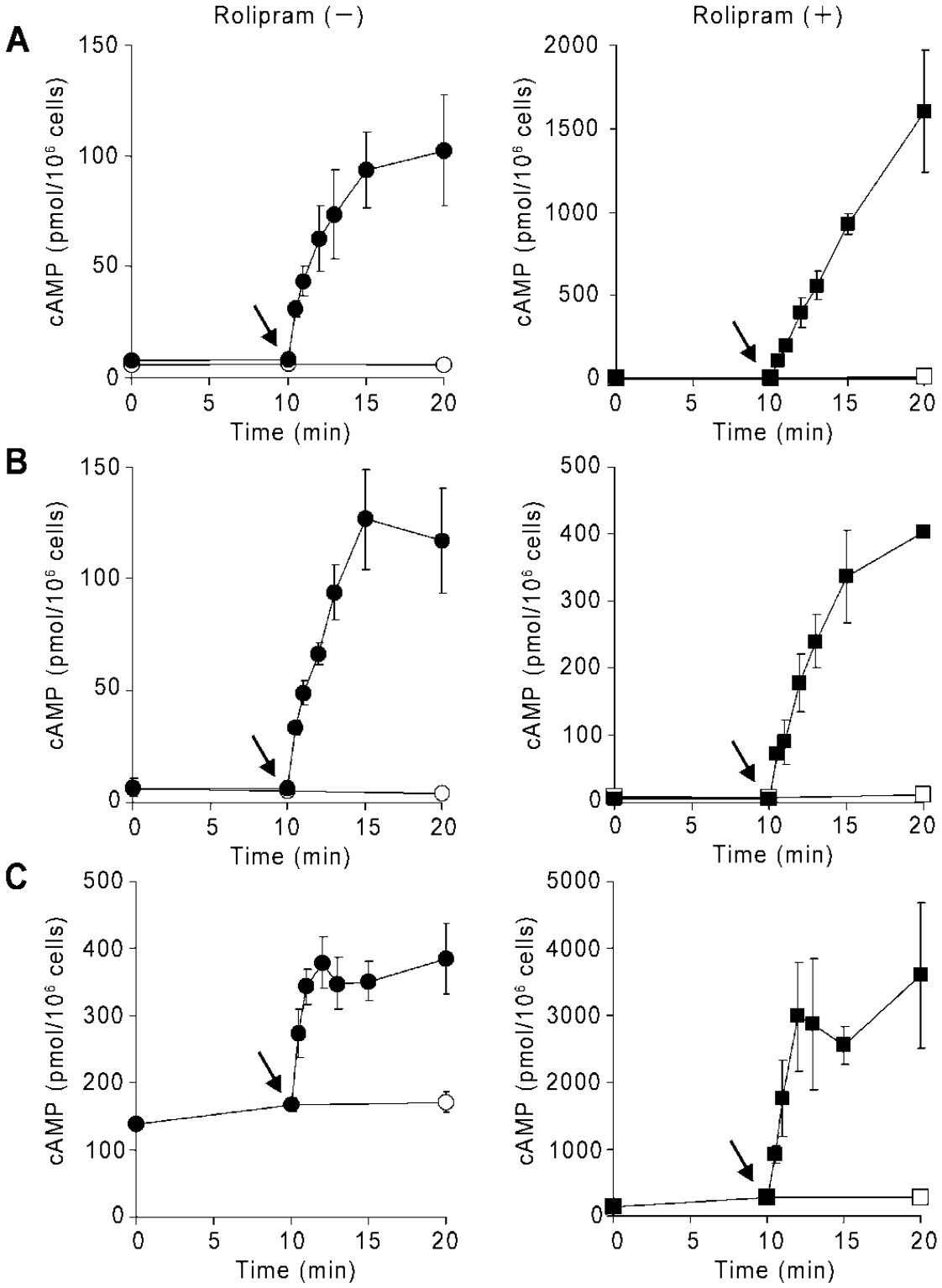

Fig. 2 Effect of rolipram on the isoproterenol-induced increase in cAMP levels in acinar cells isolated from mouse, rat and rabbit parotid glands. After preincubation without (clear and solid circles) or with (clear and solid squares) rolipram (50 μM) for 10 min, cells were stimulated with vehicle (clear circles and squares) or 1 μM isoproterenol (solid circles and squares), as indicated by the arrow. A, mouse; B, rat; C, rabbit. Results are means ± S.E.M. from three independent experiments.

