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Substance P and Mitochondrial Oxygen Consumption: Evidence for a Direct Intracellular Role for the Peptide

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PRABHAKAR, N. R., M. RUNOLD, G. K. KUMAR, N. S. CHERNIACK AND A. SCARPA. *Substance P and mitochondrial oxygen consumption: Evidence for a direct intracellular role for the peptide.* PEPTIDES **10**(5) 1003–1006, 1989.—Substance P (SP), a member of the tachykinin group of peptides, has been shown to augment the sensory discharge of the carotid body, an oxygen sensing chemoreceptor. In this study we present evidence that the excitatory effect of SP, in part, could arise from a direct effect of the peptide on mitochondrial oxidative phosphorylation. Measurement of the partition coefficient of SP showed that the peptide has a relatively high apolar partition, which could be consistent with its distribution across lipid bilayers and in intracellular organelles. In addition, the effects of three concentrations of SP were tested on oxygen consumption of mitochondria isolated from rat hearts. The results showed that while the lower concentration of the peptide (0.5 μM) did not affect O_2 consumption, higher concentrations, i.e., 1 and 2 μM, enhanced the rate of state 4 respiration by 52 and 64%, respectively. The rate of state 3 respiration, on the other hand, was unaltered with 0.5 and 1 μM, and was only slightly decreased with 2 μM of the peptide. The ADP:O ratio was unaffected by any concentrations of SP tested. The peptide-induced effect on state 4 respiration was even more pronounced with glutamate as a respiratory substrate and in presence of K^+ in the medium. These results indicate that SP, in addition to its more accepted role as a neurotransmitter or modulator in the carotid body, may elicit intracellular response by interfering directly with oxidative phosphorylation.

Substance P Peptide Partition coefficient Oxidative phosphorylation Mitochondria

SUBSTANCE P (SP) was first isolated from extracts of brain and intestine (5), subsequently characterized as an undecapeptide by Chang and Leeman (2), and is present in both neural and nonneural tissues (7,11). Administration of this peptide produces a variety of effects, including neuronal excitation, hypotension, sialogogic activity and smooth muscle contraction [see (11) for references]. Although its physiological actions are still under study, it is generally believed that SP acts as a neuromodulator or transmitter in certain peripheral and central nervous tissues (7).

The carotid bodies are sensory receptors that detect changes in arterial oxygen. Neuropharmacological studies have demonstrated that exogenously administered SP augments the frequency of carotid body neural discharge (9, 12, 13). Antagonists specific for SP-like peptides prevent the increase in carotid body activity caused by hypoxia but not by hypercapnia (12). These data suggest that SP may be a neuromodulator or transmitter in the carotid body and mediates its effects through the SP receptors, possibly located on the afferent nerve ending(s) (12).

Some observations made in recent studies are difficult to explain if SP exerts its effects on the carotid body just by binding to cell membrane receptors (13). For example, oligomycin and antimycin A, substances that interfere with mitochondrial oxidative phosphorylation not only abolish the carotid body stimulation caused by hypoxia, but also prevent SP-induced chemoexcitation (13). These findings raise the possibility that chemoreceptor responses to SP may in part arise from the action of the peptide on mitochondria, the site of oxidative phosphorylation. Therefore, in the present study we investigated a) the partition coefficient (Kpar) of the peptide to establish its ability to cross lipid bilayers, and b) tested the effect of peptide on oxygen consumption of the mitochondria isolated from rat hearts. The reason for choosing the mitochondrial preparation from rat hearts rather than the carotid bodies was the extremely small amount of the latter tissue (i.e., approximately 60 μg). The results show that SP has the ability to cross lipid bilayers and the peptide indeed increases the oxygen consumption of the isolated mitochondria in a dose-dependent manner.

