Biochemical Pharmacology, Vol. 38, No. 7, pp. 1069–1074, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00
© 1989. Pergamon Press plc

MONOVALENT ION ENHANCEMENT OF β-ADRENERGIC-STIMULATED ADENYLATE CYCLASE ACTIVITY IN MOUSE PAROTID GLAND

EILEEN L. WATSON*, KERRY L. JACOBSON and JEAN C. SINGH

Departments of Oral Biology and Pharmacology, and Center for Research in Oral Biology, University of Washington, Seattle, WA 98195, U.S.A.

(*Received* 31 *August* 1987; *accepted* 3 *August* 1988)

Abstract—Sodium activated basal adenylate cyclase at all concentrations of sodium examined (5–100 mM) and independently of GTP. Stimulation of adenhylate cyclase by the β-adrenergic agonist, isoproterenol, was enhanced at all concentrations (5–100 mM) of sodium ions tested in the presence of GTP. Maximal enzyme activation under all conditions occurred between 25 and 50 mM NaCl. Enhancement of forskolin-activated adenylate cyclase by sodium did not require GTP nor was it affected by guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS), a competitive inhibitor of GTP. The selectivity of adenylate cyclase for monovalent cations was $Na^+ \simeq K^+$. Lithium chloride produced an inhibition of hormone-activated adenylate cyclase. Sodium ions also enhanced isoproterenol- and forskolin-activated adenylate cyclase of submandibular gland membranes. In contrast to mouse parotid and submandibular glands, activation of mouse liver and brain adenylate cyclase activities by forskolin and isoproterenol was not enhanced by sodium ions. The tissue differences were not related to differences in potency of the agonists. These results suggest (1) that sodium ions may have a selective and positive regulatory role in hormonal activation of adenylate cyclase in mouse exocrine tissue, and (2) that sodium ions enhance hormonal activation of enzyme by interacting at a site on the adenylate cyclase complex which is independent of the hormone receptor (R_s) and the stimulatory guanine nucleotide binding protein (N_s).

In mouse parotid gland, β-adrenergic agonists stimulate amylase release by mechanisms involving cyclic AMP (cAMP†) [1]. Hormonal stimulation of adenylate cyclase-catalyzed cAMP formation involves the interaction of at least three membrane-associated components: hormone receptor (R_s), guanine nucleotide binding protein (N_s) and catalytic component (C). Recent studies have demonstrated that sodium ions may play a regulatory role in the stimulation of amylase release and cAMP accumulation by the β-adrenergic agonist, isoproterenol, in mouse parotid gland [2, 3]. Sodium ions were found to be required for activation of both processes. Monovalent cations, particularly sodium, have been shown to have effects in modulating both hormonal stimulation and inhibition of cAMP accumulation and adenylate cyclase activity in a variety of tissues [4–11]. Recent evidence suggests that sodium ions increase adenylate cyclase inhibition by inhibitory hormones and reduce the potency of the adenylate cyclase stimulatory hormones [6]. Jakobs *et al.* [12] demonstrated that sodium ions influence the inhibitory and stimulatory transduction processes to the adenylate cyclase in an identical manner and that the sodium effect is an inhibitory one in both systems. Evidence for a stimulatory or enhancing effect of sodium on hormonal-activated adenylate cyclase, however, is lacking.

The goal of the present study was to extend our initial observations in parotid gland [2, 3] by examining the effects of sodium on β-adrenergic and forskolin-activated adenylate cyclase in parotid membranes. The effect of sodium on forskolin-activated adenylate cyclase in the absence or presence of GDPβS, a competitive inhibitor of GTP, was also ascertained. To examine the selectivity of the effects of sodium on adenylate cyclase activity in mouse parotid membranes, we also examined the effects of sodium on mouse liver and brain membranes.

