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Enhancement of Phosphatidylinositol Turnover and Cyclic Nucleotide Accumulation by Chronic Anethole Trithione Treatment in Rat Submaxillary Glands

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Abstract—The effect of chronic treatment with anethole trithione (ANTT) on the phosphatidylinositol (PI) turnover and cyclic (c)AMP and cGMP accumulation in rat submaxillary glands (SMG) has been compared with the effect of chronic treatment with atropine and a cholinesterase inhibitor, diisopropylfluorophosphate (dyflos, DFP). Experiments were performed 24, 48 and 24 h after the last dose of ANTT, atropine and dyflos, respectively. ANTT and atropine enhanced carbachol-stimulated [^{32}P] incorporation into phosphatidic acid in the SMG slices, while dyflos showed no effect. Pilocarpine-stimulated in-vivo incorporation of [^{3}H]myoinositol into inositol phosphates was significantly enhanced by ANTT, but not by atropine or by dyflos. Phospholipase C-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate was significantly enhanced by ANTT and atropine, but not by dyflos. Pilocarpine-stimulated in-vivo accumulation of cAMP and cGMP was enhanced by ANTT and atropine, but dyflos reduced cAMP accumulation without affecting cGMP accumulation. The enhancement of PI turnover and cyclic nucleotide accumulation seems to contribute to the development of supersensitivity of the salivary gland caused by chronic treatment with ANTT and atropine, while reduction of cAMP accumulation may be responsible for the subsensitivity caused by dyflos.

Chronic treatment with 5-(*p*-methoxyphenyl)-3H-1,2-dithiole-3-throne (anethole trithione; ANTT) has been reported to be effective in the treatment of dry mouth caused by psychotropic medications with anticholinergic properties (Lelord et al 1969) and in patients with Sjögren's syndrome (Epstein et al 1983). In an animal experiment, Palmieri et al (1980) showed that ANTT prevented the inhibition of salivary secretion caused by a single dose of atropine. In a previous report we demonstrated that chronic treatment with ANTT increased the flow of submaxillary saliva induced by pilocarpine and increased the number of muscarinic cholinergic receptors in the submaxillary glands (SMG) of rats (Ukai et al 1984). An enhanced secretory response with a concomitant increase of the muscarinic receptors has also been demonstrated by Hedlund et al (1983) with chronic treatment with atropine. However, the mechanisms for the enhanced secretory response caused by ANTT seem to be different from those caused by atropine, because ANTT has no anticholinergic properties (Ukai et al 1984).

There are two stimulus-secretion coupling pathways in salivary secretion, one involving cyclic (c)AMP, which primarily regulates enzyme secretion, and the other involving turnover of phosphatidylinositol (PI), which regulates Ca^{2+} mobilization and monovalent ion fluxes and water excretion (Putney 1986; Martinez 1987). Activation of muscarinic receptors has been reported to enhance the turnover of PI in the salivary glands (Aub & Putney 1984) and to produce changes in the cellular calcium transport or cGMP levels (Michell 1975).

In the present study, the effect of chronic treatment with ANTT on [^{32}P] incorporation into phospholipids, [^{3}H]myoinositol incorporation into inositolphosphates, phospholipase C activity and accumulation of cAMP and cGMP in the rat SMG has been compared with the effect of atropine and an irreversible cholinesterase inhibitor, diisopropylfluorophosphate (dyflos), which causes subsensitivity in various tissues (Russell et al 1975; Uchida et al 1979) including rat SMGs (Ukai et al 1988).

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Materials and Methods

Male Sprague-Dawley rats, 250 to 350 g, were used. ANTT at a dose of 300 mg kg^{-1} was administered p.o. once daily for 7 days. Atropine was continuously infused subcutaneously at a daily dose of 20 mg kg^{-1} for 14 days with a mini-osmotic pump (Alzet, type 2002) implanted in the back. Dyflos was administered i.m. at a dose of 1 mg kg^{-1} on the 1st day, then 0·5 mg kg^{-1} every 3 days for 4 weeks according to the dosage regimen of Schiller (1979). Animals had free access to food and water during the period of drug treatment, and were fasted for 16 h before the following experiments. Animals were decapitated 24, 48 and 24 h after the last administration of ANTT, atropine and dyflos, respectively.

