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Substance P and epibatidine-evoked catecholamine release from fractionated chromaffin cells

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Abstract

Bovine chromaffin cells were separated by density gradient centrifugation into subfractions enriched with either > 90% adrenaline- or 70–80% noradrenaline-producing cells. Concentrations of epibatidine (an alkaloid with nicotinic receptor activity) as low as 10 nM released adrenaline and noradrenaline from both fractions of cells maintained as monolayer cultures. The maximal effect was evoked by 30 nM epibatidine and was comparable to that evoked by 10 μM nicotine. The catecholamine release from the noradrenaline fraction was 30–40% higher than from the adrenaline fraction. Initial exposure to 50 nM epibatidine reduced release induced by a second exposure to the drug. There was cross-desensitization between epibatidine and nicotine. Substance P inhibited the epibatidine-evoked catecholamine release from both fractions by up to 85% (IC_{50} = 3–5 μM). The release of noradrenaline was inhibited more than that of adrenaline. In addition, substance P protected the chromaffin cells against desensitization of the nicotinic receptor by epibatidine. The C-terminal heptapeptide sequence of substance P was 10× less active, two N-terminal sequences did not modulate the catecholamine release.

Keywords: Chromaffin cell; Epibatidine; Substance P; Noradrenaline release; Adrenaline release

1. Introduction

Epibatidine is an alkaloid which was first isolated from the skin of the Ecuadorian poison frog *Epipedobates tricolor* (Daly et al., 1980). Its structure was published in 1992 (Spande et al., 1992) and is shown in Fig. 1. Today epibatidine is produced by total synthesis and commercially available.

Epibatidine is a potent analgesic in mice and elicits the Straub-tail reaction. This phenomenon is known to be typically evoked by morphine and morphine-like analgesics. However, the analgesic effect of epibatidine is not reduced by the opiate receptor antagonist naloxone, but is effectively blocked by the neuronal nicotinic receptor antagonist mecamylamine (Spande et al., 1992). Receptor binding measurements indicate that epibatidine has a very high affinity for nicotinic receptors. Epibatidine binds to sites in rat adrenal gland (Houghtling et al., 1995) and stimulates the influx of sodium into PC-12 cells. Its agonist activity is 5× higher at ganglionic-type nicotinic receptors of PC-12 cells than at muscle-type nicotinic receptors of medulloblastoma TE671 cells (Badio and Daly, 1994).

Our present study aimed to quantitate the catecholamine-releasing activity of epibatidine in comparison to that of nicotine on two fractions of bovine chromaffin cells, one fraction containing predominantly adrenaline-producing cells (> 90%) and the other containing approx. 70% noradrenaline-producing cells (Krause et al., 1996). We were also interested in comparing the desensitization of the nicotinic response induced by epibatidine with that of nicotine.

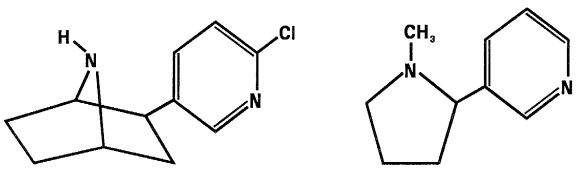

Fig. 1. Structure of epibatidine and of nicotine.

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The second part of this study examines the effect of substance P on epibatidine-evoked catecholamine release. In isolated bovine chromaffin cells substance P inhibits the acetylcholine- and nicotine-evoked release of catecholamines (Livett et al., 1979; for review see Livett and Zhou, 1991). In superfused rat adrenal gland slices, substance P inhibits the presynaptic acetylcholine release and the postsynaptic catecholamine release evoked by electrical field stimulation (Nieber and Oehme, 1987). The $^{22}Na^{+}$ efflux from acetylcholine receptor-enriched membrane vesicles of *Torpedo* electroplaque is also reduced by substance P (Min and Weiland, 1992).

A physiological role for modulation of nicotinic acetylcholine response by substance P is suggested in the adrenal gland, where substance P modulates catecholamine secretion in vivo (see Livett and Zhou, 1991) and innervation by substance-P-containing fibers, originating from the dorsal root ganglia has been demonstrated (Zhou et al., 1991). Moreover, substance-P-positive cells have been detected in the adrenal gland (Görne et al., 1984). All these findings support the hypothesis that substance P modulates the release of catecholamines within the adrenal gland in an auto- and paracrine fashion.

