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REVIEW

Modulation of neuronal nicotinic receptor function by the neuropeptides CGRP and substance P on autonomic nerve cells

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1 One classical example of how neuropeptides can affect the function of ligand-gated receptors is the modulation of neuronal nicotinic receptors (nAChRs) by substance P. The present review updates current understanding of this action by substance P and compares it with other neuropeptides more recently found to modulate nAChRs in the autonomic nervous system.

2 Calcitonin gene-related peptide (CGRP) and its N-terminal fragments have been shown to exert complex inhibitory as well facilitatory actions on nAChRs. Fragments such as $CGRP_{1-4}$, $CGRP_{1-5}$ and $CGRP_{1-6}$ rapidly and reversibly enhance agonist sensitivity of nAChRs without directly activating those receptors. Longer fragments or the full-length peptide potently inhibit responses mediated by nAChRs *via* an apparently competitive-type antagonism. This phenomenon differs from the substance P-induced block, which is agonist use-dependent and preferential towards large nicotinic responses.

3 It is argued that the full-length peptides CGRP and substance P might play distinct roles in the activity-dependent modulation of cholinergic neurotransmission, by inhibiting background noise in the case of CGRP or by reducing excessive excitation in the case of substance P. Hence, multiple neuropeptide mechanisms may represent a wide array of fine-tuning processes to regulate nicotinic synaptic transmission.

4 The availability of novel CGRP derivatives with a strong enhancing action on nAChRs may offer new leads for the drug design targeted for potentiation of nAChRs in the autonomic nervous system as well as in the brain, a subject of interest to counteract the deficit of the nAChR function associated with neurodegenerative diseases like Alzheimer's and Parkinson's diseases.
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Abbreviations: ACh, acetylcholine; APL, allosterically potentiating ligand; CGRP, calcitonin gene-related peptide, $CGRP_{1-x}$, 1 to *x* *N*-terminal fragment of calcitonin gene-related peptide, where *x* is 3, 4, 5, 6, or 7; $CGRP_{1-7A}$, *N*-terminal fragment of calcitonin gene-related peptide in which Cys7 is replaced by Ala; $CGRP_{2-7}$, *N*-terminal fragment of calcitonin gene-related peptide missing Ser1; F3, *N,N,N*-trimethyl-1-(4-trans-stilbenoxy)-2-propylammonium iodide; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; GABA, γ-aminobutyric acid; nAChRs, neuronal nicotinic receptors; $hCGRP_{8-37}$, 8–37 C-terminal fragment of human calcitonin gene-related peptide; PC12, rat pheochromocytoma cells

Neuronal nicotinic receptors as prototypes of ionotropic, ligand-gated receptors

Neuronal nicotinic receptors (nAChRs) are integral membrane proteins that mediate central and peripheral effects of endogenously released acetylcholine (ACh) as well as the action of nicotine delivered *via* tobacco smoking (for a review, see Paterson & Nordberg, 2000). Receptor activation is characterized by an opening of its central aqueous pore through which Na^+, Ca^{2+} and K^+ permeate to generate membrane depolarization with a consequent increase in neuronal excitability (Changeux & Edelstein, 1998; Itier & Bertrand, 2001). AChRs are a heterogeneous group with discrete tissue distribution (Gotti *et al*., 1997). The pleiotropy of nAChRs makes their systematic classification difficult, although a broad subdivision may be based on homomeric α7 receptors and heteromeric non-α7 receptors. Even if the relation between nAChR structure and neuronal function is poorly understood, it seems likely that different subunit compositions may determine amplitude, kinetics or rate of desensitization of nicotinic receptor-mediated responses (Groot-Kormelink *et al*., 1998; Yu & Role, 1998; Le Novère *et al*., 2002). Further details concerning nAChR mechanisms may be found in recent review articles (Karlin, 2002; Le Novere *et al*., 2002; Quick & Lester, 2002; Sine, 2002). Considerable interest in nAChRs stems from the realization that several neuropsychiatric disorders are accompanied by a

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strong deficit in nAChR function, which is probably responsible for at least part of the clinical symptomatology (Paterson & Nordberg, 2000; Picciotto & Zoli, 2002; Raggenbass & Bertrand, 2002). To obtain amelioration of these conditions, it would be potentially useful to amplify either the extracellular concentration of the endogenous neurotransmitter ACh or the nAChR signalling mechanism. The present review will discuss some novel approaches to the latter issue based on direct actions of neuropeptides, which represent a new and rapidly growing field of neuropharmacology.

Modulation of nAChRs

Receptor modulation may be defined as an up- or down-regulation of receptor function associated with direct modification of the receptor protein, often of reversible nature. In principle, it is possible to imagine at least two conditions when receptor modulation may take place. In one case, receptor upmodulation is desirable to boost the faltering activity of nAChRs during the progressive, slow onset of neurological diseases (Pereira *et al*., 2002; Picciotto & Zoli, 2002). In this instance, the aim is to enhance receptor responsiveness in view of their reduced number or decreased concentration of the endogenous transmitter. This type of modulation may be observed with administration of allosteric modulators that, by themselves, cannot significantly activate receptors, yet they facilitate agonist/receptor interaction (Le Novere *et al*., 2002; Pereira *et al*., 2002). As far as the cholinergic system is concerned, drugs like galanthamine or tacrine are thought to be allosteric modulators of nAChRs and are useful for controlling symptoms of Alzheimer's disease (Grutzendler & Morris, 2001; Pereira *et al*., 2002).

A different case of nAChR modulation may be observed with large or persistently applied concentrations of nicotinic agonists (Buisson & Bertrand, 2001). In this instance, it is likely that strong and/or persistent ligand occupation of a large receptor fraction triggers the activation of intracellular second messengers, in turn modifying nAChR responsiveness, for instance, by phosphorylation of certain nAChR domains (Wonnacott, 1990; Khiroug *et al*., 1998; Fenster *et al*., 1999; Harkness & Millar, 2002). More intriguing is the possibility that there are endogenous modulators to fine-tune the activity of nAChRs. Such modulators could be present constitutively to set the background responsiveness of nAChRs, or they could be released during intense activity to reverse receptor desensitization or, conversely, they could inhibit nAChRs to avoid excessive activation. Hence, the ability of nAChRs to mediate ACh-evoked responses might be a state-dependent phenomenon with rapid adaptive changes determined by the extent of neuronal signalling in conjunction with locally released substances. Within this framework, the present report will focus on the potential modulatory role of two endogenous neuropeptides, namely CGRP and substance P, which are often colocalized within the same neurons (Lee *et al*., 1985; Ma *et al*., 2001; Lawson *et al*., 2002). While substance P has long been known to inhibit nAChRs (Clapham & Neher, 1984; Valenta *et al*., 1993; Stafford *et al*., 1994), the demonstration of an apparently similar effect by CGRP on nAChRs is a more recent discovery (Giniatullin *et al*., 1999).