MATERIALS AND METHODS

Materials. *dl*-Isoproterenol, pyruvate kinase, phosphoenolpyruvate, ATP, mercaptoethanol, bovine serum albumin (BSA), and GTP were obtained from the Sigma Chemical Co.; GDPβS was obtained from Boehringer Manheim Biochemicals, [α-^{32}P]ATP (10–30 Ci/mmol) from Amersham, [^{3}H]cAMP from New England Nuclear, and forskolin from Calbiochem.

Preparation of membranes. Parotid glands were removed from Swiss Webster mice in the manner previously described by Watson *et al.* [13]. In addition, mouse submandibular gland, liver and whole brain were also removed, and membranes were prepared by homogenizing glands in ice-cold

* Send correspondence to: Eileen L. Watson, Ph.D., Department of Oral Biology, SB-22, University of Washington, Seattle, WA 98195.

† Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; GDPβS, guanosine-5′-*O*-(2-thiodiphosphate) N_s and N_i, the guanine nucleotide binding proteins associated with stimulation and inhibition; C, the catalytic subunit of adenylate cyclase; R_s, hormone receptor; and BSA, bovine serum albumin.

buffer containing 0.25M sucrose, 10 mM Tris–HCl and 10 mM $MgCl_2$ at pH 7.5. Homogenates were centrifuged at 20,000 *g* at 4° for 20 min. Pellets were washed once with the above buffer, recentrifuged, and resuspended in 10 mM Tris–HCl and 6 mM $MgCl_2$, pH 7.5. Fresh membranes were utilized in all experiments.

Measurement of adenylate cyclase activity. Adenylate cyclase was assayed by the methods of Chiu *et al.* [14] and Krishna *et al.* [15]. Membranes were incubated in a final volume of 0.1 ml in a reaction mixture containing 25 mM Tris–HCl buffer, pH 7.5, 6 mM $MgCl_2$, 3 mM mercaptoethanol, 0.0075% bovine serum albumin, 1 mM cAMP, [α-^{32}P]ATP (1 mM, 1 μCi), 5.4 mM phosphoenolpyruvate and 8 μg pyruvate kinase. The sodium concentration in the incubation medium, present as sodium salts of ATP, cAMP and phosphoenolpyruvate, was 7.5 mM. The sodium concentrations presented in Figs. 1–3 have had this value subtracted. Assays were initiated by addition of enzyme (50–100 μg), carried out at 37° for 10 min, and terminated by addition of 0.1 ml of a solution containing 10 mM ATP and 1 mM cyclic [^{3}H]AMP (10,000 cpm) at pH 7.4. The adenylate cyclase assay was linear with respect to time and membrane protein concentration. Assays were performed in duplicate, and activity was expressed as picomoles of cAMP per milligram of protein per 10 minutes. Protein was determined by the method of Lowry *et al.* [16].

To determine whether the effects of monovalent cations were due to specific effects on adenylate cyclase rather than non-specific changes in ionic strength, sucrose and *N*-methyl-D-glucamine, a non-ionic solute, were used to replace sodium in the incubation medium to maintain osmolality. Sucrose and *N*-methyl-D-glucamine at concentrations up to 100 mM were without effect on adenylate cyclase activity.

Statistical analysis. Data are presented as mean ± standard error. Statistical analysis was performed using a paired *t*-test. Differences with P values <0.05 were considered significant.

RESULTS

Since sodium ions have been reported to regulate β-adrenergic-stimulated cAMP accumulation in mouse parotid gland [2], the effects of sodium ions on basal, GTP and β-adrenergic (isoproterenol)-activated adenylate cyclase were examined. The results are presented in Fig. 1. In the absence of GTP, sodium stimulated adenylate cyclase in a dose-dependent manner with maximum stimulation occurring at between 25 and 50 mM sodium chloride. In the presence of GTP (10 μM), a concentration which alone produces maximal activation of adenylate cyclase in mouse parotid gland [17], sodium produced further stimulation of enzyme activity. Percent stimulations with 25, 50 and 100 mM sodium chloride plus GTP were 34.5, 37.9 and 35.1% respectively. In the presence of isoproterenol (10^{-6} M) plus GTP, adenylate cyclase was significantly ($P < 0.05$) enhanced by sodium ions with maximal activation also occurring between 25 and 50 mM NaCl. The percent increases by sodium were 54.9, 63.9 and 54.1%, with 25, 50 and 100 mM NaCl respectively. As reported for other tissues, hormone-induced stimulation of adenylate cyclase was dependent on the presence of GTP.

