Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 232-237

SUBSTANCE P INDUCES ALTERATIONS ON CEREBRAL LIPIDS INVOLVED IN MEMBRANE FLUIDITY

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Received February 18, 1987

SUMMARY: This study was undertaken to examine the variations in rat brain of cholesterol, phospholipid and phospholipid fatty acid composition induced by substance P. The cholesterol content was increased by substance P; concomitantly, an increase of the ratio cholesterol/phospholipid was observed. These changes do not appear to be responsible of the stimulation observed in Na^+,K^+-ATPase activity by substance P action. Phospholipid fatty acid analysis revealed that the peptide induced a decrease in both linoleic and arachidonic acids content.

The undecapeptide substance P is a neuroactive peptide in both central and peripheral neuronal systems. Although many effects evoked by substance P are known (1), little information concerning the effects of substance P on lipid metabolism has been reported (2-4) and the data reported in the literature are far from being a detailed analysis.

It has been reported that both membrane cholesterol content and the degree of acyl chain unsaturation are correlated with changes in membrane viscosity from a variety of biological systems (5-7). Furthermore, a prominent determinant of membrane viscosity is the cholesterol/phospholipid ratio (5-7).

Particularly interesting for us is the fact that the state of the cell membrane in which Na^+, K^+-ATPase is embedded may be an

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0006-291X/87 $1.50

important physiologic regulator of its activity (8). This enzyme is present in high concentrations in the brain and other nervous tissue (9), where it plays several roles in the complex and finely tuned control of the ionic environment which underlies nerve activity. Substance P has been shown to modulate the activity of synaptosomal membrane Na^+, K^+-ATPase from different areas of rat brain, being the cerebral cortex the area most affected (10).

Accordingly, the present studies were undertaken to determine: 1) whether the substance P can modify the cholesterol and phospholipid content as well as the phospholipid fatty acids in cerebral cortex, 2) whether substance P is able to affect of Na^+, K^+-ATPase in intact cerebral cortical slices and 3) whether such alterations might be linked, and thus, to add valuable information on the brain Na^+, K^+-ATPase modulation by substance P.

MATERIALS AND METHODS

Preparation and incubation of slices

The subjects were male Wistar rats weighing between 190 and 200 g. They were maintained on a standard laboratory diet with free access to water. Brains were rapidly removed after decapitation and all assays were done immediately. The cerebral cortex slices were obtained as previously described (11) and incubated for 30 min in Krebs-Henseleit buffer containing 10 mM D-glucose in the presence or absence of substance P at appropriate concentrations. To see the possibility that endogenous proteases can alter the substance P during the incubation, several control experiments were performed in the presence of 1 mM 1,10-phenanthroline as protease inhibitor. Results obtained in the presence of inhibitor were similar to those obtained in its absence. Slices were gassed throughout the incubation with 95% O_2-5% CO_2. Incubations were stopped by removing the tissue samples quickly and by homogenating with 2 ml of chloroform-methanol (1:1, v/v) mixture or 40 mM Tris-HCl buffer, pH 7.4 for lipid extraction or ATPase assay respectively in a Potter-Elvehjem homogenizer.

Extraction, separation and analysis of lipids.

Extraction, separation and analysis of phospholipids and cholesterol were performed as described previously (12). Analysis of the fatty acids were carried out by GLC. Methyl esters were prepared essentially according to Morrison and Smith (13). The phospholipid bands were separated into test tubes and 1 ml boron trifluoride (14% in methanol), 1 ml methanol and 0.9 ml bencene were added. After heating 90 min at 110°C, methyl esters were extracted with 6 ml hexane and 3 ml water. The extracts were washed and evaporated with nitrogen, and then diluted with suitable amount of hexane. Analysis of the methyl esters were carried out by GLC using a Perkin-Elmer model 3920 gas chromatograph. Fatty acid methyl esters were identified by comparing the retention times with those of known standards.

Assay of ATPase activity

Na^+, K^+-ATPase activity was assayed essentially according to the method of Zaheer and Talwar (14). For the total ATPase assay, the assay mixture in a final volume of 1 ml contained: 40 mM Tris-HCl buffer, pH 7.5; 1mM EDTA; 6 mM $MgCl_2$; 100 mM NaCl and 20 mM KCl.

For Mg^{2+}-ATPase assay, the assay mixture in a final volume of 1 ml contained: 40 mM Tris-HCl buffer, pH 7.5; 1 mM EDTA; 6 mM $MgCl_2$; 100 mM NaCl; 20 mM KCl and 1 mM ouabain. The samples were preincubated in these media for 10 min at 37°c and the reaction was started by the addition of 5 mM ATP. The reaction was stopped by the addition of 1 ml 12% cold trichloroacetic acid. After centrifugation for 5 min at 900 g, inorganic phosphate was determined in 1.5 ml supernatant by the method of Murray and Wild (15).

Na^+, K^+-ATPase activity was obtained from that obtained in the absence of ouabain. The protein concentration was estimated by the method of Lowry et al. (16) with bovine serum albumin as a standard.

Calculations

Student's t-test was used to test the significance of differences between means. Differences with a P value of less than 0.05 were considered statistically significant.

RESULTS

The effect of substance P on cholesterol levels in rat brain is shown in Table I. The peptide induced a significant increase on brain cholesterol only at 6.5×10^{-7} M and 6.5×10^{-11} M ($P<0.05$). 6.5×10^{-9} M substance P did not elicit any effect. On the other hand, substance P did not exert a significant effect on phospholipid levels (Table I). In addition, an overall increase in the molar ratio cholesterol/phospholipid was observed, only at the doses indicated above ($P<0.05$).

