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Inhibitory effect of diazepam on muscarinic receptor-stimulated inositol 1,4,5-trisphosphate production in rat parotid acinar cells

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1 This study examined the effect of diazepam (DZP) on phosphoinositide turnover, which plays an important role in the regulation of salivary secretion, in rat parotid acinar cells.

2 DZP (10^{-9} M to 10^{-5} M), a potent agonist of both central- and peripheral-type benzodiazepine receptors, dose-dependently decreased inositol 1,4,5-trisphosphate (IP_3) production stimulated by carbachol, a muscarinic receptor agonist, in the cells.

3 DZP produced a maximum inhibitory response at a concentration of 10^{-5} M, with IP_3 production decreased to 63% of maximal levels. The concentration inducing half maximal inhibition of IP_3 production was approximately 3.5×10^{-8} M.

4 An inhibitory response to DZP was produced by a short-term pretreatment (<3 min) of the cells and prevented by antagonist and competing ligand for the central- and peripheral-type benzodiazepine receptors, flumazenil and PK 11195, respectively.

5 DZP showed a non-competitive inhibition of carbachol-stimulated IP_3 production. It did not directly inhibit the activities of GTP-binding regulatory proteins and phosphatidylinositol 4,5-bisphosphate-specific phospholipase C (PLC) in the parotid gland membranes, though choline chloride inhibited PLC activity.

6 DZP (10^{-5} M) attenuated the increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the cells following stimulation of the muscarinic and α_1-adrenoceptors.

7 These results suggest that in the parotid acinar cells, DZP inhibits muscarinic receptor-stimulated IP_3 production through benzodiazepine receptors and that PLC activity which produces IP_3 is inhibited by chloride. The decreases in IP_3 and $[Ca^{2+}]_i$ in the cells may be connected with the suppression of salivary secretion induced by DZP.
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Abbreviations: BDZ, benzodiazepine; CCh, carbachol; DMEM, Dulbecco's Modified Eagle's Medium; DZP, diazepam; FLZ, flumazenil; G protein, GTP-binding regulatory protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HBSS, Hanks' balanced salt solution; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IP_3, inositol 1,4,5-trisphosphate; PE, phosphatidylethanolamine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PK 11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide; PMSF, phenylmethylsulphonyl fluoride; PS, phosphatidylserine

Introduction

Benzodiazepines (BDZs) such as diazepam (DZP) and clonazepam are used clinically as anxiolytic, anticonvulsant, and hypnotic drugs. Their effects are mediated by specific BDZ receptors which are classified into a central-type linked to the $GABA_A$ receptor-chloride channel complex and a peripheral-type not linked to the $GABA_A$ receptor (Braestrup & Squires, 1977; Schoemaker *et al*., 1983; Rampe & Triggle, 1986; Olsen & Tobin, 1990). Both types of BDZ receptors exist in the salivary glands as well as in the brain (Yamagishi & Kawaguchi, 1998; Yamagishi *et al*., 2000). Numerous reports in both humans and animals have confirmed that BDZs suppress salivary secretion as a serious oral side effect (Sreebny & Schwartz, 1986). We have previously reported that BDZs decreased salivary secretion caused by muscarinic receptor stimulation in rats and modified chloride transport and Ca^{2+} flux activity, which trigger the process of fluid secretion, in rat parotid cells (Kawaguchi *et al*., 1995; Kawaguchi & Yamagishi, 1996). BDZs also suppressed the release of amylase from rat parotid cells and their inhibitory responses were blocked by BDZ receptor antagonists (Okubo & Kawaguchi, 1998). These findings indicate that BDZs not only suppress the central nervous system but also act directly on the salivary glands and that BDZ receptors in the salivary glands are linked to the inhibitory responses of BDZs.

Stimulation of the m_3-muscarinic and α_1-adrenoceptors in the salivary glands leads to the hydrolysis of a plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), *via* the activation of GTP-binding regulatory protein (G protein; the G_q family) coupled to these receptors and a membrane-bound phosphoinositide-specific phospho-

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lipase C (PLC), most likely of β_3 type (Merritt & Rink, 1987; Hughes *et al*., 1988; Ambudkar *et al*., 1990; Horn *et al*., 1990; Hiramatsu *et al*., 1992; Sawaki *et al*., 1993; 1995). This results in the generation of the second messenger molecules, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 gates the release of Ca^{2+} from intracellular Ca^{2+} stores, resulting in Ca^{2+} entry from the external medium and a sustained elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Hughes *et al*., 1988; Baum & Ambudkar, 1988; Mertz *et al*., 1990). The resulting increase in $[Ca^{2+}]_i$ regulates, directly or indirectly, a number of Ca^{2+}-dependent ion channels, e.g., the Ca^{2+}-activated potassium channel in the basolateral membrane and the Ca^{2+}-activated chloride channel in the luminal membrane, which are intricately involved in salivary secretion in the salivary glands (Putney, 1986; Ambudkar *et al*., 1988; Petersen & Gallacher, 1988; Ambudkar, 2000). Thus, phosphoinositide turnover in the salivary glands plays an important role in the regulation of salivary secretion.

