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Investigation of possible interactions between substance P and transmitter mechanisms in the substantia nigra and corpus striatum of the rat

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The effect of substance P (SP) on the uptake and release of radiolabelled dopamine (^{3}H-DA), 5-hydroxytryptamine (^{3}H-5-HT) and γ-aminobutyric acid (^{3}H-GABA) was studied in slices of rat substantia nigra and corpus striatum. SP, 10^{-9} to 10^{-5}M, failed to modify the uptakes of these compounds during incubations (10–90 min) with slices of either brain region. SP, 10^{-6}M, had no apparent effect on the spontaneous output of any of these compounds in either substantia nigra or corpus striatum. In the corpus striatum, SP seemed to potentiate the potassium-stimulated outflow of ^{3}H-DA and ^{3}H-5-HT, but not ^{3}H-GABA, while the releases from substantia nigra were unaffected. Morphine (10^{-3}M), but not met-enkephalin (5×10^{-6}M), weakly antagonized K^+-evoked release of ^{3}H-DA in the corpus striatum. These results are discussed with reference to the possible interaction of SP with transmitter mechanisms at presynaptic sites in the central nervous system.

Recent evidence from immunocytochemical and lesioning experiments leaves little doubt that substance P (SP) abounds in the axon terminals of specific pallido-nigral and caudato-nigral neurons in the rat, although the peptidergic nature of these projections remains unresolved (Kanazawa & Jessell, 1976; Hong, Yang & others, 1977; Kanazawa, Emson & Cuello, 1977). Apart from SP, other prospective neurotransmitters in the substantia nigra (SN) include γ-aminobutyric acid (GABA: Kim, Bak & others, 1971; Okada & Hassler, 1973; Fonnum, Grofova & others, 1974; Dray & Straughan, 1976), dopamine (Aghajanian & Bunney, 1973; Björklund & Lindvall, 1975; Geffen, Jessell & others, 1976; Korf, Zieleman & Westerink, 1976) and 5-hydroxytryptamine (5-HT; Fuxe, 1965; Palkovits, Brownstein & Saavedra, 1974; Brownstein, Palkovits & others, 1975; Dray, Gonye & others, 1976). There is increasing evidence for the existence of a complex network of synaptic connections in the SN involving some of these transmitter substances. For example, the efflux of endogenous SP from nigral slices can be attenuated by GABA (Jessell, 1977), but is unaffected by dopamine, whereas this amine liberates GABA from intranigral stores (Reubi, Iversen & Jessell, 1977). It is possible that SP, too, in addition to its proposed transmitter role at postsynaptic receptors, may regulate neuronal activity at presynaptic sites within the SN. The present study was designed, in part, to test this hypothesis.

There is also reason to suspect that SP may similarly interfere with neuronal transmitter processes in other areas of the central nervous system (cns). Malick & Goldstein (1977) observed that the analgesia produced in the rat by injections of SP into the dorsal nucleus of the midbrain raphé could be reversed by naloxone, and they speculated that SP might be an opiate receptor agonist. Other powerful analgesic agents, such as β-endorphin and morphine, stimulate opiate receptors in the corpus striatum (CS) and elicit a potent, naloxone-sensitive inhibition of the potassium-stimulated overflow of [^{3}H]dopamine from this tissue (Loh, Brase & others, 1976). It seemed appropriate, therefore, to determine whether SP was similarly capable of modulating the release (and uptake) of this and other transmitter candidates in the CS, as well as SN, possibly via opiate receptors, in an attempt to gain some insight into the cellular mechanisms underlying the central actions of SP.

MATERIALS AND METHODS

Materials

Male Wistar albino rats, 150–250 g were used (A. J. Tuck & Son, Rayleigh, Essex). Radiochemicals were obtained from The Radiochemical Centre, Amersham, and included 4-amino-n-[G-^{3}H]butyric acid (27·2 Ci mmol^{-1}) (^{3}H-GABA), [ring-G-^{3}H]dopamine hydrochloride (8·5 Ci mmol^{-1}) (^{3}H-DA) and 5-hydroxy[G-^{3}H]tryptamine creatinine sulphate (0·5 Ci mmol^{-1}) (^{3}H-5-HT). Substance P was obtained from the Protein Research Foundation, Osaka, Japan. Butyl-PBD, toluene and 2-ethoxyethanol were supplied by Fisons Scientific Apparatus, Loughborough. Morphine was obtained through MacFarlan Smith Ltd., Edinburgh, while methionine^5-

enkephalin (met-enkephalin) was a gift from Reckitt and Colman.

