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HIGH AFFINITY RECEPTORS FOR PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE ON CHICK CILIARY GANGLION NEURONS: DETECTION, INTRACELLULAR SIGNALING AND RELEVANCE TO NICOTINIC ACETYLCHOLINE RECEPTOR FUNCTION

BY

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A Dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

HIGH AFFINITY RECEPTORS FOR PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE ON CHICK CILIARY GANGLION NEURONS: DETECTION, INTRACELLULAR SIGNALING AND RELEVANCE TO NICOTINIC ACETYLCHOLINE RECEPTOR FUNCTION

by

Desiree Pardi

Adviser: Professor Joseph Margiotta

Neuropeptide receptors that can couple to two major intracellular signaling pathways have been identified. One such receptor is an isoform of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor which elevates intracellular cAMP via stimulation of adenylate cyclase (AC), and induces release of intracellular calcium via phospholipase-C (PLC) stimulated inositol phosphate (IP) turnover. Previously, we showed that high-affinity (type I) PACAP receptors are present on chick ciliary ganglion neurons, and can stimulate the AC signaling pathway (Margiotta and Pardi, 1995) such that the resulting increase in cAMP enhances neuronal acetylcholine sensitivity (Margiotta, 1987). Using fast perfusion to identify nicotinic acetylcholine receptor (AChR) classes activated by nicotine, I now report that following AC blockade, PACAP receptor activation decreases the current produced by nicotine-induced activation of nicotinic receptors containing $\alpha 7$ subunits ($\alpha 7$-AChRs) leaving those receptors containing $\alpha 3$, $\alpha 5$, $\beta 4$ and $\beta 2$ subunits ($\alpha 3^*$-AChRs) unaffected. The selective effect of PACAP on $\alpha 7$-AChRs utilized the PLC signaling pathway since it was blocked by buffering intracellular calcium with BAPTA, or by lowering PLC activity with U73122 and was mimicked by Inositol-1,4,5-triphosphate (IP3). PACAP also induced IP turnover

in the neurons and increased intracellular calcium, assessed directly with Fluo-3AM imaging. These further indicate that the activated type I PACAP receptor can couple to PLC. Given our previous findings that PACAP receptors can couple to AC, increasing cAMP, the present results demonstrate a remarkable ability of a single neuropeptide to activate multiple neuronal signaling pathways, and in so doing, to selectively regulate specific classes of downstream ion channel targets depending on which pathway predominates.

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CHAPTER 1:

Introduction

Diversity and regulation

There is a significant amount of diversity in neuronal nicotinic acetylcholine receptors. To date, complementary DNAs (cDNAs) coding for 8 different α subunits (α2-α9) and 3 different β subunits (β2-β4) have been cloned. It has been theorized that via various subunit combinations there is potential for at least a thousand distinct neuronal AChRs (McGehee and Role, 1995). This diversity in receptor composition has translated into diversity in receptor function. Studies involving expression of various subunit combinations in *Xenopus* oocytes and mammalian cell lines as well as studies of native receptors in various neuronal preparations have demonstrated differences in agonist and antagonist sensitivities, calcium permeabilities, and desensitization properties (reviewed in Role, 1992; McGehee and Role, 1995). Much less is known about how different receptor subtypes are regulated on neurons. Does diversity in receptor composition also translate into diversity in regulation? In this regard, receptors of different composition have been localized to distinct regions globally within the nervous system and also locally, on neurons themselves (Role, 1992) and a differential expression of kinases and phosphatases within the nervous system has been suggested (reviewed in Walaas and Greengard, 1991; Wang et al., 1997). Accordingly, receptors of different combinations could be regulated solely based on the different subcellular milieu in which they are located within the nervous system. Furthermore, the sequences of the various subunits vary in the presence of known consensus sites for phosphorylation by kinases such as protein kinase A (PKA) and protein kinase C (PKC). Thus, it is possible that AChRs assembled with different subunits can exhibit different properties depending on the presence or absence of potential phosphorylation sites in the specific subunits making up

the receptor (McGehee and Role, 1995). Different neuropeptides, located in diverse parts of the nervous system, have been shown to induce disparate intracellular signaling cascades that can activate the various kinases and phosphatases (reviewed in Walaas and Greengard, 1991; Probst et al., 1992). Hence, the presence or absence of a particular neuropeptide, the specific signaling cascade activated, the particular subunit composition of the AChR present at the specific locale and the presence or absence of a particular regulatory site within the different subunits can determine the modulation of AChRs composed of distinct subunits.

