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Isoproterenol-stimulated labelling of particulate proteins by using [*adenylate*-^{32}P]NAD$^+$ independent on a cAMP-dependent protein kinase in parotid acinar cells

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When saponin-permeabilized rat parotid acinar cells were incubated with [*adenylate*-^{32}P]NAD$^+$, labelling of proteins (33, 27 and 23 kDa) in particulate fractions of the cells was stimulated by isoproterenol. The effect of isoproterenol was completely blocked by a β-antagonist. Both forskolin or cAMP mimicked the effect of isoproterenol on the labelling. However, an inhibitor of cAMPdPK failed to induce complete inhibition of the effects of isoproterenol, forskolin and cAMP. When the labelled proteins were treated with snake venom phosphodiesterase, neither [^{32}P]5′-AMP nor [^{32}P]phosphoribosyladenosine was released. These results suggest that covalent modification of proteins with NAD$^+$, which is distinct from ADP-ribosylation and cAMPdPK-dependent phosphorylation, is coupled to β-receptor-cAMP signalling system in rat parotid acinar cells.

Isoproterenol; NAD$^+$; Cyclic AMP; Phosphorylation; ADP-ribosylation; Parotid

1. INTRODUCTION

In parotid acinar cells, isoproterenol stimulates exocytotic amylase release [1,2]. Without β-receptor activation, the accumulation of cellular cAMP by stimulation of catalytic subunit of adenylate cyclase by forskolin [3] or inhibition of cyclic nucleotide phosphodiesterase can also induce amylase release from parotid cells [4,5]. Therefore, the release of amylase by isoproterenol is considered to be due to the accumulation of cellular cAMP. In this paper, we demonstrate that isoproterenol, forskolin and cAMP stimulated labelling of proteins in particulate fractions by incubating saponin-permeabilized acinar cells of rat parotid gland with [*adenylate*-^{32}P]NAD$^+$.

2. EXPERIMENTAL

2.1. *Materials*

Forskolin, cAMP, propranolol, IBMX, trypsin and trypsin inhibitor were purchased from Sigma (St. Louis, MO, USA). Collagenase and snake venom phosphodiesterase I (*Crotarus durissus*) were from Boehringer Mannheim GmbH (Germany). H-8 was from Seikagaku Kogyo (Tokyo, Japan). [*adenylate*-^{32}P]NAD$^+$ (800 Ci/mmol) was purchased from Du Pont/New England Nuclear (Boston, MA, USA). All other reagents were the highest grade commercially available.

Abbreviations: cAMP, cyclic AMP; cAMPdPK, cyclic AMP-dependent protein kinase; IBMX, 3-isobutyl-1-methylxanthine; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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2.2. *Preparation of permeabilized cells*

Acinar cells of rat parotid gland were prepared as described previously [6]. The cells were suspended in the solution containing 100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 0.96 mM NaH_2PO_4, 1 mM EGTA, 0.435 mM $CaCl_2$ (free [Ca^{2+}] = 140 nM), 11.1 mM glucose, 25 mM HEPES (pH 7.2), 2% bovine serum albumin, 0.01% soybean trypsin inhibitor, and 10 mM thymidine to inhibit poly-ADP-ribosylation [7]. The cells (5×10^7 cells/ml) were incubated with saponin (20 μg/ml) for 5 min at 37°C to prepare saponin-permeabilized cells.

2.3. *Labelling of proteins*

The permeabilized cells were incubated with [^{32}P]NAD$^+$ (0.2–0.4 mCi/ml) with reagents described below at 37°C. After removing the medium for termination of the reaction, the cells were homogenized with 20 mM HEPES/Tris buffer (pH 7.2) containing 0.3 M sucrose, 0.1 mM EGTA, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 750 × *g* for 10 min for removing nucleus-rich fractions, and the supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C. The resulting pellet was subjected to SDS-PAGE (8–16% gradient or 15% gel) [8]. Gels were dried and labelled proteins were visualized by autoradiography.