[^{32}P] incorporation into phospholipids in SMG slices
Experiments were performed by the method of Farese et al (1982) and Miyamoto et al (1986). SMGs were removed immediately after decapitation of the animals, placed in a small quantity of chilled Krebs-Ringer bicarborate (KRB) buffer, and cut into slices of approximately 1 mm^3. Approximately 40 mg of tissue was preincubated in 2 mL of the KRB buffer containing 200 μ Ci of [^{32}P]phosphate at 37°C for 60 min in an atmosphere of 95% O_2 and 5% CO_2. Following the preincubation, the tissue slices were washed three times with 3 mL of ice-cold KRB buffer, incubated with 180 μL of KRB buffer at 37°C for 5 min, and then 20 μL of KRB buffer containing carbachol (final concentration; 10^{-4} M) was added and incubation was continued. Incubation was terminated by the addition of 2 mL of ice-cold $CHCl_3$–MeOH–conc: HCl (200:100:0·75, v/v/v). The incubation mixture was

then homogenized and stood overnight at 4°C. After the addition of 0·175 mL of 1·3M HCl, the tubes were shaken and centrifuged. The lower phase containing the phospholipids was washed three times with 1 mL of $CHCl_3$–MeOH–0·6M HCl (3:48:47, v/v/v) and dried in an evaporator. The tissue phospholipid concentrations were determined by thin-layer chromatography with silica gel plates (Silica Gel 60 F254, Merck). The solvent system was $CHCl_3$–MeOH–28% NH_4OH (53:38:9, v/v/v). Phospholipids on the plates were visualized by exposure to I_2 vapour and scraped into vials, and the radioactivities were measured by a liquid scintillation counter.

In-vivo [^{3}H]myo-inositol incorporation into inositol phosphates in SMG

Experiments were performed by the method of Sherman et al (1985). In a preliminary study, [^{3}H]myo-inositol incorporation into phospholipids in the SMG was confirmed to attain its maximum level 24 h after an i.p. injection of [^{3}H]myo-inositol, so the following schedule was used: 10 μCi of [^{3}H]myo-inositol was administered i.p. and 24 h later, 3 mequiv kg^{-1} of LiCl was administered s.c. Three h later, pilocarpine at a dose of 1·0 mg kg^{-1} was administered i.p., and 10 min after decapitation of animals, the SMGs were removed rapidly. The glands were homogenized in 10 volumes of 6% trichloroacetic acid with Polytron and centrifuged at 1000*g* for 10 min. Supernatants containing inositol phosphates were washed three times with two volumes of water saturated ether, lyophilized, and dissolved in water again. Aliquots were developed on PEI-cellulose plates (Merck) with distilled water to remove non-charged (non-phosphorylated) inositol. After development, an origin containing inositol phosphates was scraped into vials, 0·5 mL of water was added and radioactivity was measured by a liquid scintillation counter.

Phospholipase C activity

Immediately after decapitation of animals, the SMGs were removed. Assays were performed by the method of Melin et al (1986). The glands were homogenized with Polytron in 10 volumes of a buffer containing 1 mM of EGTA, 5 mM of imidazole, 1 mM of dithiothreitol and 0·32 M of sucrose at pH 7·5. The homogenates were centrifuged at 1000 *g* for 10 min. The pellets were discarded, and the supernatants were centrifuged at 45 000 *g* for 20 min. Cytosolic fractions were obtained from the supernatants. The resultant pellets were homogenized with 10 volumes of the buffer and centrifuged at 45 000 *g* for 20 min. The pellets were suspended in 10 volumes of the buffer. Thus membrane fractions were obtained.