Methods

Rats were stunned and killed by cervical dislocation. Brains were quickly removed into ice-cold Krebs bicarbonate solution (for composition see Starr, 1973), and the SN and CS dissected free. Slices of these regions, 0·2 mm thick, were prepared using a mechanical tissue chopper (McIlwain & Buddle, 1953). These were carefully teased apart and allocated randomly to siliconized conical flasks containing 10 ml Krebs solution. Slices were given a 10 min preincubation at 37°, with or without added drugs, and then incubated for various times at this temperature in the presence of ^{3}H-DA, ^{3}H-5-HT or ^{3}H-GABA (0·5 μCi ml^{-1} for perfusion experiments, 0·05 μCi ml^{-1} for uptake experiments). The flasks were shaken continuously and gassed with 5% carbon dioxide in oxygen throughout. For the uptake experiments the incubation was terminated after 15 min and the slices washed, weighed on a torsion balance and dissolved in 0·2 ml Soluene-350 (Packard). The solutions were neutralized with 5 drops glacial acetic acid and mixed with 6 ml 2-ethoxyethanol and 10 ml 0·5% butyl-PBD in toluene in preparation for liquid scintillation spectrometry. Small aliquots (10 μl) of the incubation media were also counted and uptakes expressed as tissue: medium ratios (d min^{-1} g^{-1} tissue: d min^{-1} ml^{-1} incubation medium).

For the perfusion experiments, the slices were recovered after 30 min incubation, washed and placed randomly into an eight compartment Perspex perfusion chamber. Each chamber was perfused with Krebs solution at 37° from a continuously gassed reservoir at the rate of 0·8 ml min^{-1} by means of a roller pump. The perfusates were collected automatically every 10 min by a fraction collector, their volumes measured and 0·5 ml removed for scintillation counting. The radioactivity present in each 10 min sample was expressed as a percentage of the amount remaining in the tissue and the average quantity released per min calculated (% min^{-1}). Results were analysed statistically by Student's *t*-test.

RESULTS

Uptake experiments

It can be seen from Table 1 that ^{3}H-DA, ^{3}H-5-HT and ^{3}H-GABA were actively accumulated by rat SN and CS *in vitro*. At the chosen substrate concentration (10^{-7} M), ^{3}H-GABA and ^{3}H-5-HT were transported by SN much more avidly than was ^{3}H-DA, whereas in CS the tissue: medium ratios were of the same order for all three substrates. In both structures amino-oxyacetic acid (AOAA, 10^{-5} M), at a concentration which inhibited completely the metabolism of GABA (Neal & Starr, 1973), potentiated the accumulation of ^{3}H-GABA by about 40% (P <0·005). On the other hand, nialamide (3 × 10^{-5} M) prevented the oxidative deamination of dopamine and 5-HT without modifying the uptake of either amine by these two brain regions (Starr, James & Gaytten, 1978). These enzyme inhibitors were routinely included in the appropriate bathing medium in subsequent perfusion studies to eliminate breakdown of the compound being investigated.

Table 1. *Influence of SP on uptakes of ^{3}H-DA, ^{3}H-5-HT and ^{3}H-GABA in rat substantia nigra (SN) and corpus striatum (CS).* All incubations were conducted for 15 min at 37° and at final substrate concentrations of 10^{-7} M. Where used, drugs were included in the bathing medium throughout the experiment. Each result is the mean of the number of experiments shown in parentheses. All s.e.m. lay in the range ± 4–11%.