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METHOD

Partition Coefficients

The partition coefficient (Kpar) of SP was determined at room temperature in a n-octanol/water system, essentially as described by Kontoghiorghes *et al.* (8). Five hundred μl of water or physiological buffered solution [potassium (50 mM) or sodium phosphate (50 mM)] containing various concentrations of I^{125}-SP were stirred with 500 μl of n-octanol for 5 min by means of a Vortex mixer. The two layers were then allowed to separate and the mixture was centrifuged for 5 min at 2100 × g. One hundred μl aliquots of the octanol and aqueous layers were analyzed for radioactivity by gamma-counter. The Kpar was determined as the ratio of radioactivity in n-octanol and aqueous layer. In addition, Kpar for SP was also determined in a chloroform/water system using the following procedure. Different concentrations of SP were added to a mixture of chloroform and water (500 μl each). The layers were mixed for 5 minutes and were separated by centrifugation as described above. The chloroform and aqueous layers were transferred to separate vials and lyophilized. The residues were dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) and analyzed by high performance liquid chromatography (HPLC). For HPLC peptide analysis, a two solvent system, i.e., 0.1% (v/v) TFA/water (solvent A) and 0.1% (v/v) TFA/acetonitrile (solvent B) was employed. SP was eluted 11 minutes after solvent B. The concentration of SP in the sample was determined using a standard curve generated from known concentrations of SP.

Preparation of Rat Heart Mitochondria

Rat heart mitochondria (subcellular fraction) were isolated by the method described by Palmer *et al.* (10). The heart tissues obtained from adult rats were transferred to an isolation medium at 0–4°C consisting of mannitol (225 mM), sucrose (75 mM), ethylene diaminotetracetic acid (EDTA; 0.5 mM), 3-N-morpholino-propane sulfonic acid (MOPS; 5 mM); pH adjusted to 7.4. Tissues were rinsed in 10 ml of the medium until they were free of blood. Thereafter they were finely minced and subjected to homogenization in isolation medium with a Polytron homogenizer (rheostat setting at 4.0 for 3 sec). The homogenate was centrifuged at 500 × g for 6 min at 4°C. The resulting supernatant was filtered and kept at 0–4°C. The pellet was resuspended in an equal volume of isolation buffer, homogenized by a prechilled glass tube and was again centrifuged at 500 × g for 6 min. The supernatants were combined and centrifuged at 12000 × g for 10 min. The resulting pellet was gently dispersed in 1 ml of the suspension medium (KCl 100 mM, MOPS 50 mM and ethyleneglycoltetracetic acid, EGTA, 2 mM, pH 7.4). Protein determinations were made by Rose Bengal method using bovine serum albumin as standard (3).

Respiratory Control and ADP:O Ratio

The uptake of oxygen was measured polarographically with a Clark type oxygen electrode (4). One–1.5 mg of mitochondrial protein was added to 800 μl of the reaction medium (KCl or NaCl 80 mM, MOPS 50 mM, EGTA 1.05 mM and sucrose 225 mM, pH 7.0). State 3 respiration was initiated by adding 300 nmol of adenosinediphosphate (ADP) to the reaction medium in the presence of either glutamate or succinate (20 mM). Mitochondrial respiration was recorded in presence of vehicle matching the highest concentration of SP (i.e., Control) and with three concentrations of peptide (i.e., SP 0.5, 1.0 and 2.0 μM). The following variables were measured according to Estabrook (4); 1) the rates of state 3 and 4 (nanoatoms of oxygen per mg of protein); 2) the respiratory control index, RCI (state 3/state 4); and 3) the ratio of added ADP over extra oxygen consumed (ADP:O). Statistical comparisons were made among groups with analysis of variance followed by a multiple comparisons test (Tukey) and p values less than 0.05 were considered significant.

TABLE 1

PARTITION COEFFICIENT OF SUBSTANCE P

Organic Phase	pH of the Phase	Kpar Medium	Aqueous
Water	n-octanol	7.0	0.3
Water	chloroform*	7.0	0.1
$KHPO_4$ (50 mM)	n-octanol	6.8	0.01
$NaHPO_4$ (50 mM)	n-octanol	6.8	0.01

Partition coefficient with chloroform (*) was determined by HPLC and n-octanol with I^{125}-SP. Each value is the mean of four measurements.