It has been demonstrated that DZP decreased somatostatin-induced IP_3 accumulation in the rat frontoparietal cortex after its administration *in vivo* (Martínez-Ferrer *et al*., 2000), while IP_3 levels in mouse spleen lymphocytes were not altered after exposure to DZP *in vitro* (Lachowicz *et al*., 1992). In the salivary glands, the actions of BDZs on phosphoinositide turnover are not yet clear. To elucidate the inhibitory mechanisms, we examined the effect of DZP, which has a high affinity for both central- and peripheral-type BDZ receptors, on muscarinic receptor-stimulated IP_3 production and Ca^{2+} response in the parotid glands.

Methods

Animals

Male Wistar rats, 210–250 g, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were kept at a constant room temperature ($23 \pm 2^{\circ}C$) under a 12 h light-dark cycle (0600–1800 h) and maintained on commercial laboratory chow and tap water for one week before the experiments. The treatment of animals was performed according to the 'Guiding principles for the care and use of laboratory animals' of The Japanese Pharmacological Society and Tokyo Dental College.

Materials

ATP, bovine testicular hyaluronidase (type 1-S), phenylephrine, BSA, carbachol (CCh), EGTA, HEPES, PIP_2, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), choline chloride, phenylmethylsulphonyl fluoride (PMSF), and sodium fluoride were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Aluminium chloride, atropine sulphate, DZP, and sodium cholate were obtained from Wako Pure Chemical (Osaka, Japan). Collagenase and 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide (PK 11195) were purchased from Worthington Biochemical (Freehold, NJ, U.S.A.) and Research Biochemicals International (Natick, MA, U.S.A.), respectively. Dulbecco's Modified Eagle's Medium (DMEM) and Hanks' balanced salt solution (HBSS) were from Gibco BRL (Grand Island, NY, U.S.A.). Compound 48/80 and fura-2 acetoxymethyl ester (fura-2/AM) were from Biomol Research Labs., Inc (Plymouth Meeting, PA, U.S.A.) and Dojindo Laboratories (Kumamoto, Japan), respectively. [^{3}H]IP_3 radioreceptor assay kit and [^{3}H]PIP_2 were purchased from Du Pont/New England Nuclear (Boston, MA, U.S.A.). Phosphatidylethanolamine (PE) and phosphatidylserine (PS) were from Avanti Polar-Lipids, Inc. (Birmingham, AL, U.S.A.). Flumazenil (FLZ) was a gift from Yamanouchi Pharmaceuticals (Tokyo, Japan). All other reagents used were of the highest grade commercially available. HBSS-H was composed of HBSS containing 1.27 mM $CaCl_2$, 0.81 mM $MgSO_4$, and 30 mM HEPES (pH 7.4). DZP, FLZ, and PK 11195 were dissolved in dimethyl sulphoxide. The other drugs were dissolved in distilled water.

Preparation of acinar cells

Parotid acinar cells were prepared according to a method described previously (Okubo & Kawaguchi, 1998). Briefly, the parotid glands were excised from rats anaesthetized with diethyl ether, cleaned, and finely minced in DMEM containing 1% BSA, collagenase (100 U ml^{-1}), and hyaluronidase (0.15 mg ml^{-1}). The minced glands were further incubated for 1 h at 37°C, with constant shaking at 100 cycles min^{-1} under an atmosphere of 95% O_2 and 5% CO_2. At 20-min intervals, the mince was dispersed by gently pipetting and gassed. Following enzyme digestion, the cells were washed three times with HBSS-H containing 0.01% BSA and further incubated for 20 min at 37°C in the same medium. The cells were filtered through a Nytex nylon screen (105 μm^2) and resuspended in fresh HBSS-H. More than 90% of the cells obtained were living. The cell preparation was usually carried out between 0900 and 1130 to avoid circadian variations among intracellular signalling factors.

Preparation of membranes

The membranes were prepared by homogenizing rat parotid glands in 10 volumes of cold 50 mM Tris/HCl (pH 7.4) containing 1 mM PMSF (buffer A), according to a method described previously (Hiramatsu *et al*., 1992). The homogenate was centrifuged at $3000 \times g$ for 10 min. The supernatant was filtered through gauze and then recentrifuged at $40{,}000 \times g$ for 10 min. The resulting pellet (membrane fraction) was washed once in buffer A and recentrifuged as above. Finally, the pellet was resuspended in buffer A and stored at −80°C until use. All preparative steps were performed at 4°C. The membranes prepared were used within one week.

