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Cation-Dependent Substance P Activation of the Enzyme Guanylate Cyclase

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Summary: Substance P enhanced guanylate cyclase (E.C.4.6.1.2) two - to - fourfold in pancreas, small intestine, cerebellum, liver, kidney, and lung. Dose response relationship revealed that substance P caused a maximal augmentation of guanylate cyclase activity at concentration of 1 micromolar. Increasing substance P's concentration to the millimolar range caused no further increase in activity. There was an absolute cation requirement for substance P's enhancement of guanylate cyclase activity. Substance P could increase guanylate cyclase activity with either calcium or manganese in the incubation medium but more augmentation was observed with manganese. The data in this investigation suggest that guanylate cyclase may play a role in the mechanism of action of substance P.

Substance P was discovered over 50 years ago by Von Euler and Gaddum (1), but its mechanism of action at the cellular level has still not been completely elucidated. Substance P has numerous pharmacological effects including excitation of spinal motorneurons, contraction of intestinal smooth muscles and the stimulation of salivary and pancreatic secretion (2). With respect to the mechanism of action of substance P, this peptide has been reported to increase cyclic AMP levels (3) and to stimulate adenylate cyclase activity in the brain (4). In the pancreas, substance P does not increase cyclic AMP levels (5,6) but increases another second messenger cyclic GMP (5,6). In dispersed pancreatic acinar cells the increase in cyclic GMP secondary to substance P is associated with efflux of ^{45}Ca and release of amylase (6). The present investigation was designed to determine if activation of guanylate cyclase (E.C. 4.6.1.2), the enzyme that catalyses the conversion of guanosine triphosphate to cyclic GMP, is part of substance P's mechanism of action.

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Methods

Tissues utilized in this investigation were obtained from male Sprague-Dawley rats weighing 150-200 g that had been maintained ad libitum on Purina laboratory chow. Substance P was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Substance P was dissolved immediately before use in triple distilled water to prepare the concentrations specified in the text. Substance P was added at zero time (ie, without any preincubation) to the various particulate and soluble enzyme preparations in the present *in vitro* investigation. Alumina, neutral activity I for column chromatography, was obtained from E. Merck, Darmstadt, Germany. The (α^{32}P)-GTP was from International Chemical and Nuclear Corporation, Irvine, California.

Guanylate cyclase activity was measured as previously described (7,8). The respective tissues were homogenized with a Polytron homogenizer using 10-s bursts in cold 0.03 M Tris HCl (pH 7.6) and centrifuged at 37,000 g in a refrigerated centrifuge at 4°C for 15 minutes. The supernatant and particulate fractions were then assayed at 37°C for 10 minutes for guanylate cyclase activity. The reaction mixture consisted of 20 mM Tris HCl (pH 7.6), 4 mM $MnCl_2$ (unless specified otherwise), 2.67 mM cyclic GMP (used to minimize destruction of (^{32}P)-GMP, a GTP regenerating system (5mM creatine phosphate and 11.2 U creatine phosphokinase (E.C.2.7.3.2), 100 µg bovine serum albumin, 20 mM caffeine, and 1.2 mM (α^{32}P)-GTP, approximately $5x10^5$ cpm. The final pH of the reaction mixture was 7.6. The volume of the supernatant or particulate fractions was 25 µl and the final volume of the cyclase assay which included the above supernatant or particulate fractions, the reaction mix and the radioactive isotopes was 75 µl. The enzyme preparations contained 0.1 to 0.2 mg/ml protein. The reaction was terminated by adding 10 µl of 0.1 M EDTA (pH 7.6) containing about 30,000 cpm of (^{3}H)-cyclic GMP (to estimate recovery in the subsequent steps) and boiling for 3 minutes. After cooling in an ice bath, the (^{32}P)-cyclic GMP formed was isolated by sequential chromatography on Dowex 50 Wx4 H^+ (200-400 mesh) and alumina using the modification described in detail previously (7). In this assay system, cyclic GMP production was linear with time for 20 minutes and with added protein from 50 to 400 µg. All of the ^{32}P-containing material was identifiable as cyclic GMP as determined by thin-layer chromatography on PEI-cellulose (Brinkman, Westbury, NY) using 1 M LiCl as solvent and on Chromar sheets (Mallinckrodt Chemical Works, St. Louis, MO) developed with absolute alcohol and concentrated NH_4OH (5:2 v/v). All reagents were of analytical grade and from the same sources as described previously (7,8).

