0014-4754/93/100881-04$1.50 + 0.20 © Birkhäuser Verlag Basel, 1993

Effects of receptor agonists on polyamine concentrations and spermidine/spermine N^1-acetyltransferase activity in rat parotid gland

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Received 13 April 1993; accepted 6 July 1993

Abstract. The polyamine putrescine might be formed via a degradation (catalyzed by spermidine/spermine N^1-acetyltransferase, SSAT) of the higher polyamines spermidine and spermine to putrescine. The involvement of different intracellular signal pathways in the regulation of putrescine formation was studied in explants and in cultured cells of rat parotid glands by using receptor agonists that activate separate second messenger systems, and measuring their effects on the concentrations of putrescine, spermidine and spermine and on the SSAT activity. The β-adrenoceptor agonist isoprenaline, which is an activator of cAMP formation, increased the putrescine concentration and stimulated the SSAT activity. Pilocarpine, a drug that activates the muscarinic receptors and thereby enhances the phosphoinositide turnover, had no effect on either the polyamine concentrations or on the SSAT activity. Epidermal growth factor (EGF), which induces activation of a protein tyrosine kinase, had no effect on the polyamine concentrations or on the SSAT activity. The adenylate cyclase activator forskolin increased the glandular levels of putrescine. Taken together, these findings suggest that increases in putrescine concentration in cultured rat parotid gland cells are accompanied by accumulation of cAMP.
Key words. Epidermal growth factor; forskolin; isoprenaline; pilocarpine; polyamines; spermidine/spermine N^1-acetyltransferase.

The polyamines putrescine, spermidine and spermine are found in all living cells, and they are associated with growth and protein synthesis[1]. Putrescine is formed from ornithine via a decarboxylation (catalyzed by ornithine decarboxylase, ODC). However, putrescine can also be formed via a degradation of the higher polyamines spermidine and spermine. This process is catalyzed by the two enzymes spermidine/spermine N^1-acetyltransferase (SSAT) and polyamine oxidase. The formation of putrescine from spermidine and spermine is usually called polyamine interconversion. SSAT is induced in response to a variety of growth stimuli, for example in regenerating rat liver and after adding growth hormone to cells[2]. The physiological importance of the induction of SSAT leading to putrescine formation is still not understood.

The present study deals with changes in the polyamine concentrations and in the induction of SSAT in primary cultures of rat parotid gland tissue. SSAT activity and polyamine concentrations in the gland cells were studied in response to the β-adrenergic receptor agonist isoprenaline, the muscarinic receptor agonist pilocarpine, and epidermal growth factor (EGF). Since these agents activate and use different intracellular mediators we used them as tools to investigate the possible intracellular signal pathways regulating putrescine formation via induction of SSAT. As our results indicated that cAMP was involved in the regulation of putrescine formation, we also studied the effects of the adenylate cyclase activator forskolin.

Materials and methods

Adult rats of both sexes, weighing about 300 g, were killed by cervical fracture and exsanguinated. The parotid glands were dissected free of connective tissue and placed on cold Petri dishes. A few ml of medium was instantly added. The medium used was Ham's F10 supplemented with glutamine and antibiotics. The tissue was cut into small pieces (1–2 mm^3) and transferred to culture flasks which were placed in an incubator under 5% CO_2 in air at 37 °C for 3 h in the presence of either isoprenaline (10 μM), forskolin (10 μM or 100 μM) or EGF (10 ng/ml). The control explants received the corresponding solvents which were either ascorbic acid, bovine serum albumin or dimethyl sulfoxide (DMSO). At the end of the incubation the tissue pieces were removed from the culture flasks, gently dried on a filter paper and weighed. The explants were sonicated in ice-cold Tris-sucrose buffer for 20 s and aliquots of the homogenates were then withdrawn and prepared for determination of polyamine contents, while the rest of the homogenate was centrifuged at 20 000 × g for 30 min at 4 °C. The supernatant obtained was frozen at −80 °C until determination of spermidine/spermine N^1-acetyltransferase (SSAT) activity was performed.