The modulatory effect of substance P on acetylcholine receptors is specific for the nicotinic acetylcholine receptor. Substance P does not block secretion induced by high potassium or veratridine (Livett et al., 1979; Role et al., 1981). It also fails to alter the excitatory effects of glutamate, aspartate, or muscarinic agonists (Belcher and Ryall, 1977; Krnjevic and Lekic, 1977). Up to now, there are no reports about a modulatory effect of substance P on the epibatidine-induced release of catecholamines from isolated chromaffin cells.

Additionally to its modulatory effects, substance P protects chromaffin cells against desensitization of the acetylcholine receptor by nicotine (Boksa and Livett, 1984). In the present study we have examined whether substance P has such an activity against epibatidine. To test this hypothesis, we exposed chromaffin cells pre-treated with epibatidine and a range of concentrations of substance P to a second stimulation with epibatidine in the absence of substance P and measured the catecholamine release. Finally, we tested the modulatory activity of truncated C- and N-terminal sequences of substance P to obtain more information about the relative importance of the C- and N-terminal sequence of substance P in this response.

2. Materials and methods

2.1. Preparation of adrenal medullary cells

Bovine adrenal gland were obtained from a local slaughterhouse and were processed for cell culture within 2 h of slaughter. The chromaffin cells were isolated by digestion with collagenase A as described by Livett et al. (1987).

The principal steps were as follows: The adrenal glands were trimmed of fat and washed with Ca^{2+}- and Mg^{2+}-free buffer containing 154 mM NaCl, 2.6 mM KCl, 0.85 mM KH_2PO_4, 2.15 mM K_2HPO_4, 10 mM glucose and 12.7 mM HEPES buffer at pH 7.4. The buffer was injected into the adrenal vein to rinse out the remaining blood from the gland and then the glands were digested with a solution consisting of this buffer containing 0.25% collagenase A plus 0.01% DNAase I. Five ml of the collagenase solution was injected into the adrenal vein of each gland. This procedure was repeated every 15 min with the glands incubated at 37°C. After 1 h the digested medullary tissue was separated mechanically from the adrenal cortex and filtered through a 500-μm nylon mesh. The filtered cells were washed 3 times with Ca^{2+}-Mg^{2+}-free buffer to remove the collagenase/DNAase and then filtered through a 60-μm nylon mesh.

2.2. Separation of adrenaline- and noradrenaline-producing cells

Approximately 4×10^8 cells were resuspended in 84 ml of the Ca^{2+}-Mg^{2+}-free buffer and mixed with 76 ml isotonic Percoll (68.4 ml Percoll plus 7.6 ml 10-fold concentrated Ca^{2+}-Mg^{2+}-free buffer). The cell-Percoll suspension with a final pH of 7.4 was then added to four polycarbonate centrifuge tubes each of 40 ml. The tubes were centrifuged at $30\,000 \times g$ (= 16 000 rpm in a Sorvall centrifuge model RC 5C Plus with rotor SS-34) for 22 min at 22°C to produce a self-generating Percoll gradient. After centrifugation the content of each tube was fractionated by aspirating the gradient from the bottom of the tubes using a peristaltic roller pump. For further details, see Krause et al. (1996).

2.3. Cell culture

Cell fractions containing preferentially one of the catecholamines (adrenaline or noradrenaline) were collected from the gradient, washed three times to remove the Percoll and transferred into culture medium consisting of 45% Dulbecco's modified Eagle's medium, 45% nutrient mixture F-12 Ham and 10% foetal calf serum. Additionally, the medium contained antibiotics (penicillin and streptomycin; 100 μg/ml of each), antimitotics (fluorodeoxyuridine, cytosine arabinoside and uridine; 2.5 μg/ml of each) and glutamine (0.365 g/l). The cell density was adjusted to 1000 cells/μl culture medium and 400 μl were plated into each well of a 24-well plate and incubated at 37°C in an atmosphere consisting of 5% carbon dioxide in air for 3–6 days before use in functional experiments. After that time more than 90% of all cells were viable as tested by staining with Trypan blue.