Cholate extraction of membranes

The cholate extraction of membranes was carried out as described previously (Sawaki *et al*., 1993). The parotid gland membranes (approximately 5.0 mg of protein) were incubated with 2 M KCl in buffer A for 2 h. The concentration of KCl was diluted to 1 M with buffer A and the suspension was centrifuged at $105{,}000 \times g$ for 1 h. The pellet was suspended in buffer A containing 1% sodium cholate and 0.1 M NaCl, incubated for 2 h, and recentrifuged at $105{,}000 \times g$ for 1 h. This supernatant (cholate extract) was dialyzed overnight

against buffer A and stored at −80°C until use. All preparative steps were performed at 4°C.

Measurement of IP_3

The suspension of parotid cells (equivalent to 1.0–1.2 mg protein in pellet; see below) was preincubated with DZP or the receptor-related drugs for given periods at 37°C and then stimulated with CCh in a total volume of 0.5 ml. The CCh stimulation was terminated by the addition of 0.1 ml of ice-cold 20% perchloric acid and the sample was kept on ice for 20 min before centrifugation at $1000 \times g$ for 5 min. The pellet obtained was dissolved in 0.5 N NaOH and used for protein measurement. The supernatant was neutralized with 5 M KOH plus 0.5 M HEPES, kept on ice for 20 min, and recentrifuged to remove $KClO_4$ precipitate. A 0.1 ml aliquot of each sample was used in the IP_3 assay system. The amount of IP_3 was quantitatively determined from the calibration curve using a [^{3}H]IP_3 radioreceptor assay kit.

Measurement of $[Ca^{2+}]_i$

Dispersed parotid cells were seeded onto the nonfluorescence glass plate, loaded with 10 μM fura-2/AM in HBSS-H, and further incubated at 37°C for 1 h in the CO_2 incubator. After incubation, the cells were washed three times with HBSS-H and resuspended in the same medium. The fluorescence of fura-2 was measured by an ARGUS 50 imaging analysis system (Hamamatsu Photonics K.K., Hamamatsu, Japan) and monitored in 'ratio mode' by alternating the excitation wavelength between 340 and 360 nm and measuring the fluorescence emitted at 510 nm. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence using the calibration curve. Details of experiments are presented in the figure legends.

Measurement of PIP_2-specific PLC activity

The activity of PIP_2-specific PLC was measured according to the method described by Hiramatsu *et al*. (1992). The parotid gland membranes and the cholate extracts were added to 0.1 ml of assay medium containing (mM) HEPES 20 (pH 7.2), KCl 120, NaCl 20, $MgSO_4$ 1, LiCl 10, ATP 2, sodium deoxycholate 0.8, EGTA 1, $CaCl_2$ 0.37 to obtain a 100 nM free calcium concentration, and sonicated micelles of 0.1 mM [^{3}H]PIP_2, 0.1 mM PE, and 0.1 mM PS. For choline chloride experiments, the KCl, NaCl, and $CaCl_2$ in the assay medium were replaced with gluconates of K^+, Na^+, and Ca^{2+}. LiCl was omitted from the medium. Assays were performed at 30°C for 10 or 20 min in the absence or presence of various agents and stopped by the addition of 0.75 ml of $CHCl_3$/CH_3OH/HCl (100/200/2), 0.25 ml of $CHCl_3$, and 0.25 ml of 0.1 N HCl. The mixture was centrifuged at $1000 \times g$ for 5 min and the radioactivity of the hydrophilic products in the aqueous phase was measured by liquid scintillation counter. Enzymatic activity is expressed as nmol of PIP_2 hydrolysis mg protein^{-1} min^{-1}.

Measurement of protein concentration

Protein concentrations were determined according to the method of Lowry *et al*. (1951) using BSA as the standard.

Statistical analysis

Statistical analyses were performed with Student's *t*-test or Dunnett's multiple comparison test. *P* values of less than 5% were considered statistically significant.

Results

IP_3 production after muscarinic receptor stimulation

The time-course of IP_3 production stimulated by CCh in rat parotid cells is shown in Figure 1a. The production increased transiently, reaching a maximum in the first 5 s of stimulation, and then rapidly decreased. CCh stimulated IP_3 production in the cells according to concentration (Figure 1b). At 10^{-3} M, it induced a maximum response and caused about a 2-fold increase in IP_3 over the basal level. The concentration of CCh inducing half maximal IP_3 production was approximately 2.5×10^{-5} M. In subsequent experiments, IP_3 levels were measured 5 s after treatment with 10^{-4} M CCh.

Effect of DZP on CCh-stimulated IP_3 production

DZP dose-dependently decreased CCh-stimulated IP_3 production in rat parotid cells and elicited significant decreases at $> 5 \times 10^{-7}$ M (Figure 2). The IP_3 production was decreased to 63% of the maximal level at a concentration of 10^{-5} M DZP. The concentration inducing half maximal inhibition of IP_3 production was approximately 3.5×10^{-8} M. The inhibitory effect of DZP was produced by a short-term pretreatment (< 3 min) (data not shown). DZP alone had no observable effect on IP_3 levels (Figure 2). Unlike DZP, atropine, an antagonist of muscarinic receptor, completely abolished the effect of CCh on IP_3 production (Figure 4a).