2.4. *Snake venom phosphodiesterase digestion*

To prepare the labelled samples, cells were stimulated by 1 mM cAMP/100 μM IBMX and treated as described above. Each of labelled 33, 27 and 23 kDa proteins were electro-eluted, concentrated using Centricon 10 (Amicon) and suspended in 100 mM Tris-HCl buffer (pH 9) containing 10 mM $MgCl_2$. The labelled proteins were, then, incubated with snake venom phosphodiesterase (0.2 mg/ml final concentration) for 30 min at 37°C. The samples were subsequently subjected to paper chromatography (solvent: 0.1 M sodium phosphate buffer (pH 6.8)/ammonium sulfate/*n*-propanol; 100:60:2, v/w/v) [9] and to autoradiography. As a reference for ADP-ribosylated material, pertussis toxin-treated GTP-binding proteins from bovine brain (mixture of G_i and G_o [10] were used.

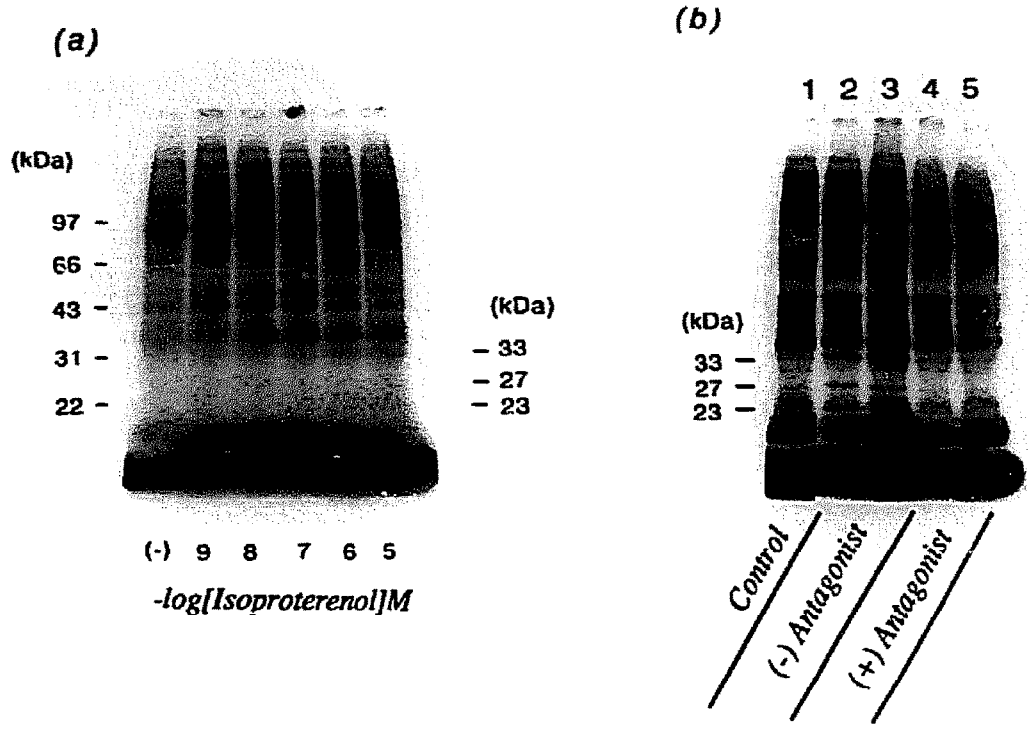

Fig. 1. Effects of isoproterenol and propranolol on labelling of proteins in particulate fractions of parotid acinar cells. (a) Saponin-permeabilized parotid acinar cells were incubated without or with various concentrations of isoproterenol in the medium containing [^{32}P]NAD$^+$ at 37°C for 20 min. (b) Saponin-permeabilized cells were incubated with [^{32}P]NAD$^+$ in the absence of agonist for 30 min (lane 1) or the presence of 1 μM isoproterenol for 20 min (lane 2) or 30 min (lane 3). After stimulation by 1 μM isoproterenol for 10 min, 10 μM propranolol was added in the reaction mixture and the cells were further incubated for 10 min (lane 4) or 20 min (lane 5).

