FURTHER OBSERVATION ON THE LACK OF ACTIVE UPTAKE SYSTEM FOR SUBSTANCE P IN THE CENTRAL NERVOUS SYSTEM

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Abstract—Crude mitochondrial P2 fractions from bovine hypothalamus and substantia nigra, slices from rabbit spinal cord and mesencephalon and glial fractions from rabbit brain were incubated with [3H]-substance P and the uptake was measured and compared with those for 5-HT and GABA. Substance P was to some extent taken up into the fractions but this uptake was neither temperature nor time dependent and the pellet/medium ratios were less than 1. Similar results were obtained in high potassium treated slices from rabbit mesencephalon. The rate of uptake for [3H]-substance P increased linearly in proportion to the medium concentration, suggesting a non-saturable binding. These results, together with our previous observations provide strong evidence that nerve terminals and glial cells lack a temperature sensitive, active uptake system capable of terminating transmitter action of substance P at the synapse.

There is considerable evidence that substance P is a neurotransmitter candidate in the mammalian central nervous system. Substance P is observed in primary sensory neurons and nerve terminals in many brain areas (1) and is highly localized in subcellular organelles containing nerve ending particles (2). Substance P-like activity is released when the spinal cord isolated from newborn rats is stimulated electrically or soaked in a high potassium medium (3). Synthetic substance P exerts strong depolarizing action on the spinal motoneurons of the rat (4).

If substance P does indeed act as a neurotransmitter, rapid inactivation at the site of action would be necessary. Benuck and Marks (5) have partially purified the enzyme responsible for inactivation of substance P in rat brain. However, extensive studies on the biogenic amines suggest that the uptake system, rather than the enzymatic inactivation may account for termination of transmitter action at the synapse (6).

In a previous communication Segawa et al. (7) reported that uptake of labelled substance P into crude mitochondrial fraction from rabbit brain and spinal cord differs from those of known putative transmitters. The amount of substance P taken up at 37°C was almost the same as that taken up at 0°C. The pellet/medium ratios scarcely exceed 1. Furthermore, when the fraction incubated with labelled substance P was further subfractionated, the radioactivity was predominantly localized in the mitochondrial fraction. These results suggest that the uptake is not an active high affinity transport but rather a non-specific
binding.

To confirm these observations the following experiments were performed. As marked regional differences in the uptake of the putative transmitter have been reported, the uptake of substance P was re-investigated in crude mitochondrial P₂ fractions prepared from bovine hypothalamus and substantia nigra which are particularly rich in this substance (2, 8). Furthermore, since the high affinity uptake system has been demonstrated only with a low concentration of the transmitter in incubation medium, P₂ fraction was incubated with a low concentration of substance P. To exclude the possibility that substance P nerve endings are fragile and are destroyed during homogenization and incubation we re-examined substance P uptake into slices prepared from rabbit mesencephalon and spinal cord. The uptake into glial cells fraction was also investigated since it has become increasingly evident that glial cells may participate in the termination of certain transmitter actions at the synapse. For purposes of comparison, we studied the uptake of 5-hydroxytryptamine (5-HT) and γ-aminobutyric acid (GABA) since the evidence for their role as transmitters in the mammalian central nervous system is relatively good, if not conclusive.

MATERIALS AND METHODS

Substance P synthesized by the conventional method described in a previous paper (9), was labelled with tritium and purified as described previously (7). The specific activity is 1.31 Ci/m mole. 4-Amino-n-[U-14C]-butyric acid (224 mCi/m mole) and [3H]-5-HT creatinine sulfate (0.5 Ci/m mole) were obtained from The Radiochemical Centre, Amersham.

Preparation of crude mitochondrial P₂ fractions

Bovine brain was obtained at a local slaughterhouse and hypothalamus and substantia nigra were minced and homogenized in 9 vol. of ice cold 0.32 M sucrose using a Teflon pestle. The crude mitochondrial P₂ fraction was prepared by the method of Segawa and Kuruma (10).