Complex mechanism of action of CGRP or substance P

The most typical action by neuropeptides on target neurons is slow neurotransmission (often referred to as 'volume transmission') *via* activation of G-protein coupled receptors to which they bind with very high affinity after having travelled a considerable distance from their site of release (Agnati *et al*., 1995; Zoli & Agnati, 1996; Jansson *et al*., 2000). These properties usually confer long latency to peptide-mediated responses that are expressed through changes in membrane conductance and cell excitability (Hokfelt, 1991; Otsuka & Yoshioka, 1993). Peptides can exert a modulatory role on fast transmitter-gated channels through at least two distinct processes: (1) an indirect mechanism mediated by the peptide G-protein-coupled receptors that, through changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and other intracellular second messengers, control the phosphorylation state of the fast transmitter receptors and their ability to bind receptor agonists (Huganir & Greengard, 1990; Levitan, 1994; Smart, 1997). This phenomenon is just one example of the process of cross talk which often occurs between different transmitter systems (Kotter, 1994; Barbour & Hausser, 1997) and will not be further examined here: (2) An incompletely understood effect that involves direct interaction of the neuropeptide with certain subunits of the fast transmitter receptor with subsequent alteration in receptor signalling (Clapham & Neher, 1984; Stafford *et al*., 1994; Giniatullin *et al*., 1999; Di Angelantonio *et al*., 2002). We consider this property as representative of direct receptor modulation and will discuss it in relation to nAChRs. The aim of the present review is to discuss evidence pertaining to the action of CGRP and substance P on autonomic neurons for the main reason that the direct accessibility of such cells makes high-resolution studies of the molecular mechanisms underlying the observed phenomena possible. It is also useful to consider such studies as a model for expanding and interpreting current data obtained with brain neurons.

Cholinergic transmission is a target for CGRP modulation

Calcitonin gene-related peptide (CGRP), cloned in the early 1980 s from the gene encoding calcitonin (Amara *et al*., 1982), is a 37 amino-acid peptide, with a characteristic six amino-acid ring made by a disulphide bridge between Cys 2 and Cys 7 at the N-terminal region.

Although at the rodent neuromuscular junction, CGRP is contained in large, dense-core vesicles distinct from ACh-filled ones, these two substances can be coreleased (Matteoli *et al*., 1988) with the result that CGRP prolongs the open time of muscle nicotinic channels (Lu *et al*., 1993). Furthermore, on the same receptors, CGRP facilitates desensitization by phoshorylating certain receptor subunits (Mulle *et al*., 1988; Miles *et al*., 1989; but see Lu *et al*., 1993) and, ultimately, it increases receptor biosynthesis (Changeux *et al*., 1992). Traditionally, the analysis of the modulatory role of CGRP was limited to muscle nicotinic receptors (Eusebi *et al*., 1988; Mulle *et al*., 1988; Miles *et al*., 1989; Lu *et al*., 1993). A convenient model for investigating the modulatory action of CGRP on nAChRs is the adrenal chromaffin cell because it

expresses a large population of nAChRs (predominantly of the $\alpha3\beta4$ type; Campos-Caro *et al*., 1997; Di Angelantonio *et al*., 2003) normally activated by ACh released from the splanchnic nerve terminals (Douglas & Rubin, 1961; Douglas *et al*., 1967). In the adrenal medulla, CGRP is present in nerve fibres (Costa *et al*., 1994; Heym *et al*., 1995) and in the chromaffin cells themselves (Kuramoto *et al*., 1987).

Slow effects of CGRP on $[Ca^{2+}]_i$ and membrane current induced by nicotine

Chromaffin cells provide a clear example (Figure 1) of how CGRP can produce a delayed $[Ca^{2+}]_i$ rise (without associated change in membrane current) due to Ca^{2+} release from internal stores *via* activation of G-protein coupled receptors (Mazzocchi *et al*., 1996a,b; Ayar *et al*., 1999). Unlike CGRP, the action of nicotine (applied by brief pressure pulses from a puffer pipette to mimic the synaptic activity of the natural transmitter ACh) is much faster and accompanied by large, rapid $[Ca^{2+}]_i$ increases (due to Ca^{2+} influx) and fast inward currents (see also Vernino *et al*., 1994; Khiroug *et al*., 1997). Note, however, that responses induced by nicotine after the application of CGRP are clearly depressed *versus* control, demonstrating that the neuropeptide had induced a transient downregulation of nAChRs. The question next arises whether such a downregulation is contingent upon elevated $[Ca^{2+}]_i$ or independent from it. The CGRP-mediated $[Ca^{2+}]_i$ rise is prevented by the CGRP receptor antagonist $hCGRP_{8-37}$ (Quirion *et al*., 1992, Giniatullin *et al*., 1999, Poyner *et al*., 2002), a pharmacological agent selective against G-protein coupled CGRP receptors (Bell & McDermott, 1996). Conversely, $hCGRP_{8-37}$ does not change either nicotine-evoked responses or their depression by CGRP. These results confirm that the reduction of nicotine-induced currents by CGRP is not due to persistent $[Ca^{2+}]_i$ rise or activation of CGRP G-protein receptors.

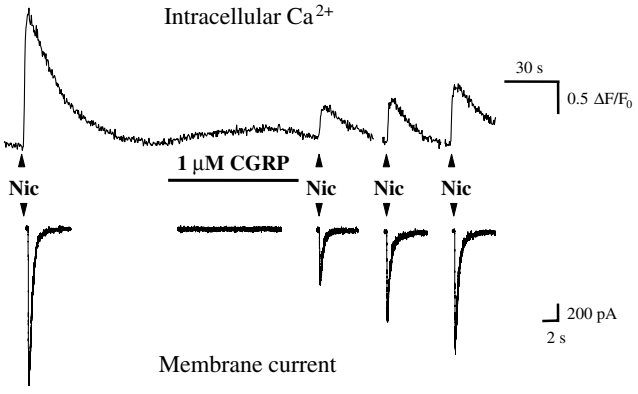

Figure 1 Changes in $[Ca^{2+}]_i$ and membrane current induced by CGRP or nicotine. Top: $[Ca^{2+}]_i$ fluorescence signals induced by pressure application of nicotine (Nic; 20 ms; 0.1 mM; see arrowheads) or CGRP (1 min; 1 μM; horizontal filled bar). Note that the $[Ca^{2+}]_i$ rise evoked by CGRP is smaller and slower than the one evoked by nicotine, but it induces a lasting depression of nicotine responses evoked at 30 s intervals. Bottom: Membrane currents induced by the same application of nicotine. CGRP did not change baseline current, but depressed subsequent responses to nicotine. Note different time scales between the top and bottom records. All traces are from the same cell (Giniatullin *et al*., 1999 with permission from the Journal of Neuroscience. Copyright 1999 Society for Neuroscience).

Fast action of CGRP on nicotine-mediated responses

Any depression of nicotinic responses occurring independent from metabotropic receptor activity should prompt studying the basis of this phenomenon in a much faster time domain. In fact, when CGRP is briefly applied for just a few seconds prior to nicotine, even without changing $[Ca^{2+}]_i$, it strongly (and reversibly) depresses the inward current induced by nicotine (Figure 2a). The extent of this block does not intensify during continuous application of CGRP, and is unrelated to $[Ca^{2+}]_i$ buffering (Giniatullin *et al*., 1999). This observation suggests that CGRP interacts directly with nAChRs on a rapid timescale. In support of this notion is the finding that CGRP acts as fast as nicotine itself, because the nicotine current response is decreased even when CGRP and nicotine are briefly coapplied (Giniatullin *et al*., 1999). Since nAChR desensitization has a time constant of approximately 100 ms (Khiroug *et al*., 1997, 1998; Quick & Lester, 2002), the inhibitory action in CGRP takes place before receptors can be significantly desensitized. Thus, either CGRP can dramatically speed up desensitization kinetics or it can act *via* a different mechanism to depress nicotinic responses. It seems unlikely that the first possibility holds true because large responses to nicotine (which should be more prone to desensitization; Valenta *et al*., 1993; Khiroug *et al*., 1997, 1998) are actually less affected by the peptide as indicated by the plots in Figure 2b (Giniatullin *et al*., 1999). Note also that the ability of CGRP to modulate receptors does not extend to ionotropic γ-aminobutyric acid ($GABA_A$) receptors (which belong to the same superfamily comprising nAChRs; Barnard, 1996), indicating nAChR specificity to the blocking action of CGRP.