To determine whether the enhancing effect of sodium on β-adrenergic-stimulated cAMP levels was related to receptor interaction, the effects of sodium were examined on forskolin-stimulated adenylate

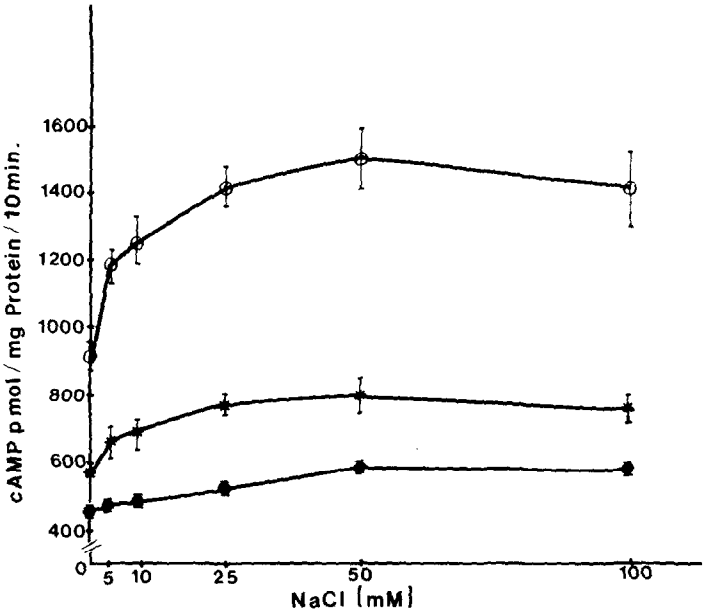

Fig. 1. Effect of sodium ions on isoproterenol (10^{-6} M) stimulated adenylate cyclase activity. Enzyme activity was determined in the absence (●—●), and presence (*—*) of 10 μM GTP and in the presence of isoproterenol plus GTP (○—○). Each point is the mean ± SE of six to eight experiments performed in duplicate.

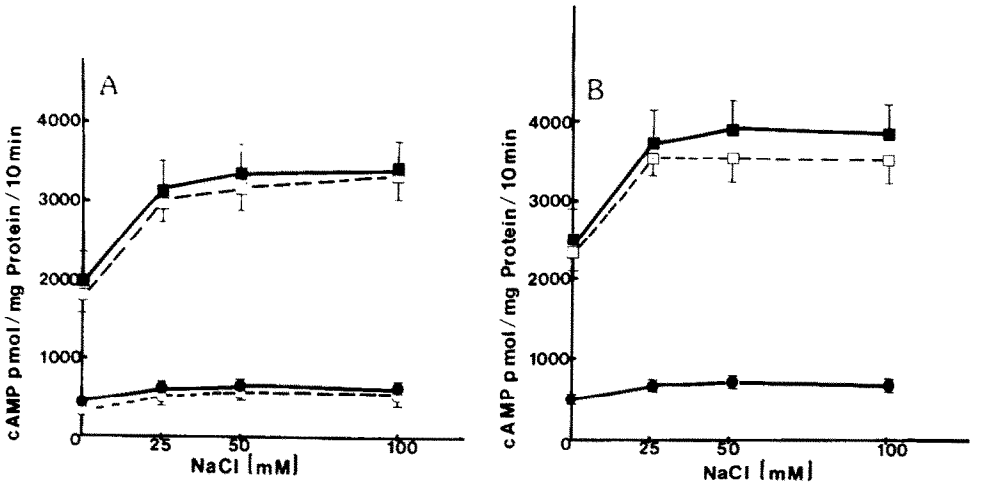

Fig. 2. Effect of sodium ions on forskolin (10 μM) stimulated adenylate cyclase activity. (A) Enzyme activity was determined in the presence (●—●) and absence (○—○) of 10 μM GTP, and in the presence of forskolkin with (■—■) and without (□—□) GTP. (B) Enzyme activity was determined in the presence of GTP (●—●), and in the presence of forskolin plus GTP wioth (□—□) and without (■—■) GDPβS (100 μM). Each point is the mean of ± SE of three experiments.