In a second series of experiments we used enzymes to disperse the gland cells in order to study the effects of some of the agonists on dispersed cells and to compare these results with those obtained from the explants. The tissue pieces were placed in culture flasks which contained the medium with the addition of collagenase (final conc. 1 mg/ml) and hyaluronidase (final conc. 0.2 mg/ml). The flasks were placed in an incubator for 2 h and then gently shaken before the cells were separated from their medium by centrifugation at 250 × *g* for 5 min. The medium was removed and the cells were washed once with medium containing 10% serum and centrifuged as above. The remaining pellet was suspended in serum-free medium and thereafter the cells were seeded in culture flasks for 24 h so that the cell-membrane receptors would be re-established. Then one of the agents isoprenaline (10 μM), pilocarpine (10 μM) or EGF (0.1–10 ng/ml) was added to the culture flasks. After 3 h the cells were separated from the medium by centrifugation at 1000 × *g* for 10 min, washed once with phosphate buffered saline (PBS) and centrifuged as described above. The resulting pellets were sonicated in Tris-sucrose buffer for 20 s and aliquots of the homogenates were withdrawn and prepared for determination of polyamine concentrations and protein contents. The rest of the homogenate was centrifuged at 20 000 × *g* for 30 min at 4 °C and the supernatant was kept frozen at −80 °C until SSAT activity was assayed.

Determination of SSAT activity, polyamine concentrations and protein content. The SSAT activity was determined by measuring the acetylation of spermidine using ^{14}C-acetyl CoA as acetyl group donator as described by Persson and Pegg[3]. The radioactivity was measured in a Kontron liquid scintillation spectrometer. To determine polyamine concentrations the homogenate was precipitated with sulfosalicylic acid. After centrifugation and then filtration of the supernatant the amine content was measured by liquid chromatography, using a cation exchange resin coupled to an automatic amino-acid analyzer. After passing down the column the eluate was mixed with fluorescent reagent and the fluorescence was measured in a spectrofluorometer. The protein content of the cell cultures was determined using the method described by Bradford[4]. At each occasion of protein determination a standard curve with bovine serum albumin was prepared.

Statistical evaluation was performed using Student's t-test for unpaired comparisons. P-values less than 0.05 were considered statistically significant.

Results

Spermidine/spermine N^1-acetyltransferase (SSAT) activity and polyamine concentrations in parotid gland explants. The effects of isoprenaline, forskolin and epidermal growth factor (EGF) on the SSAT activities and polyamine contents are shown in table 1. The putrescine concentration was increased almost 4-fold and the SSAT activity between 2- and 3-fold in response to isoprenaline (10 μM). Incubating the explants with forskolin resulted in an increase in putrescine concentration by about 75% (10 μM) and 2-fold (100 μM). When a higher dose (500 μM) of forskolin was tested (n = 2), then no changes in putrescine content and SSAT activity compared to control values were observed (data not shown). EGF (10 ng/ml) had no effect on either the putrescine concentration or the SSAT activity. The spermidine and spermine concentrations were not changed in response to either isoprenaline, forskolin or EGF.

SSAT activity and polyamine concentrations in cultured rat parotid gland cells. As can be seen in table 2, the SSAT activity was increased about 3-fold in response to isoprenaline (10 μM). Putrescine concentration tended to be increased in the dispersed cells, but the increase was not significant. Both spermidine and spermine concentrations were unchanged after treatment with isoprenaline. Pilocarpine (10 μM) had no effect on the SSAT activity or on the content of any of the three amines under study. Four different concentrations (0.1, 1, 5 and 10 ng/ml) of EGF were tested. No change in either

Table 1. Spermidine/spermine N^1-acetyltransferase (SSAT) activity and concentrations of the polyamines putrescine (PUT), spermidine (SPD) and spermine (SPN) in explants of rat parotid gland tissue in response to isoprenaline (iso), forskolin (forsk) and epidermal growth factor (EGF); c = controls