Mechanism of CGRP fast block on nAChRs

CGRP does not apparently act as an open channel blocker of nicotinic receptors such as, for instance, local anaesthetics (Neher & Steinbach, 1978) because its block is neither agonist use-dependent nor voltage-dependent (Giniatullin *et al*., 1999). It is apparent that CGRP preferentially inhibits small responses to nicotine, and that increasing the amount of nicotine delivered to the cell counteracts the inhibitory effect of CGRP (Figure 2b). In fact, plotting the fractional response amplitude *versus* the amount of nicotine shows a rightward, parallel shift of the dose–response curve in the presence of CGRP, consistent with an apparently competitive antagonism (Figure 2b). Hence, the mode of action of CGRP is similar to the one of the competitive antagonist *N,N,N*-trimethyl-1-(4-trans-stilbenoxy)-2-propylammonium iodide (F3; Di Angelantonio *et al*., 2000); indeed, coapplication of CGRP and F3 produces linear summation of antagonism. It is also unlikely that the blocking effect of CGRP is caused by negative allosteric modulation of nAChRs like the one reported for substance P, which elicits a downward shift of the agonist dose–response curve (Akasu *et al*., 1983; Stafford *et al*., 1994).

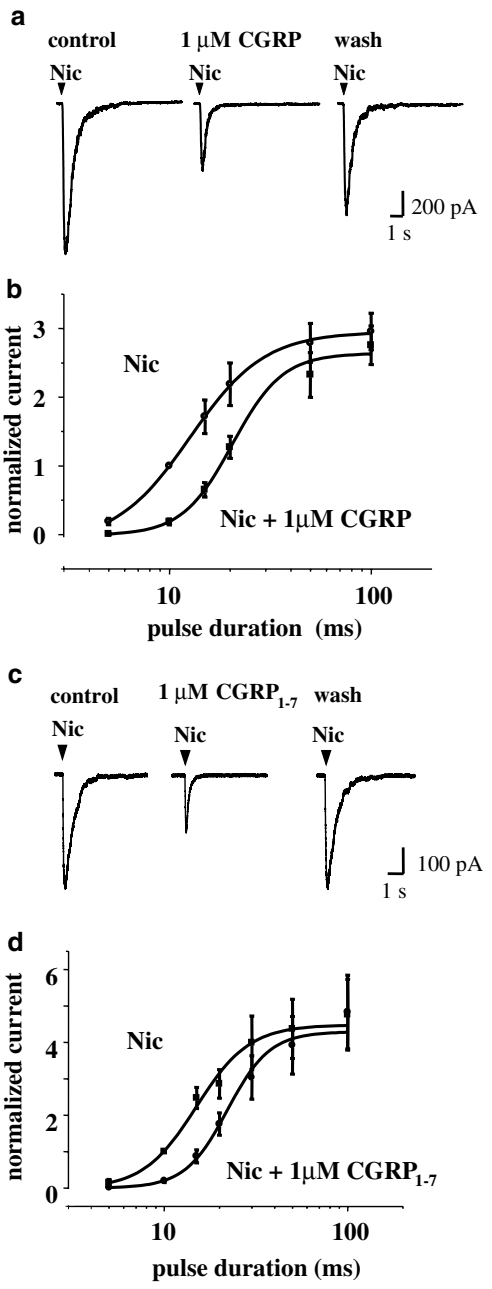

Figure 2 Rapid downregulation of nicotine-induced responses by CGRP and its N-terminal fragment $CGRP_{1-7}$. (a) Current records obtained with 20 ms nicotine (0.1 mM; left), 15 s after starting pressure application of CGRP (1 μM, middle) and 45 s after washout of CGRP. Note the reduction in nicotine current amplitude. (b) Plot of nicotine current amplitudes *versus* increasing duration of nicotine pressure pulses in control solution, or the presence of CGRP. Ordinate, current amplitude normalized with respect to the response evoked by 10 ms nicotine in control solution for each cell. Abscissa, pulse duration of nicotine (0.1 mM) applications. CGRP (1 μM) was applied for 15 s before each nicotine response ($n = 6-9$ cells). Note the rightward shift of the plot without decrease in maximal response. (c) Current records obtained with 20 ms nicotine (0.1 mM; left), 15 s after starting pressure application of $CGRP_{1-7}$ (1 μM; middle) and 45 s after washout of $CGRP_{1-7}$. Note the reduction in nicotine current amplitude. (d) Plot of nicotine current amplitudes *versus* increasing duration of nicotine pressure pulses in control solution, or the presence of $CGRP_{1-7}$, constructed as in b ($n = 8$ cells). Note the rightward shift of the plot without decrease in maximal response. Data are based on results by Giniatullin *et al.* (1999).

Molecular determinants of the fast blocking action of CGRP

Since $hCGRP_{8-37}$ is inactive on nicotine-induced currents, it follows that the N-terminal sequence series of seven amino acids is actually the main determinant for the CGRP modulation of nAChRs. Indeed, $CGRP_{1-7}$ modulates nicotine-induced currents (Figure 2c) like full-length CGRP, with analogous effectiveness and shift in the dose–response curve (Figure 2d). Sustained application of $CGRP_{1-7}$ fails to raise $[Ca^{2+}]_i$, confirming that the N-terminal sequence of the peptide is responsible for rapid block of nAChRs, while the full-length molecule is necessary for the slow $[Ca^{2+}]_i$ increase mediated by metabotropic CGRP receptors (Giniatullin *et al.*, 1999).

Potentiation of nAChRs by $CGRP_{1-6}$ and other short-length peptide fragments

Structure–activity studies aimed at identifying the minimal CGRP fragments capable of interacting with nAChRs have revealed further, unexpected effects (Di Angelantonio *et al.*, 2002). In fact, deleting Cys7 from $CGRP_{1-7}$ generates $CGRP_{1-6}$ which rapidly and reversibly enhances responses mediated by nAChR activation (Figure 3a). $CGRP_{1-6}$ is not a partial agonist on nAChRs because it evokes no change in baseline current or input conductance. Its potentiating action is not use-dependent and rapidly manifested even with the first response to nicotine in $CGRP_{1-6}$ solution, suggesting that this peptide fragment can bind nAChRs in the absence of their agonist. The action of $CGRP_{1-6}$ is voltage- and agonist-independent, as responses to cytisine or nicotine are equally increased at various membrane potentials (Di Angelantonio *et al.*, 2002). Figure 3b shows that the curve relating the fractional response amplitude to the amount of nicotine is displaced to the left (with unchanged maximum response) in the presence of $CGRP_{1-6}$. The $CGRP_{1-6}$ concentration threshold is 0.1 μM and its enhancing action levels off at 50 μM (Nistri & Di Angelantonio, 2002). Thus, this peptide increases the sensitivity of nAChRs to their agonist nicotine without changing the agonist efficacy on them. It is, however, clear that $CGRP_{1-6}$ cannot modulate responses mediated by muscle-type nicotinic receptors or $GABA_A$ receptors (Di Angelantonio *et al.*, 2002).