Isolation and cloning of the first AChR

The AChR was the first ligand-gated ion channel to be purified and subsequently cloned. The receptor was purified from *Torpedo californica* electric organ membrane extracts which contain abundant receptors (Hille, 1992). This purification took advantage of the receptor's high affinity binding to α-bungarotoxin (αBgt), a toxin isolated from snake venom. Electrophoretic separation of the purified channel protein by SDS-polyacrylamide gel electrophoresis revealed the presence of four polypeptides with molecular weights of 40, 50, 60 and 65kD (Conti-Tronconi and Raftery, 1982). Insertion of the purified proteins into artificial planar lipid bilayers resulted in functional acetylcholine-activated channels (Tank et al., 1983), indicating that sufficient components necessary for channel function were present. Sequencing of the first 54 amino acids of one of the subunits provided a deduced nucleotide sequence that was then used as a probe to screen a *Torpedo* electric organ cDNA library. Eventually the cDNAs corresponding to all four polypeptides were obtained and sequenced (Noda et al., 1983). In order to determine whether the products of these cDNAs could assemble and function

as an AChR, the cDNAs were expressed in *Xenopus* oocytes (Gunderson et al., 1984). It was then concluded that formation of a functional channel required co-expression of all four subunits. The cloned channel exhibited electrophysiologic and pharmacologic properties similar to the *in vivo* receptor. The Torpedo electric organ AChR is a heteropentamer comprised of the four subunits in the stoichiometry: $\alpha_2\beta\gamma\delta$. Subsequently, the skeletal muscle homologue was cloned and also shown to be a heteropentamer comprised of two α, one β, one δ, and either one γ or one ε subunit depending on whether the tissue is fetal or adult, respectively (reviewed in Schuetze and Role, 1987). To date, isoforms have only been identified for the α subunit from *Xenopus* skeletal muscle.

The ACh binding site is thought to reside on the α subunits between the N-terminus and the first transmembrane (TM) segment in a region that contains a pair of cysteine residues that are conserved in all α subunits isolated to date (Schuetze and Role, 1987; Role, 1992). This determination has come from results of ligand binding studies as well as mutational analyses. The presence of these paired cysteines in the sequence of an AChR subunit has become the defining feature of an α subunit (McGehee and Role, 1995).

The structural similarity of the sequences encoding the different subunits of the AChR to sequences of other ligand-gated ion channels has allowed their assignment into a family that includes the γ-aminobutyric acid (GABA) type A receptor, the glycine receptor and the 5-hydroxytryptophan type 3 receptor. Hydrophobicity plots have suggested the presence of five hydrophobic stretches that are of sufficient length to traverse the membrane: the first is the signal sequence and the other four are thought to

be present in the mature protein subunits as transmembrane segments. The location of the TM segments within the sequences is conserved among family members. They exhibit a characteristic topology, with the first three TM segments clustered together near the amino (N-) terminus and the last TM, TM4, close to the carboxy (C-) terminus, leaving a long stretch of amino acids between TM3 and TM4, that is thought to be located intracellularly. Depending on the subunit, this intracellular loop contains consensus sites for phosphorylation by various kinases (Swope et al., 1992). Thus, the receptor that bound acetylcholine (ACh) in skeletal muscle was finally cloned.

Isolation and cloning of neuronal AChRs

High affinity ^{3}H-nicotine and ^{3}H-ACh binding, specifically inhibited by cholinergic agonists, is found throughout the mammalian nervous system (Clarke, 1985), suggesting the existence of AChRs there. However, a difference between receptors in skeletal muscle and those in the nervous system was suspected, since in contrast to the neuromuscular junction, αBgt was unable to block cholinergic transmission in several neuronal systems, even though toxin binding had been observed there (reviewed in Schuetze and Role, 1987). Also, even though αBgt bound to brain membranes, the pattern of binding did not always overlap that of nicotine and ACh (Clarke, 1985), and often did not coincide with regions known to be cholinergic (Hunt and Schmidt, 1987). However, it was believed that the two, (neuronal and muscle), receptor types were structurally related since antibodies directed at the skeletal muscle type receptor could block cholinergic transmission via an αBgt-insensitive receptor in rat pheochromocytoma-12 (PC12) cells (Whiting, 1987). Eventually, an antibody raised against the 'main immunogenic region' of the α subunit of the *Torpedo* and