Phospholipase C activity was determined by the hydrolysis of [^{3}H]phosphatidylinositol 4,5-bisphosphate (PIP_2). Assays were carried out in a final volume of 40 μL containing 32 μL of incubation buffer, 4 μL of micellar solution and 4 μL of the enzyme (appropriately diluted cytosolic and membrane fractions). The standard incubation buffer containing 50 mM imidazole, 10^{-6} M of free Ca^{2+}, pH 7·5, 1 mM EGTA (EGTA-$CaCl_2$ mixture as described by Owen 1976). Micellar solution was prepared as follows: 1 μmol of PIP_2 and 0·2 μCi of [^{3}H]PIP_2 were dissolved in 1 mL of $CHCl_3$–MeOH–H_2O (75:25:2, v/v/v) and evaporated to dryness under a N_2 gas stream. 80 μL of buffer composed of 1 mM of EGTA, 5 mM of imidazole and 1 mM of dithiothreitol was added and PIP_2 was dispersed by shaking. The reaction was initiated by the addition of the enzyme, and incubation was continued for 10 min at 37°C. The reaction was terminated by adding 1 mL of ice-cold $CHCl_3$–MeOH–conc. HCl (200:100:0·75, v/v/v). After the addition of 250 μL of 0·6 M HCl, vortex mixing and 30 s centrifugation with a Beckman microfuge were done and the radioactivity in the water phase was measured by a liquid scintillation counter.

In-vivo cAMP and cGMP accumulation

Before or after an i.p. injection of 1 mg kg^{-1} of pilocarpine, the SMGs were removed immediately after decapitation of animals and plunged into 5% trichloroacetic acid and homogenized. cAMP and cGMP were extracted from the homogenates by the method of Robbereched et al (1974). The homogenates were centrifuged and the proteins in the precipitates were determined by the method of Lowry et al (1951). The supernatant was acidified with hydrochloride and trichloroacetic acid was eliminated by five successive extractions with water-saturated ethylether. The residue obtained after lyophilization was dissolved in an appropriate volume of water, and cAMP and cGMP were assayed radioimmunologically with a commercial cAMP or cGMP assay kit obtained from Amersham Co.

Drugs

The drugs used were anethole trithione, atropine sulphate, diisopropylfluorophosphate, pilocarpine hydrochloride and carbachol. Except for anethole trithione (Felviten, Nippon Shinyaku), they were purchased from Sigma Chemical. [^{32}P]Phosphoric acid (carrier free), [^{3}H]myo-inositol (17·1 Ci $mmol^{-1}$) and [^{3}H]L-3-phosphatidylinositol 4,5-bisphosphate (4·0 Ci $mmol^{-1}$) were purchased from New England Nuclear.

Statistics

Statistical analysis was done by analysis of variance followed by Dunnett's test (Dunnett 1964).

Results

[^{32}P] incorporation into phospholipids of the SMG

Fig. 1 shows the time course of the incorporation of [^{32}P]phosphate into phospholipids in the SMG slices after the addition of 10^{-4} M carbachol. By 2 min after the addition of carbachol, marked increases in the incorporation of [^{32}P] into PI and phosphatidic acid (PA) were observed, but no changes in the incorporation of [^{32}P] into phosphatidylcholine or phosphatidylethanolamine. Fig. 2 shows the effects of chronic treatment with ANTT, atropine and dyflos on the carbachol-stimulated [^{32}P] incorporation into PA and PI. The [^{32}P] incorporation into phospholipids was determined 1 min after the addition of 10^{-4} M carbachol. Both ANTT and atropine significantly increased [^{32}P] incorporation into PA, while dyflos showed no significant effect on the incorporation of [^{32}P] into phospholipids. The [^{32}P] incorporation into PI was not affected by any of these drugs.