Comparison of data in Figure 2d with those in Figure 3b shows that $CGRP_{1-7}$ and $CGRP_{1-6}$ have mirror-like actions on nicotinic currents. This finding suggests that a discrete change in the amino-acid sequence, consisting of a single amino-acid deletion, could transform an antagonist into a potentiating substance. This observation outlines the possibility that these fragments interact with similar sites located at a discrete (although yet unidentified) region of nAChRs. In support of this notion, equimolar concentrations of $CGRP_{1-6}$ and $CGRP_{1-7}$, coapplied to the same cell, leave nicotine-induced submaximal currents unchanged, while solutions containing mixtures of these peptides in dissimilar concentration evoke facilitation or depression of nicotinic currents depending on the prevailing fragment concentration (Di Angelantonio *et al.*, 2002).

Further reduction in the amino-acid sequence length has been tested to identify the minimal structure for receptor

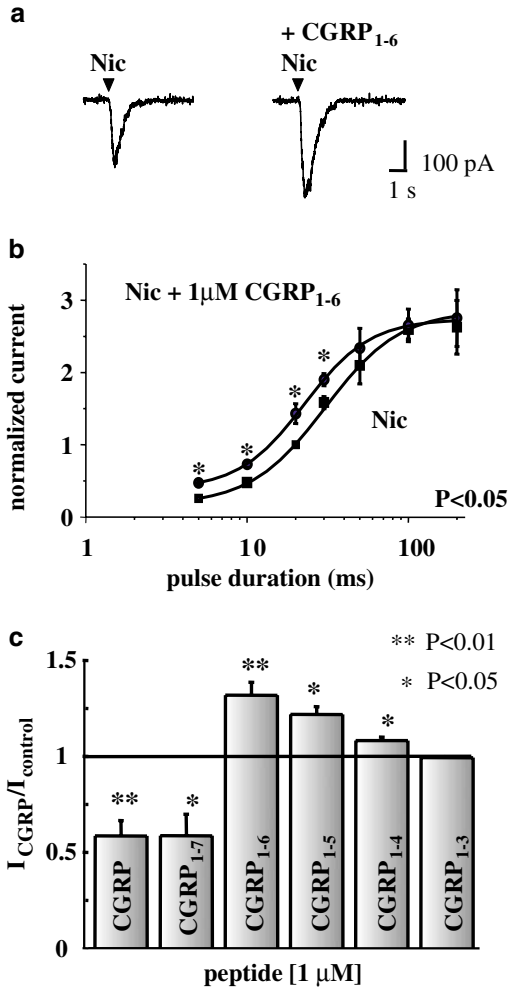

Figure 3 Effect of $CGRP_{1-6}$ and shorter CGRP fragments on nicotine-evoked currents. (a) Submaximal currents induced by a short nicotine pulse (20 ms) are potentiated by 1 μM $CGRP_{1-6}$ fragment. (b) Dose–Response curve (expressed as pulse duration *versus* response) for nicotine in control and in the presence of $CGRP_{1-6}$ (1 μM); the plot in the presence of the peptide is shifted to the left in a parallel manner without altering maximal responses ($n = 5-12$). Currents are normalized with respect to response to 20 ms pulse in control solution and fitted with the logistic equation. *P* refers to significant difference between control and $CGRP_{1-6}$ data indicated by asterisks. (c) Histograms summarizing the action of different fragments of CGRP (all compounds applied at 1 μM concentration) on nicotine-mediated responses. The efficacy of $CGRP_{1-5}$ is less than the one observed with 1–6 fragments. $CGRP_{1-4}$ maintains a slight potentiating effect, whereas $CGRP_{1-3}$ is inactive. For comparison, data corresponding to the antagonism exerted by CGRP and $CGRP_{1-7}$ are reported. Responses are expressed as ratios of currents (I_{CGRP}) in the presence of CGRP or its fragment with respect to controls. *P*-values refer to significant differences with respect to control responses. Data are based on results by Di Angelantonio *et al*. (2002).

modulation and to outline some structural characteristics of the peptide molecules, which might be exploited with molecular dynamics studies to unveil analogies or differences in spatial conformation.

Deleting one amino acid from the carboxyl end of the $CGRP_{1-6}$ sequence yields a compound ($CGRP_{1-5}$) still endowed with potentiating activity on nAChRs (although with reduced potency). Even the $CGRP_{1-4}$ fragment retains a slight, yet significant potentiation, absent, however, with $CGRP_{1-3}$ (Figure 3c). Such a potentiation of nicotinic receptors suggests $CGRP_{1-6}$ and its derivatives to be prototypes of a new class of molecules capable of enhancing responses mediated by nAChRs.

The mechanism of action of $CGRP_{1-6}$ differs from the one of a typical allosteric potentiator

One possibility is that $CGRP_{1-6}$ might act as an allosterically potentiating ligand (APL) on nAChRs (Changeux & Edelstein, 1998). When used at submicromolar concentration APLs, like physostigmine, facilitate nicotine-induced responses even if generated by desensitized receptors, while a 10 times higher dose of the same APL depresses nAChR-mediated responses (Maelicke *et al*., 1997; Maelicke & Albuquerque, 2000). Coapplication of enhancing concentrations of physostigmine and $CGRP_{1-6}$ leads to linear summation of the individual effects, while $CGRP_{1-6}$ can partly reverse the depression induced by a large concentration of physostigmine (Di Angelantonio *et al*., 2002). Thus, $CGRP_{1-6}$ and physostigmine have pharmacologically different effects on nicotine-induced currents, suggesting functionally distinct sites of action for $CGRP_{1-6}$ and physostigmine. These data, however, do not preclude that $CGRP_{1-6}$ might exert a different type of allosteric enhancement as they simply show an action distinct from that of a typical APL.

Structure/function studies of CGRP fragments

Inspection of the primary structure of the CGRP fragments reveals one major difference between $CGRP_{1-7}$ and $CGRP_{1-6}$, namely the presence of a disulphide bridge between Cys2 and Cys7 responsible for the closed ring structure of $CGRP_{1-7}$. Molecular dynamics simulations indicate that the ring structure of $CGRP_{1-7}$ (Figure 4a) is stabilized by an inner ring hydrogen bond as confirmed by circular dichroism spectra (Di Angelantonio *et al*., 2002). This rather rigid structure might be responsible for blocking agonist binding to nAChRs. Conversely, molecular dynamics studies of $CGRP_{1-6}$ show it to be a flexible molecule with considerable freedom to assume various spatial conformations (Figure 4b). Both $CGRP_{1-6}$ (blue) and $CGRP_{1-5}$ (yellow) turn out to preferentially adopt a flexible structure partly with an α-helix conformation (Figure 4c). The helical conformations of $CGRP_{1-6}$ and $CGRP_{1-5}$ may be essential for peptide/receptor interaction and are probably responsible for the enhancing action of the agonist on nAChRs, without any direct activity on the agonist binding site. A helical motif has also been shown to be the spatial conformation assumed by the $CGRP_{8-18}$ fragment (Howitt *et al*., 2003) responsible for the high-affinity binding of the antagonist $CGRP_{8-37}$ to the metabotropic CGRP type 1 receptor (Lynch & Kaiser, 1988).

