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Biochemical Pharmacology, Vol. 26, pp. 991–994. Pergamon Press, 1977. Printed in Great Britain.

Correlation between gastric protein kinase and secretion in the pylorus ligated rat

(*Received* 24 *July* 1976; *accepted* 15 *October* 1976)

Protein kinase has been shown, in many tissues, to mediate some of the biological effects of cyclic nucleotides [1]. Investigators have implicated cyclic AMP (cAMP) and/or cyclic GMP (cGMP) in the regulation of gastric secretion [2, 3]; however, evidence suggesting the mediation of this physiological effect by cAMP and/or cGMP activated protein kinase activity *in vivo* has not been reported. The isolation and characterization of protein kinase in the rabbit gastric mucosa [4, 5] raised a possibility that these enzymes mediate the effects of cyclic nucleotides on gastric secretion.

We have undertaken an investigation of the effects of certain anti-secretory agents on gastric protein kinase activity in order to demonstrate a possible relationship between enzyme activity and acid secretory levels. For this investigation we have selected the pylorus ligated rat, commonly used in gastric secretory studies.

Male Sprague-Dawley rats (body wt 160–180 g) were fasted for 18 hr with free access to water until compound administration. Thirty minutes after compound treatment the pylorus was ligated. Two hr following pylorus ligation the animals were sacrificed by cervical dislocation and their stomachs removed and emptied for analysis of gastric juice volume and acidity. The stomachs were placed in ice-cold saline prior to removal of the mucosa. The mucosal layer was scraped and pooled (two animals/group) and then homogenized with a glass-to-glass homogenizer in 20 vol. of 0.25 M sucrose containing 5 mM $MgCl_2$ and 25 mM KCl. Homogenates were centrifuged at 27,000 *g* for 15 min and the resulting clear supernatant was used as the enzyme source. Protein kinase activity was determined according to the procedures of Miyamoto *et al.* [6] and Hiestand *et al.* [7] in a total vol. of 0.2 ml. The standard incubation mixture contained DL-α-glycerophosphate buffer (pH 7.5), 50 μM; protamine sulfate, 40 μg; (γ-^{32}P) ATP, 10 μM; $MgCl_2$, 10 mM; NaF, 10 mM; theophylline, 4 mM; dithiothreitol, 10 mM; mucosal enzyme, 20 μg; and cyclic nucleotide as indicated. Trichloroacetic acid (TCA)-precipitable proteins were collected on Millipore membranes (Type HA, 0.45 μm) and the incorporation of pMoles of ^{32}P into these proteins was calculated after enzyme blank subtraction. For this crude enzyme extract we determined the optimum pH, optimum cyclic nucleotide concentration, time course and K_m. Finally, the effects of the anti-secretory agents, 2-pyridylthioacetamide (CMN-131) [8], metiamide [9] and propantheline [10] on this enzyme were determined *in vivo* with the pylorus ligated rat preparation.

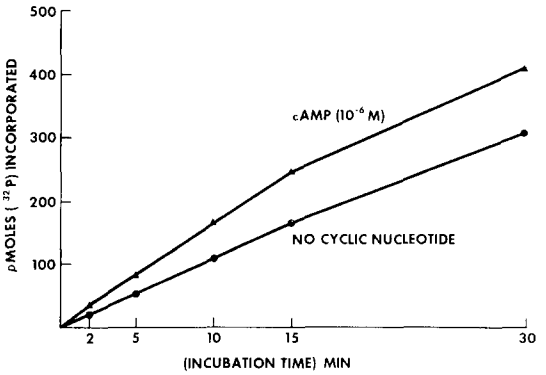

Fig. 1. Protein kinase time response. ATP = 10 μm.