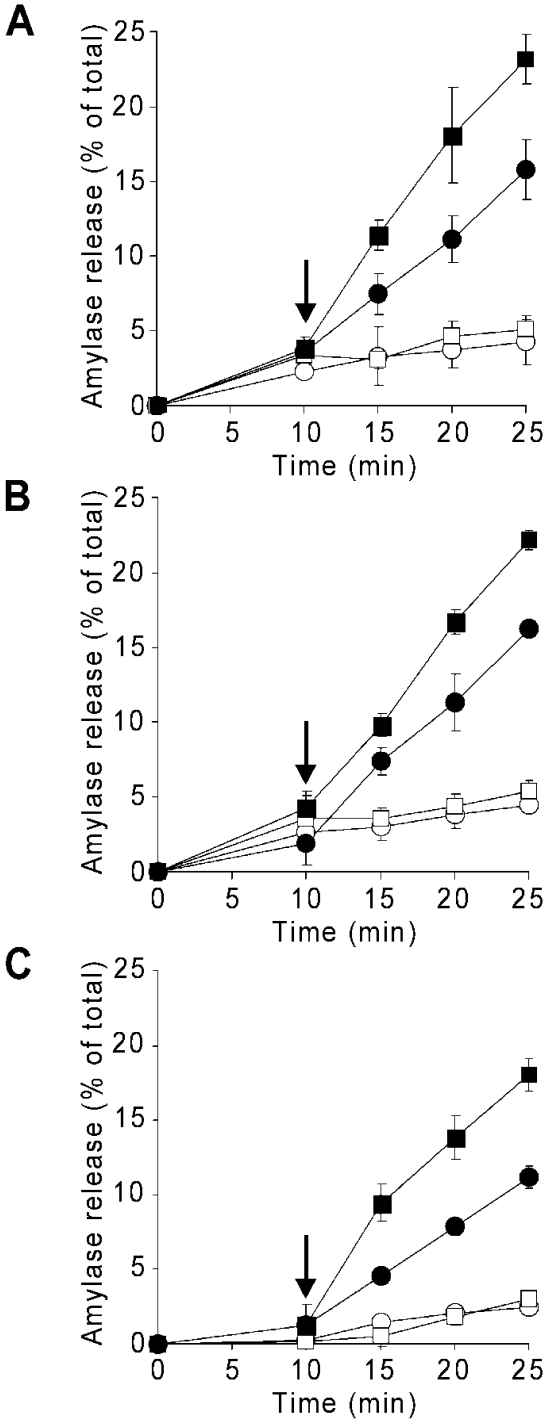

Fig. 3 Effect of rolipram on isoproterenol-induced amylase release in acinar cells isolated from mouse, rat and rabbit parotid glands. After preincubation without (clear and solid circles) or with (clear and solid squares) rolipram (50 μM) for 10 min, cells were stimulated with vehicle (clear circles and square) or 1 μM isoproterenol (solid circles and squares) as indicated by the arrow. Amylase release was expressed as a percentage of the total. Results are means ± S.E.M. from three independent experiments.

Discussion

Various PDE isozymes have been reported to be present in the parotid glands of rodents (15). In this study, we demonstrated involvement of PDE4 in amylase release induced by β-adrenoceptor activation in acinar cells isolated from mouse, rat and rabbit parotid glands. Although the specific activities of cAMP-PDE were different, the inhibitory effect of the PDE4 specific inhibitor rolipram on the activities was comparable, being about 60-70%. These results imply that PDE4 plays a dominant role in the regulation of cAMP breakdown in parotid acinar cells. In fact, β-adrenoceptor agonist-induced cAMP formation was drastically enhanced in the presence of rolipram.

In the presence of rolipram, β-adrenoceptor agonist-induced amylase release was enhanced 1.5-1.8-fold in mouse, rat and rabbit parotid acinar cells, but constitutive release was not affected. It has been reported that exocytosis comprises both constitutive and regulatory exocytosis (7). Therefore, these observations suggest that PDE4 contributes to regulatory amylase release but not to constitutive release.