	SSAT (nmol/g h)	Polyamine concentrations (nmol/g) PUT	SPD	SPN
c	8.9 ± 2.0	16.2 ± 1.5	843 ± 53	395 ± 46
iso 10 μM	23.1 ± 4.9*	60.2 ± 8.8**	882 ± 30	408 ± 31
c	7.2 ± 1.1	13.5 ± 1.6	994 ± 59	403 ± 34
forsk 10 μM	7.1 ± 1.9	23.3 ± 3.4*	1095 ± 40	402 ± 49
forsk 100 μM	11.5 ± 1.6	26.0 ± 1.5***	1050 ± 94	405 ± 11
c	10.0 ± 1.4	20.4 ± 3.0	691 ± 60	390 ± 16
EGF 10 ng/ml	9.9 ± 1.3	23.2 ± 3.9	625 ± 51	362 ± 17

Values are means ± SEM of 4–9 determinations. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared to control values.

Table 2. Spermidine/spermine N^1-acetyltransferase (SSAT) activity and the concentrations of the polyamines putrescine (PUT), spermidine (SPD) and spermine (SPN) in cultured rat parotid gland cells in response to isoprenaline (iso), pilocarpine (pilo) and epidermal growth factor (EGF); c = controls

	SSAT (nmol/mg protein h)	Polyamine concentrations (nmol/mg protein) PUT	SPD	SPN
c	0.10 ± 0.02	5.3 ± 0.7	4.3 ± 0.4	3.2 ± 0.4
iso 10 μM	0.28 ± 0.04**	7.5 ± 0.7	5.0 ± 0.4	4.0 ± 0.6
c	0.57 ± 0.14	9.6 ± 1.5	9.8 ± 0.9	11.4 ± 1.9
pilo 10 μM	0.55 ± 0.04	7.0 ± 1.3	8.9 ± 1.3	9.2 ± 1.5
c	0.19 ± 0.03	6.6 ± 1.4	4.5 ± 0.4	4.4 ± 0.5
EGF 0.1 ng/ml	0.19 ± 0.04	4.4 ± 1.4	4.1 ± 0.6	3.8 ± 0.7
EGF 1 ng/ml	0.27 ± 0.04	8.6 ± 1.5	4.7 ± 0.4	4.9 ± 0.9
EGF 5 ng/ml	0.25 ± 0.04	7.3 ± 1.2	4.3 ± 0.3	4.5 ± 0.4
EGF 10 ng/ml	0.16 ± 0.06	5.0 ± 2.7	4.2 ± 0.9	3.8 ± 1.2

Values are means ± SEM of 3–9 determinations. **$p < 0.01$ when compared to control values.

the SSAT activity or in the polyamine concentrations were observed at any of the EGF concentrations tested. It seems evident, when comparing the results in table 1 with the corresponding ones in table 2, that the dispersed cells and the explants responded to isoprenaline and EGF in a similar way.

Since isoprenaline is not only an inducer of growth in salivary glands but also stimulates secretion, it was interesting to investigate whether this agent stimulates secretion in our system. In a series of experiments we measured the amylase release (according to the method described by Dahlqvist[5]) from the parotid gland cells in response to isoprenaline (10 μM). In the cultured cells that had been incubated for 24 h we were not able to detect any stimulation of amylase release by isoprenaline, probably because of a spontaneous release of amylase that totally masked the stimulation by isoprenaline. Therefore we tested the amylase release in freshly dispersed parotid gland cells. The amylase activity in isoprenaline-treated cells was lower than in control cells (145 ± 3, n = 4 vs 186 ± 13, n = 4 U/mg protein, $p < 0.05$), while the amylase activity in the medium of the isoprenaline-stimulated cells tended (but not significantly) to be higher (80 ± 4, n = 4 vs 60 ± 9, n = 4) than in the medium of control cells.