Preparation of brain slices and glial cell enriched fractions

Rabbits weighing 1.9–2.6 kg of both sexes were decapitated and spinal cord and brain were rapidly removed. The spinal cord or midbrain excised from the whole brain was placed on wet (modified Krebs-Ringer solution) filter paper on ice, and was dissected with a razor blade into cubic slices of approximately 0.7 mm side. These slices were suspended in ice-cold modified Krebs-Ringer solution (11) bubbled previously with 95% O₂-5%CO₂ for 10 min and were then allowed to stand for several min. The modified Krebs-Ringer solution was composed of 118 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 0.5 mM MgCl₂, 25.0 mM NaHCO₃ and 11.1 mM glucose. After removing the supernatant by decantation the suspension was sieved through Nylon mesh with a pore size of 680 μm and the resulting filtrate was used for the study.

Fractions enriched in glial cells were prepared according to the method of Nagata et al. (12) with slight modification. The rabbit brain (without cerebellum) was immersed immediately in ice-cold 10% Ficoll-basic buffer solution of 100 mM KCl in 10 mM potassium phosphate buffer (pH 7.4) and then chopped with a razor blade. This preparation was then suspended in 2.5 vol. of ice-cold 10%, Ficoll-buffer solution and sieved successively
through Nylon mesh with progressively finer pore sizes: 800 μm, 680 μm, 430 μm, 174 μm and 82 μm. To the filtrate an equal volume of 30% Ficoll in 10 mM potassium phosphate buffer was added to make a final Ficoll concentration of 20%. An aliquot of the filtered suspension was layered on the discontinuous sucrose (50%)-Ficoll (30%) gradient and followed by 15% and 10% Ficoll solution. Centrifugation was carried out in a Hitachi Ultracentrifuge-55P with a SW-25 rotor at 41,000 g for 120 min. The glial cells enriched fraction was aspirated, diluted with 20-30 vol. of 0.32 M sucrose and re-centrifuged at 11,500 g for 20 min. The resulting pellets were suspended in 95% O2-5% CO2 saturated modified Krebs-Ringer Solution.

Uptake of [3H]-substance P, [3H]-5-HT and [14C]-GABA

Experiments were carried out by the modified method of Segawa et al. (7). Portions of tissue suspension containing 0.6-1.3 mg protein (P2), 10-20 mg wet tissue (slices) or 0.5-1.0 mg protein (glial cells) were transferred into 10 ml polyethylene tubes. After pre-incubation for 10 min at 37°C, 0.1 ml of [3H]-substance P, [3H]-5-HT or [14C]-GABA (final concentrations are given in Table and Fig.) was added and a further incubation was carried out at 37°C. At the end of the incubation period, 6 ml of cold saline was added to the reaction mixture and centrifugation at 12,300 g was carried out for 20 min at 4°C after which the supernatant fluid was collected. The pellet was re-suspended in 6 ml of the cold saline solution and was re-centrifuged at 12,300 g for 20 min. The washing was repeated. The radioactivity in the supernatant and the pellet was determined in a model 3320 Packard Tri-Carb liquid scintillation spectrometer and corrected for efficiency by external standardization.

RESULTS

Uptake of [3H]-substance P into crude mitochondrial P2 fractions from bovine hypothalamus and substantia nigra

The results are presented in Fig. 1 and Table 1. When crude mitochondrial P2 fractions from bovine hypothalamus or substantia nigra were incubated with 1.46 × 10^{-9} M of [3H]-substance P for 1, 10 and 20 min, substance P was to some extent taken up into the fractions. Initial rate of uptake was rapid. Thus, within 1 min after incubation, substance P uptake reached a steady-state level which was maintained for 20 min (Fig. 1). However, since the fractions took up almost the same amount of substance P both at 0°C and 37°C, the net uptake calculated by subtracting the value at 0°C from the value at 37°C was minute or nil. The pellet/medium ratios, calculated from the net uptake at 37°C were less than 1 (Table 1). These results are similar to those obtained with 2.3 × 10^{-9}–2.3 × 10^{-8} M of [3H]-substance P in crude mitochondrial P2 fractions from rabbit mesencephalon, diencephalon and spinal cord (7).