These data raise two possibilities, namely that either the overall amino-acid length or the absence of a disulphide bridge is responsible for the nAChR enhancing activity. This issue can be investigated by testing custom designed peptides with the following characteristics: (1) a six amino-acid peptide $CGRP_{2-7}$ retaining the rigid ring structure of the disulphide bridge through deletion of Ser1 from $CGRP_{1-7}$; (2) a seven amino-

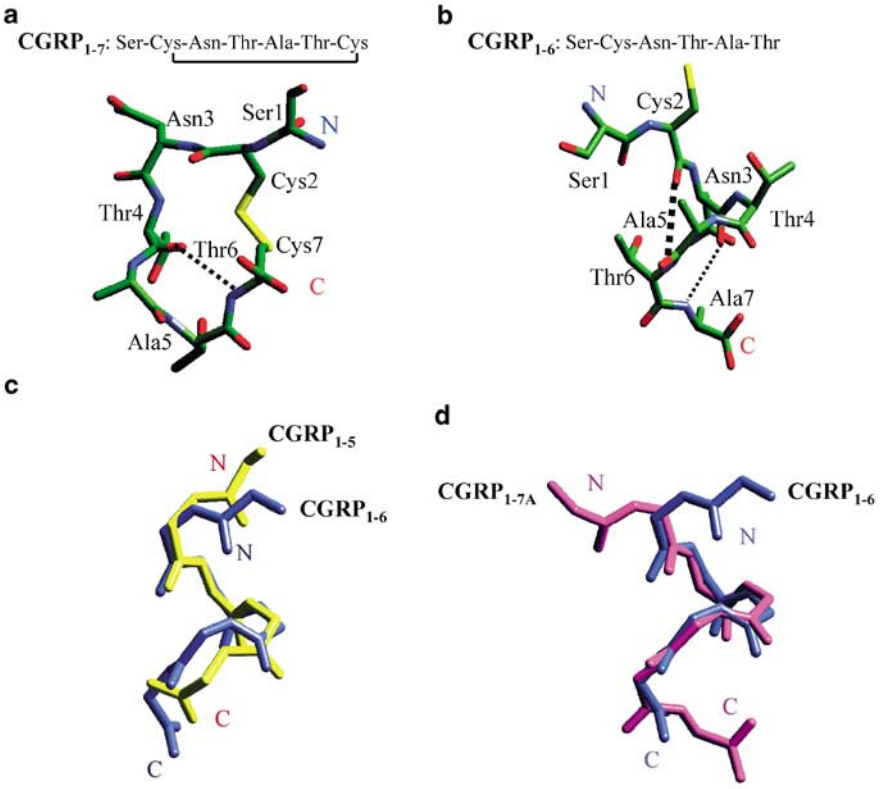

Figure 4 Molecular dynamics analysis of CGRP fragments. Top: Schematic conformations of $CGRP_{1-7}$ and $CGRP_{1-6}$ in an aqueous solution as obtained with molecular dynamics calculations. Hydrogen atoms are not displayed for the sake of clarity. Hydrogen bonds are indicated as dashed lines of different thickness according to the strength of the bond: (a) $CGRP_{1-7}$; (b) $CGRP_{1-6}$. Atoms are coloured using the following code: oxygen (red), nitrogen (blue), carbon (green) and sulphide (yellow). Bottom: Spatial alignment of $CGRP_{1-6}$, $_{1-5}$, or $_{1-7A}$ backbones. (c) Comparison of $CGRP_{1-6}$ backbone (blue) with the one of $CGRP_{1-5}$ (yellow). (d) Comparison of $CGRP_{1-6}$ backbone (blue) with the one of $CGRP_{1-7A}$ (magenta). Data are based on results by Di Angelantonio *et al*. (2002).

acid peptide $CGRP_{1-7A}$ analogous to $CGRP_{1-7}$, except that the terminal Cys7 is replaced by Ala and thus devoid of the disulphide bridge. The conformation of these new compounds assumed after the molecular dynamics simulations is shown in Figure 5a and c. $CGRP_{2-7}$, as expected, maintains the rigid ring conformation of the $CGRP_{1-7}$, while the 3D structure of the $CGRP_{1-7A}$ (magenta) is similar to the one of $CGRP_{1-6}$ (blue; Figure 4d). In particular, they differ only in terms of the conformation assumed by their N-terminal residues that, in the case of $CGRP_{1-6}$, is constrained to the backbone by the two hydrogen bonds.

Functional tests confirm the molecular dynamics simulations by indicating that $CGRP_{2-7}$ behaves like a rapid and reversible blocker of nicotine-evoked responses (Figure 5b), whereas $CGRP_{1-7A}$ behaves like its shorter length counterpart $CGRP_{1-6}$ in potentiating nAChRs, although with somewhat reduced potency (Figure 5d; Di Angelantonio *et al*., 2002).

At present, it is difficult to identify endogenous peptides which would share a facilitatory action on nAChRs similar to the one of $CGRP_{1-6}$. However, the recently discovered Lynx-1 protein that is widely expressed by neurons in the cortex, hippocampus and cerebellum, coexists with $\alpha4\beta2$ and $\alpha7$ receptors and can enhance the activity of nAChRs expressed by Xenopus oocytes (Miwa *et al*., 1999), although a subsequent report demonstrates that the main action of Lynx-1 is to facilitate nAChR desensitization and recovery from it (Ibanez-Tallon *et al*., 2002). Thus, the function of Lynx-1 remains to be established.

The results of CGRP derivatives may provide design leads for developing powerful, nonpeptide modulators of nAChRs.

Electrophysiological modulation by substance P of peripheral nAChRs

Most studies dealing with the effects of substance P on nicotinic systems have used adrenal chromaffin and rat pheochromocytoma cells (PC12) cells. In fact, substance P, which belongs to the tachykinin peptide family (Leeman & Ferguson, 2000), has been shown to be colocalized with ACh within the splanchnic nerve terminals in the adrenal gland from which it can be released in response to stress (Livett & Boksa, 1984). The action of substance P on chromaffin cells appears complex. Electrophysiological studies have consistently demonstrated that substance P inhibits membrane currents generated by nAChR activation (Clapham & Neher, 1984; Role, 1984; Boyd, 1987) *via* a mechanism not mediated by G-protein coupled tachykinin receptors (Stafford *et al*., 1994). nAChRs containing $\beta4$ subunits display the highest sensitivity to substance P-induced block with certain residues present on β subunits responsible for the action of the peptide (Stafford *et al*., 1998). These data suggest that substance P

