A cytoplasmic protein that promotes nucleo-cytoplasmic RNA transport in rat liver

ALEXANDER R. MCDONALD
CHARLES A. STEWART, CHARLES D. GLEED and PAUL S. AGUTTER

Department of Biological Sciences, Napier College, Colinton Road, Edinburgh EH10 5DT, Scotland, U.K.

It is well established that specific cytoplasmic proteins modulate the rate of nucleo-cytoplasmic mRNA transport in liver and hepatoma (Yu et al., 1972; Schumm et al., 1973a,b; Lemaire et al., 1981) and in other tissues (Weck & Johnson, 1978). These cytoplasmic protein factors have resisted purification, and their mechanism of action is not understood. However, the problem is interesting, not least because both the mechanism and the cytoplasmic regulation of RNA transport seem to be different in hepatoma and in normal liver (Schumm et al., 1977). The work described in the present communication was carried out as part of a study of the mechanism of action of the factors. Nuclei from rat liver and from liver containing 4,4'-dimethylaminobenzoic acid- and benzene-induced hepatoma were isolated by the method of Blobel & Potter (1966). In some experiments, the tissues were prelabelled by intraperitoneal injection of [5-3H]uridine (0.5 µCi/g body wt.) 30 min before the animals were killed. Nuclear envelopes were isolated by the method of Harris & Milne (1974). Nucleoside triphosphatase activity (EC 3.6.1.15) was assayed, and efflux of RNA from isolated nuclei was measured, by the methods of Agutter et al. (1979), and protein was determined by the method of Lowry et al. (1951).

At least 50% of the RNA-efflux-promoting activity of dialysed rat liver cytosol (the 100000g supernatants of homogenates) was present in a 2M-NaCl extract of polyribosomes (Moffett & Webb, 1981). 0.5M-NaCl did not solubilize most of the activity from polyribosomes. The 2M-NaCl extract was incubated at pH 9.0 for 15 min at 20°C to hydrolyse any residual RNA, and the active material was precipitated by 30–40% saturation with (NH4)2SO4, redissolved in a small volume of 10 mM-Tris-HCl, pH 7.6, and dialysed against two changes of this buffer. Purification of the active material from the cytoplasm by this method, judged from measurements of the stimulation of RNA efflux and of total protein, was approx. 2500-fold.

The active material was found to stimulate the nucleoside triphosphatase in isolated liver nuclear envelopes by about 15% in the presence of exogenous total yeast RNA. This enzyme, which supplies the energy for RNA transport (Agutter et al., 1976), catalyses the sequential phosphorylation and dephosphorylation of a nuclear-envelope polypeptide that co-migrates electrophoretically with one of the lamins; the rate-limiting dephosphorylation step is coincident with RNA binding at the nucleoplasmic face of the envelope (McDonald & Agutter, 1980). Therefore, it now seems likely that the cytoplasmic factor that has been largely purified by the method described (a) binds tightly to RNA (hence its association with cytoplasmic polyribosomes), (b) facilitates the interaction of the RNA with the nuclear-envelope binding site, so that dephosphorylation and hence the overall NTPase are accelerated, (c) accelerates nucleo-cytoplasmic RNA transport by virtue of the increase of this rate-limiting energy-utilizing step.

NTPase specific activity in hepatoma nuclear envelopes is higher than that in normal liver envelopes (cf. Clawson et al., 1980), and is also enhanced by 20% by the cytoplasmic stimulating factor from normal liver. Total hepatoma cytoplasm stimulates RNA efflux, even from normal liver nuclei, more markedly than does normal liver cytoplasm. Thus the higher rate of nucleo-cytoplasmic RNA transport associated with carcinogenesis (Schumm et al., 1973a,b, 1977) may result from higher activities of both the NTPase and the cytoplasmic stimulating factor. In view of the differences in RNA transport between hepatoma and normal liver, it would be interesting to know if the major stimulating factor hepatoma cytoplasm is (a) identical with its counterpart in normal liver but present in greater quantities, or (b) a different polypeptide.