In-vivo [^{3}H]myo-inositol incorporation into inositol phosphates

In rats pretreated with 3 mequiv kg^{-1} of LiCl, [^{3}H]myo-

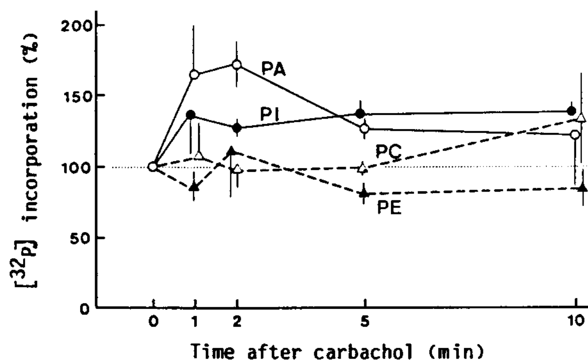

FIG. 1. Time course of [^{32}P] incorporation into phospholipids in submaxillary gland slices after addition of carbachol. Submaxillary gland slices were labelled with [^{32}P]phosphate for 60 min before the addition of carbachol at a concentration of 10^{-4}M, and incubation was continued for the time indicated. Values are the mean ± s.e. of triplicate determinations. Abbreviations: PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

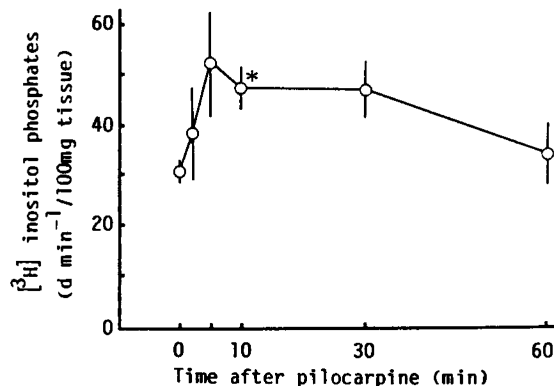

FIG. 3. Time course of [^{3}H]myo-inositol incorporation into inositol phosphates in submaxillary glands of rats after pilocarpine injection. Pilocarpine at a dose of 1 mg kg^{-1} was administered i.p. 3 h after 3 mEq kg^{-1} of LiCl. LiCl was administered s.c. 24 h after an i.p. injection of 10 μCi of [^{3}H]myo-inositol. Values are the mean ± s.e. of 4 to 5 experiments. Significantly different from the value at time 0 (before pilocarpine): * $P<0{\cdot}05$.

inositol incorporation into inositol phosphates of the SMG was significantly increased 10 min after an i.p. injection of 1 mg kg^{-1} of pilocarpine (Fig. 3).

Fig. 4 shows the effects of chronic treatment with ANTT, atropine and dyflos on the incorporation of [^{3}H]myo-inositol into inositol phosphates of the SMG 10 min after pilocarpine. In the group treated with ANTT, a significant increase was observed in the accumulation of [^{3}H]inositol phosphates, while atropine and dyflos showed no effect.

Phospholipase C activity

In a preliminary experiment, the effect of free-Ca^{2+} in the medium on [^{3}H]PIP_2 hydrolysis was examined. At pH 7·5, both cytosolic and membrane fractions produced hydrolysis of [^{3}H]PIP_2 at Ca^{2+} concentrations of 10^{-7} to 10^{-4} M. At a pH below 6, no hydrolysis was observed in the cytosolic or membrane fractions at a Ca^{2+} concentration of 10^{-6} M (data not shown).

Table 1 shows the effect of chronic treatment with ANTT, atropine and dyflos on the hydrolysis of [^{3}H]PIP_2 (phospholipase C activity) in cytosolic and membrane fractions, examined at pH 7·5 and a Ca^{2+} concentration of 10^{-6} M. ANTT caused significant increases of PIP_2 hydrolysis in both the cytosolic and membrane fractions. Atropine caused a significant increase in PIP_2 hydrolysis in the membrane fraction without affecting PIP_2 hydrolysis in the cytosolic fraction. Dyflos had no effect on PIP_2 hydrolysis.