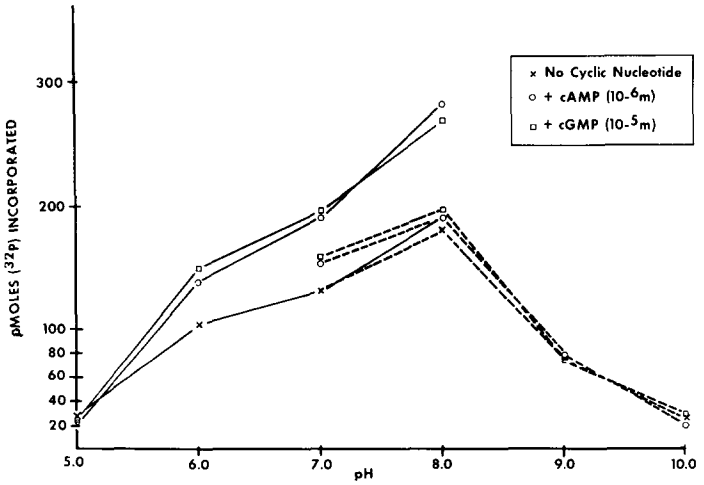

Fig. 2. Activity of rat gastric mucosal protein kinase as a function of pH, in 0.05 M DL-α-glycerophosphate buffer (solid lines) and in 0.05 M Tris-HCl buffer (hatched lines). ATP = 100 μM (2.5 × K_m), t = 15 min.

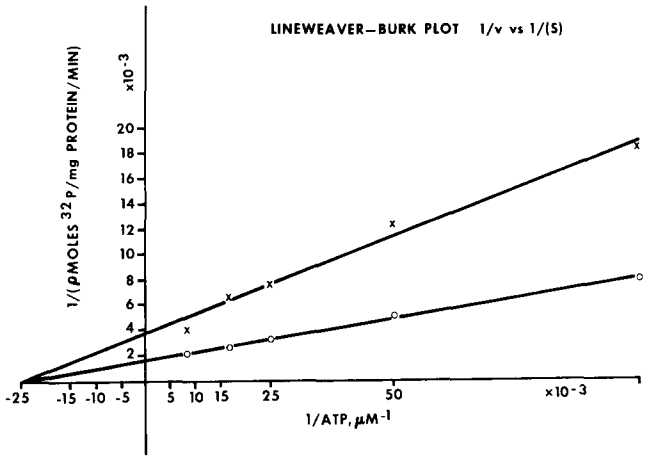

Fig. 3. K_m determination for ATP. The phosphorylation of protamine sulfate was measured in the absence (—×—) or presence (—○—) of cyclic AMP (10^{-6} M) with varied ATP concentrations.

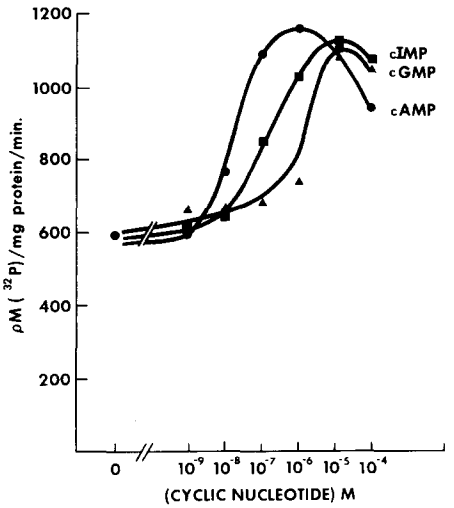

Fig. 4. Effect of cyclic nucleotide concentration on protein kinase activity. cIMP used as reference for cAMP and cGMP activation curves. ATP = 10 μM.

Incubation of rat gastric mucosal extracts with (γ^{32}P) ATP and protamine sulfate results in the incorporation of TCA precipitable radioactivity. Under the assay conditions, phosphorylation of the protamine sulfate proceeded linearly for 15 min. Cyclic AMP (1 μM) exerted a stimulatory effect (30–50% stimulation) at all incubation times tested (Fig. 1). The pH optimum of the gastric mucosal protein kinase either in the presence or absence of cyclic nucleotide was in the range of 7.0 to 8.0 (Fig. 2). The buffer of choice appears to be DL-α-glycerophosphate since Tris-HCl in our system did not support exogenous cyclic nucleotide activation of protein kinase. Similar observations have been reported using bovine adrenal ribosome preparations [11]. The K_m of the enzyme for ATP was found to be 41.7 μM by Lineweaver-Burk plot. Cyclic AMP (1 μM) had no effect on the K_m of ATP (Fig. 3). The optimum concentration of cyclic AMP, cyclic GMP and cyclic IMP for our system are 10^{-6} M, 10^{-5} M and 10^{-5} M, respectively (Fig. 4). Cyclic IMP activation of protein kinase usually falls between the cyclic AMP and cyclic GMP activation curves, thus serving as an *in vitro* reference curve.