Electrophorus electric organ receptor (mAb35) was found to cross-react with an ACh binding protein on brain membranes and used to purify an AChR from chick brain (Whiting and Lindstrom, 1986). Interestingly, when the purified receptor protein was subjected to SDS-PAGE analysis, only two polypeptides with molecular weights of 48 and 59kD, were visualized. This supported the idea that the two receptor types were indeed different. Amino acid sequencing revealed that the neuronal proteins were similar but not identical to each other or to the muscle type AChR subunits. Finally, immunization of rats with the purified neuronal receptor produced antibodies that were unable to recognize either the electric organ or skeletal muscle receptor, supporting the contention that the neuronal receptor was a different type of AChR. Another group (Boulter et al., 1986) used a cDNA encoding the mouse α subunit as a probe to screen a PC12 cDNA library under low stringency conditions. As a result of such efforts, the first neuronal α subunit was cloned. As the previous experimental results had suggested, this subunit, designated α2 (α1 being the electric organ and skeletal muscle subunit) had several similarities to α1 as well as some clear differences. The sequence was predicted to contain five hydrophobic stretches, (the first being the signal sequence), with the characteristic topology expected of a ligand-gated channel subunit. It also contained the two conserved cysteines considered to be diagnostic of an AChR α subunit. The neuronal clone did not hybridize to messenger RNA (mRNA) derived from skeletal muscle. Conversely, mRNA from PC12 cells could be protected from S1 nuclease digestion by the cDNA of α2 but not by the α1 cDNA. Furthermore, Southern hybridization experiments using mouse genomic DNA illustrated that the two different clones hybridized to fragments of different lengths, supporting the argument that the

proteins were products of different genes. This same study provided the first hint of the diversity of the neuronal AChR subfamily when it was observed that the α2 clone hybridized to several other fragments under low stringency conditions, suggesting the presence of other genes encoding AChR subunits within the mouse genome. To date, 11 different neuronal subunit cDNAs have been cloned; eight α subunits designated α2-α9 and three β subunits, β2-β4. The α subunits are so named due to their sequence homology with each other and with α1. All contain the conserved pair of cysteines that operationally define α subunits. The β subunits share little homology with the skeletal muscle β subunit, and also do not contain the vicinal cysteines present in α subunits. Therefore, they have been designated as β simply because they are not α; consequently, they are also known as 'non-α1-3'. No subunits equivalent to the muscle and electric organ β, δ, γ or ε have been found in nervous tissue (in McGehee and Role, 1995).

Neuronal AChRs are pentamers formed by α and β subunits

By analogy with the muscle receptor, the neuronal receptor is thought to form a pentamer in the membrane. Evidence in support of a pentameric structure for the neuronal receptor, formed from only two types of subunits, comes from expression studies showing that injection of a single class of α with a single class of β gives multiple channels with different subunit stoichiometries. More direct evidence comes from an elegant study from the Ballivet lab (Cooper et al., 1991; see also Anand et al., 1991). This group mutated a residue in the α4 and β2 subunits that confer different electrophysiologic properties to each. They then injected the mutant subunits into *Xenopus* oocytes along with the wild type subunits and analyzed the ratios of the different conductance classes obtained. Analyses of this type indicate that the stoichiometry of

receptors containing these two subunits, are pentamers formed by two α4 and three β2 subunits. This is consistent with the finding that ACh binding curves have Hill coefficients for ACh binding close to two, suggesting the presence of two ACh binding subunits per receptor protein.

The αBgt-binding protein is a functional AChR

Prior to the cloning era, whether the αBgt-binding site on neurons was an AChR was a matter of controversy. When it was discovered that the toxin could bind neuronal membranes (Morely and Kemp, 1981) but could not block synaptic transmission (reviewed in Schuetze and Role, 1987) and that both nicotinic and cholinergic agonists could block this binding (Oswald and Freeman, 1981), the search for the identity and the function of the αBgt binding protein began.