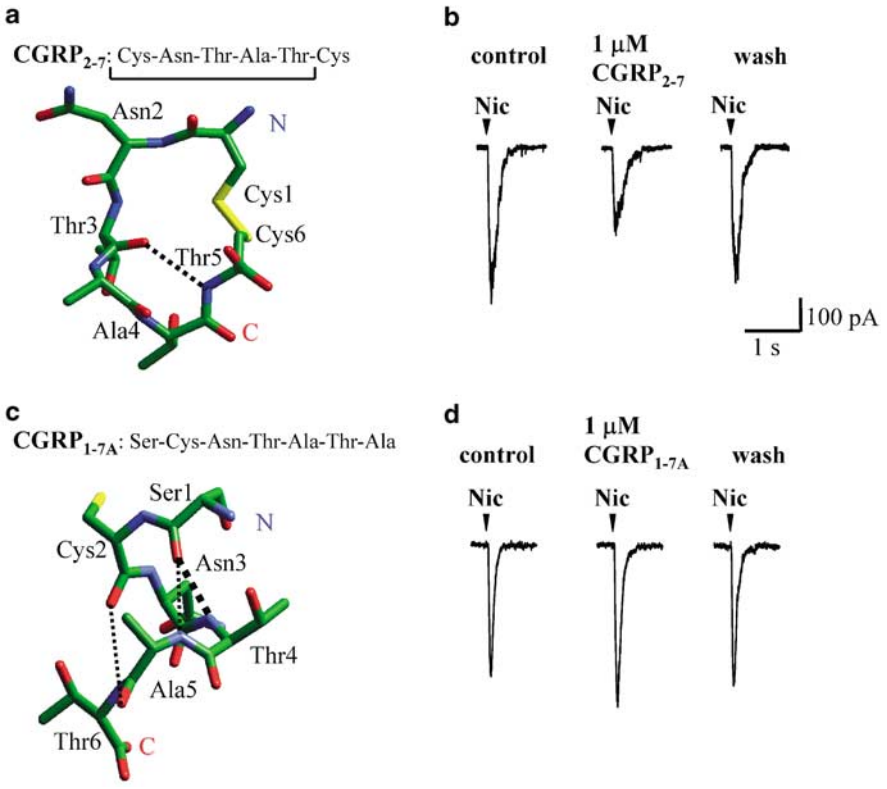

Figure 5 Structure/function studies of CGRP fragments. (a) Schematic conformations of $CGRP_{2-7}$ in an aqueous solution as obtained with molecular dynamics calculations. Hydrogen atoms are not displayed for the sake of clarity. Hydrogen bonds are indicated as dashed lines of different thicknesses according to the strength of the bond. Atoms are coloured using the following code: oxygen (red), nitrogen (blue), carbon (green) and sulphide (yellow). (b) Current records obtained with 20 ms nicotine (0.1 mM), 15 s after starting pressure application of $CGRP_{2-7}$ (1 μM) and 45 s after washout of $CGRP_{2-7}$. Note the reversible reduction in nicotine current amplitude. (c) Schematic conformations of $CGRP_{1-7A}$ in an aqueous solution as obtained with molecular dynamics calculations (for colour legend see a). (d) Example of current (induced by 20 ms nicotine) potentiated by 1 μM $CGRP_{1-7A}$ (preapplied for 15 s). This effect is reversible after peptide washout (right). Data are based on results by Di Angelantonio *et al*. (2002).

binds to an allosteric site on the nAChR to generate transient downregulation of nicotinic receptor activity and have prompted a number of investigations into the molecular mechanisms underlying this phenomenon.

Mechanism of substance P action on nAChRs

While CGRP as well as substance P rapidly downregulate nAChR function, it is clear that the depressant action by substance P is phenomenologically different from that of CGRP as shown by an example of rat chromaffin cell nAChRs (see filled squares for control in Figure 6a; details in Khiroug *et al*., 1998). Repeated test applications of a low dose of nicotine generate stable peak responses, whereas a large dose of the same agonist depresses the response to subsequent test applications of low-dose nicotine (see also Katz & Thesleff, 1957). Subsequent test pulses are used to monitor the time course of nAChR recovery from desensitization. When the same protocol is applied in the presence of 10 μM CGRP (Figure 6a; open triangles), the peak amplitude of currents is depressed by the peptide (cf. Figure 2a and b), yet the extent and time course of the recovery from desensitization is not different from control. Conversely, in the presence of 10 μM substance P (Figure 6a; grey circles), not only is the peak current value more strongly depressed than with CGRP, but also recovery from desensitization is largely delayed. This result illustrates, on the same cell, the clear separation between the effects produced by CGRP or substance P on nicotine-evoked currents.

Unlike CGRP, substance P binds to the lumen of nicotinic channels and blocks them in a use-dependent fashion (Boyd & Leeman, 1987; Arias, 1998). Such observations suggest that substance P may modulate nAChR desensitization which is a strong characteristic of adrenal chromaffin cell receptors (Marley, 1988; Ochoa *et al*., 1989). However, another possibility is that substance P induces nicotinic channel block. In fact, in the presence of substance P, there is a large reduction in ACh-evoked current due to increased channel interburst intervals plus decreased channel burst duration and number of channel openings per burst, while single-channel activation, conductance and ion permeation are not changed by substance P (Clapham & Neher, 1984). Thus, the peptide either allosterically binds to the nicotinic receptor and stabilizes it in its desensitized state or induces channel block, which indirectly enhances desensitization (Clapham & Neher, 1984). It is interesting that the action of substance P does not apparently require nAChR activation, thus implying that the peptide can bind agonist-free receptors and convert them into a nonresponsive state (Valenta *et al*., 1993).

Since Blanton *et al*. (1994) have shown photo-incorporation of a radioiodinated analogue of substance P into the δ subunit

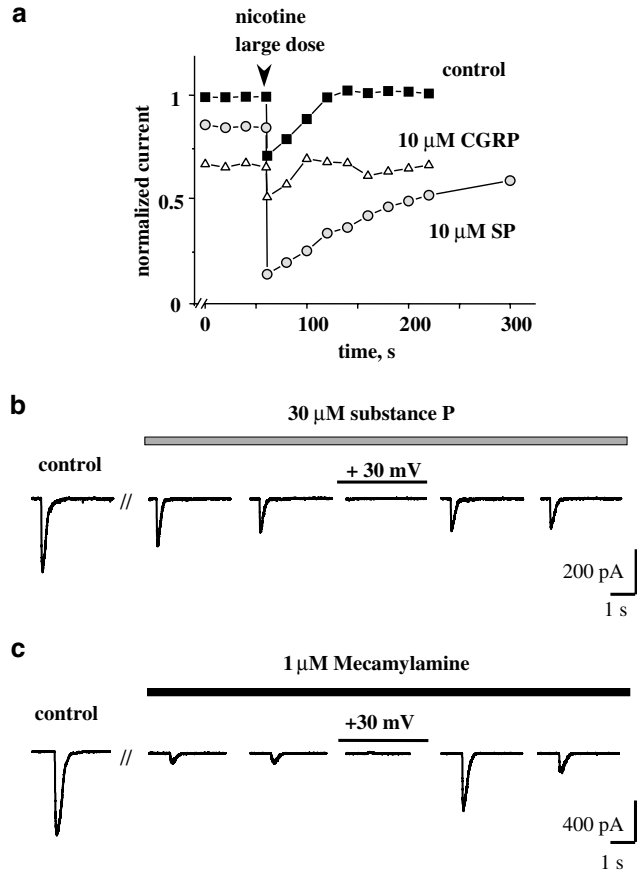

Figure 6 Allosteric modulation of substance P on nAChRs. (a) Plot of nicotine-induced peak currents obtained with a protocol consisting of repeated test applications of nicotine before the conditioning (desensitizing; 2 s) dose. After the conditioning pulse, test pulses are resumed at the same rate to monitor the time course of nAChR recovery from desensitization (filled squares). The same protocol is applied in the presence of 10 μM CGRP (open triangles) or 10 μM substance P (grey circles). Reponses to test doses of nicotine are more strongly reduced by CGRP than substance P, while the desensitized current is more intensively depressed by substance P rather than CGRP. The time course of the recovery from desensitization is left unchanged by CGRP and largely prolonged by substance P (R. Giniatullin and E. Sokolova, unpublished). (b) Substance P is not trapped inside nicotinic channels. Control current evoked by nicotine pulses is reduced at steady state by substance P; combination of membrane depolarization to +30 mV (horizontal line) with nicotine pulse does not change the extent of the block tested with repeated application of nicotine (C. Matteoni, unpublished). (c) Trapping of the nicotinic antagonist mecamylamine inside nicotinic channels is revealed by rapid relief of block. Control current evoked by nicotine pulses is reduced at steady state by mecamylamine. After combining depolarization to +30 mV (horizontal line) with nicotine pulse, the subsequent application of nicotine transiently generates a strong inward current, which is then blocked again. Data are based on results by Giniatullin *et al.* (2000).