	Tissue: medium ratio (d min^{-1} g^{-1} tissue: d min^{-1} ml^{-1} medium)					
	^{3}H-DA		^{3}H-5-HT		^{3}H-GABA	
	Nialamide concn (M)				AOAA concn (M)	
SP concn (M)	0	3 × 10^{-5}	0	3 × 10^{-5}	0	10^{-5}
SN 0	4·60 (12)	4·93 (15)	28·11 (17)	26·97 (22)	42·94 (20)	59·44 (32)*
10^{-6}	5·04 (12)	4·77 (16)	26·08 (11)	25·44 (22)	46·86 (17)	66·22 (33)*
CS 0	6·59 (16)	6·52 (9)	8·73 (8)	9·04 (8)	11·51 (16)	16·62 (15)*
10^{-6}	6·68 (10)	6·44 (10)	8·91 (8)	8·83 (9)	12·21 (10)	17·40 (10)*

* P <0·005 *vs* controls.

The effects of SP on these uptake processes were studied at 37° using incubation times lasting 10–90 min and at SP concentrations ranging from 10^{-9} to 10^{-5} M. Results for 10^{-6} M SP and 15 min incubation are presented in Table 1. In no instance was the uptake of ^{3}H-DA, ^{3}H-5-HT or ^{3}H-GABA altered significantly in the continued presence of SP, or when SP was added to the flasks at the start of incubation and omitted from the preincubation stage, irrespective of whether the metabolism of the substrate had been impaired or not.

^{3}H-DA release

The outflow of radioactivity from both SN and CS slices was initially rapid, but settled down to a

steady rate after 30–40 min superfusion. Nigral tissues released ^{3}H-DA spontaneously at a significantly faster proportional rate (0·42% min^{-1}) than did striatal slices (0·12% min^{-1}, P <0·005; Table 2). Brief exposure to high potassium (10–40 mM) evoked a concentration-dependent release of ^{3}H-DA in slices of both brain regions. Omission of calcium ions from the medium perfusing over SN tissue reduced the response to 20 mM K^+ from 1·20 ± 0·07 to 0·40 ± 0·04% min^{-1} (P <0·005, n = 6). Identical results were obtained in the presence of SP (10^{-8}–10^{-5} M) no matter whether the peptide was superfused continuously over the slices, or administered as 1 or 10 min pulses, delivered either singly or repeatedly at 30 min intervals.

Morphine and met-enkephalin were included in this study to establish the susceptibility of the potassium effects in CS to opiate receptor activation. In contrast to the earlier findings of Loh & others (1976), 10^{-5} and 10^{-4} M morphine did not suppress ^{3}H-DA release provoked by potassium depolarization (20 mM, Table 3). Only when CS slices were exposed to 10^{-3} M morphine for 20 min was any subsequent inhibition of the response observed (36%, P <0·005). At this dose, however, morphine itself caused a pronounced, though gradual increase in ^{3}H-DA overflow, reaching 0·56 ± 0·08% min^{-1} after 20 min administration. Met-enkephalin (5×10^{-6} M) was similarly devoid of inhibitory activity in this test system.

^{3}H-5-HT release

The effects of SP on the efflux of ^{3}H-5-HT from SN and CS *in vitro* are presented in Table 2. The basal release rate of ^{3}H-5-HT after 30 min superfusion was higher in CS (0·63 ± 0·05% min^{-1}) than in SN (0·28 ± 0·02% min^{-1}, P <0·005). SP, over the concentration range 10^{-8} to 10^{-5} M, had no effect on the resting output of ^{3}H-5-HT from either SN or CS when the peptide was presented briefly to the tissues for one or more periods of 1 or 10 min every 30 min, or when it was administered continuously. As can be seen from Table 2, the ^{3}H-5-HT accumulated by CS slices was more sensitive to release by potassium than was that taken up by SN. Calcium ions were found to be necessary for this ^{3}H-5-HT release in SN, since in their absence the release obtained with 30 mM K^+ fell from 1·23 ± 0·07 to 0·45 ± 0·03% min^{-1} (P <0·005, n = 5). Pretreatment with SP (10^{-6} M) for 20 min had no discernible effect on the potassium-stimulated efflux of ^{3}H-5-HT in either SN or CS.