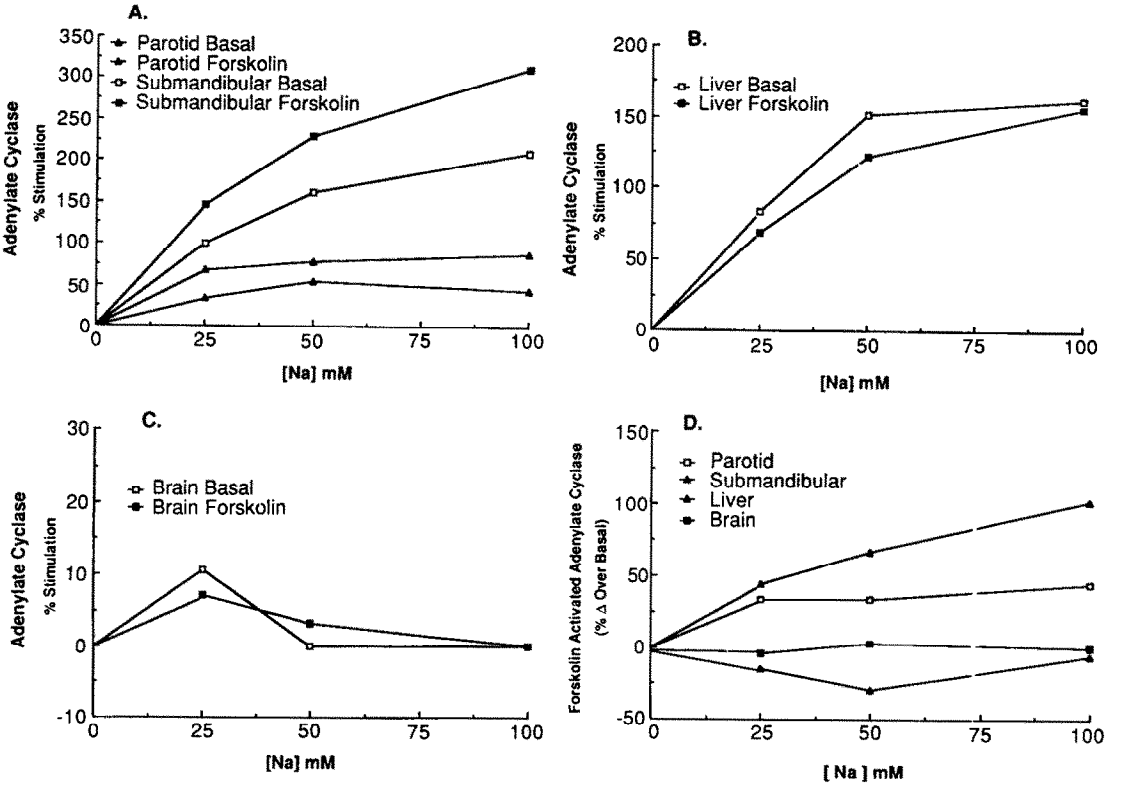

Fig. 3. Effect of sodium ions on adenylate cyclase activity. Enzyme activity was determined in the absence of GTP and in the absence or presence of forskolin (1 μM). (A, B and C) Percent stimulation of adenylate cyclase in mouse parotid, submandibular, liver and brain tissues. Note difference in scales. (D) Forskolin-activated adenylate cyclase, percent over basal. Basal specific activities were 387.5 ± 50, 51.6 ± 6.0, 18.1 ± 1.0 and 714.5 ± 92.0 pmol/mg protein/10 min for parotid, submandibular, liver and brain respectively. Results are presented as the mean ± SE of three to six experiments with duplicate determinations.