Methods

Male CFY rats (230–270 g body wt.) were stunned and killed by cervical dislocation. The nuclei accumbens and corpus striatum was dissected out from the brain as described previously (de Bellefrocke & Gardiner, 1982a). Four tissue slices from the nucleus accumbens and corpus striatum of each side were cut in a plane parallel to the coronal section (approx. 0.35 mm thickness). The tissue slices were incubated in Krebs

Substance P facilitates the release of dopamine in the nucleus accumbens and corpus striatum of rat

I. M. GARDINER and J. S. DE BELLEFROCHE

Departments of Biochemistry and Neurology, Charing Cross Hospital Medical School, Fulham Palace Road, London W6 8RF, U.K.

Substance P is an undecapeptide with a potent excitatory action on central neurons such as spinal motor neurons (Takahashi et al., 1974). It is found in many regions of the mammalian central nervous system and is particularly concentrated in the dorsal horn of the spinal cord, trigeminal nerve nucleus, basal ganglia, and limbic system (Kanazawa & Jessell, 1976). Both Substance P immunoreactive cells and a prominent Substance P innervation are found in the nucleus accumbens and corpus striatum (Ljungdahl et al., 1978; Cuello & Kanazawa, 1978). This led us to investigate the effect of Substance P on two major transmitters present in these regions, dopamine (3,4-dihydroxyphenethylamine) and acetylcholine.

Methods

Male CFY rats (230–270g body wt.) were stunned and killed by cervical dislocation. The nucleus accumbens and corpus striatum were dissected out from the brain as described previously (de Bellefrocke & Gardiner, 1982a). Four tissue slices from the nucleus accumbens and corpus striatum of each side were cut in a plane parallel to the coronal section (approx. 0.35 mm thickness). The tissue slices were incubated in Krebs
bicarbonate medium of the following composition (mM): NaCl, 118; KCl, 4.7; MgSO<sub>4</sub>, 7H<sub>2</sub>O, 2; NaHCO<sub>3</sub>, 25; KH<sub>2</sub.PO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.6; glucose, 11.1; ascorbate, 2.3; pargyline, 1; also containing 0.065m-[Me-<sup>3</sup>H]choline chloride (sp. radioactivity 77 Ci/mmol; Amersham International) and 4.4µM-[<sup>14</sup>C]dopamine (sp. radioactivity 56 Ci/mol; Amersham International), pH 7.4, and gassed with O<sub>2</sub>/CO<sub>2</sub> (19:1) at 37°C for 30 min. The tissue slices were then superfused with isotope-free medium for 30 min and incubated first for a 10 min period under control or test conditions and then for a second period in the presence of 34m~-K+ for 5 min. Portions from the incubation medium and the solubilized tissue were taken for analysis of <sup>14</sup>C and <sup>3</sup>H. Release of [<sup>3</sup>H]acetylcholine and [<sup>14</sup>C]dopamine is expressed as fractional release/min. A fuller description is published elsewhere (de Bellerocche & Neal, 1982; de Bellerocche & Gardiner, 1982). Substance P was obtained from Cambridge Biochemicals.

Results and discussion

The release of [<sup>14</sup>C]dopamine from tissue slices of both nucleus accumbens and corpus striatum was enhanced by the presence of Substance P. In the nucleus accumbens, the release of [<sup>14</sup>C]dopamine was increased 5.2%, 15.6% and 49.7% above control levels at concentrations of Substance P of 10µM, 30µM and 50µM respectively. A similar enhancement (39.6%) of release of 34m~-K+ for 5 min. Portions from the incubation medium and the solubilized tissue were taken for analysis of <sup>14</sup>C and <sup>3</sup>H. Release of [<sup>3</sup>H]acetylcholine and [<sup>14</sup>C]dopamine is expressed as fractional release/min. A fuller description is published elsewhere (de Bellerocche & Neal, 1982; de Bellerocche & Gardiner, 1982). Substance P was obtained from Cambridge Biochemicals.