Table 2. *Effect of substance P on spontaneous and potassium-stimulated overflow of ^{3}H-DA and ^{3}H-5-HT from slices of rat substantia nigra (SN) and corpus striatum (CS).* Slices were preloaded with ^{3}H-DA (0·5 μCi ml^{-1}, $5·9 \times 10^{-8}$ M) or ^{3}H-5-HT (0·5 μCi ml^{-1}, 10^{-6} M) for 30 min at 37°, then washed and superfused with Krebs bicarbonate solution at 37° at the rate of 0·8 ml min^{-1}. Values for the resting release correspond to average amount of radioactivity recovered over the period 30–60 min after the start of superfusion. Slices were exposed to a 5 min pulse of high potassium medium at 60 min, and to SP at 40–65 min after commencing superfusion. Nialamide (3×10^{-5} M) was present throughout. Each result is the mean ± s.e.m. of the number of experiments shown in parentheses.

	K+ concn (mM)	Radioactivity released (% min^{-1} of total tissue stores)			
		Substance P concentration (M)			
		0	10^{-6}	0	10^{-6}
		^{3}H-DA		^{3}H-5-HT	
SN	0	0·42 ± 0·02 (37)	0·44 ± 0·05 (19)	0·28 ± 0·02 (6)	0·26 ± 0·02 (6)
	10	0·66 ± 0·04 (8)	0·62 ± 0·06 (8)	—	—
	20	1·20 ± 0·07 (26)	1·09 ± 0·08 (9)	0·46 ± 0·01 (6)	0·47 ± 0·01 (6)
	30	—	—	1·14 ± 0·09 (8)	1·19 ± 0·07 (7)
	40	1·78 ± 0·13 (9)	1·68 ± 0·16 (9)	—	—
CS	0	0·12 ± 0·01 (49)	0·07 ± 0·01 (28)	0·63 ± 0·05 (6)	0·49 ± 0·08 (8)
	10	0·44 ± 0·04 (24)	0·37 ± 0·04 (11)	—	—
	20	1·26 ± 0·16 (10)	1·73 ± 0·26 (10)*	1·87 ± 0·11 (6)	2·20 ± 0·10 (6)*
	40	3·32 ± 0·19 (15)	3·71 ± 0·26 (7)	—	—

* P <0·1 compared to release in the absence of SP.

^{3}H-GABA release

In the absence of GABA catabolism a typically slow efflux of tritium occurred from SN and CS slices preloaded with ^{3}H-GABA. Spontaneous efflux rates were closely similar in both tissues and approximated to 0·1% of the tissue stores min^{-1}. High potassium (+30 mM) raised the release rates by roughly fivefold in both brain regions, but in neither case was the resting or potassium-evoked release of ^{3}H-GABA modified in the presence of SP (10^{-6} M). Similar results were also obtained with lower (10^{-8} M) and higher (10^{-5} M) SP concentrations applied to the slices for periods of 1–60 min.

In four experiments the release rate of ^{3}H-GABA with 20 mM K^+ and normal calcium (2·54 mM) was 0·67 ± 0·05% min^{-1} compared with 0·62 ± 0·04% min^{-1} without calcium, and 1·90 ± 0·26% min^{-1} in

Table 3. *Effects of morphine and met-enkephalin on potassium stimulated ^{3}H-DA release from rat striatal slices.* Experimental details as for Table 2. Morphine and met-enkephalin were applied to the tissues 40–65 min, and high potassium medium 60–65 min following the onset of superfusion. Nialamide (3×10^{-5} M) was present throughout. Each result is the mean ± s.e.m. of the number of experiments shown in parentheses.

Pretreatment	Concn (M)	Release of ^{3}H-DA evoked by 20 mM K^+ (% min^{-1} of total tissue stores)
None (controls)	—	13·69 ± 1·14 (8)
Morphine	10^{-5}	13·92 ± 0·81 (12)
Morphine	10^{-4}	12·31 ± 0·92 (10)
Morphine	10^{-3}	8·73 ± 1·05 (8)*
Met-enkephalin	10^{-6}	14·10 ± 0·56 (8)
Met-enkephalin	5×10^{-6}	11·22 ± 0·38 (4)

*$P<0.005$ compared to controls.

a Ca^2-free medium containing 10 mM EDTA. At this concentration EDTA also raised the resting output of ^{3}H-GABA approximately threefold (0·20 ± 0·03% min^{-1}). When the Mg^{2+} concentration of the Ca^{2+}-free medium was raised to 13·2 mM, the K^+-evoked release fell to 0·18 ± 0·04% min^{-1} ($P<0.005$, n = 6). These findings could explain the discrepancy between the apparent insensitivity to changes in calcium ions alone of the electrically-evoked release of ^{3}H-GABA from SN (Okada & Hassler, 1973), and the abolition of depolarization-induced ^{3}H-GABA release in a low calcium, high magnesium medium (Geffen & others, 1977).