Further studies revealed that DZP decreased maximal CCh-stimulated IP_3 production in rat parotid cells and the concentration-response curves for CCh were shifted down-

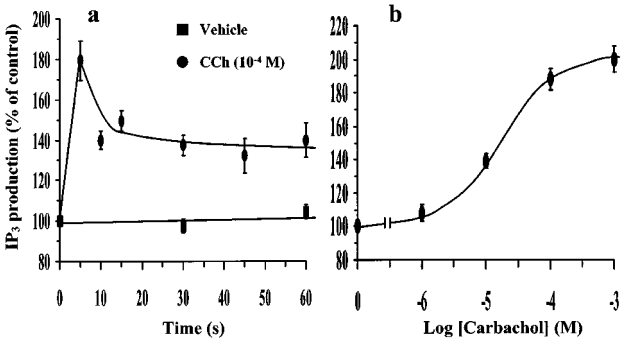


Figure 1 Time response effect of CCh (a) and effect of doses of CCh (b) on IP_3 production in rat parotid cells. (a) Parotid cells were incubated with or without CCh (10^{-4} M) for the periods indicated at 37°C after which IP_3 levels were measured. (b) Parotid cells were incubated with the indicated concentrations of CCh for 5 s at 37°C after which IP_3 contents were measured. The IP_3 level is shown as a percentage of the basal value taken as 100%. Basal IP_3 values of (a) and (b) experiments were 8.92±0.76 and 8.66±0.51 pmol mg protein^{-1}, respectively. The data are expressed as the mean±s.e.mean of three experiments.

wards by DZP (Figure 3a). Furthermore, double reciprocal plots of the data showed an apparent convergence of CCh concentrations (approximately 3×10^{-4} M) and a decrease in the maximal velocity for IP_3 production: the values for vehicle, 10^{-7} M DZP, and 10^{-5} M DZP were approximately 32.3, 27.8, and 23.8 pmol mg protein^{-1}, respectively (Figure 3b). These results indicate that DZP exhibits a noncompetitive inhibition of CCh-stimulated IP_3 production in rat parotid cells.

Effect of BDZ receptor-related compounds on DZP inhibitory response

To clarify whether specific BDZ receptors are involved in the inhibitory effect of DZP on CCh-stimulated IP_3 production in rat parotid cells, we examined the effects of antagonist and competing ligand for the central- and peripheral-type BDZ receptors. At the concentrations studied, PK 11195, a competing ligand for the peripheral-type receptor, and FLZ, an antagonist for the central-type receptor, suppressed >40% of the inhibitory effect of DZP (Figure 4). However, when both agents were present simultaneously, the inhibitory response was completely blocked (Figure 4b). These results suggest that both central- and peripheral-type BDZ receptors contribute to the inhibitory effect of DZP on CCh-stimulated IP_3 production in rat parotid cells.

Effect of DZP on G protein-dependent PIP_2-specific PLC activity

We further examined whether DZP directly modifies the activity of G protein-dependent PIP_2-specific PLC in rat parotid gland membranes. AlF_4^- ($NaF + AlCl_3$) binds to the GDP-bound form of the G proteins and, by mimicking the terminal phosphate of GTP, activates G_α subunits which can

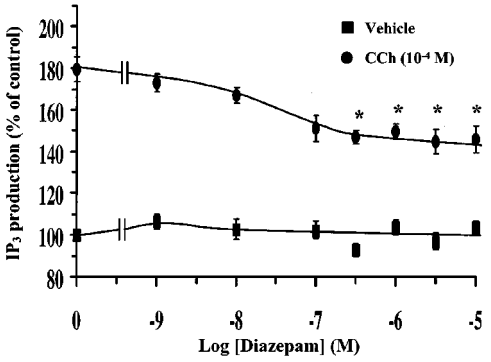


Figure 2 Effect of DZP on CCh-stimulated IP_3 production in rat parotid cells. After preincubation in the absence or presence of the indicated concentrations of DZP for 5 min at 37°C, parotid cells were further incubated with or without CCh (10^{-4} M) for 5 s at 37°C. The basal IP_3 value was 8.56 ± 0.50 pmol mg protein^{-1}. The data are expressed as the mean ± s.e.mean of three to four experiments. $^*P < 0.05$, compared to the values obtained with CCh alone in the absence of DZP.