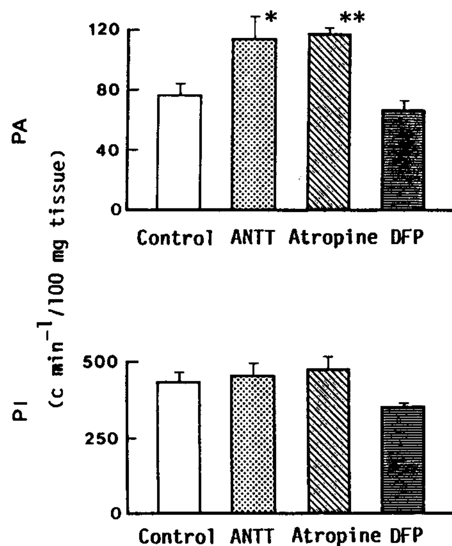

FIG. 2. Effect of chronic treatment with ANTT, atropine and dyflos (DFP) on [^{32}P] incorporation into PA and PI in submaxillary gland slices after addition of carbachol. Submaxillary gland slices were labelled with [^{32}P]phosphate for 60 min before the addition of carbachol. One min after the addition of carbachol at a concentration of 10^{-4} M, incubation was terminated. Values are the mean ± s.e. of 8 experiments. Significantly different from control: * $P<0{\cdot}05$, ** $P<0{\cdot}01$.

In-vivo cAMP and cGMP accumulation

Fig. 5 shows the time course of the accumulation of cAMP and cGMP in the SMG of the naive animals before and after an i.p. injection of 1 mg kg^{-1} of pilocarpine. The tissue cAMP levels were slightly, but not significantly, increased 4

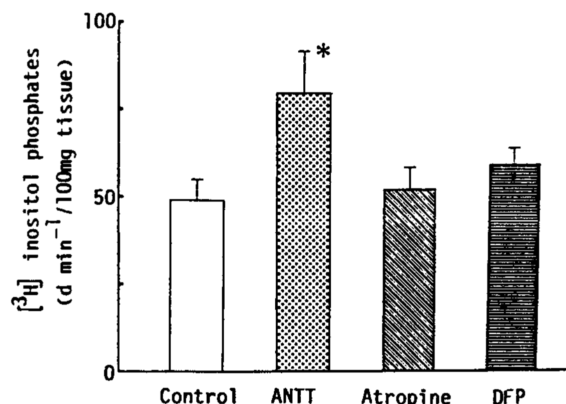

FIG. 4. Effect of chronic treatment with ANTT, atropine and dyflos (DFP) on pilocarpine-stimulated [^{3}H]myo-inositol incorporation into inositol phosphates in submaxillary glands of rats. Ten min after the i.p. injection of 1 mg kg^{-1} of pilocarpine, animals were killed and the accumulation of [^{3}H]inositol phosphates in the glands was determined. Values are the mean ± s.e. of 4 to 6 experiments. Significantly different from control: * $P<0{\cdot}05$.

Table 1. Effects of chronic treatment with ANTT, atropine and DFP on phospholipase C activity in cytosolic and membrane fractions prepared from the rat submaxillary glands.

	PIP_2 hydrolysed (nmol (mg tissue^{-1}) 10 min)	
	Cytosol	Membrane
Control	39·24±0·66 (5)	12·14±0·41 (7)
ANTT	43·24±1·46* (4)	13·58±0·29* (7)
Atropine	41·32±0·70 (6)	13·32±0·27* (8)
Dyflos	39·02±0·78 (6)	13·17±0·22 (8)

Phospholipase C activities were examined at pH 7·5 and a Ca^{2+} concentration of 10^{-6} M.
Values are the mean ± s.e.
Number of experiments is indicated in parentheses.
Significantly different from control: * $P<0·05$.

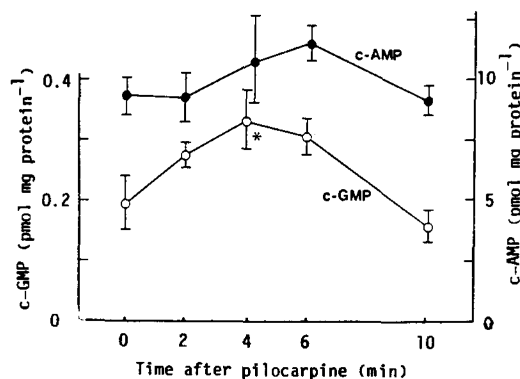

FIG. 5. Time course of cAMP and cGMP accumulation in the rat submaxillary glands after pilocarpine. Pilocarpine was administered i.p. at a dose of 1 mg kg^{-1}. Values are the mean ± s.e. of 5 to 6 experiments. Significantly different from the levels at time 0 (before pilocarpine): * $P<0·05$.

to 6 min after pilocarpine injection. The cGMP levels were increased consistently and attained a peak level 4 min after pilocarpine injection.