It can be seen in Table II that substance P modified the phospholipid fatty acid composition. The most striking

Table I

Effect of substance P on cholesterol and phospholipid levels in rat brain cortical slices

Substance P (M)	Cholesterol	Phospholipid	Molar ratio
0	139.2 ± 10.4	23.9 ± 0.9	0.46 ± 0.01
6.5×10^{-7}	167.0 ± 3.2*	22.6 ± 0.4	0.59 ± 0.02*
6.5×10^{-9}	142.0 ± 6.4	23.2 ± 0.7	0.49 ± 0.07
6.6×10^{-11}	180.9 ± 7.2*	23.6 ± 0.8	0.62 ± 0.04*

* $P<0.05$ relative to the control value. Cholesterol levels are expressed as µg P/mg total lipids. Results are shown as mean ± SD from five separate experiments.

Table II

Effect of substance P on phospholipid fatty acid composition in rat brain cortical slices

Fatty acid	Substance P (M)			
	0	6.5×10^{-7}	6.5×10^{-9}	6.5×10^{-11}
16:0	22.20±0.90	22.91±0.30	26.41±0.20*	25.51±0.02*
18:0	22.90±0.70	23.62±0.50	27.25±0.10*	24.27±0.61
18:1	24.00±0.50	24.96±0.40	29.76±0.03*	26.64±0.40
18:2	0.60±0.10	0.31±0.02*	0.28±0.02*	0.20±0.01*
20:4	3.80±0.60	3.46±0.30	2.85±0.15*	3.04±0.02*
22:6	8.21±0.42	8.31±0.21	5.33±0.22	6.16±0.24
others	18.29±1.00	16.43±0.50	8.12±0.70	14.18±0.91

* $P<0.001$ relative to the control value. Fatty acid levels are expressed as percentage of total phospholipid fatty acid methyl esters. Results are expressed as percent ± SEM of total fatty acid methyl esters derived from 4-6 separate experiments.

differences between controls and treated brain slices affected linoleic acid (18:2), at all concentrations tested. Substance P provoked a significant reduction in the level of this fatty acid ($P<0.001$). It can be observed that 6.5×10^{-9} M substance P, the most effective concentration, evoked a decrease in arachidonic acid (20:4) and docosahexanoic acid (22:6) and a concomitant increase in palmitic acid (16:0), oleic acid (18:1) and stearic acid (18:0) ($P<0.001$). The same pattern of variation was observed when the concentration of substance P was 6.5×10^{-11} M. No significant changes were found at 6.5×10^{-7} M substance P.

In Table III the effects of different concentrations of substance P on Na^+, K^+-ATPase activity are shown. It can be seen that the neuropeptide provoked a significant increase on enzyme activity at 6.5×10^{-9} M and 6.5×10^{-7} M concentrations.

Table III

Effect of substance P on Na^+, K^+-ATPase activity in rat brain cortical slices

	Substance P (M)			
	0	6.5×10^{-7}	6.5×10^{-9}	6.5×10^{-11}
Enzyme activity	0.31±0.01	0.42±0.02*	0.48±0.01*	0.38±0.03

* $P<0.05$ relative to the control value. Enzyme activity is expressed as μmol phosphorus released/min/mg protein. Results are shown as mean ± SD from five separate experiments.

DISCUSSION

Our data demonstrate that substance P is capable of increasing the cholesterol level in cerebral cortex and more important, that a significant increase in the molar ratio cholesterol/phospholipid is observed; this fact has special interest since it has been demonstrated that an increased molar ratio of cholesterol to phospholipids contributes to a lowered membrane lipid fluidity (5,6,7). Moreover, and although the exact mechanism of regulation of HMG-CoA reductase in brain is still unknown, a possible linkage between phosphorylation-dephosphorylation of the enzyme, cholesterol levels and substance P action cannot be excluded. On the other hand, our study demonstrates that substance P caused a decrease in both linoleic and arachidonic acids in brain phospholipids, whereas the saturated and monounsaturated fatty acids were enhanced. Linoleic acid is an essential fatty acid that adult brain can utilize it for the synthesis of arachidonic acid by elongation and desaturation mechanisms (17). The concomitant decrease of both fatty acids reported here could indicate that their incorporation into and/or their release from phospholipids are modified by substance P. Since arachidonic acid is the predominant precursor for synthesis of prostaglandins by brain tissue (18), the decrease of arachidonic acid could involve a modification of prostaglandin synthesis. This idea, however, awaits further investigations.

In an attempt to correlate the substance P-induced alterations in cerebral lipids and in brain Na^+,K^+-ATPase activity, we also studied the effect of substance P on this enzymatic system in our experimental conditions. The results demonstrate that the peptide increases the Na^+,K^+-ATPase activity. This effect is in agreement with data reported previously in synaptosomal membranes (10). However, our data indicate that a direct relationship between substance P-elicited stimulation of Na^+,K^+-ATPase activity and lipid alterations cannot be firmly established.

We would like to enfasize that although progress in research on substance P in the past few years has been tremendous, our knowledge on the effectuations mechanism of substance P is still limited. In this regard, this first study presenting data on substance P action on cerebral cortex lipids contributes to a further understanding of biological actions of substance P.

ACKNOWLEDGMENTS: The authors are indebted to Mr J. Palacín for his valuable assistance. We gratefully acknowledge support and encouragement given by Prof. F. Mayor. This work was supported in part by grants from FIS and CAICYT.

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