Studies comparing the distribution of αBgt binding sites with that of mAb35 binding sites (Jacob and Berg, 1982) on ciliary ganglion neurons, showed that mAb35 binding was associated with post-synaptic membranes whereas αBgt binding was mostly extrasynaptic. These results also indicated that the binding sites for the two labels were present on different proteins. Another group (Whiting and Lindstrom, 1986), while purifying the neuronal AChR from chick brain extracts, also studied the ability of mAb35 affinity columns to remove ^{125}I-αBgt binding sites from the extract, as well as the ability of αBgt affinity columns to remove mAb35 binding sites. Neither column removed the other binding protein, thus increasing the evidence in favor of the existence of two different proteins. However, when the αBgt binding protein was purified from chick brain (Whiting, 1987) the N-terminal amino acid sequence exhibited homology with the muscle AChR, providing evidence for the protein being a possible AChR.

In the meantime, results suggesting a trophic role for these putative αBgt binding AChRs began to accumulate. One study correlated the increase in acetyl transferase, (the enzyme involved in ACh formation from acetyl-coenzymeA and choline), with the appearance of αBgt binding sites, and therefore inferred a role for the protein in guiding incoming presynaptic cholinergic fibers (Chiappinelli and Giacobini, 1978). Support for this conclusion comes from the results of another study (Freeman, 1977) showing that addition of αBgt to the developing optic tectum shifts the incoming preganglionic terminals away from the toxin binding sites.

In 1990 the field was blown wide open when a cDNA encoding the α7 subunit was cloned from chick brain (Couturier et al., 1990). This subunit exhibits relatively low overall homology with other α subunits (~40%), although its sequence does contain the topology expected of a ligand-gated ion channel subunit. The largest degree of homology exists in the first three TM domains of the various α subunits, which is relevant since these regions are considered to be important in receptor assembly and insertion into the membrane (McGehee and Role, 1995). The sequence of the α7 subunit also contains the conserved cysteines that are believed to make up the ACh binding site and which identify it as an α subunit. The intracellular loop between TM3 and TM4 contains several potential serine/threonine and tyrosine phosphorylation sites. The gene structure is also different from that of any other known AChR subunit gene. Most interesting were its properties when expressed in *Xenopus* oocytes. Unlike any other α subunit known at the time, when the mRNA was injected alone, functional agonist-activated channels were formed and these currents were blocked by αBgt. The N-terminal sequence was then used to create a bacterial fusion protein, which was

demonstrated to bind αBgt. This was the first conclusive evidence that the αBgt binding protein was a functional AChR (Couturier et al., 1990).

Examination of its electrophysiological and pharmacological properties offered clues as to why these receptors had previously been undetectable. Pharmacological studies of the various nicotinic receptor agonists revealed that nicotine was 5-fold more potent than ACh in activating the αBgt-sensitive AChR (Couturier et al., 1990). Most prior experiments not only utilized ACh as the agonist but often at concentrations, which were unlikely to activate these receptors. Additionally, it was discovered that the receptor activated and desensitized with kinetics which were faster (τ_f~13ms) than the resolution of the experimental systems at the time, limited by the relatively slow delivery of agonist. Thus it was eventually concluded that the α7 subunit could form functional homo-oligomeric AChRs that had distinctive kinetic and pharmacological characteristics. In 1994, armed with the knowledge of these kinetic properties and agonist preference profiles, Berg and colleagues (Zhang et al.,1994) used a perfusion system that was able to deliver agonist in under 10ms and for the first time, the electrophysiological properties of the αBgt-binding AChR were determined *in vivo*. Using this 'fast perfusion' system, two components of the nicotine-induced currents were clearly observed. One, a rapidly desensitizing component (fast component), was completely blocked by αBgt and the other; a more slowly desensitizing component (slow component) had the well-characterized kinetic properties of the αBgt-insensitive AChRs known to be present on ciliary ganglion neurons. All agonist-induced current was blocked by d-tubocurare, confirming that it was indeed due to activation of AChRs. As selective inhibitors of

mAb35 receptors are lacking at the moment, it is unknown whether the entire slow component is due solely to currents produced on activation of these receptors.