Fig. 6 shows the effect of chronic treatment with ANTT, atropine and dyflos on the accumulation of cAMP. Both ANTT and atropine enhanced the cAMP accumulation induced by pilocarpine, but did not affect the cAMP levels before pilocarpine injection. On the other hand, chronic

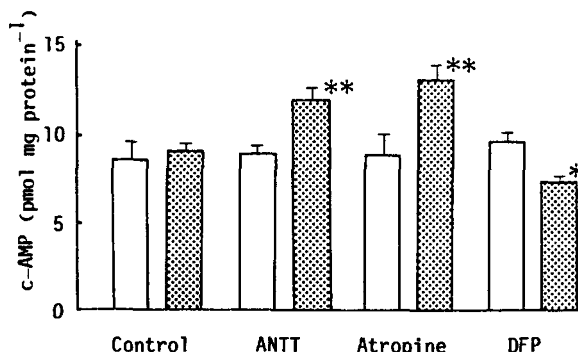

FIG. 6. Effect of chronic treatment with ANTT, atropine and (dyflos) (DFP) on the cAMP accumulation in the rat submaxillary glands before and after pilocarpine. Before (open column) and 4 min after (dotted column) an i.p. injection of 1 mg kg^{-1} of pilocarpine, animals were killed and cAMP was determined. Values are the mean ± s.e. of 8 to 13 experiments. Significantly different from corresponding control: * $P<0·05$, ** $P<0·01$.

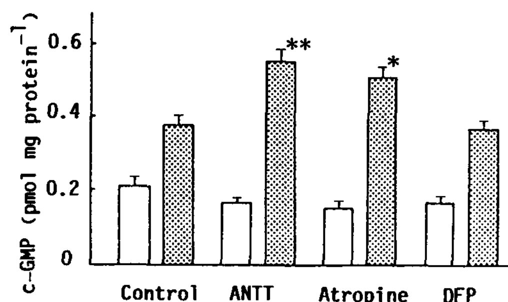

FIG. 7. Effect of chronic treatment with ANTT, atropine and (dyflos) (DFP) on the cGMP accumulation in the rat submaxillary glands before and after pilocarpine. Before (open column) and 4 min after (dotted column) an i.p. injection of 1 mg kg^{-1} of pilocarpine, animals were killed and cGMP was determined. Values are the mean ± s.e. of 7 to 11 experiments. Significantly different from corresponding control: * $P<0·05$, ** $P<0·01$.

dyflos reduced the cAMP accumulation induced by pilocarpine. Without pilocarpine, dyflos showed no effect on cAMP levels.

Fig. 7 shows the effect of chronic treatment with ANTT, atropine and dyflos on the cGMP accumulation induced by pilocarpine. Both ANTT and atropine enhanced the cGMP accumulation induced by pilocarpine, but did not affect the cGMP levels before pilocarpine injection. Dyflos showed no effect on cGMP accumulation with or without pilocarpine injection.

Discussion

In the salivary glands, it is evident that the release of water and electrolytes is mediated via muscarinic and α-adrenoceptors (Putney 1986). It has been reported that [^{32}P] incorporation into PA and PI is increased by cholinergic stimulation (Farese et al 1982; Godfrey & Putney 1984), and secretagogue-induced accumulation of inositol phosphates in the salivary glands has been considered to correlate well with the secretory response (Hanley et al 1980; Jacobson et al 1985). Our finding that ANTT enhanced ^{32}P incorporation into PA, and [^{3}H]inositol phosphates accumulation induced by carbachol, suggests that such an enhancement of PI-turnover may contribute to the increase in the secretory response. The same is true for atropine, in part. Chronic atropine treatment enhanced ^{32}P incorporation into PA, but not '[^{3}H]inositol phosphates accumulation.