Uptake of [3H]-substance P and [3H]-5-HT into rabbit brain slices

Slices prepared from rabbit spinal cord were incubated with [3H]-substance P (7.5 × 10^{-8} M) for 1 and 10 min and the pellet/medium ratios were measured (Table 2). The results were similar to those found in crude mitochondrial P2 fractions. The uptake was neither
Fig. 1. Time course of [$\text{H}$]-substance P uptake into crude mitochondrial P2 fractions from bovine hypothalamus and substantia nigra. The fractions are incubated for 1, 10 and 20 min with $1.46 \times 10^{-9}$ M [$\text{H}$]-substance P at 0°C (○) or 37°C (●). Each point represents the mean of three determinations, vertical line indicates S.E.M.

**Table 1.** Uptake of [$\text{H}$]-substance P into crude mitochondrial P2 fractions from bovine hypothalamus and substantia nigra

| Incubation Time (min) | Hypothalamus | | Substantia nigra | |
|-----------------------|--------------|-----------------|-----------------|-----------------|
|                       | cpm/mg protein (37°C-0°C) | Pellet/medium | cpm/mg protein (37°C-0°C) | Pellet/medium |
| 1                     | 30±15        | 0.146±0.058     | 0               | 0               |
| 10                    | 43±17        | 0.211±0.084     | 102±37          | 0.465±0.185    |
| 20                    | 18±7         | 0.089±0.035     | 21±9            | 0.096±0.042    |

Incubation procedure follows the description in methods. Pellet/medium ratios are calculated as cpm in 100 mg protein per cpm in 1 ml of medium. Concentration of [$\text{H}$]-substance P is $1.46 \times 10^{-9}$ M. Values are presented as the mean±S.E.M. of three determinations.

temperature nor time dependent and the pellet/medium ratios, calculated as cpm in one gram of tissue per cpm in one ml of medium were less than 1. The uptake of [$\text{H}$]-5-HT is also presented in Table 2. A considerable uptake of [$\text{H}$]-5-HT occurred at 37°C, giving a pellet/medium ratio of 3.341 after 10 min incubation.

Uptake of [$\text{H}$]-substance P and [$\text{H}$]-5-HT into rabbit mesencephalon slices is shown
**TABLE 2.** Uptake of [3H]-substance P and [3H]-5-HT into rabbit spinal cord slices

| Compound and concentration | Incubation | Pellet/medium |
|----------------------------|------------|---------------|
|                            | Temperature (°C) | Time (min)   |
| [3H]-substance P (7.5 \times 10^{-8} M) | 0          | 1            |
|                            | 10         | 0.400±0.024  |
|                            | 37         | 0.555±0.113  |
|                            | 10         | 0.559±0.047  |
| [3H]-5-HT (1 \times 10^{-7} M) | 0          | 1            |
|                            | 10         | 0.588±0.029  |
|                            | 37         | 0.775±0.075  |

Incubation procedure follows the description in methods. Pellet/medium ratios are calculated as cpm in 1 g of tissue per cpm in 1 ml of medium. Values are presented as the mean±S.E.M. of three determinations.