Results

Substance P activated soluble guanylate cyclase *in vitro* when added to this enzyme isolated from a variety of tissues (Table I). Thus, in addition to the pancreas where it has been reported to increase cyclic GMP levels (5,6), substance P enhanced soluble guanylate cyclase activity two - to - four fold in small intestine, cerebellum, liver, kidney, and lung (Table I). Substance P caused a similar enhancement of guanylate cyclase activity in particulate as well as soluble fractions from these various tissues. Dose response-relationships revealed that the half-maximal activation of soluble guanylate cyclase by substance P was at the 10 nanomolar concentra-

Table I

Substance P enhances soluble guanylate cyclase activity in a variety of tissues

Tissue	Guanylate cyclase* (pmol cyclic GMP / mg protein / 10 min) Without substance P added	With substance P	P§
Pancreas	176 ± 9	390 ± 14	<0.001
Small Intestine	596 ± 25	1193 ± 20	<0.001
Cerebellum	488 ± 22	982 ± 29	<0.001
Liver	280 ± 13	601 ± 18	<0.001
Kidney	291 ± 15	655 ± 32	<0.001
Lung	1296 ± 36	5191 ± 43	<0.001

* Each value is the mean ± S.E.M. of triplicate samples on three animals confirmed in three separate experiments (N = 9). Assay conditions are as described in the text.

§ Significance of comparisons with controls was determined by Student's t test for unpaired values.

tion (Figure 1). Maximal enhancement of guanylate cyclase activity was observed with micromolar concentrations of substance P. There was no further augmentation of this enzyme with increasing substance P's concentra-

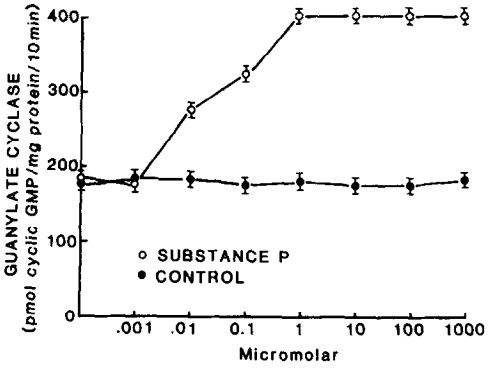

FIGURE 1. Dose response relationships of substance P on soluble pancreatic guanylate cyclase activity. Each value represents the mean ± S.E.M. of triplicate samples from 3 animals in each group in 3 separate experiments.

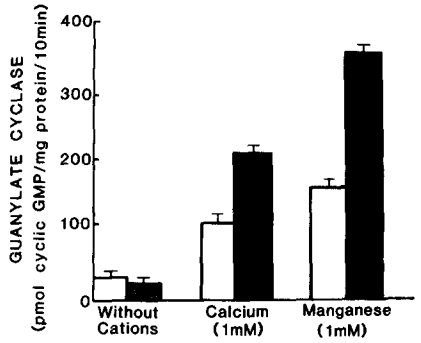

FIGURE 2. Cation requirement for substance P enhancement (at the 1 micromolar concentration) of soluble pancreatic guanylate cyclase activity. Each bar represents the mean ± S.E.M. of triplicate samples from three animals in each group in three separate experiments. The groups without any cations also had 2.5 mM EGTA added to them. With calcium (1 mM) or manganese (1 mM), substance P's augmentation of guanylate cyclase activity was significant ($p < 0.001$) when compared to controls by the Student's t test for unpaired values.

tion to the millimolar range (Figure 1). Similar dose-response relationships were observed with the particulate preparations of this enzyme.