cyclase since forskolin has been shown not to require receptor interaction for its effects [18]. As shown in Fig. 2, sodium ions also enhanced forskolin (10 μM) stimulated adenylate cyclase activity. Adenylate cyclase activity in the presence of 25, 50 and 100 mM NaCl plus GTP (Fig. 2A) was increased by 55.0, 65.1 and 67.5% respectively. Enhancement of forskolin-activated adenylate cyclase by sodium ions also occurred in the absence of GTP (Fig. 2A), and was not influenced by GDPβS, a competitive inhibitor of GTP [19, 20] (Fig. 2B). In other experiments we determined whether sodium ions would alter the isoproterenol concentration–response curve for adenylate cyclase stimulation. Concentration–response curves for isoproterenol were similar in the absence or presence of sodium; the EC_{50} values were 1.5×10^{-7} M and 1.2×10^{-7} M in the absence and presence of sodium respectively.

To assess whether sodium selectively enhances parotid gland adenylate cyclase, other sources of enzyme were examined including another salivary gland i.e. submandibular. Results presented in Fig. 3 show that, in the absence of GTP, sodium ions stimulated basal mouse parotid, submandibular, and liver adenylate cyclase activities; basal values were 387.5 ± 50, 51.6 ± 6.0, and 18.1 ± 1.0 pmol/mg protein/10 min respectively. For mouse brain membranes, increasing the sodium concentration did not produce a significant change in basal activity (714.5 ± 92.0 pmol/mg protein/10 min). In the presence of forskolin, adenylate cyclase was stimulated markedly in all tissues examined; adenylate cyclase activities of parotid, submandibular, liver and brain increased by 361, 374, 647 and 276%, respectively, above basal values. In the presence of various sodium concentrations, the percent stimulation of forskolin-activated adenylate cyclase in mouse parotid and submandibular glands (Fig. 3A) was significantly ($P < 0.05$) greater than the percent stimulation of basal activities at all concentrations of sodium examined. Different results were obtained for both liver and brain membranes. Although sodium increased enzyme activity of mouse liver membranes (Fig. 3B), the percent stimulation of forskolin-activated adenylate cyclase was less than that observed for basal activities at all sodium concentrations. The inhibitory effect was most pronounced with 50 mM sodium. For mouse brain membranes (Fig. 3c), varying the sodium concentration produced no significant change in percent stimulation of basal or forskolin-activated adenylate cyclase. Figure 3D is a composite of data presented in A, B, and C expressed as percent change over basal for forskolin-activated adenylate cyclase activity.

To determine whether the tissue differences noted in Fig. 3 were related to differences in potency of the agonist concentrations employed, the effect of 50 mM sodium on adenylate cyclase activity of parotid and submandibular glands, brain and liver was examined in the presence of various concentrations of isoproterenol and forskolin. The results presented in Fig. 4 (expressed as a percent Δ over basal) show that in the presence of 50 mM sodium activation of parotid, submandibular and brain adenylate cyclases by either isoproterenol or forskolin was independent of the concentration of agonist utilized. Some dif-