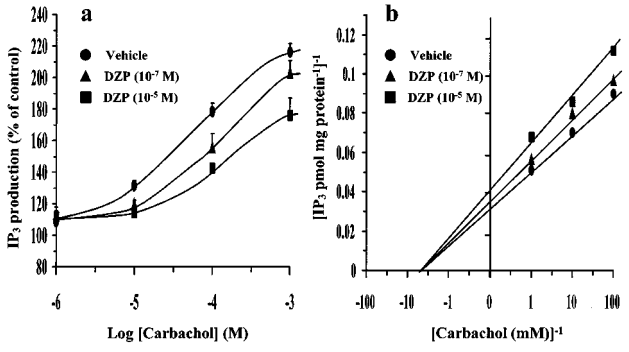
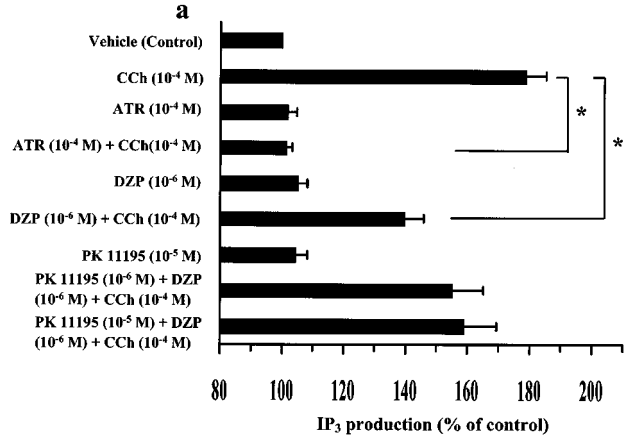


Figure 3 Effect of DZP on the concentration-response curves for CCh-stimulated IP_3 production in rat parotid cells. (a) After preincubation in the absence or presence of DZP (10^{-7} and 10^{-5} M) for 5 min at 37°C, parotid cells were further incubated with the indicated concentrations of CCh for 5 s at 37°C. The basal IP_3 value was 8.09 ± 0.44 pmol mg protein^{-1}. The data are expressed as the mean ± s.e.mean of four experiments. (b) Double reciprocal plots are taken from the data of experiment (a). The ordinate and abscissa represent the reciprocals of IP_3 production and CCh concentration, respectively.



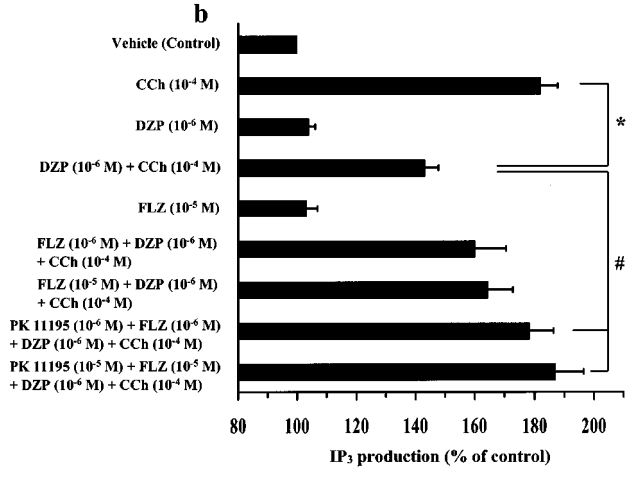


Figure 4 Effects of PK 11195 (a) and flumazenil (b) on the inhibitory effect of DZP on CCh-stimulated IP_3 production in rat parotid cells. After preincubation in the absence or presence of DZP, atropine (ATR), PK 11195, and flumazenil (FLZ) for 5 min at 37°C, parotid cells were further incubated with or without CCh (10^{-4} M) for 5 s at 37°C. Basal IP_3 levels of (a) and (b) experiments were 9.03 ± 0.65 and 9.22 ± 0.74 pmol mg protein^{-1}, respectively. The data are expressed as the mean ± s.e.mean of three to five experiments. $^*P < 0.05$ and $\#P < 0.05$, compared to the values obtained with CCh alone or DZP plus CCh, respectively.

then interact with PIP_2-specific PLC (Gilman, 1987). Table 1 shows that AlF_4^- stimulated by about 2-fold, PLC activity in the membranes, compared to control levels obtained with $NaCl + AlCl_3$ and that DZP inhibited only slightly this enzymatic activity (<10%) at a high concentration of 10^{-5} M. These results suggest that DZP does not directly modify G proteins which activate PLC.

We next examined the effect of DZP on PIP_2-specific PLC. Cholate extract of the membranes was prepared to measure direct PLC activity. It was reported that when rat parotid gland membranes were treated with 1% sodium cholate, more than 80% of this enzymatic activity was obtained in the cholate extract uncoupled from the receptor and G protein; e.g., the solubilized enzyme was not stimulated by CCh and $GTP_\gamma S$ (Sawaki *et al*., 1993). PLC activity in the cholate extract was not inhibited by DZP, but significantly inhibited by compound 48/80, a potent inhibitor of PLC (Table 2). These results also suggest that DZP does not directly inhibit the activity of PIP_2-specific PLC.

Effect of chloride on PIP_2-specific PLC activity

We previously reported that DZP increased the influx and decreased the efflux of chloride in rat parotid cells (Kawaguchi *et al*., 1995). Consequently, the chloride increased in the cells might directly affect the activity of PIP_2-specific PLC. To clarify more the inhibitory mechanism of DZP, we examined the effect of chloride on PLC activity. For this experiment, we used choline chloride and measured the enzymatic activity in the modified assay medium (see Methods). As shown in Table 3, choline chloride dose-dependently inhibited the enzymatic activity in the parotid gland membranes and cholate extract of the membranes. The activity was decreased to approximately 80% maximal control levels. This result suggests that PIP_2-specific PLC in the parotid cells might be inhibited by the intracellular chloride concentration.