The activation of guanylate cyclase by some hormones requires the presence of a cationic co-factor (9). A series of experiments were done to determine whether calcium or manganese, the usual guanylate cyclase co-factor, affected substance P's activation of guanylate cyclase. Without calcium or manganese and in the presence of 2.5 mM EDTA, substance P was unable to stimulate soluble guanylate cyclase activity (Figure 2). With 1 mM calcium as the cationic co-factor, pancreatic guanylate cyclase could be enhanced by substance P, but both basal and substance P stimulated guanylate cyclase activity were less with calcium than when 1 mM manganese was utilized (Figure 2). Utilizing 2,3 and 4 mM calcium as the co-factor revealed no increased stimulation of guanylate cyclase by substance P compared to the 1 mM calcium concentration (data not shown). Both basal and substance P stimulated guanylate cyclase were increased somewhat with 4 mM Mn^{2+} compared to 1 mM Mn^{2+}. The data shown are on soluble pancreatic guanylate cyclase activity but similar results were found with particulate pancreatic guanylate cyclase and both forms of guanylate cyclase isolated from the other tissues utilized in this investigation.

Discussion

Although substance P has only been investigated in one tissue (pancreas) previously with respect to cyclic GMP metabolism (5,6), the present investigation indicates that substance P can activate the enzyme guanylate cyclase in the pancreas but in a number of other tissues as well. The present findings are what one might expect given the ubiquitous nature of substance P (2), ie, with substance P being present and having effects in so many tissues it would be very unlikely for its mechanism of action to involve effects on a enzyme (that is also ubiquitous) in only one tissue. A number of substance P's effects appear to be related to its possible role as a neurotransmitter. Local application of substance P induces a depolarization of motorneurons in the isolated mammalian spinal cord, accompanied by high frequency spike discharges of the neurons (10). Dibutyryl cyclic GMP, likewise, itself is able to cause depolorization when applied to the rabbit superior cervical ganglion (11). The depolarizing potency of substance P is much higher than that of glutamate (10) which along with the amino acid glycine is thought to mediate the excitatory parallel and climbing fibers modulations of Purkinje cells. Both glutamate and glycine have been shown to increase cyclic GMP levels in the cerebellum (12). Synaptic transmission in bullfrog sympathetic ganglion has also been demonstrated to be associated with a doubling of cyclic GMP (13). Substance P activation of guanylate cyclase, thus, is consistent with substance P's possible role as a neurotransmitter.

With respect to how substance P interacts with guanylate cyclase, the present investigation would indicate that there is an absolute cation requirement for substance P to be able to activate either particulate or soluble guanylate cyclase. There was no enhancement of either guanylate cyclase when all cations were removed from the incubation media. This cation requirement for activation of guanylate cyclase by substance P distinguishes it from some hormones such as the glucocorticoids which can enhance guanylate cyclase activity without any divalent cation present

(14). Although the enhancement of guanylate cyclase with glucocorticoids does not obtain the maximal augmentation of activity that is seen when a divalent cofactor is present, glucocorticoids do cause a statistically significant activation without any divalent cations (14). The activation of guanylate cyclase by substance P, on the other hand, requires the presence of a cationic cofactor. This cofactor requirement was not specific for calcium, as has been reported previously for the carbamylcholine-stimulated increase in cyclic GMP levels in the kidney (15). In the present investigation the augmentation by substance P was actually greater when manganese was the cofactor. The ability of various agents to activate guanylate cyclase thus depends on the ionic mileau since acetylcholine (carbamylcholine) requires the presence of calcium to be able to increase cyclic GMP levels (15); vitamin D and its metabolites need manganese to able to augment guanylate cyclase (9), and substance P requires either manganese or calcium to enhance guanylate cyclase activity, whereas glucocorticoid hormones can activate this enzyme without any specific ionic requirement. The respective ionic environment, therefore, helps to determine which agents that activate guanylate cyclase will do so at a specific point in time. The respective ionic mileau at each target tissue may therefore contribute to the specificity of different hormones and other agents that activate the guanylate cyclase-cyclic GMP system.

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