Properties of AChRs

AChRs form non-selective cation channels with a reversal potential close to zero millivolts as expected of a channel that does not select between cations. Many neuronal AChRs have calcium to sodium permeability ratios of about 1. One exception is the supposed homo-pentameric receptor made up of the αBgt-binding α7 subunit, whose calcium to sodium permeability ratio of about 20 is the largest measured for any ligand-gated ion channel, including the N-methyl-d-Aspartate (NMDA) receptor (McGehee and Role, 1995; Role and Berg, 1997). Recently, activation of a recombinant human α7 has been shown to raise intracellular calcium levels significantly on its own (Delbono et al., 1997). All AChRs also increase intracellular calcium through depolarization of the membrane thereby activating voltage-dependent calcium channels (VDCC) (Vijayaraghavan et al.,1992; Rathouz and Berg, 1994; Role and Berg, 1997), but many studies have disclosed a significant consequence of the increased intracellular calcium through AChRs, even in the absence of VDCC activation. In this regard, activation of presynaptic AChRs, in the absence of membrane depolarization, has been shown to lead to neurotransmitter release in the interpeduncular and lateral geniculate nucleus of the chick (Wonnacott, 1997). Thus it is widely believed that AChRs could serve as a major route of calcium entry during times when the neuron is hyperpolarized or at rest (Role and Berg, 1997). Moreover, activation of postsynaptic receptors has been demonstrated to augment calcium channel-mediated exocytosis (Harkins and Fox, 1998). Thus, it appears that calcium entering through AChRs can raise intracellular calcium to levels

high enough to have significant effects, regardless of the polarization state of the membrane.

The chick ciliary ganglion

The embryonic chick ciliary ganglion is part of the parasympathetic division of the autonomic nervous system. The ganglion contains about 3000 neurons composed of two populations. Half are the larger ciliary neurons that innervate the striated ciliary and iris muscles. The other half is composed of the smaller choroid neurons that innervate the vascular smooth muscle of the choroid coat (Landmesser and Pilar, 1978). Both populations receive their innervation from the neurons in the Eddinger-Westphal nucleus. This innervation is entirely cholinergic and both neuronal cell types have been shown by electrophysiological criteria to express functional nicotinic AChRs on their membranes (Role, 1992; McNerney and Margiotta, manuscript in preparation). Using co-precipitation experiments with subunit specific antibodies, two major AChR classes have been identified and characterized in the ciliary ganglion (Conroy and Berg, 1995). One class contains α3, α5, and β4 but not the α7 subunit and is recognized by mAB35 and is consequently termed mAB35-AChRs or α3*-AChRs. This class is localized primarily at the synapse (Jacob and Berg, 1983; Horch and Sargent, 1995) and were previously thought to be solely responsible for synaptic transmission in the ganglion since transmission appeared to be unaffected by the presence of αBgt (Zhang et al.,1996). The other class contains α7 but not α3, α5 or β4 subunits and are recognized and blocked by αBgt and is therefore termed αBgt-AChRs or α7-AChRs. In contrast to α3*-AChRs, these receptors desensitize rapidly, prefer nicotine to ACh, are primarily extrasynaptic, and are thought to be formed by the homomeric association of α7 subunits (Zhang et al,

1996; Vicente-Agullo et al., 1996). However, it is still unknown whether, in these neurons, α7 subunits form homo-oligomeric channels exclusively or if it can associate with other subunits. In rat brain, α7 does not associate with other subunits (Cheng and Patrick, 1997) while in chick brain, some of the channels that contain α7 subunits also contain α8 subunits (Schoepfer et al., 1990). As mentioned above, these receptors had not previously been thought to participate in synaptic transmission within the ciliary ganglia. However, a recent report has demonstrated that a rapidly activating and desensitizing component of the synaptic current with the properties expected of activated α7-AChRs can be blocked by αBgt (Zhang et al., 1996). Thus, it is now believed that, due to their particular attributes, such as their rapid activation and desensitization kinetics, α7-AChRs may be important in decreasing the time to reach the threshold necessary for triggering an action potential as αBgt was found to slow action potential generation. Another distinguishing feature of α7-AChRs is their high permeability to calcium (Vijayaraghavan et al., 1992), which is larger than any other known ligand-gated ion channel, including the N-Methyl-D-Aspartate (NMDA) receptor (reviewed in McGehee and Role, 1995).

Since their discovery, the α7-AChRs have been implicated in the regulation of many cellular processes. These include roles in growth and development (Chan and Quick, 1993; Deltoro et al., 1997) as well as in synaptic plasticity (McGehee et al., 1995; Alkondon et al., 1996; Liang and Vizi, 1997). In addition to the well known effects of nicotine on behavior (Levin et al., 1996b) and learning and memory (Warburton, 1992; Levin, 1996a), nicotine and the nicotine-preferring α7-AchRs have also been linked to several human illnesses, including schizophrenia (Adler et al., 1992; Freedman et al.,

1997), Alzheimer's disease (Newhouse et al., 1996; Chessell, 1996) and Parkinson's disease (Newhouse et al., 1996). An increased intracellular calcium concentration is believed to be an important factor in these processes (Kandel, 1991; Clapham, 1995; Mark et al., 1996). Since the activation of α7-containing receptors has been shown to increase intracellular calcium to levels high enough to produce physiological effects (Dolezal et al., 1996), it seems likely that modulation of these receptors would be of major importance to cells.