Table 1. The effect of Substance P on the release of dopamine from rat brain

<table>
<thead>
<tr>
<th>Substance P (30µM)</th>
<th>Control</th>
<th>Substrate P (30µM)</th>
<th>34m~-K+</th>
<th>Substance P (30µM)</th>
<th>34m~-K+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate P (50µM)</td>
<td>0.870 ± 0.058 (18)</td>
<td>0.931 ± 0.087 (12)</td>
<td>1.050 ± 0.272 (9)</td>
<td>0.870 ± 0.058 (18)</td>
<td>0.931 ± 0.087 (12)</td>
</tr>
<tr>
<td>34m~-K+</td>
<td>2.400 ± 0.272 (9)</td>
<td>3.790 ± 0.386 (7)</td>
<td>3.396 ± 0.402 (10)</td>
<td>4.855 ± 0.472 (7)</td>
<td></td>
</tr>
<tr>
<td>Substance P (30µM)</td>
<td>3.396 ± 0.402 (10)</td>
<td>4.855 ± 0.472 (7)</td>
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<td>4.855 ± 0.472 (7)</td>
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Values are means ± S.E.M. for the numbers of experiments in parentheses. * indicates that the value is significantly increased by Substance P, using Student's t test (P < 0.01).

facilitating release, as has also been shown to occur in substantia nigra (Reubi et al., 1978). The present results also indicate that the facilitatory action of Substance P on the resting release of dopamine is not dependent on the presence of exogenous Ca<sup>2+</sup>.

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The role of neutral endopeptidase in neuropeptide metabolism

REBECCA MATSAS, IAN S. FULCHER, A. JOHN KENNY and ANTHONY J. TURNER
Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

Substance P and the enkephalins are believed to function as neurotransmitters in the central nervous system. Their post-synaptic actions are rapidly terminated by metabolism rather than by a transport mechanism (Iversen et al., 1976; Lane et al., 1977). Current discussion of the inactivation of these peptides has implied the existence of peptide-specific enzymes, e.g. Substance P-degrading enzyme (Lee et al., 1981) or enkaphalinase (Malfroy et al., 1978). Such nomenclature suggests an exclusive relationship between peptidase and substrate, and has consequently suppressed comparisons with well-characterized peptidases from other tissues. However, we have previously shown that kidney neutral endopeptidase (EC 3.4.24.11) and the enzyme responsible for hydrolysis of the Gly<sub>γ</sub>-Phe<sub>δ</sub> bond of [Leu]enkephalin by synaptic membranes show similar sensitivity to phosphoramidon and other inhibitors (Fulcher et al., 1982). In the present communication we provide further evidence to support the functional identity of the two enzymes.

Synaptic membranes were prepared from pig caudate nuclei (15–30g), as previously described for cortex (Fulcher et al., 1982), to yield 200µg of membrane/g of tissue. The hydrolysis of an enkephalin analogue (Tyr-d-Ala-Gly-Phe-Leu) by this preparation was completely inhibited by phosphoramidon. The IC<sub>50</sub> concentration was 8nM, comparable with that reported for the hydrolysis of [Leu]enkephalin by purified kidney endopeptidase (Fulcher et al., 1982). The kidney endopeptidase hydrolysed Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH<sub>2</sub>) producing at least seven peptides. The products of Substance P hydrolysis were separated by high-pressure liquid chromatography and identified by amino acid analysis of the individual peptides. The products were consistent with hydrolysis occurring at the bonds Gly<sub>γ</sub>-Phe<sub>δ</sub>, Phe<sub>γ</sub>-Phe<sub>δ</sub> and Gly<sub>γ</sub>-Leu<sub>δ</sub> (Table 1). All are predictable from the known specificity of the endopeptidase, which hydrolyses peptide bonds involving the amino groups of hydrophobic amino acid residues (Kerr & Kenny, 1974). Hydrolysis of Substance P by synaptic membranes yielded peptides with the same high-pressure-liquid-chromatographic retention times, provided that the aminopeptidase inhibitor bestatin (0.1mM) was present to inhibit further degradation by aminopeptidases of the products. Phosphoramidon (10µM) totally abolished the hydrolysis of Substance P by kidney endopeptidase and synaptic membranes, indicating the similarity of the two activities. Aminopeptidases could not initiate hydrolysis of Substance P, but could further degrade products formed by the primary attack of the enzyme.