that lines up the channel of *Torpedo californa* AChRs, it is possible that this peptide might work as a 'trapped blocker' inside the channel from which it could be released during subsequent channel opening coincident with inverted ion flow (Lingle, 1983; Gurney & Rang, 1984). The prototypic agent producing such a transient channel inhibition is the antagonist mecamylamine (Giniatullin *et al.*, 2000), the blocking action of which is temporarily removed whenever the membrane potential is shifted to positive values (so as to generate an outward current) together with nicotine application. One example of this phenomenon is depicted in Figure 6c. When an analogous protocol is applied to the block of nAChRs by substance P, it is apparent that membrane depolarization plus nicotine application cannot transiently reverse the block by substance P (Figure 6b). These data suggest that the mechanism responsible for such a block is different from the typical channel block by mecamylamine. Together with the observation that the blocking action by substance P is voltage independent (Cuevas & Adams, 2000), these findings concur to make it unlikely that the mechanism of action of substance P on nAChRs is to block their open channels. Similar conclusions have also been reached for amphibian ganglion neurons (Akasu *et al.*, 1983).

An indirect component of the action by substance P?

A number of investigations have also considered the possibility that the blocking action by substance P involves intracellular second messengers. In particular, while recording from cell-attached patches of avian ganglion cells, Simmons *et al.* (1990) have confirmed the facilitation of nAChR desensitization by bath-applied substance P, but have also reported that it must have acted *via* an intracellular second messenger system, probably requiring the activation of protein kinase C, as the peptide was not applied to the region under the recording pipette (see also Downing & Role, 1987, for the role of protein kinase C in desensitization). This phenomenon has not been corroborated with studies of PC12 cells on which substance P directly facilitates desensitization (Andoh *et al.*, 2001). On rat intracardiac ganglion cells, Cuevas & Adams (2000) have shown that substance P inhibits ACh-activated unitary currents even in the outside-out membrane patches, thus ruling out the contribution by intracellular messengers. Since substance P does not affect binding of ACh to nAChRs (Lukas & Eisenhour, 1996; Weiland *et al.*, 1987), it seems likely that its inhibitory effects are due to a decrease in the number of functional nicotinic channels in accordance with the proposal of facilitation of receptor desensitization.

Biochemical studies of the interaction of substance P with nAChRs

Electrophysiological studies have predominantly addressed the issue of the action of substance P within a relatively short time domain. Biochemical techniques have conversely examined how substance P may influence the release of catecholamines from chromaffin cells either directly or in response to cholinergic agonists. Substance P *per se* cannot stimulate catecholamine release (Zhou & Livett, 1990; Valenta *et al.*, 1993). Low (nM) concentrations of substance P are reported to enhance the stimulatory action of nicotine on catecholamine release during the first few min and subsequently to inhibit it (Zhou *et al.*, 1991). Conversely, μM concentrations of substance P strongly and consistently inhibit the action of nicotine, although rebound facilitation of release is observed

after substance P washout (Zhou *et al*., 1991). Note that substance P does not change the releasing property of muscarinic agonists, indicating peptide specificity towards nAChR-mediated responses (Zhou *et al*., 1991; Valenta *et al*. 1993). When catecholamine release is evoked by electrical field stimulation, substance P is reported to act at various sites: presynaptically to facilitate release of endogenous ACh, and postsynaptically to modulate nAChR desensitization and to protect them from this process over a longer time scale (Zhou & Livett, 1990). These complex data apparently stand against electrophysiological results.

To resolve this discrepancy, Lyford *et al*. (1990) have used on-line electrochemical detection of catecholamine release to investigate the action of substance P with a more rapid time resolution. They have confirmed that, at short latency after substance P application, there is accelerated desensitization of the release process induced by nicotinic agents. However, over a longer time and after substance P washout, desensitization to nicotinic agonists is much less intense, thus implying that the peptide had 'protected' receptors from their more profound desensitization (Lyford *et al*., 1990). This complicated phenomenon probably involves coactivation of two distinct mechanisms, namely tachykinin receptors responsible for protecting nAChRs from desensitization (presumably *via* intracellular second messengers) and direct interaction of substance P with nAChRs to enhance their desensitization (Khalil *et al*., 1988). It is noteworthy that studies of $^{22}Na^{+}$ influx into PC12 cells have also indicated two phases of nAChR desensitization (Boyd & Leeman, 1987). The first one is a fast process always inhibited by substance P (Stallcup & Patrick, 1980) and possibly corresponds to the phenomenon usually investigated electrophysiologically. The second phase is much slower and actually inhibited by substance P (Boyd & Leeman, 1987). While electrophysiological and imaging studies have indicated that, on rat chromaffin cells, nAChR desensitization does not depend on intracellular second messengers, recovery from desensitization does depend on intracellular protein kinase/phosphatase activity (Khiroug *et al*., 1998). Hence, it is likely that the recovery from desensitization is the target through which substance P indirectly modulates desensitization of nAChRs responsible for catecholamine release. This process may be species-specific as, unlike mammalian tissues, on chick sympathetic neurons, substance P consistently blocks catecholamine release induced by nicotinic agonists (Valenta *et al*., 1993).

In conclusion, reconciling biochemical and electrophysiological data is difficult because the two different approaches employ diverse techniques in terms of single *versus* population responses, fast or slow data sampling, animal species differences, measurements directly related to nAChR occupancy or nonlinearly related to receptor activation (like catecholamine release), etc. Biochemical studies have extended the current understanding of the action by substance P to encompass integrated tissue responses over longer times not usually accessible to high-resolution, yet time constrained, electrophysiological records.

Examples of other endogenous peptides modulating nAChRs

In recent years, several endogenously occurring neuropeptides have been shown to inhibit nAChRs. For the sake of brevity, the present review will not discuss the nAChR blocking action exerted by these substances (listed in Table 1). Evidence in support of their direct effect was obtained with heterologous expression of nAChRs in the absence of native peptide receptors (Herrero *et al*., 2002; Lioudyno *et al*., 2002; Grassi *et al*., 2003), recording from excised membrane patches (Pettit *et al*., 2001), or insensitivity to G-protein blockers (Oka *et al*., 1998). Future studies are required to fully understand the molecular mechanism of action of such substances and disclose any structural analogies among them.