Modulation of neuronal AChRs

As the role of the AChR in diseases such as Alzheimers (Gallagher and Colombo, 1995) has become clear and the importance of calcium in many cellular functions including growth, secretion and synaptic plasticity (Clapham, 1995; Berridge, 1997) has been recognized, the modulation of these receptors has become an area of intense research. Over the past twenty years, several different intracellular signaling cascades have been elucidated and their effects on various ion channels have been studied (Levitan, 1994). In particular, elevated levels of cyclic adenosine monophosphate (cAMP) have been shown to decrease the probability of opening of voltage activated sodium channels through a step involving phosphorylation of the channel by protein kinase A (Li et al., 1993), and activating the cystic fibrosis transmembrane regulator (CFTR) (reviewed by Fuller and Benos, 1992). In skeletal muscle, cAMP has been shown to increase the rate of AChR desensitization and this was shown to be due to phosphorylation of the δ and γ subunits by PKA (Swope et al., 1992). Intracellular elevations of cAMP also have effects on neuronal AChRs. Incubation of ciliary ganglion neurons with the membrane permeant analog 8-Bromo-cAMP increased the whole-cell

ACh sensitivity, measured as an increase in peak ACh-induced whole-cell current amplitude, by 200% (Margiotta et al., 1987). This increased sensitivity was shown to occur without changes in the single channel properties, or in the affinity of the receptor for the agonist. Even more interesting is the fact that this increase occurred in the presence of cyclohexamide, a protein synthesis inhibitor, and without a change in the number of mAb35 or neuronal-Bgt (nBgt) binding sites, both markers specific for the αBgt-insensitive receptor. Calculations revealed that the number of functional receptors present on the membrane doubled in the presence of cAMP. This lead to the conclusion that the neurons have the ability to recruit functional receptors from a pool of non-functional receptors through some type of post-translational modification, possibly involving a phosphorylation of the receptor itself. These results are consistent with later reports showing that these neurons maintain receptor numbers in much larger quantities than their mRNA transcripts and that culture conditions which change the receptor numbers do not change the levels of mRNA (Corriveau and Berg, 1994). Another report demonstrated that the α3 subunit can be phosphorylated by the catalytic subunit of PKA (Vijayaraghavan et al.,1990) and is phosphorylated when incubated with 8-Bromo-cAMP and the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX). More recently the α7 subunit of rat and chick was shown to be phosphorylated by the catalytic subunit of PKA, but not by protein kinase C or calcium/calmodulin kinase II (cAMKII), on a conserved serine residue located in the major intracellular loop between TM3 and TM4 (Moss et al., 1996). Taken together, these results suggest that ciliary ganglion neurons can regulate their receptors through post-translational modifications and are in keeping with the notion that there is a balance between phosphorylated states of cellular proteins

at rest. In this regard, it has been suggested that the balance between the activity of protein kinases and protein phosphatases is reflected in the phosphorylation state of cellular proteins (Kurosawa, 1994). Evidence in favor of such a balance affecting ion channels comes from the results of several recent studies demonstrating the effects of phosphatase and kinase inhibitors. In one report of a calcium-activated potassium channel in pyramidal neurons, the authors concluded that there exists a balance between PKA and protein phosphatase activity (Pederzani et al., 1998). In another, the calcium-dependent protein phosphatase-2B (PP2B), was involved in the tempering of CFTR activity (Fischer et al., 1998). Given that elevations in cAMP could affect agonist sensitivity of ciliary ganglion neurons, and that the two receptor classes could be phosphorylated by cAMP-dependent protein kinase (PKA), it became important to search for first messengers (e.g. transmitters or neuropeptides) that by binding to the appropriate receptor, could cause elevations in cAMP levels.