2.4. Functional studies: release of catecholamines

The cells were washed 2 × for 5 min with buffer (154 mM NaCl, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 1.18 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM D-glucose, 0.5% (w/v) bovine serum albumin (Serva, fraction V), pH 7.4) and then incubated with epibatidine or nicotine for 5 min. To test whether substance P has a modulatory effect on epibatidine-evoked secretion of catecholamines, the cells were exposed to substance P or to N- and C-terminal sequences for 5 min and for a second period of 5 min to epibatidine (50 μM). All incubations were at room temperature (20–22°C). After each incubation, the buffer was collected and acidified to 0.4 M with perchloric acid for catecholamine analysis. At the end of the experiment, the cells were treated with 0.4 ml of 0.01 M perchloric acid for 10 min to release the remaining cellular catecholamines and then with an additional 0.4 ml of 0.8 M perchloric acid for 5 min. Finally, the two perchloric acid washes were combined and assayed for catecholamines.

Desensitization of the nicotinic response was investigated as follows: After the two washes with buffer, the cells were incubated with various concentrations of epibatidine or nicotine for 5 min followed by two 10-s washes with buffer. The media from the 5-min incubation period and the two short washes were pooled and analyzed for catecholamines. The degree of desensitization was then tested by incubating all cells with the same concentration of epibatidine (50 nM) or nicotine (5 μM). Again, the cells were exposed to the stimulus for 5 min, washed 2 × for 10 s, and all three samples were pooled for analysis of catecholamines.

For evaluating the effect of substance P on epibatidine-evoked secretion and desensitization, various concentrations of substance P were already present in the buffer used for the second wash and during the first incubation with epibatidine (100 nM). After two washes, a second incubation with 50 nM epibatidine applied to all wells revealed the extent the prior substance P treatment had protected against desensitization.

All samples were centrifuged at 20 000 × *g* for 8 min to precipitate proteins. The supernatants were analysed for catecholamines by high-performance liquid chromatography (125 mm × 3 mm I.D. column, packed with Nucleosil 100C18; 3 μm particle size) and electrochemical detection (model Waters 460). The mobile phase consisted of 5% acetonitrile, 10 g/l citric acid, 4 g/l KH_2PO_2 and 0.17 g/l octanesulfonic acid; pH 3.0.

Substance P (SP) and its derivatives (SP_{1-4}, SP_{1-7} and SP_{5-11}) were synthesized and supplied by M. Bienert from our Institute. Epibatidine was purchased from Research Biochemical International, Natick, USA; collagenase A and DNAse I from Boehringer, Mannheim, Germany; bovine serum albumin from Serva, Heidelberg, Germany; all other drugs from Sigma-Aldrich Chemie, Deisenhofen, Germany.

3. Results

Since (−)-epibatidine and (+)-epibatidine are reported to be nearly equipotent (Badio and Daly, 1994) we used the racemic (±)-mixture. Epibatidine induced strong release of catecholamines, even at low concentrations. The left diagram of Fig. 2 shows the concentration-response curve of epibatidine-induced catecholamine release from a fraction enriched with noradrenaline cells. In comparison to nicotine which induced a half-maximal release from both fractions at 5 μM (Krause et al., 1996), epibatidine evoked comparable effects at about 250 times lower concentration. The shape of the concentration-response curves corresponded to that of nicotine: cells exposed to a higher concentration of epibatidine displayed desensitization of the nicotinic response, i.e., lower catecholamine release. The adrenaline-rich cell fraction released significantly less catecholamines, noradrenaline as well as adrenaline, in comparison to the fraction enriched with noradrenaline