Functional implications

The chromaffin cell synaptic organization consists of a single axon innervating clusters of postsynaptic cells (Iijima *et al*., 1992) from which multiple peaks of miniature currents can be recorded indicating heterogeneity of release sites (Kajiwara *et al*., 1997) with a potential for transmitter spillover to adjacent sites. The observation of consistent subpopulations of nAChRs at perisynaptic or extrasynaptic areas on cardiac (Wilson Horch & Sargent, 1996) and ciliary (Horch & Sargent, 1995) ganglion cells provides an example of likely membrane targets for such a transmitter spillover at autonomic ganglia, even though the demonstration of analogous sites on chromaffin cells is currently lacking. Electrical stimulation of

Table 1 List of endogenous peptides with direct inhibitory action on nAChRs

Peptide	*Amino-acid length*	*Cell type*	*Type of inhibition*	*References*
β-amyloid peptide and peptide $A_{12\text{-}28}$	42 17	Hippocampal interneurons, α7 nAChRs expressed in oocytes	Noncompetitive	Pettit *et al*. (2001) Grassi *et al*. (2003)
Catestatin	15	Chromaffin cells, nAChRs expressed in oocytes	Noncompetitive, voltage- and use-dependent	Herrero *et al*. (2002)
Proadrenomedullin	20	PC12 cells	Noncompetitive	Mahata *et al*. (1998)
Dynorphin A	17	PC12 cells	Noncompetitive	Oka *et al*. (1998)
Dynorphin A fragments		PC12 cells	Direct, noncompetitive, voltage-independent	Itoh *et al*. (2000)
(1–13)	13			
(2–13)	12			
(1–8)	8			
Endomorphin-1 and Dynorphin B	17	Hair cells and α9/α10 nAChRs expressed in oocytes	Direct, noncompetitive, voltage-independent	Lioudyno *et al*. (2002)
Thymopentin	5	Chromaffin cells	Noncompetitive	Afar *et al*. (1993)

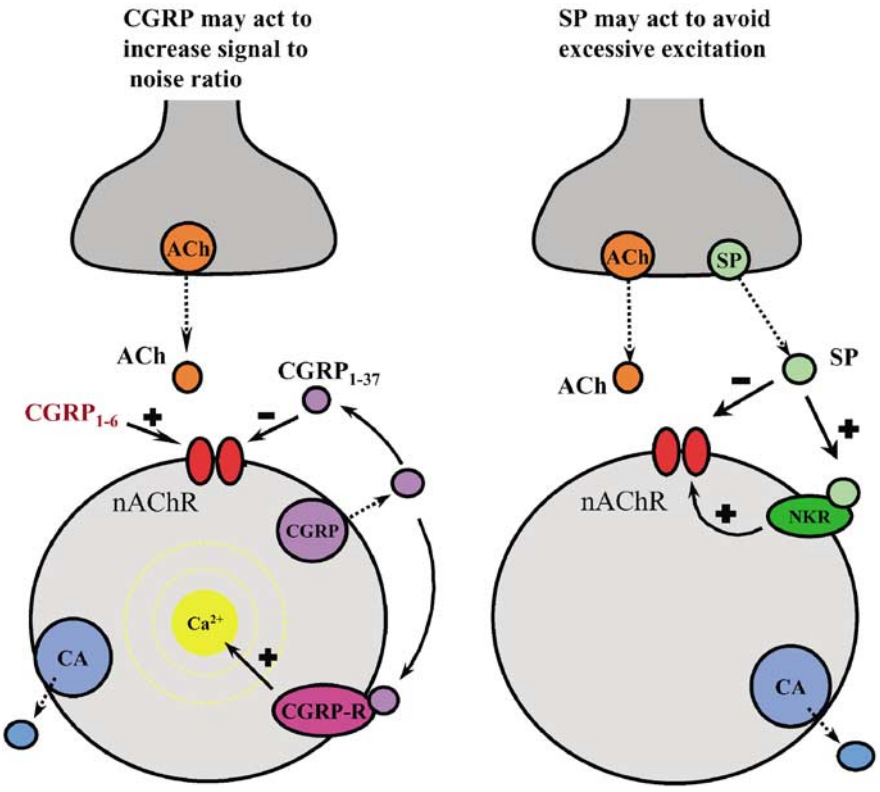

Figure 7 Idealized scheme of neuropeptide modulatory sites at synapses between splanchnic nerve terminals and chromaffin cells. Left: Modulatory action by CGRP and its derivative $CGRP_{1-6}$. CGRP (purple) can be released by the chromaffin cell itself and/or other nearby cells (not shown). CGRP binds to G-protein coupled receptors (CGRP-R; magenta) to increase intracellular free Ca^{2+}. Furthermore, CGRP directly blocks nAChRs (red; decrease in nAChR function is indicated by a negative sign) activated by release of ACh from presynaptic vesicles (orange). Since CGRP preferentially inhibits small rather than large responses mediated by nAChRs, we postulate that this effect can improve the signal-to-noise ratio at this synapse. We also assume that CGRP might be broken down into shorter peptides like $CGRP_{1-6}$ with facilitatory action on nAChRs (this phenomenon is indicated by a positive sign). The main consequence of chromaffin cell activation is the release of catecholamines (CA; blue) into the bloodstream. Right: Modulatory action by substance P (SP; green) released by presynaptic terminals. Substance P activates G-protein coupled neurokinin receptors (NKR), which exert a positive modulatory role on nAChRs *via* intracellular messengers. In addition, substance P directly binds to an allosteric site on nAChRs to enhance desensitization so that ACh-mediated responses are depressed (negative signs). Since large responses are preferentially reduced by this action of substance P, this phenomenon is assumed to be useful in preventing excessive depolarization of the chromaffin cell membrane. Other abbreviations and symbols as in left panel.

single afferent axons induces variable postsynaptic currents which are not always associated with action potential generation (Holman *et al*., 1994). Thus, it seems likely that there are spontaneous fluctuations in the extracellular concentration of ACh, especially because presynaptic cells can fire phasically or tonically (Cassell *et al*., 1986) with presumably different degrees of activation of synaptic and extrasynaptic receptors. These properties suggest that the strength of synaptic transmission between splanchnic nerve fibre and chromaffin cell is potentially susceptible to pharmacological up- or downregulation. We suspect that activation of nAChRs by ACh released from splanchnic nerve terminals could be modulated differentially by CGRP or substance P, thus leading to significant changes in synaptic efficacy.

Figure 7 is an idealized scheme to summarize the complex actions by CGRP and substance P on chromaffin cells taken as representative of autonomic neurons. In the first instance, CGRP (possibly *via* endogenous release from chromaffin cells and non-neuronal cells) may block postsynaptic responses to relatively low, ambient concentrations of ACh. In this way, CGRP would help to filter out unnecessary signalling. Like most endogenous peptides, CGRP is expected to be broken down by peptidases. It is, however, postulated that, perhaps under certain conditions of intense and persistent CGRP release, short fragments like $CGRP_{1-6}$ endowed with nAChR enhancing activity might be generated to counteract any decline in cholinergic neurotransmission. At the same time, CGRP could activate its own metabotropic receptors, leading to $[Ca^{2+}]_i$ rise and possibly release of catecholamines and modulation of other cell functions.

The action of substance P might be complementary to (yet distinct from) that of CGRP on cholinergic transmission. It could block nAChRs activated by low ACh concentrations, yet protecting them from desensitization (*via* an indirect pathway) so as to make them fully available to rapid signalling and catecholamine release. However, larger release of substance P may protect cells from excessive activation of nAChRs *via* facilitation of their desensitization. The extent of nAChR desensitization in chromaffin cells during physiological activity *in vivo* is currently unknown, and its role remains, therefore, elusive. Assuming that desensitization *in vivo* can be as strong as the one demonstrated *in vitro*, the phenomenon induced by

substance P might even be paradigmatic for peptide neuroprotection against Ca^{2+} overloading (Ochoa *et al*., 1989), especially in view of the fact that nAChRs are highly permeable to Ca^{2+} (Vernino *et al*., 1994; McGehee & Role, 1995) and activation of mutant forms of highly Ca^{2+} permeable α7 receptors is strongly neurotoxic (Lukas *et al*., 2001).

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