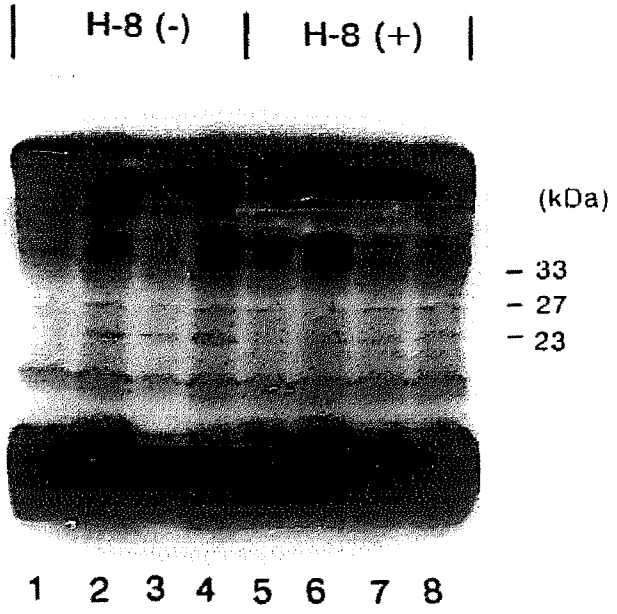

Fig. 2. Effect of isoproterenol, forskolin, cAMP/IBMX on labelling of the proteins in parotid particulate fractions in the absence or presence of H-8. Saponin-permeabilized cells were preincubated without or with 200 μM H-8 for 5 min. The cells were incubated with [^{32}P]NAD$^+$ in the absence (lanes 1 and 5) or the presence of 1 μM isoproterenol (lanes 2 and 6), 10 μM forskolin (lanes 3 and 7) or 1 mM cAMP/100 μM IBMX (lanes 4 and 8) at 37°C for 20 min.

3. RESULTS AND DISCUSSION

When saponin-permeabilized cells were incubated with [*adenylate*-^{32}P]NAD$^+$, labelling of 33 kDa proteins in the particulate fractions was stimulated by 1 pM up to 10 μm isoproterenol in a dose-dependent manner (Fig. 1a). Although it is not that clear, labelling of 23 and 27 kDa proteins was also stimulated by the same concentrations of isoproterenol. The dose- and the time-dependencies of isoproterenol on the labelling of the proteins coincided with that on amylase release [11,12]. Neither a Ca^{2+}-mobilizing agonist nor high concentrations of free Ca^{2+} (up to 1.2 μM) in the reaction medium stimulated labelling of the proteins (data not shown), indicating that the labelling of the proteins is independent of Ca^{2+} mobilization.

Fig. 1b summarizes the effect of propranolol, a β-adrenergic antagonist, on isoproterenol-induced labelling of the proteins. In the presence of 1 μM isoproterenol, the labelling of 33, 27 and 23 kDa proteins was stimulated up to 30 min (lanes 2–3 in Fig. 1b). When 10 μM propranolol was added 10 min after stimulation by 1 μM isoproterenol, the effect of isoproterenol was blocked and the labelling was decreased (lanes 4–5 in Fig. 1b). These results imply that isoproterenol-stimulated labelling is attributed to the activation of β-adrenergic receptors.