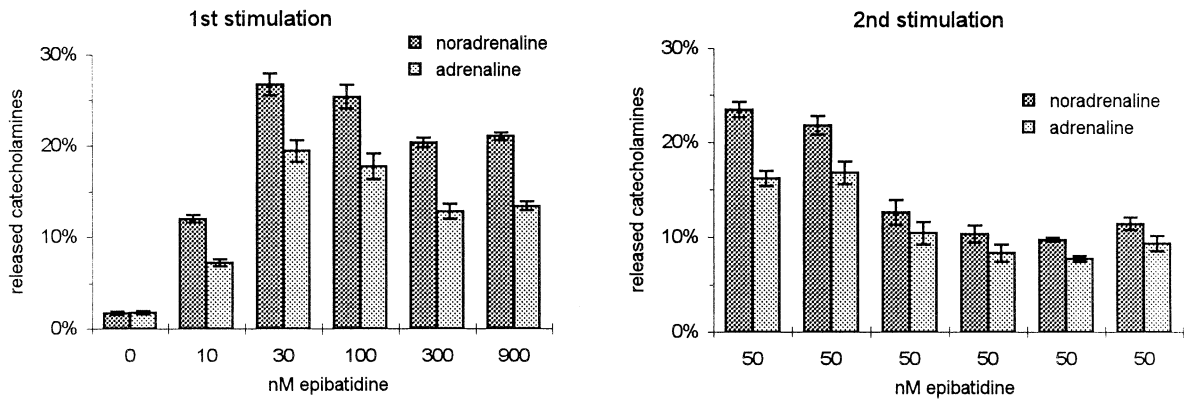

Fig. 2. Concentration–response curve of epibatidine-induced catecholamine release from bovine chromaffin cells (left diagram). Dependent on the first concentration of epibatidine (1st stimulation) the cells were less responsive to a second stimulation with 50 nM epibatidine (right diagram). Release of catecholamine expressed as percentage of total content/5 min. Mean ± S.E.M., $n = 4$.

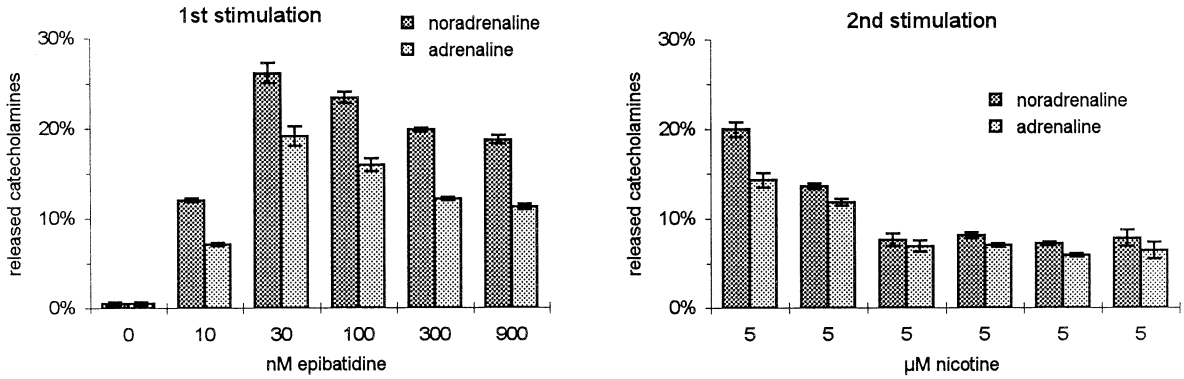

Fig. 3. Concentration–response curve of catecholamine release from bovine chromaffin cells: cross-desensitization epibatidine/nicotine. Pretreatment with epibatidine (left diagram) desensitizes the cells to an EC_{50} concentration of nicotine (right diagram). Release expressed as percentage of total content/5 min. Mean ± S.E.M., $n = 4$.

cells. This difference was seen at all concentrations of epibatidine, the maximal catecholamine release was approx. 30% lower from the adrenaline-enriched cells. On the other hand, more noradrenaline was released than adrenaline from both fractions. As a percentage of the total cell content, approx. 30% more noradrenaline than adrenaline were secreted. An analogous effect was observed in earlier experiments (Krause et al., 1996) when the two fractions were stimulated with nicotine.

The nicotinic receptor antagonist hexamethonium blocked the response of epibatidine (data not shown). The effects of epibatidine and nicotine were antagonized by comparable concentrations of hexamethonium. The catecholamine release evoked by 30 nM epibatidine was half-maximally reduced by 60 µM hexamethonium, an equieffective concentration of 10 µM nicotine by 50 µM hexamethonium.

The data presented in Fig. 2 (right-hand side) show a diminished catecholamine release from cells stimulated a second time with epibatidine. If the cells were stimulated with epibatidine for 5 min followed by two washes and exposed to 50 nM epibatidine, the chromaffin cells desensitized. Moreover, there was a clear correlation between the concentration of epibatidine present during the first incubation period and the reduced catecholamine release during the second incubation period. Reduced catecholamine release was observed if the cells were treated first with nicotine followed by epibatidine (data not shown) or vice versa (Fig. 3). As mentioned above, approx. 30% more noradrenaline than adrenaline was released in response to the first stimulation. This difference decreased during repeated (or long-lasting) stimulation of the cells: the noradrenaline-releasing cells desensitized faster than the adrenaline-releasing cells.