Table 1 Effect of DZP on $A1F_4^-$-stimulated PIP_2-specific PLC activity in rat parotid gland membranes

Drug		*PIP_2 hydrolysis* (nmol mg protein^{-1} min^{-1})
10 mM NaCl + 30 μM $A1Cl_3$		3.74 ± 0.09 (100)
+ DZP	10^{-7} M	3.77 ± 0.11 (101)
	10^{-5} M	3.66 ± 0.13 (98)
10 mM NaF + 30 μM $A1Cl_3$		7.46 ± 0.23 (100)
+ DZP	10^{-8} M	7.48 ± 0.20 (100)
	10^{-7} M	7.56 ± 0.21 (101)
	10^{-6} M	6.99 ± 0.14 (94)
	10^{-5} M	6.77 ± 0.06* (91)

PLC activity was measured in the presence of either 10 mM NaCl + 30 μM $A1Cl_3$ (control) or 10 mM NaF + 30 μM $A1Cl_3$ (AlF_4^-). Parotid gland membranes (10 μg) were incubated with or without DZP in the presence of NaCl, NaF, and $A1Cl_3$ for 20 min at 30°C. All values are expressed as the mean ± s.e.mean of three to four experiments. The values in parentheses indicate the percentage of the value obtained with DZP, with the no-addition condition taken as 100%. *P<0.05, compared to the values in the absence of DZP.

Table 2 Effects of DZP and compound 48/80 on PIP_2-specific PLC activity in cholate extract of rat parotid gland membranes

Drug		*PIP_2 hydrolysis* (nmol mg protein^{-1} min^{-1})
Vehicle		25.80 ± 1.36 (100)
+ DZP	10^{-8} M	26.42 ± 1.45 (103)
	10^{-7} M	26.26 ± 1.44 (102)
	10^{-6} M	25.86 ± 1.20 (100)
	10^{-5} M	26.19 ± 1.34 (101)
Vehicle		24.99 ± 0.53 (100)
+ Compound 48/80	10^{-6} M	24.68 ± 1.03 (99)
	5×10^{-6} M	19.41 ± 0.49* (78)
	10^{-5} M	12.18 ± 1.27** (49)

PLC activity was measured in the absence of muscarinic receptor agonist and G protein stimulators. Cholate extract (10 μg) was incubated with DZP, compound 48/80 or vehicle for 10 min at 30°C. All values are expressed as the mean ± s.e.mean of three experiments. The values in parentheses indicate the percentage of the value obtained with DZP or compound 48/80, with the no-addition condition taken as 100%. *P<0.05; **P<0.01, compared to the values in the absence of compound 48/80.

Table 3 Effect of choline chloride on PIP_2-specific PLC activity in rat parotid gland membranes and cholate extracts

Drug		*PIP_2 hydrolysis* (nmol mg protein^{-1} min^{-1})
Membrane		
1 mM CCh + 10 μM $GTP\gamma S$		7.41 ± 0.70 (100)
+ Choline chloride	50 mM	6.90 ± 0.42 (93)
	100 mM	6.21 ± 0.41* (84)
	150 mM	5.80 ± 0.47** (78)
Cholate extract		
Vehicle		29.17 ± 0.57 (100)
+ Choline choloride	50 mM	28.74 ± 1.37 (98)
	100 mM	27.06 ± 1.00 (93)
	150 mM	24.29 ± 0.70** (83)

PLC activity in the membrane and cholate extract was measured in the presence of 1 mM CCh + 10 μM $GTP\gamma S$ or in the absence of muscarinic receptor agonist and G protein stimulators. Parotid gland membranes (10 μg) and cholate extract (10 μg) were incubated with or without choline chloride for 10 or 20 min at 30°C in the modified assay medium described in 'Methods'. All values are expressed as the mean ± s.e.mean of three to four experiments. In the membranes, the basal PIP_2 hydrolysis was 2.68 ± 0.52 nmol mg protein^{-1} min^{-1}. The values in parentheses indicate the percentage of the value obtained with choline chloride, with the no-addition condition taken as 100%. *P<0.05; **P<0.01, compared to the values in the absence of choline chloride.