The enhancement of amylase release by rolipram was not linearly correlated with the increase in cAMP levels. This observation implies the existence of a maximum level of cAMP-dependent amylase release in parotid acinar cells. In parotid acinar cells, cAMP-dependent protein kinase (PKA) is activated by the cAMP levels induced by β-adrenoceptor activation, which provokes phosphorylation of many proteins (6). It has been reported that vesicle-associated membrane protein 2 (VAMP2) is essential for cAMP-dependent amylase release and that VAMP2 may be activated by PKA activation (16,17). Rab3D and Rab27, small molecular GTP-binding proteins, are also reportedly expressed in secretory granules containing amylase, and are suggested to contribute to amylase release via regulation of the size of secretory granules and maintenance of the cytoskeleton, respectively, in β-adrenoceptor agonist-stimulated parotid acinar cells (18-22). Such proteins, which function downstream of the increase in cAMP levels induced by β-adrenoceptor activation, appear to regulate the maximum levels of cAMP-dependent amylase release, although the precise mechanisms are still obscure.

The PDE4 family represents the largest PDE family, being constituted by 4 genes (PDE4A, PDE4B, PDE4C and PDE4D). These four PDE4 genes generate over 20 different variants by means of alternative start sites and alternative splicing (3-5). Expression of the PDE4 family members has been reported in various tissues and cell types. Multiple different targeting molecules reactive with PDE4 family members, such as myomegalin, arrestins and A-kinase anchoring proteins, have also been identified (23-26). Furthermore, it has been demonstrated that the

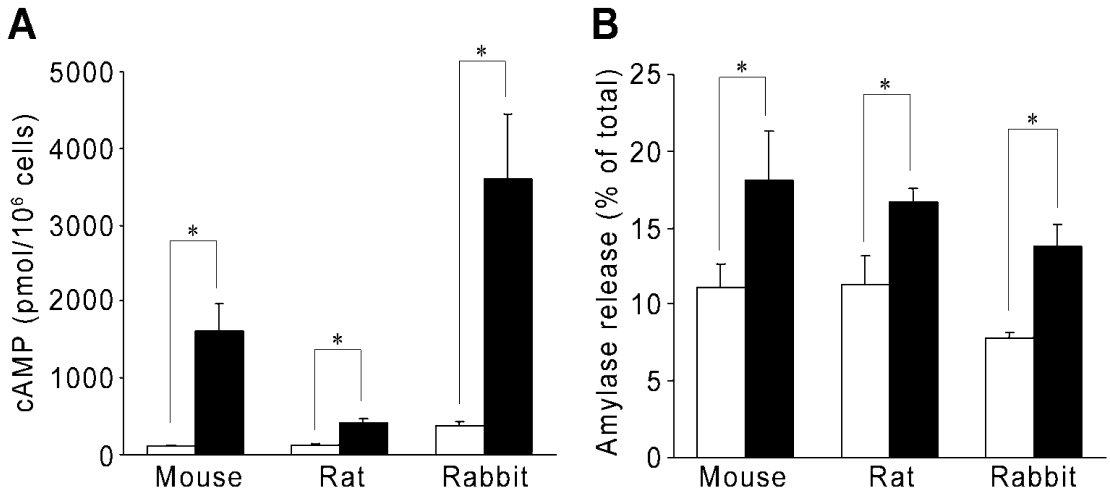

Fig. 4 Comparison of cAMP elevation and amylase release. Effects of rolipram on intracellular cAMP levels (A) or amylase release (B) in mouse, rat and rabbit parotid acinar cells stimulated by isoproterenol for 10 min. Clear column, without rolipram; solid column, with rolipram. Results are means ± S.E.M. from three independent experiments. Statistical analysis: *, $P < 0.05$.

subcellular distribution of the PDE4 family is correlated with their function (27,28). Therefore, further studies of the PDE4 family and its subcellular distribution in parotid acinar cells are warranted.

In conclusion, the rolipram-sensitive cAMP-PDE, PDE4, is present in mouse, rat and rabbit parotid acinar cells, where it regulates intracellular cAMP levels and is involved in β-adrenoceptor agonist-induced amylase release.

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