Chemicals and Peptides

All chemicals and solvents were obtained commercially and were of reagent grade. Synthetic substance P (SP amide) was purchased from Sigma Chemicals (St. Louis, MO) and I^{125}-SP from Incstar Corporation (Stillwater, MN). Stock solutions of the peptide (10 μmol/ml) were prepared in 0.01 M acetic acid, pH adjusted to 6.8 and stored at −20°C. The purity of the peptide was assessed by HPLC analysis using the solvent system described above.

RESULTS

Partition Coefficients of Substance P

The partition coefficient (Kpar) of SP was measured under different conditions. As shown in Table 1, SP can be distributed in both organic and aqueous media. The relative partitioning of the peptide in the organic medium ranged between 10 and 30% and depended on a) the presence of ions in the aqueous medium, and b) the type of organic solvent. Thus, the distribution of the peptide in n-octanol was low in presence of phosphate buffer containing K^+ (50 mM) or Na^+ (50 mM) compared to water alone. Similarly, the Kpar was 3 times higher in n-octanol system compared to chloroform/water (Table 1). These results are in agreement with a recent report by Seelig and McDonald (16) and indicate that the peptide is partially lipophilic and, therefore, may penetrate cell membranes and partition in intracellular organelles.

Effect of SP on Mitochondrial Oxygen Consumption

When glutamate was used as a respiratory substrate, control ADP:O ratios of different preparations averaged 2.7 ± 0.1, indicating that the function of the isolated mitochondria was well preserved (15). The effect of 1.0 μM of SP on mitochondrial oxygen consumption is illustrated in Fig. 1. As compared to the solvent control, the rate of state 4 respiration was enhanced by the peptide. Average results obtained with three different concentrations of SP are summarized in Table 2. Addition of 0.5 μM of SP did not alter either state 4 or state 3 respiration; as a result RCI was unaffected. Increasing the SP concentration to 1 μM significantly increased state 4 respiration by 52%, $F(24) = 11.18$, $p < 0.0027$, but not that of state 3, so that RCI decreased by 36% as compared to controls, $F(20) = 8.9$, $p < 0.0065$. Increasing the concentration of SP to 2 μM, the maximum concentration used in this study,

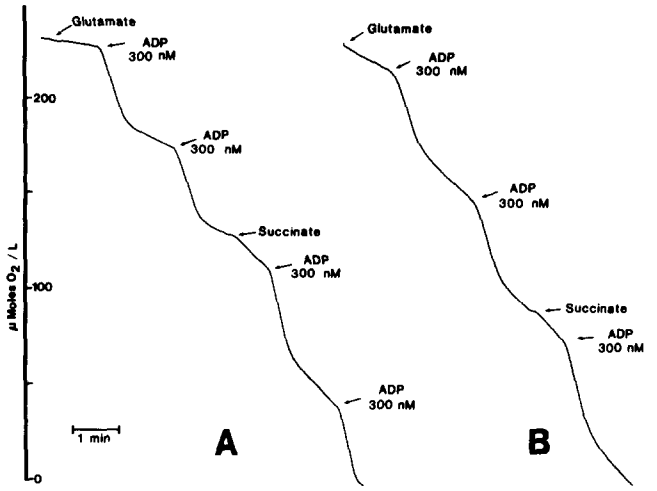

FIG. 1. Effect of substance P (SP) on oxygen consumption of mitochondria isolated from rat heart. (A) solvent control, (B) in the presence of SP 1 µM. Volume of the medium was 800 µl containing 1.5 mg of mitochondrial protein. Temperature was 25°C. Note the enhanced rate of state 4 in presence of SP.