Effect of DZP on the intracellular Ca^{2+} response

We examined the effect of DZP on the intracellular Ca^{2+} response, which is mobilized by IP_3, after stimulation of the muscarinic receptor in rat parotid cells. At 10^{-5} M, a concentration which inhibits CCh-stimulated IP_3 production, DZP clearly attenuated the rapid increase in $[Ca^{2+}]_i$, which reached a maximum in <10 s, after the stimulation with CCh (Figure 5a,b). Decreases in $[Ca^{2+}]_i$ were observed in both the presence and absence of extracellular Ca^{2+} (i.e., Ca^{2+}-free HBSS-H + 3 mM EGTA), to approximately 72 and 67% of

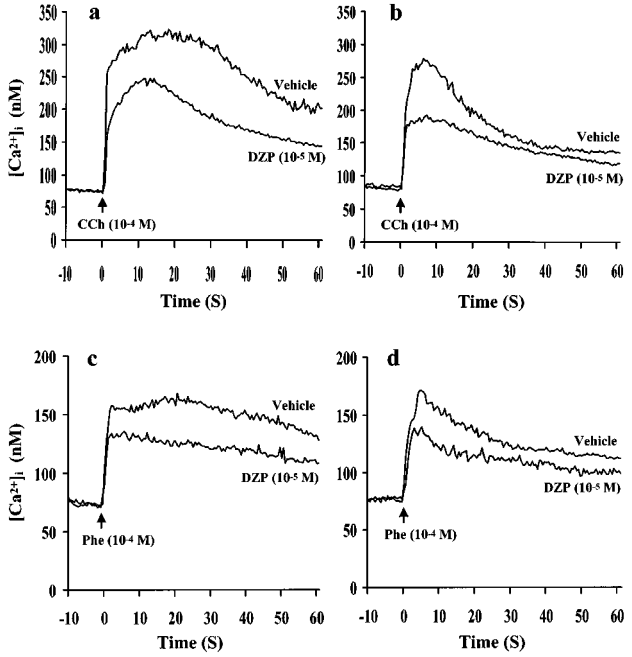


Figure 5 Effect of DZP on the CCh- and phenylephrine-stimulated Ca^{2+} response in rat parotid cells. Parotid cells loaded with fura-2/AM were preincubated with or without DZP (10^{-5} M) for 5 min and then $[Ca^{2+}]_i$ following stimulation of CCh (10^{-4} M) or phenylephrine (Phe; 10^{-4} M) was measured as described under 'Methods'. CCh and Phe were added at the arrow. Cells were incubated in HBSS-H (a, c) or Ca^{2+}-free HBSS-H + 3 mM EGTA (b, d). The traces are presented as the average of results obtained with six or seven cells.

the maximal control level, respectively. DZP alone did not induce any detectable changes in $[Ca^{2+}]_i$.

Similar decrease in $[Ca^{2+}]_i$ was also observed when the cells were stimulated with phenylephrine, an agonist of α_1-adrenoceptor (Figure 5c,d). This result suggests that DZP also suppresses phosphoinositide turnover linked to α_1-adrenoceptor as well as that to the muscarinic receptor.

Discussion

The regulation by neurotransmitters of fluid secretion in the salivary glands is achieved by a coordinated sequence of Ca^{2+} signalling events (Baum, 1987; Ambudkar, 2000). An increase in $[Ca^{2+}]_i$ is a consequence of the hydrolysis of membrane phospholipid PIP_2 *via* activation of G protein and PIP_2-specific PLC after stimulation of m_3-muscarinic and α_1-adrenoceptors (Tanimura *et al*., 1999). The generation of IP_3 releases Ca^{2+} from internal stores, and induces Ca^{2+} entry (Merritt & Rink, 1987; Hughes *et al*., 1988; Ambudkar *et al*., 1990; Horn *et al*., 1990; Hiramatsu *et al*., 1992; Sawaki *et al*., 1993; 1995). Thus, IP_3 produced immediately after the stimulation of receptors plays a very important role in triggering, as well as maintaining, sustained fluid secretion in the salivary glands.

The present results show that DZP has a significant inhibitory effect on muscarinic receptor-stimulated IP_3 production in rat parotid cells at the concentrations of $>5\times10^{-7}$ M (Figure 2). At 10^{-5} M DZP, IP_3 production was decreased to 63% of the maximal level. The concentration of DZP inducing half maximal inhibition was approximately 3.5×10^{-8} M, which was very similar to that of its binding affinity for rat parotid central- and peripheral-type BDZ receptors, namely 18×10^{-8} M and 1.2×10^{-8} M, respectively (Yamagishi & Kawaguchi, 1998). In this study, the inhibitory concentration of DZP was higher than the concentration which was necessary to saturate the receptor, but in the range of circulating levels in patients taking DZP at the clinical dosage commonly used for sedation; e.g., 5 mg twice daily (Trevor & Way, 2001). However, it has been reported that it is not unusual for the functional inhibitory concentration of a drug to be different from its concentration for binding to a receptor (Möhler & Okada, 1977).

DZP also attenuated the increase in $[Ca^{2+}]_i$ caused by CCh and phenylephrine, which immediately occurs subsequent to IP_3 production in rat parotid cells, in both the presence and absence of extracellular Ca^{2+} (Figure 5). In the CCh stimulation, the decrease in $[Ca^{2+}]_i$, at approximately 28 and 33%, was very similar to that in IP_3 production. The results shown in Figure 5 suggest that DZP suppresses phosphoinositide turnover linked to the muscarinic and α_1-adrenoceptors, and that not only the Ca^{2+} released from intracellular stores by IP_3, but DZP inhibits the entry of Ca^{2+} from the external medium. Therefore, the present results clearly indicate that in rat parotid cells, the decrease in IP_3 production induced by DZP is closely correlated to that in $[Ca^{2+}]_i$ and that BDZ receptors in this gland are likely associated with these inhibitory effects of DZP.