Discussion

In this study we used the β-adrenoceptor agonist isoprenaline, the muscarinic receptor agonist pilocarpine and the polypeptide growth factor epidemal growth factor (EGF). These agents activate different receptor-coupled intracellular reactions leading to the formation of different second messengers. When isoprenaline is coupled to its receptor, activation of adenylate cyclase activity is induced and the cAMP concentration is raised[6]. Activation of the muscarinic receptor, by using for example pilocarpine, enhances the phosphoinositide turnover and subsequently an elevation of intracellular [Ca^{2+}] and an activation of protein kinase C[7]. EGF binding to its receptor increases its protein tyrosine kinase activity[8,9]. The main purpose of this study was to examine changes in polyamine metabolism using agonists which utilize different intracellular signal pathways.

The present results showed that the putrescine concentration was increased in response to isoprenaline, whereas both pilocarpine and EGF were without effect. The increase in putrescine concentration was accompanied by an increase in spermidine/spermine N^1-acetyltransferase (SSAT) activity. Despite the fact that the SSAT activity was increased in response to isoprenaline, no changes in spermidine and spermine concentrations were observed. As seen in table 1 the concentrations of spermidine and spermine were much higher than the putrescine concentration, so that increases in putrescine might correspond to very small and easily masked changes in spermidine and spermine. SSAT is an important enzyme in the interconversion of the higher polyamines spermine and spermidine to putrescine, which is a process that is induced in response to a variety of growth stimuli. Increases in SSAT activity have previously been shown in rat parotid glands in vivo[10] as well as in vitro[11] in response to isoprenaline. Since we only observed a stimulation of putrescine formation in response to isoprenaline, we performed a series of experiments using forskolin. This agent activates the catalytic subunit of adenylate cyclase[12] and thereby stimulates accumulation of cAMP. Forskolin increased the putrescine concentration several-fold, but only a small, not significant, increase in SSAT activity was detected. As putrescine is not solely formed via degradation of spermine and spermidine but also via decarboxylation of ornithine (catalyzed by ornithine decarboxylase, ODC), it is possible that forskolin stimulated the putrescine formation by activation both of SSAT and of ODC. The concentrations of forskolin (10 and 100 μM) that we used were of the same magnitude as those used by Watson and Dowd[13]. They measured the cAMP formation in isolated mouse parotid gland cells and showed that forskolin (1.5 and 10 μM) increased the cAMP content in a dose-depen-

dent way. Further, these authors showed that forskolin (100 μM) stimulated the amylase release by about 800%. Our results showed that the putrescine concentration was increased by both isoprenaline and forskolin while the other agents used had no effect, indicating that accumulation of cAMP might be a prerequisite for putrescine formation.

We have previously shown that isoprenaline (10 μM) stimulates DNA synthesis in rat parotid gland explants[11]. In vivo, isoprenaline is regarded as being a potent inducer of growth in rodent salivary glands[14]. Our present results show that isoprenaline stimulates the secretion of amylase from the dispersed parotid gland cells, which indicates that these cells have a secretory capacity. Pilocarpine is a powerful secretagogue, without any reported effects on cellular growth in salivary glands[15]. The third agent that we used was EGF. This substance (0.5–5 ng/ml) has been shown to enhance isoprenaline-stimulated DNA synthesis in cultured rat parotid gland explants without having any secretory effect[16]. Mouse plasma contains 1–5 ng/ml of immunoreactive EGF[17], indicating that the concentrations used in our study are nearly physiological. It is a well-known fact that EGF stimulates growth in a variety of epidermal and epithelial cells[8]. Since putrescine formation was only induced by isoprenaline and forskolin, which are substances leading to the accumulation of cAMP, it might be possible that the increasing putrescine concentration reflects the secretory effect and/or the stimulation of growth by isoprenaline which in turn is probably mediated by cAMP. In this context it is interesting to note that putrescine has been identified as a growth factor[18]. Further, in cells treated with the specific ODC inhibitor α-difluoromethylornithine (DFMO), the inhibitory effect on DNA synthesis exerted by DFMO is reversed by addition of putrescine[19].

Acknowledgments. This study was supported by grants from the Swedish Medical Research Council (04X-10352), the Medical and Odontological Faculties at the University of Lund and Åke Wibergs Foundation.

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