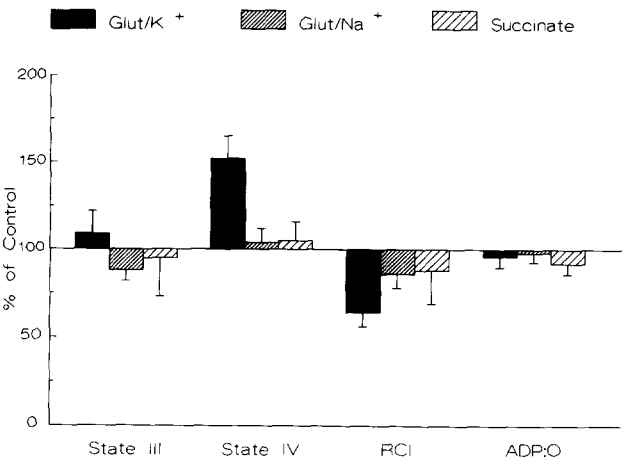

FIG. 2. Influence of respiratory substrates and monovalent cations on SP (1 µM)-induced changes in oxygen consumption of isolated mitochondria. Data are expressed as percent (mean ± SEM) of solvent control (without SP). Note the reduced response of state 4 in the presence of succinate and Na^+.

affected both the state 4 and state 3 respirations. State 4 respiration increased by 66%, $F(20) = 13.01$, $p < 0.0018$, whereas state 3 respiration decreased by 18%, $F(20) = 41.08$, $p < 0.0001$. As a consequence, RCI fell by 50% of its control value, $F(20) = 77.48$, $p < 0.0001$. The magnitude of SP-induced changes were dependent on the concentration of the peptide. ADP:O ratio, on the other hand, was unaffected by any of the three doses studied.

Effect of Succinate on SP-Induced Responses

It is known that glutamate oxidation by the respiratory chain occurs at the level of site 1 in the electron transfer chain, i.e., NAD level, whereas substrates like succinate enters at site 2, i.e., coenzyme Q level (15). In order to determine whether SP-induced changes in oxygen consumption depend on the type of respiratory substrate, the effects of the peptide were assessed with succinate and compared with glutamate (n = 8). The control ADP:O ratio in the presence of succinate was 1.8 ± 0.1 as compared to 2.6 ± 0.1 with glutamate, $F(18) = 10.02$, $p < 0.006$. In presence of glutamate SP increased state 4 respiration by 55 ± 6% of the controls. By contrast in presence of succinate the peptide-induced increase averaged only 5 ± 8% of the controls and was significantly less than glutamate, $F(18) = 5.23$, $p < 0.03$. On the other hand, SP-induced changes in state 3, RCI and ADP:O ratios were the same with succinate compared to glutamate [state 3, $F(18) = 0.36$, $p > 0.55$; RCI, $F(18) = 3.16$, $p > 0.1$; ADP:O, $F(18) = 0.155$, $p > 0.70$] (Fig. 2).

TABLE 2

EFFECT OF DIFFERENT CONCENTRATIONS OF SUBSTANCE P ON MITOCHONDRIAL RESPIRATION

	Solvent	Substance P
	Control (n = 12)	0.5 µM
State 3	112 ± 11	131 ± 13
State 4	23 ± 2	27 ± 3
RCI	5 ± 0.2	5 ± 0.3
ADP:O	2.7 ± 0.1	2.5 ± 0.1
	Control (n = 13)	1.0 µM
State 3	140 ± 14	154 ± 18
State 4	31 ± 3	47 ± 4*
RCI	5 ± 0.4	3 ± 0.4*
ADP:O	2.7 ± 0.1	2.4 ± 0.1
	Control (n = 11)	2.0 µM
State 3	138 ± 6	113 ± 4*
State 4	25 ± 2	41 ± 2*
RCI	6 ± 0.3	3 ± 0.1*
ADP:O	2.6 ± 0.1	2.6 ± 0.1

State 3 and state 4 are expressed as µmol of O_2/min/mg of protein. RCI = respiratory control index; n = number of mitochondrial preparations. Data are mean ± SEM. *Indicates p values less than 0.05 (see text for details).