As summarized in Table 1, the results obtained with the antisecretory agents reveal significantly greater levels

Table 1. *In vivo* effect of anti-secretory agents on gastric secretion and protein kinase activity in the 2 hr pylorus ligated rat*

Treatment	Gastric secretion Vol. (ml)	Acidity (mEq/L)	Acidity (μEq/2 hr)	Protein kinase activity pM(^{32}P)/mg/min No exogenous cyclic nucleotide	cAMP (10^{-6} M)	cGMP (10^{-5} M)
Control	5.7	113.3	650.5	5.9	11.4	9.4
(0.5% Methocel,	±0.3	±2.4	±40.6	±2.3	±4.9	±3.9
2 ml/kg, p.o.)	(24)	(24)	(24)	(12)	(12)	(9)
2-Pyridyl-	2.1†	10.6†	27.9‡	148.5†	389.3‡	342.2‡
thioacetamide	±0.3	±4.1	±10.8	±32.3	±52.4	±50.1
(10 mg/kg, p.o.)	(11)	(8)	(8)	(6)	(6)	(3)
Metiamide	3.2†	45.5‡	147.5‡	66.7†	155.4§	171.1§
(50 mg/kg, p.o.)	±0.7	±10.5	±38.1	±8.3	±9.8	±22.3
	(6)	(6)	(6)	(3)	(3)	(3)
Propantheline	3.7	79.2‡	336.1‡	33.4§	101.6§	83.5
(25 mg/kg, p.o.)	±0.5	±7.0	±60.1	±11.2	±23.6	±25.3
	(12)	(11)	(11)	(6)	(6)	(6)

* Compound given 30 min prior to ligation and values represent mean ± S.E., n = ( ).
† Significant difference from control, $P < 0.05$, t-test.
‡ Significant difference from control and both drug groups, $P < 0.05$, t-test.
§ Significant difference from control and 2-pyridylthioacetamide treated groups, $P < 0.05$, t-test.

Table 2. *In vivo* effect of gastrin pentapeptide on protein kinase activity in the intact rat

Treatment	Protein kinase activity pM(^{32}P)/mg/min No exogenous cyclic nucleotides	cAMP (10^{-6} M)	cGMP (10^{-5} M)
Control*	123.1	261.3	231.8
(Iso-saline 4 ml/kg, S.C.)	±10.4	±36.3	±38.2
Gastrin Pentapeptide*	80.5	141.6	133.4
(400 μg/kg, S.C.)	±23.2	±32.0	±39.9
Control†	102.0	316.5	244.8
(Iso-saline 4 ml/kg, S.C.)	±16.4	±19.0	±28.1
Gastrin Pentapeptide†	82.2	238.6	188.8
(400 μg/kg, S.C.)	±15.7	±67.9	±62.6

* Compound/vehicle given 30 min prior to sacrifice.

† Compound/vehicle given 60 min prior to sacrifice.

Values represent mean ± S.E. (n = 3). Statistically significant variation between control and treatment groups could not be ascertained due to the small sample size; however, lower values following treatment with gastrin pentapeptide were consistent on two separate occasions.

of protein kinase activity ($P < 0.05$) when compared to controls. CMN-131 (10 mg/Kg, p.o.) produced a greater than thirty-fold increase in enzyme activity, while inhibiting acid output by greater than 90 per cent of controls. Likewise, metiamide (50 mg/Kg, p.o.) produced a greater than ten-fold increase in enzyme activity, while inhibiting acid output by greater than 75 per cent of controls. Finally, propantheline (25 mg/Kg, p.o.) produced a greater than six-fold increase in enzyme activity with inhibition of acid output by nearly 50 per cent. The cyclic nucleotide dependency as indicated by the degree of exogenous cyclic nucleotide stimulation was similar between control and treatment groups.

Further studies of the relationship between acid secretion and mucosal protein kinase were carried out with various doses of CMN-131. The results in Fig. 5 indicated a significant inverse correlation between protein kinase activity and gastric anti-secretory response ($P < 0.0001$); the higher the protein kinase activity, the lower the acid output.