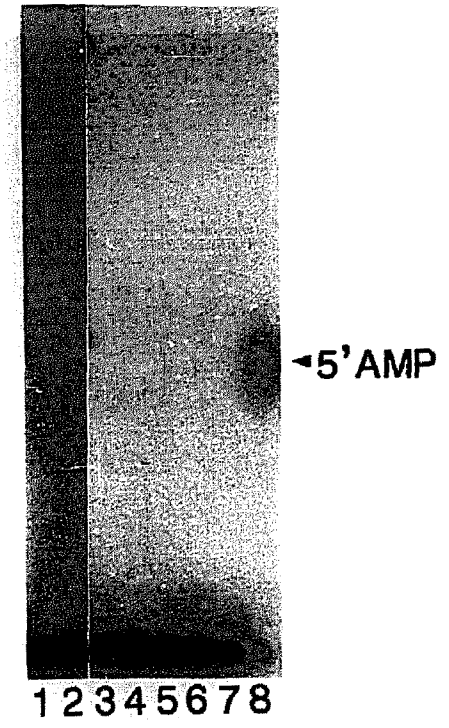

Fig. 3. No release of radioactive moiety from the labelled proteins in parotid particulate fractions by snake venom phosphodiesterase treatment. Labelled proteins were incubated without (lanes 1, 3, 5 and 7) or with (lanes 2, 4, 6 and 8) snake venom phosphodiesterase as described in the Experimental section. (Lanes 1 and 2) 23 kDa protein; (lanes 3 and 4) 27 kDa protein; (lanes 5 and 6) 33 kDa protein; (lanes 7 and 8) GTP-binding protein.

It is well known that isoproterenol activates adenylate cyclase in plasma membranes [13,14] and subsequently induces an increase in the concentration of cAMP [15,16]. Therefore, the effects of a direct activator of adenylate cyclase, forskolin, and simultaneous addition of cAMP and an inhibitor of cyclic nucleotide phosphodiesterase, IBMX, on the labelling by using [^{32}P]NAD$^+$ were investigated (Fig. 2). The addition of forskolin (lane 3) or cAMP/IBMX (lane 4) in the medium resulted in the labelling of the similar molecular mass proteins as that observed following isoproterenol stimulation. Therefore, it is most likely that isoproterenol-stimulated labelling is dependent on cAMP.

Role of cAMPdPK in the exocytotic amylase release has long been postulated [11,17–21]. However, Takuma [12,22] has demonstrated that inhibitors of protein kinase including H-8 inhibit cAMP-dependent phosphorylation, but not amylase release. Then, the effect of H-8 was investigated (Fig. 2). The labelling of proteins stimulated by isoproterenol- (lane 6), forskolin- (lane 7) or cAMP/IBMX-stimulation (lane 8) was not completely inhibited by 200 μM H-8, which was reported to inhibit completely cAMP-dependent phosphorylation in parotid [12]. These results suggest that a part of the labelling stimulated by isoproterenol or cAMP is independent of the activation of cAMPdPK.

To elucidate whether cAMP-dependent labelling is ADP-ribosylation, the particulate proteins labelled using [^{32}P]NAD$^+$ were treated with snake venom phosphodiesterase. As shown in Fig. 3, the radioactive moieties such as [^{32}P]5′-AMP and [^{32}P]phosphoribosyladenosine were not released from the labelled 23, 27 and 33 kDa proteins by the phosphodiesterase treatment. In contrast, this enzyme treatment released [^{32}P]5′-AMP from bovine brain GTP-binding proteins previously mono-ADP-ribosylated by pertussis toxin.

Structurally, NAD$^+$ has two high energy linkages, glycosidic and phosphodiester linkages. In the case of ADP-ribosylation, the glycosidic linkage is cleaved and an ADP-ribosyl moiety is transferred to proteins. However, snake venom phosphodiesterase treatment had no effect on releasing the labelled moiety from the labelled proteins in parotid (Fig. 3). Thus, the covalent modification observed in this study is distinct from ADP- ribosylation. Another possibility is that cleavage of the phosphodiester linkage results in the labelling of proteins by transfer of [^{32}P]5′-AMP moiety in [*adenylate*-^{32}P]NAD$^+$. Transfer of 5′-AMP, adenylation, is one of the post-translational modifications of proteins in prokaryotic cells. Recently, adenylated proteins were also found in rat liver plasma membranes [23]. Therefore, importance of adenylation process remains to be elucidated in β-receptor-cAMP signalling system, especially in relation to amylase release in rat parotid.

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