The left-hand side of Fig. 4 shows the modulatory effect of substance P on the catecholamine release from a fraction of adrenaline-enriched chromaffin cells which were stimulated with 100 nM epibatidine. The data have been corrected by subtraction of the basal release which was approx. 1% of the total cell content. During the 5 min period of pre-treatment with substance P, the basal catecholamine release remained constant. This confirms that substance P by itself had no effect on the release of adrenaline or noradrenaline from the chromaffin cells.

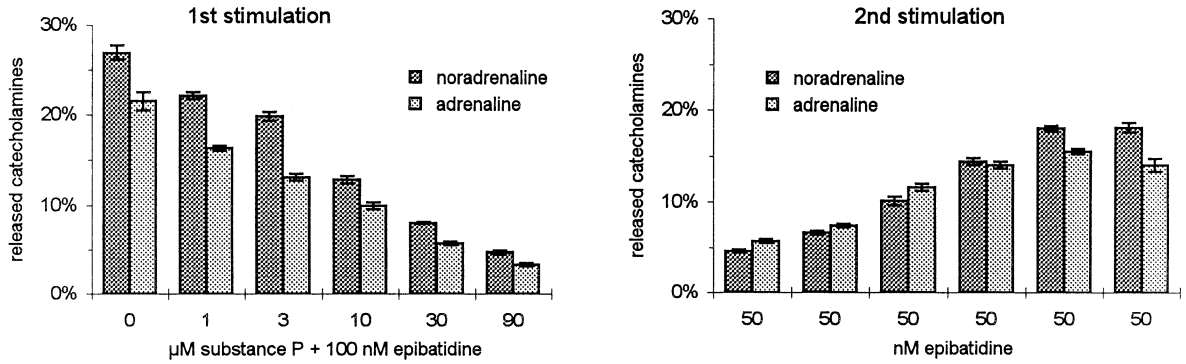

Fig. 4. Modulation of epibatidine-induced catecholamine release by substance P (left diagram) and protection against nicotinic receptor desensitization in cells pre-exposed to substance P in the first stimulation when re-exposed to of epibatidine (no substance P) in the second stimulation (right diagram). Release expressed as percentage of total content/5 min. In each case basal release, which was approx. 1%, has been subtracted. Mean ± S.E.M., $n = 4$.

Substance P inhibited the epibatidine-evoked release of adrenaline and noradrenaline from cells of both fractions up to $>85\%$. This indicates that the cells in both fractions have binding sites for substance P which modulate the release of adrenaline and noradrenaline. The catecholamine release was inhibited half-maximally (IC_{50}) at a concentration of 5–10 μM substance P. A higher concentration of epibatidine (up to 3 μM) did not require more substance P for half-maximal inhibition of the catecholamine release (data not shown). This implies a non-competitive interaction between substance P and epibatidine on the chromaffin cells. Moreover, the catecholamine secretion evoked by 5 μM nicotine was also inhibited half-maximally by approx. 5 μM substance P. This was true in all experiments for the release of adrenaline as well as noradrenaline from cells in both fractions. The IC_{50} values estimated in our experiments compare favourably to data obtained on a mixed adrenaline/noradrenaline cell population (Boksa and Livett, 1984).

The C-terminal heptapeptide sequence of substance P (substance P_{5-11}) was about $10\times$ less effective in modulating the nicotinic evoked catecholamine release than the full sequence (data not shown). Substance P_{1-4} and substance P_{1-7}, two N-terminal sequences, did not significantly modulate the nicotine-evoked release of adrenaline or noradrenaline. The amidated N-terminal tetrapeptide substance P_{1-4} amide was also ineffective.

As presented in Figs. 2 and 3, repeated or long-lasting stimulation of chromaffin cells with epibatidine results in diminution of the catecholamine release. Substance P prevents this process (Fig. 3, right diagram). Cells treated during a first stimulation period with 100 nM epibatidine together with various concentrations of substance P desensitized less, as shown by a second stimulation with 50 μM epibatidine (without substance P). The degree of desensitization was inversely related to the concentration of substance P.