The results reported here demonstrate the presence of protein kinase in the rat gastric mucosa and reveal an inverse relationship between gastric acid output and gastric mucosal protein kinase activity. Since acid secretion is believed to be near maximum in the pylorus ligated rat preparation, we could not use this model to study the effect of secretagogues on protein kinase levels. Nevertheless, in the intact rat, we did observe a decrease in protein kinase activity after pentagastrin administration (Table 2). The methods employed do not differentiate between the various cyclic nucleotide activated kinases; therefore, it is likely that the stimulation of protein kinase activity by cyclic GMP was unspecific, and required higher levels for activation.

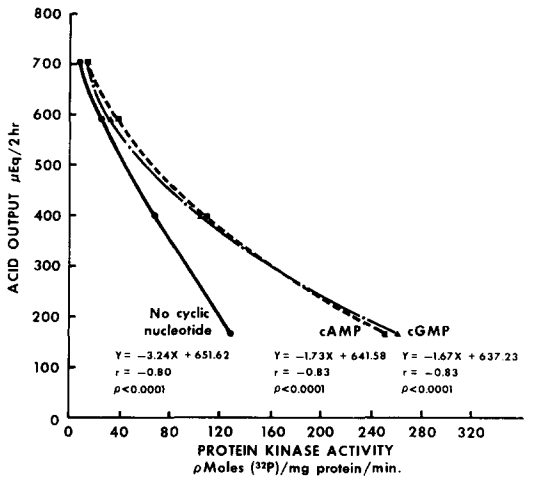

Fig. 5. Correlation between acid output and protein kinase activity. ATP = 10 μM.

The significance of these findings in relation to the regulation of gastric secretion is yet to be elucidated. One might hypothesize that protein kinase, whose activity is regulated by the levels of cyclic nucleotides, plays a role as activator and/or inactivator of other enzyme systems responsible for acid production (carbonic anhydrase system) and secretion (transport systems such as Na^+-K^+ or HCO_3^- dependent ATPase). Narumi and Miyamoto [12] reported that isoenzymes of carbonic anhydrase I can be activated *in vitro* by cyclic AMP dependent protein kinase and that this activation is associated with the phosphorylation of the carbonic anhydrase. They speculated that cyclic AMP dependent protein kinase in the gastric mucosa may stimulate gastric juice secretion by activation of carbonic anhydrase. The data presented in this report does not support the contention that increased protein kinase activity is associated with increased gastric secretion. The isolation and characterization of endogenous substrates that can be differentially phosphorylated by gastric protein kinase may clarify the mechanisms related to changes in secretion.

Recent findings of Rosenfeld *et al.* [13], using rat gastric mucosal membrane preparations, revealed that adenylyl cyclase activity was not altered by known secretagogues but was increased by several inhibitors of gastric secretion. Their data was supportive of the hypothesis that increased cyclic AMP levels correlate, in general, with inhibition of gastric acid secretion [14]. Since endogenous cyclic nucleotide levels were not determined in our investigation, an unqualified link between cyclic nucleotide activation and gastric acid secretion was not established. However, our findings tend to favor the contention that gastric acid secretion may be inversely related to intracellular cyclic nucleotide levels.

Acknowledgements—We would like to thank Mr. C. Yuan, Dept. of Applied Mathematics, for his help in the statistical analysis of the data and Les Laboratories Servier, Neuilly,

France for kindly supplying the 2-pyridylthioacetamide (CMN-131).

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Biochemical Pharmacology, Vol. 26, pp. 994–996. Pergamon Press, 1977. Printed in Great Britain.

The activation of cyclic 3′, 5′-adenosine monophosphate-dependent protein kinase on sarcoplasmic reticulum fractions of various smooth muscles and its related novel relaxants

(*Received* 22 *April* 1976; *accepted* 27 *August* 1976)

The mechanisms of smooth muscle relaxants have been found to be connected with an increase in the cyclic AMP (cAMP) level of the tissues concerned, partially caused by an inactivation of phosphodiesterase (for example, papaverine) or as a consequence of stimulation of adenylcyclase (for example, isoproterenol). Recently Nillson *et al.* [1] have suggested that the basic mechanism by which drugs, increasing the cAMP levels, exert their relaxing effect connects with stimulation of the protein kinase system. We studied on the phosphorylation of sarcoplasmic reticulum fractions (SR-F) in various smooth muscles of domestic pigs using cAMP-dependent protein kinase (PK) of cardiac or tracheal muscles or bile ducts, in order to clarify the site of action of a potent relaxant different from papaverine for Oddi's sphincters.