Influence of Potassium Ions on SP-Induced Responses

We evaluated the influence of K^+ ions on SP-induced responses. In the presence of K^+ the ADP:O was 2.6 ± 0.1. Substituting Na^+ with K^+ did not alter the ADP:O ratio [2.5 ± 0.1; $F(19) = 0.54$, $p < 0.48$], suggesting that presence of sodium itself did not alter the coupling state of mitochondria. Replacing sodium for potassium in the reaction medium, however, markedly attenuated the SP-induced increases in state 4 O_2 consumption of the mitochondria [K^+ 55 ± 6 vs. Na^+ 4 ± 8%; $F(19) = 6.64$, $p < 0.02$] (Fig. 2). SP-induced changes in other variables like state 3, RCI and ADP:O ratios were not affected by Na^+ as compared to K^+ in the reaction medium [state 3, $F(19) = 1.33$, $p < 0.26$; RCI, $F(19) = 3.44$, $p > 0.1$; ADP:O, $F(19) = 0.06$, $p > 0.8$].

DISCUSSION

The results presented in this study demonstrate that SP enhances the O_2 consumption of the isolated mitochondria. That this effect could be of physiological significance is suggested by the experiments which show that SP is relatively lipophilic and has the potential to partition effectively in cellular membranes and directly affecting mitochondria in situ.

The concentrations of SP necessary for this effect are far higher

than those physiologically present in extracellular fluids. However, because of its localization in tissues like the carotid bodies and its lipophilic partition, the amount of SP interacting with intracellular organelles, such as mitochondria, could be in situ far higher than that predicted in simple concentration and binding equilibrium isotherms.

SP induced a dose-dependent enhanced rate of oxygen consumption in state 4 respiration. Although this property is shared by a broad range of uncoupling agents, it was also accompanied by a decrease in state 3 respiration with site 1 respiratory substrates and, more remarkably, by the absence of any effect in ADP:O ratios. There is a close resemblance of this effect to that described in the literature for thiobarbiturates (1).

Another notable difference between the effect of SP and that of other metabolic inhibitors or uncouplers is the fact that the effect of SP on respiration rates is dependent on K^+ in the medium. Various K^+ ionophores such as valinomycin (6,14) induce an increase of state 4 respiration in the presence of K^+ but this effect is accompanied by the accumulation of K^+, collapse of $\Delta\psi$ and consequently uncoupling. Furthermore, this effect of K^+ ionophores is observed equally well with site 1 and site 2 substrates of respiration.

Taken together, these observations are inconsistent with SP action as an uncoupler, a site 1 inhibitor or an electrogenic K^+ ionophore. It is possible that SP may have multiple effects at different sites of substrate or nucleotide transport, or individual respiratory components. This explanation does not reconcile with the necessity of K^+ for the action shown. However, one can speculate that the conformation and/or partition of the SP may depend on the presence of K^+ or, alternatively, that SP promotes an electroneutral exchange of K^+ with H^+ or other intramitochondrial ions.

While additional studies are necessary to elucidate the precise mechanism of these observations, this report clearly shows for the first time that SP has a profound effect on rates of oxygen and substrate utilization by isolated mitochondria. Based on the finding of the high lipid partition coefficient of SP, the possibility that it may act directly in mitochondria of the carotid body and target tissues is a real and challenging possibility that requires additional testing. This explanation is also consistent with our previous studies that the effect of SP in carotid bodies could be mimicked in vivo by hypoxia (12,13).

Hence, a working hypothesis and a source of partially justifiable speculation is that SP, in addition to its established role through receptor binding in signal transduction, could act directly on energy metabolism in cells in close proximity to the source of SP release.

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