4. Discussion

Since epibatidine mimics several effects of nicotine and inhibits the nicotine binding to brain membranes it is generally accepted that its effects are mediated by excitation of nicotinic acetylcholine receptors. Moreover, epibatidine is known as a nicotinic agonist with a very high potency (nanomolar activity). The present results confirm that epibatidine, at relatively low concentrations, induces a rapid and strong secretion of catecholamines from chromaffin cells. In our experiments, a half-maximal catecholamine release was induced by 20 nM (±)-epibatidine, whereas the equieffective concentration of (−)-nicotine was 5 μM. This confirms results obtained with PC-12 cells in which epibatidine stimulated the influx of sodium and was 200–300-fold more potent than nicotine (Badio and Daly, 1994).

A maximal release of adrenaline and noradrenaline was evoked by 30 nM epibatidine, higher concentrations were less active. This implies that the lower catecholamine release after strong or repeated stimulation of the cells is not caused by exhaustion of the releasable catecholamine pool.

Our results showing cross-desensitization between epibatidine and nicotine provide additional evidence that both compounds stimulate catecholamine release from chromaffin cells by similar mechanisms. Cells desensitized by pretreatment with epibatidine responded less when stimulated with nicotine and vice versa.

Epibatidine is reported to bind with high affinity to cells or tissues that possess nicotinic receptors with distinct subunit combinations. In brain homogenates, [^{3}H]epibatidine binds with high affinity to two classes of sites (Houghtling et al., 1995). This is in contrast to other nicotinic agonists which label only a single binding site in brain. In our experiments, there were no significantly different IC_{50} values estimated from the concentration-response curves for the secretion of adrenaline and noradrenaline from separated fractions of chromaffin cells. This suggests that epibatidine does not bind to the diverse chromaffin cell types or subpopulations with different affinity.

Epibatidine induced a higher release of noradrenaline in comparison to adrenaline. The same effect was seen in response to nicotine, but also to potassium and several other agents (Krause et al., 1996). Moreover, the catecholamine release was generally higher from the fraction enriched with noradrenaline cells, even the approx. 30% adrenaline-producing cells present in this fraction released more of their adrenaline than did cells in the adrenaline-enriched fraction. We suggest that these quantitative differences are based on differences in the receptor-effector coupling rather than a different receptor distribution.

The mechanism by which substance P inhibits the nicotinic response is not fully understood. There is some evidence that substance P binds to a regulatory site on the nicotinic receptor distinct from the neurokinin type 1 receptor. This hypothesis is supported by molecular pharmacological investigations on cloned subunits of the neuronal acetylcholine receptor which were expressed in various combinations in *Xenopus* oocytes. The results indicate that substance P interacts with different nicotinic receptor subunit combinations to inhibit cation currents induced in the oocytes by nicotinic stimulation (Stafford et al., 1994). The IC_{50} differed by approx. 20–30-fold, depending whether β_2 or β_4 was expressed with an α subunit. The effect of substance P was generally higher in receptors containing the β_4 subunit than in those containing the β_2 subunit, whereas the α_3 and α_4 subunits were less important for this activity of substance P. Therefore, the β subunit is assumed to play an important role in determining the effect of substance P, whereas the nicotinic agonist binding sites are located on the α subunits. The concentra-

tion-response curves found in our experiments were of the non-competitive type. This supports the evidence for distinct substance P and nicotinic agonist binding sites on the acetylcholine receptor complex in the adrenal gland.

Substance P_{1-4} and substance P_{1-7}, two N-terminal partial sequences, did not modulate the nicotinic evoked release of catecholamines, although they are known to compete for binding of [^{3}H]substance P to adrenal medullary cells (Geraghty et al., 1990). Moreover, in functional studies, substance P_{1-7} is reported to inhibit the nicotine-stimulated release of ATP (which is co-released stoichiometrically with the catecholamines) from mixed chromaffin cells by 25% (Cheung et al., 1994). We suggest that this effect of substance-P_{1-7} could be mediated indirectly, for example by affecting non-chromaffin cells. Our results indicate that the C-terminus of substance P is essential for the modulatory effect of substance P and that the N-terminus facilitates full activity.

In conclusion, epibatidine-evoked secretion from chromaffin cells was modulated by substance P, requiring both the C- and N-terminus sequence for full activity. With the recent availability of [^{3}H]epibatidine it will be of interest to compare the binding sites for functional activity and modulation by substance P and analogues.

Acknowledgements

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