The relaxant, a novel compound 3-(2′-hydroxy-4′, 5′-diethoxybenzoyl) propionic acid (AA373), did not have any inhibitory effect on the intestinal phosphodiesterase of guinea-pigs, and also the response on adrenergic β-receptor, because of no inhibition by β-adrenergic antagonist on bile ducts. The fact was observed, however, that PK was activated by its compound both in the presence of and in the absence of saturated dose of cAMP on a substrate histone.

Recently Katz *et al.* [2] have shown the possibility of controlling calcium transport in the myocardium by PK system. This paper is presented on the possible mechanism of relaxing the Oddi's sphincter by AA373, by which the phosphorylation of biliary sarcoplasmic reticulum may accelerate relaxation in the smooth muscles of bile ducts.

Microsomal fractions (M-F) and SR-F were prepared by the method of Katz and Repke [3] as follows. After a removal of most of the fat and vessels, 16 g of every tissue from domestic pigs were minced and then homogenized in a Waring Blender for 90 sec with 2.5 vol. of ice cold 0.3 M sucrose, 5 mM Tris–oxalate and 5 mM histidine at pH 7.4. After centrifugation for 30 min at 12,000 *g*, the supernatant was filtered through four layers of gauze, if necessary, and centrifuged for 90 min at 105,000 *g*. The pellet was taken up in 3 ml of 0.3 M sucrose, 1 mM Tris–oxalate and 5 mM histidine at pH 7.4 and homogenized by ten gentle strokes of a Teflon–glass Potter homogenizer. This M-F diluted with a 0.3 M sucrose to contain about 60 μg of protein were used to the assay of protein kinase. Homogenate (0.25 ml), containing approximately 3.6–2.1 mg protein of crude microsomes, was applied to the top of a sucrose gradient consisting of 2 ml of 35% sucrose and 2 ml of 20% sucrose, layered in a polyallomer tube. The gradient tubes were centrifuged for 2 hr at 25,000 rpm in RPS 40 swinging rotor (Hitachi Co. Ltd.). The purified microsomes (corresponding to SR-F) appeared in one prominent middle layer, of which we used about 2 ml. These SR-F had been reported to show a lower contamination by both soluble and mitochondrial enzymes one ninth of crude microsomes and to have Ca^{2+} uptake capacity and Ca^{2+}-activated ATPase activity [3]. These fractions were heated to 78–84° for 2 min to inactivate several interfering enzymes [4].

The preparation of protein kinase was supernate fractions obtained from 0.9 g cardiac muscles, terminal bile ducts and gall bladders of domestic pigs minced, incubated in 9 ml Tyrode solution with or without drugs for 10 min, homogenized for 3 min, and supercentrifuged at 105,000 *g* for 1 hr.

Protein kinase assay was as follows: the reaction medium (0.25 ml) consisted of 8 mM phosphate buffer (pH 7.0), 1.6 mM theophylline, 8 mM NaF, 12 mM Mg acetate, 1 μCi/ml ATP-γ-^{32}P (The Radiochemical Centre Ltd., England), 10 μM ATP, SR-F (20–48 μg protein) or M-F (53–81 μg protein) and PK (13–68 μg protein) with 4 μM cAMP incubated at 37° for 5, 10 and 15 min. The phosphorylation reactions at the concentrations of SR-F, M-F and PK were ascertained to be almost linear. The reaction was stopped by the addition of 5 ml of 10% cold trichloracetic acid (TCA) containing 20 mg 'Hyflosuper celite'. The mixture was filtered through a paper ('Toyo' No. 2) and a thin layer of 50 mg 'Hyflosuper celite'. The residue was washed with 15 ml of 10% TCA, 3 ml acetone, 10 ml of