**TABLE 3.** Uptake of [3H]-substance P and [3H]-5-HT into rabbit mesencephalon slices

| Compound and concentration | Incubation | Pellet/medium |
|----------------------------|------------|---------------|
|                            | Temperature (°C) | Time (min)   |
| [3H]-substance P (7.5 \times 10^{-8} M) | 0          | 1            |
|                            | 10         | 1.108±0.038  |
|                            | 37         | 1.172±0.386  |
|                            | 10         | 1.070±0.105  |
| [3H]-5-HT (1 \times 10^{-7} M) | 0          | 1            |
|                            | 10         | 0.873±0.166  |
|                            | 37         | 0.387±0.055  |
|                            | 10         | 0.494±0.073  |
|                            | 1          | 1.154±0.064  |
|                            | 10         | 5.884±0.437  |

Incubation procedure follows the description in methods. Pellet/medium ratios are calculated as cpm in 1 g of tissue per cpm in 1 ml of medium. Values are presented as the mean±S.E.M. of three determinations.

**TABLE 4.** Uptake of [3H]-substance P and [3H]-5-HT into high potassium treated rabbit mesencephalon slices

| Compound and concentration | Incubation | Pellet/medium |
|----------------------------|------------|---------------|
|                            | Temperature (°C) | Time (min)   |
| [3H]-substance P (7.5 \times 10^{-5} M) | 0          | 1            |
|                            | 10         | 1.024±0.074  |
|                            | 1          | 0.866±0.193  |
|                            | 37         | 0.886±0.117  |
|                            | 10         | 0.945±0.108  |
| [3H]-5-HT (1 \times 10^{-7} M) | 0          | 1            |
|                            | 10         | 0.569±0.065  |
|                            | 37         | 0.737±0.046  |
|                            | 10         | 2.020±0.194  |

The slices, preincubated with 40 mM KCl in modified Krebs-Ringer solution at 37°C for 10 min were incubated with [3H]-substance P or [3H]-5-HT. Pellet/medium ratios are calculated as cpm in 1 g of tissue per cpm in 1 ml of medium. Values are presented as the mean±S.E.M. of three determinations.
in Table 3. The results here were similar to those obtained in spinal cord slices.

To exclude the possibility that the high concentration of endogenous substance P in the preparation interferes with the uptake of exogenous substance P, rabbit mesencephalon slices were incubated at 37°C for 10 min in modified Krebs-Ringer solution containing 40 mM KCl and thereafter, the slices were submitted to the uptake experiment as described above. As is shown in Table 4, the pellet/medium ratios for [3H]-substance P were similar to, or somewhat lower than those in non-treated slices. On the other hand, when high potassium treated slices were incubated with [3H]-5-HT (1 x 10^-7 M) the pellet/medium ratio increased considerably at 37°C, giving a value of 10.615 after 10 min incubation (Table 4).

To further characterize the uptake of substance P, the rate of initial uptake for [3H]-substance P from 3.75 x 10^-8 M to 7.5 x 10^-6 M was measured. Since the uptake was fast and appeared to reach a steadystate level within 1 min, substance P uptake at 1 min was taken as a measure of initial uptake rate. The rate of uptake for [3H]-substance P increased linearly in proportion to the medium concentration, suggesting a non-saturable binding (Fig. 2).

**Uptake of [3H]-substance P, [3H]-5-HT and [14C]-GABA into glial fractions from rabbit brain**

As shown in Table 5, when glial fractions were incubated with 7.5 x 10^-8 M of [3H]-substance P, the pellet/medium ratios were about 0.7 whereas with 7.5 x 10^-7 M the ratios were 0.3 or less. The values presented in Table 5 for pellet/medium ratios were calculated as cpm in one g of pellet (wet weight) per cpm in one ml of medium. In some experiments the extraparticulate space of the pellet was estimated by the inulin method and was found to be approx. 80%. The ratios would therefore be considerably higher than those shown in Table 5. However, since the uptake was the same order of magnitude both at 0°C and 37°C, the net uptake was practically nil, irrespective as to whether or not this space correction
TABLE 5. Uptake of \[^3\text{H}\]-substance P, \[^3\text{H}\]-5-HT and \[^{14}\text{H}\]-GABA into glial fractions from rabbit brain