DISCUSSION

The above results describing the uptake and release of ^{3}H-DA and ^{3}H-GABA in SN and CS are in broad agreement with the findings of other workers (e.g. Okada & Hassler, 1973; Geffen & others, 1977). The avid acquisition and slow, calcium-dependent re-release of ^{3}H-5-HT by SN are reminiscent of the amine entering and leaving a protected transmitter store, and lend additional support to the concept of a serotoninergic input to this region from the mid-brain raphé nucleus (e.g. Dray & others, 1976). Furthermore, the quantitatively greater uptake and tighter binding of ^{3}H-5-HT compared with ^{3}H-DA in SN suggests the former amine is being accumulated by true serotoninergic terminals as opposed, say, to storage vesicles present in nigral dopaminergic nerve endings or dendrites.

There is growing evidence that SP may have a physiological role as an excitatory transmitter in the SN. For example, depolarizing stimuli induce SN slices to release SP (Reubi & others, 1977), while intranigral injections of SP are accompanied by behavioural and biochemical manifestations of increased nigrostriatal dopamine cell activity (Cheramy Nieoullon & others, 1977; James & Starr, 1977; Starr & others, 1978). It seems highly likely, therefore, that SP afferents to the SN terminate post-synaptically on nigrostriatal dopamine neurons and help to control their firing rate. Activation of SP receptors in the brain appears to involve an SP-sensitive adenylate cyclase (Duffy, Wong & Powell, 1975), but the present results give little indication of a presynaptic modulatory function of SP at GABA-ergic, dopaminergic or serotoninergic synapses in the SN, akin to those suggested for GABA (Jessell, 1977) and dopamine (Reubi & others, 1977), since none of the aspects of transmitter function studied here was modified by the peptide.

Morphine behaves differently from SP inasmuch as it interacts extensively with a wide variety of transmitter systems in the CNS. In spite of its wide spectrum of pharmacological activity, many of the behavioural and biochemical effects of morphine appear to be centred on the CS (Iwamoto, Ho & Way, 1973; Pert, Pasternak & Snyder, 1973; Bergmann, Chaimovitz & others, 1974; Berney & Buxbaum, 1974; Kuschinsky & Hornykiewicz, 1974). The striatum contains the highest density of opiate receptors (Pert & others, 1973; Lee, Akera & others, 1974), stimulation of which powerfully attenuates the accelerated outflow of ^{3}H-DA that accompanies potassium depolarization of the tissue (Loh & others, 1976). Although these authors achieved 100% suppression of this phenomenon with 10^{-5} M morphine, no such inhibition was apparent at this dose in the present study, and even 10^{-3} M morphine caused only partial inhibition. In agreement with these investigators, met-enkephalin had no such effect, although Subramanian, Mitznegg & others (1977) have claimed this opioid peptide inhibits potassium-evoked overflow of ^{3}H-DA from CS by as much as 50% when used at micromolar concentrations. The reasons for these differences are not yet clear, but there is no doubt that SP failed to mimic these compounds in this test system.

SP's apparent non-affinity for striatal opiate receptors does not necessarily exclude the existence of other species of this receptor for which SP acts as a ligand, say in the substantia gelatinosa of the spinal cord, where SP is believed to be the transmitter released by primary sensory neurons (e.g. Cuello, Polak & Pearse, 1976). However, considerations of the structural dissimilarity between SP and

the endogenous opioid peptides, as well as the potentiation by SP of the responses to noxious stimuli (Randić & Miletic, 1977), in sharp contradistinction to morphine (Zieglgänsberger & Bayerl, 1976; Duggan, Hall & Headley, 1977), do not favour this view. The mechanism(s) by which SP produces analgesia, therefore, continues to remain an intriguing and unanswered question.

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