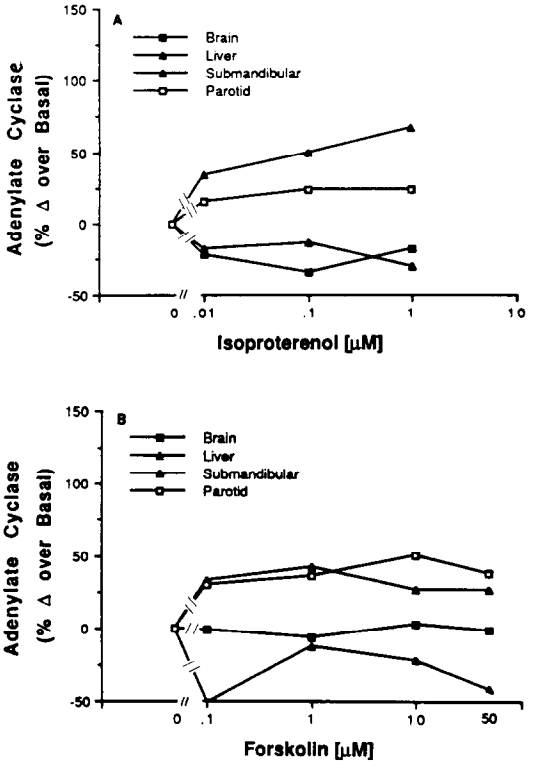

Fig. 4. Effect of sodium ions (50 mM) on adenylate cyclase activation in the presence of various concentrations of (A) isoproterenol and (B) forskolin. Enzyme activities were determined in the presence of 10 μM GTP (A) and in the absence of GTP (B). Results are expressed as a percent over basal activity and presented as the mean of four experiments with duplicate determinations. Basal activities in panel A were: 425.7 ± 74.3, 247.4 ± 44.0, 63.2 ± 3.4, and 871.7 ± 79.0 pmol/mg protein/10 min for parotid, submandibular, liver and brain respectively. Basal activities in panel B were: 362.0 ± 61.0, 88.2 ± 18.8, 18.6 ± 1.2, and 791.7 ± 94.0 pmol/mg protein/10 min for parotid, submanidibular, liver and brain respectively.

Table 1. Effect of monovalent cations on isoproterenol-stimulated adenylate cyclase

Drug	Cation	Adenylate cyclase Stimulation (%)	Adenylate cyclase Inhibition (%)
None	Na^+	51.3 ± 10.7	
	K^+	74.5 ± 17.3	
	Li^+	4.4 ± 2.4	
Isoproterenol	Na^+	64.9 ± 7.6*	
	K^+	112.5 ± 12.9*	
	Li^+		6.2 ± 2.1

Adenylate cyclase activity was measured in the presence of GTP (10 μM) in the absence and presence of isoproterenol (10^{-6} M). Each cation was present as the chloride salt at 25 mM. The specific activity was 335.2 ± 45.3 pmol/mg protein/10 min and 747.2 ± 140.0 pmol/mg protein/10 min in the absence and presence of isoproterenol respectively. Results are the means ± SE of five to seven experiments with duplicate determinations.

* $P < 0.05$ (isoproterenol vs control).

ferences were noted in liver adenylate cyclase activity in the presence of forskolin (Fig. 4B), however, the end result was an inhibition of enzyme activity.

We next examined the effects of other monovalent cations, K^+ and Li^+, on GTP- and β-adrenergic-stimulated adenylate cyclase in mouse parotid membranes; the results are shown in Table 1. The stimulatory effects noted with NaCl were also observed with KCl (25–100 mM). KCl was as effective as NaCl in stimulating adenylate cyclase. LiCl slightly stimulated basal adenylate cyclase while inhibiting isoproterenol-stimulated activity. The inhibitory response of LiCl on isoproterenol-stimulated activity was concentration dependent up to 100 mM LiCl (data not shown).

DISCUSSION

Hormones and drugs have been demonstrated to inhibit as well as stimulate cAMP levels in various tissues. It has been postulated that hormonal attenuation of cAMP levels in intact cells results from either decreased synthesis of cAMP or increased phosphodiesterase activity. In hormonal systems coupled to inhibition of adenylate cyclase activity, sodium ions have been shown to have a regulatory role in enhancing hormonal attenuation of adenylate cyclase [4, 7, 21]. Aktories *et al.* [5] demonstrated that sodium ions also affect hormonal stimulation of adenylate cyclase. Sodium was found to reduce the potency of the adenylate cyclase stimulatory hormones, thus influencing the stimulatory coupling process in a negative manner. Aktories *et al.* [6] did note, however, that sodium leads to a further increase in the maximal activation of adenylate cyclase by ACTH in hamster adipocytes, although only at high ACTH concentrations. The present report represents the first study to describe in some detail an effect of sodium in activating (enhancing) hormone-stimulated adenylate cyclase activity.