It has been established that activation of a broad spectrum of receptors stimulates a specific phospholipase C the primary target of which is PIP_2 (Berridge & Irvine 1984; Putney 1987). With references to the studies of Irvine et al (1984) and Melin et al (1986), we employed PIP_2 as a substrate for phospholipase C. At pH 7·5, phospholipase C in the SMG was very active at Ca^{2+} concentrations between 10^{-6} and 10^{-4} M. These results are similar to those reported by Hirasawa et al (1981) and Irvine et al (1984). Chronic treatment with ANTT enhanced phospholipase C activity in both membrane and cytosolic fractions. Atropine enhanced phospholipase C activity in the membrane fraction. Although there seem to be some differences between the modes of actions of ANTT and atropine regarding the PI turnover pathway, it could be concluded that both ANTT

and atropine enhance PI turnover by stimulating phospholipase C activity which leads to supersensitivity of the salivary glands.

The subsensitivity caused by chronic dyflos treatment might not be mediated by changes in the PI turnover pathway, because dyflos does not change PI turnover or phospholipase C activity.

The present study also demonstrated that both ANTT and atropine enhanced the cAMP and cGMP accumulation induced by pilocarpine, and dyflos reduced the cAMP accumulation induced by pilocarpine. Since the basal levels were not affected by these drugs, adenylate and guanylate cyclase activities appear to be enhanced by ANTT and by atropine, while dyflos seems to reduce adenylate cyclase activity.

Studies of several tissues, including parotid glands, hearts and pancreas, have shown that stimulation of muscarinic receptors inhibits adenylate cyclase (Harper & Brooker 1977; Butcher 1978; Brown 1979; Lichtshtein et al 1979; Gil & Wolfe 1985) and facilitates phosphatidylinositol (PI) turnover (Gil & Wolfe 1985). In contrast to the tissues mentioned above, in cat SMGs an increase of cAMP accumulation induced by cholinomimetics has been demonstrated (Enyedi et al 1982; Fredholm & Lundberg 1982; Enyedi & Fredholm 1984), and stimulation of a calcium-calmodulin sensitive adenylate cyclase has been suggested to be involved in the accumulation of cAMP in the glands (Enyedi & Fredholm 1984).

Chronic ANTT, as well as atropine, treatment has been proved to enhance the secretory responses to cholinergic stimuli associated with the increased numbers of muscarinic receptors in the salivary glands (Hedlund et al 1983; Ukai et al 1984), and it therefore appears that the increased number of the muscarinic receptors might contribute to the enhancement of cAMP accumulation as well as PI turnover, which is responsible for the greater secretory response to cholinergic stimuli.

Vasoactive intestinal polypeptide (VIP), which coexists in post ganglionic nerves innervating the SMGs (Lundberg 1981), has been shown to stimulate cAMP formation synergestically with cholinomimetics (Fredholm & Lundberg 1982), so VIP might mediate the enhancement of cAMP accumulation by chronic ANTT or atropine.

Chronic dyflos treatment has been found to reduce secretory response to pilocarpine without affecting the muscarinic receptors in the SMG (Ukai et al 1988 data). The present finding that chronic dyflos treatment reduced the cAMP accumulation induced by pilocarpine might account for the reduced secretory response to pilocarpine.

As muscarinic secretagogues can increase cellular cGMP as a result of increased intracellular calcium levels (Berridge 1980), the present finding that chronic ANTT and atropine treatment enhanced the accumulation of cGMP might explain the role of cGMP in the development of supersensitivity of the salivary glands.

The present results suggest that enhancement of PI turnover and cAMP and cGMP accumulation, associated with the increase of the number of muscarinic receptors, may contribute to the development of supersensitivty of the salivary glands, while reduction of cAMP accumulation or adenylate cyclase activities may be responsible for the subsensitivity caused by dyflos, which might be independent of alterations of the muscarinic receptors.

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