Central-type BDZ receptors linked to the $GABA_A$ receptor-chloride channel complex are located on the plasma membrane and are related to the anticonvulsant, anxiolytic, and hypnotic actions of BDZs (Skolnick & Paul, 1982; Greenblatt *et al*., 1983). On the other hand, peripheral-type BDZ receptors are localized typically in the mitochondrial outer membrane but can also be associated with non-mitochondrial (including plasma) membrane (Anholt *et al*., 1986; Olson *et al*., 1988; O'Beirne *et al*., 1990; Woods & Williams, 1996). In rat parotid glands, the existence of both central- and peripheral-type BDZ receptors has been clearly demonstrated in crude plasma membrane (Yamagishi & Kawaguchi, 1998; Yamagishi *et al*., 2000). However, the exact subcellular location(s) of the peripheral-type BDZ receptors in this gland has not yet been established. Thus, conceivably, both types of BDZ receptors could be present in the same cell membrane. The actions of peripheral-type BDZ receptors have been described in a variety of biological systems, some having direct effects on the physiological control of anxiety or stress independent of the central-type receptors, or on cardiac function (Gavish *et al*., 1992). A physiologic role for mitochondrial BDZ receptors in steroidogenesis and the release of Ca^{2+} from heart and kidney mitochondria is recognized (Moreno-Sanchez *et al*., 1991; Krueger & Papadopoulos, 1993). The exact role of non-mitochondrial BDZ receptor-mediated effects in several other peripheral tissues including the salivary glands has not yet been established (Woods & Williams, 1996).

Antagonist and competing ligand for the central- and peripheral-type BDZ receptors, FLZ and PK 11195, respectively, suppressed more than 40% of the inhibitory effect of DZP on muscarinic receptor-stimulated IP_3 production and a combination of both agents completely blocked the inhibitory activity (Figure 4). At the most effective concentrations, DZP

did not completely inhibit CCh-stimulated IP_3 production, whereas atropine, an antagonist of muscarinic receptor, completely inhibited the production (Figures 2 and 4). Further, DZP did not directly inhibit the activities of G protein and PIP_2-specific PLC in rat parotid gland membrane (Tables 1 and 2). In radioreceptor binding assays, DZP did not affect the binding of [^{3}H]quinuclidinyl benzilate to muscarinic receptors in this membrane (our unpublished data). These results strongly suggest that the inhibitory effect of DZP on CCh-stimulated IP_3 production is not a direct action on muscarinic receptors, G protein and PIP_2-specific PLC in the membranes, but one mediated through both types of BDZ receptors. This is supported by the results in that: DZP shifted downwards the concentration-response curves for CCh-stimulated IP_3 production and showed a noncompetitive inhibition of the production (Figure 3).

It has been reported that DZP decreased somatostain-stimulated IP_3 accumulation in the rat brain after its administration *in vivo* and this inhibitory effect might be a consequence of the decrease in the number of somatostain receptors coupled with the activation of phosphoinositide-specific PLC (Martínez-Ferrer *et al*., 2000). In the parotid cells, however, the exact mechanism by which DZP inhibited CCh-stimulated IP_3 production is not clear. We have previously found that, *in vitro*, DZP enhanced the influx and decreased the efflux of chloride in rat parotid cells (Kawaguchi *et al*., 1995). Therefore, a change in the intracellular chloride concentration could influence phosphoinositide turnover related to IP_3 production in the cells (e.g., Higashijima *et al*., 1987). To examine the effect of chloride on PIP_2-specific PLC, we measured the enzymatic activity in the presence of choline chloride (Table 3). The results strongly suggested that in the parotid cells, the chloride increased by DZP might directly inhibit PIP_2-specific PLC activity, resulting in the decreases in IP_3 production and elevation of $[Ca^{2+}]_i$. This is because the intracellular chloride concentration in rat parotid cells is approximately 69 mM (Turner, 1993) and PLC activities in the membranes and cholate extract were inhibited at relatively high concentrations of choline chloride (>100 mM) (Table 3). Another possible explanation is that DZP-induced interactions of the receptor with clathrin-coated vesicles (Tehrani *et al*., 1997) or annexins (Hofmann *et al*., 1998) may perturb the exocytotic routing pathway.

In conclusion, the present results demonstrated that DZP acted directly on rat parotid cells and inhibited muscarinic receptor-stimulated IP_3 production through BDZ receptors. Further, the decreases in IP_3 and $[Ca^{2+}]_i$ in the cells may be connected with the suppression of salivary secretion induced by DZP.

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