The specificity of the effects of sodium ions on parotid and submandibular adenylate cyclase activities is noted when the results are compared to those obtained for mouse liver and brain adenylate cyclases. For liver it was also noted that GTP inhibited the forskolin response by approximately 57%. In previous studies utilizing rat liver membranes, GTP was found to inhibit the activation of adenylate cyclase by fluoride which like forskolin does not require receptor interaction for stimulation of cyclase activity [22].

In common with other tissues, stimulation of mouse parotid adenylate cyclase by the β-adrenergic agonist, isoproterenol, requires the presence of GTP [17]. However, there were several differences between our data and data from other tissues. Unlike inhibitory-linked systems reported by Jakobs *et al.* [8] where GTP-induced inhibition of adenylate cyclase was antagonized by NaCl, the stimulation of parotid adenylate cyclase by GTP was further stimulated by NaCl. We also found that the effects of other monovalent cations on β-adrenergic-stimulated adenylate cyclase in parotid gland were different from effects described previously in systems where sodium was required for hormonal inhibition [6, 11]. In these systems $Na^+ > Li^+ > K^+ >$ choline. In parotid gland, K^+ was as effective as Na^+ in enhancing enzyme activity, although basal adenylate cyclase in the presence of K^+ was greater than that for Na^+. In contrast to Na^+ and K^+, Li^+ had a small stimulatory effect on basal enzyme activity but inhibited β-adrenergic-stimulated adenylate cyclase. Li^+, however, has been observed to have inhibitory effects in other tissues as well [23, 24].

The mechanism by which sodium ions enhance hormonal stimulation of adenylate cyclase is not known. Based on our data, it is tempting to speculate on the mechanism(s) underlying the enhancement of β-adrenergic-stimulated adenylate cyclase of mouse parotid gland by sodium ions. Although binding studies were not performed, the ability of sodium ions to enhance adenylate cyclase activation by forskolin, a diterpene, which bypasses the receptor suggests that sodium may be acting independently of the receptor. Further, sodium did not alter the isoproterenol concentration–response curve for activation of adenylate cyclase. In contrast, Jakobs *et al.* [12] reported that sodium reduces the potency of hormones and GTP to stimulate enzyme activity in tissue such as human platelets by decreasing the binding of agonist to stimulatory hormone receptors. Our data also do not appear to support an effect of sodium on the guanine nucleotide regulatory protein (N_s) since GDPβS, a competitive inhibitor of GTP, shown to block isoproterenol-stimulated adenylate cyclase activity in rat and mouse parotid glands [17, 19], failed to affect sodium enhancement of forskolin-activated adenylate cyclase. Further studies will be required, however, to substantiate this conclusion.

These results suggest, therefore, that sodium may be acting on a component of the adenylate cyclase complex which is distinct from N_s and the receptor. One of the potential sites of interaction is the catalytic subunit (C). If sodium is interacting at C, it would appear to be at a site on C different from the site of forskolin interaction since sodium stimulation occurs to the same extent in the presence of forskolin concentrations producing minimal or maximal activation of adenylate cyclase (Fig. 4B). Alternatively, sodium may be interacting with an inhibitory guanine nucleotide-binding protein (N_i). Jakobs *et al.* [12] offer support for such an interaction from studies in platelets where sodium inhibited the activation process of N_i. In mouse parotid gland, we recently identified an N_i site (unpublished observation).

The significance of the effects of sodium in modulating hormonal stimulation of adenylate cyclase is not clear. However, this effect is consistent with findings in intact mouse parotid cells showing that sodium ions are required for hormone-stimulated cAMP accumulation and amylase release [2, 3]. That sodium ions can enhance adenylate cyclase activation in submandibular as well as parotid gland suggests that fundamental similarities exist between exocrine glands. Cyclic AMP appears to play a key role in parotid and submandibular amylase and submandibular mucin release.