| Compound and concentration | Incubation Temperature (°C) | Time (min) | Pellet/medium |
|---------------------------|----------------------------|------------|---------------|
| \[^3\text{H}\]-substance P | 0                          | 10         | 0.684±0.105   |
| (7.5×10^{-8} M)           | 37                         | 10         | 0.671±0.117   |
|                           |                            | 1          | 0.660±0.010   |
| (7.5×10^{-7} M)           |                            | 10         | 0.793±0.161   |
| \[^3\text{H}\]-5-HT       | 0                          | 10         | 0.214±0.086   |
| (1×10^{-7} M)             | 37                         | 10         | 0.296±0.071   |
|                           |                            | 1          | 0.256±0.091   |
| \[^{14}\text{H}\]-GABA   | 0                          | 10         | 0.312±0.079   |
| (1×10^{-7} M)             | 37                         | 10         | 0.641±0.112   |
|                           |                            | 1          | 0.910±0.191   |
|                           |                            | 10         | 1.463±0.296   |
|                           |                            |            | 2.681±0.166   |
|                           |                            | 1          | 5.541±0.692   |
|                           |                            | 10         | 32.449±3.835  |

Incubation procedure follows the description in methods. Pellet/medium ratios are calculated as cpm in 1 g of pellet (wet weight) per cpm in 1 ml of medium. Values are presented as the mean±S.E.M. of three determinations.

was applied. A considerable uptake into glial fractions was observed for \[^3\text{H}\]-5-HT. In contrast to substance P and 5-HT, GABA was taken up much more actively into glial fractions at 37°C, giving a pellet/medium ratio of 32 after a 10 min incubation.

DISCUSSION

Crude mitochondrial P2 fractions from substance P enriched brain areas such as the hypothalamus and substantia nigra were incubated with a low concentration of substance P. The results were almost the same as those obtained in a previous study (7), in which P2 fractions from mesencephalon, diencephalon and spinal cord were incubated with relatively high concentrations of substance P. Therefore, active uptake of substance P could not be demonstrated even in nerve terminals which contain a high concentration of endogenous substance P.

Since studies with brain slices have an advantage over those with synaptosomes in good preservation of tissue during preparation and incubation, we carried out similar uptake experiments with slices from rabbit mesencephalon and spinal cord. The existence of an efficient uptake mechanism for substance P could not be demonstrated.

Uptake experiments with high potassium treated slices gave similar results. Endogenous substance P can be released by high potassium from isolated nerve-endings (13, 14) and the spinal cord (15) in vitro, therefore the possibility could be excluded that the high concentration of endogenous substance P in preparations interfereed with the uptake of exogenous
substance P.

Kinetic analysis of substance P uptake into rabbit mesencephalon slices indicated that the uptake is a non-saturable binding. The precise cellular site and the nature of substance P uptake (or binding) remain to be elucidated. In a previous paper (7) we reported that when crude mitochondrial P2 fraction was incubated with substance P, only a small amount of the substance was found to be associated with synaptosomes. Therefore, a non-specific binding of substance P by cellular components other than nerve endings may account for the pellet/medium ratios observed in this experiment.

Henn and Hamberger (16), Sellström and Hamberger (17) observed that GABA could be accumulated much more actively by glial cell fraction than by synaptosomal or neuronal fraction. These authors suggested that a certain glial cell is actively concerned with the removal of GABA from the extracellular space that surrounds neurons. In the present study, with substance P, glial uptake was extremely low with a pellet/medium ratio of 0.3-0.7. Therefore, it is improbable that glial cells contribute to the termination of transmitter action of substance P.

In summary, the present results, together with our previous findings provide strong evidence that nerve terminals and glial cells lack a temperature sensitive, active uptake system capable of terminating transmitter action of substance P at the synapse. While preparing this manuscript, Iversen et al. (13) reported similar findings that rat brain slices do not take up substance P, though the concentration employed in their experiment was considerably high (10^-7 M).

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