In summary, it seems likely that the stimulatory effects of sodium in hormone-stimulated adenylate cyclase in mouse parotid gland are specific and may be of functional significance as changes in sodium

ions also modify β-adrenergic-stimulated cAMP accumulation and amylase release in intact cells [2, 3]. The molecular mechanism(s) underlying these findings appears to be different in most cases from those involved in the inhibition of hormone responses by sodium. Future studies will help to elucidate the molecular basis for the effects of sodium or β-adrenergic-mediated responses in parotid gland.

Acknowledgements—The authors would like to thank Luella Parsons for her expert typing. This work was supported by NIH Grant DE 05249.

REFERENCES

1. A. Bdolah and M. Schramm, *Biochem. biophys. Res. Commun.* **18**, 452 (1965).
2. E. L. Watson and F. Dowd, *Life Sci.* **30**, 1631 (1982).
3. E. L. Watson, C. J. Farnham, J. Friedman and W. Farnham, *Am. J. Physiol.* **240**, C189 (1981).
4. K. Aktories, G. Schultz and K. H. Jakobs, *Fedn Eur. Biochem. Soc. Lett.* **107**, 100 (1979).
5. K. Aktories, G. Schultz and K. H. Jakobs, *Naunyn-Schmiedeberg's Archs Pharmac.* **312**, 167 (1980).
6. K. Aktories, G. Schultz and K. H. Jakobs, *Biochim. biophys. Acta* **676**, 59 (1981).
7. K. H. Jakobs, *Molec. cell. Endocr.* **16**, 147 (1979).
8. K. H. Jakobs, K. Aktories, M. Minuth and G. Schultz, in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Eds. D. M. F. Cooper and K. B. Seamon), Vol. 17, p. 137. Raven Press, New York (1985).
9. S. Jard, B. Cantau and K. H. Jakobs, *J. biol. Chem.* **256**, 2603 (1981).
10. L. E. Limbird, *Biochem. J.* **195**, 1 (1981).
11. M. L. Steer and A. Wood, *J. biol. Chem.* **256**, 9990 (1981).
12. K. H. Jakobs, M. Minuth and K. Aktories, *J. Receptor Res.* **4**, 443 (1984).
13. E. L. Watson, J. A. Williams and I. A. Siegel, *Am. J. Physiol.* **236**, C333 (1979).
14. H. I. Chiu, D. J. Franks, R. Rowe and D. Malamud, *Biochim. biophys. Acta* **451**, 29 (1976).
15. G. Krishna, B. Weiss and B. Brodie, *J. Pharmac. exp. Ther.* **163**, 379 (1968).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. E. L. Watson and K. J. Jacobson, *Life Sci.* **39**, 693 (1986).
18. K. B. Seamon, W. Padgett and J. W. Daly, *Proc. natn. Acad. Sci. U.S.A.* **78**, 3363 (1981).
19. D. Cassel, F. Eckstein, M. Lowe and Z. Selinger, *J. biol. Chem.* **254**, 9835 (1979).
20. F. Eckstein, D. Cassel, H. Levkovitz, M. Lowe and Z. Selinger, *J. biol. Chem.* **254**, 9829 (1979).
21. A. J. Blume, D. Lichshtein and G. Blume, *Proc. natn. Acad. Sci. U.S.A.* **76**, 5626 (1979).
22. M. Rodbell, L. Birnbaumer, S. L. Pohl and M. J. Krans. *J. biol. Chem.* **246**, 1877 (1971).
23. J. Forn and F. G. Valdecass, *Biochem. Pharmac.* **20**, 2773 (1971).
24. Y. C. Wang, G. N. Pandey, J. Mendels and A. Froza, *Biochem. Pharmac.* **23**, 845 (1974).