Pituitary Adenylate Cyclase-activating Polypeptide (PACAP)

Many neuropeptide hormones increase cAMP levels in neurons, including Vasoactive Intestinal Polypeptide (VIP) (reviewed in Gozes and Brenneman, 1989). A VIP-like immunoreactivity has been localized to the terminals ending on the ciliary neurons in the chick (Reiner, 1987). A study testing the effects of VIP on the ACh sensitivity of ciliary ganglion neurons found that VIP could elevate cAMP levels in these neurons and that a 10-minute pre-incubation with peptide increased the whole-cell sensitivity to ACh by 50% (Gurantz et al., 1994). In 1989 a novel neuropeptide was purified from ovine hypothalamus on the basis of its ability to increase cAMP levels in cultured pituitary cells (Miyata et al., 1989) and consequently was named Pituitary

Adenylate Cyclase-Activating Polypeptide (PACAP). Two C-terminal amidated forms of this peptide exist which are identical in their first 27 amino acids: a 38 amino acid form (PACAP38) and one of 27 amino acids (PACAP27). Both PACAPs are derived from the same precursor (Kimura et al., 1990) and PACAP27 is thought to be obtained by post-translational processing of PACAP38 (Miyata et al., 1989). PACAP shares 68% homology with VIP, leading to its assignment to the VIP/Secretin/Glucagon peptide family (Christophe, 1993). Despite its high homology with VIP, PACAP has been shown to elevate cAMP levels with a 1000-fold greater potency in many of the same cell systems previously shown to respond to VIP (Tatsuno et al., 1990; reviewed in Christophe, 1993).

Since their discovery, much information on the tissue distribution of the two forms of PACAP has been obtained. PACAP38 immunoreactivity has been observed in neuronal cell bodies, terminals and fibers throughout the central and peripheral nervous systems of rat, mouse, sheep and human, as well as in the testes. The tissue distribution of PACAP27 is very different, with very little peptide being observed in the nervous system. Rather, abundant levels of PACAP27 are found in several peripheral tissues, such as lung, and liver and throughout the gastrointestinal system (reviewed in Christophe, 1993). The physiological importance of PACAP is underscored by the fact that the sequence of PACAP in rat, sheep and human are identical, suggesting a strong evolutionary pressure placed on the gene encoding it (Ghatei et al., 1992; Christophe, 1993).

Three types of PACAP receptors were originally identified on the basis of their characteristic differences in binding affinities for PACAP38, PACAP27 and VIP. These

characteristic binding affinities now operationally define the three receptors. The type I receptor was first cloned from a pancreatic carcinoma cell line, AR4-2J (Pisegna and Wank, 1993). Hydrophobicity plots revealed the 7 TM topology expected of a G-protein coupled receptor (GPCR), which these receptors had been believed to be. Thus, their activation was associated with increased activity of adenylate cyclase (Deutsch and Sun, 1992); subsequent elevations in cAMP in several cell systems (Miyata et al., 1989; Robberecht et al., 1992; Deutsch and Sun, 1992) and ligand binding affinity could be modified by guanine nucleotides (Tatsuno et al., 1990; Schafer et al., 1991). Heterologous expression of the cDNA in COS-7 cells resulted in a receptor that bound PACAP38 and PACAP27 with equal and high affinity but which bound VIP with an affinity about 1000X lower, as expected of the type I binding site (Shivers et al., 1991). Prior to the cloning of the type I receptor, several reports had shown that PACAP could also increase IP (Deutsch and Sun, 1992) or intracellular calcium (Canny et al., 1992; Tatsuno et al., 1992; Deutsch et al., 1993) levels as well as cAMP levels in the same cells. Thus it appeared that a single receptor, the type I receptor could couple to two different signaling pathways. This idea was confirmed when a second group cloned five different splice variants of the type I receptor from a newborn rat superior colliculi cDNA library (Spengler et al., 1993). The variants differed only in the presence or absence of one or two cassettes containing an additional 84 nucleotides, in the C-terminal portion of the third intracellular loop. This loop is thought to be crucial for receptor/G-protein coupling for some G-protein coupled receptors (Probst et al., 1992; Neer, 1994) and an insertion in exactly the same position also occurs in the human gonadotropin releasing hormone receptor (Mayo, 1992) pointing to the importance of this region. Moreover,

placing the third intracellular loop from the Platelet Activating Factor receptor into another receptor that does not normally couple to PLC converts it to one that is now able to activate PLC (Carlson et al., 1996). Importantly, when the PACAP receptor variants were individually expressed in *Xenopus* oocytes, it was found that a single receptor could couple to a path involving elevation of cAMP and also one involving increased intracellular calcium. Thus, a single receptor, when expressed in this way, not only increased cAMP levels but also activated a calcium-dependent chloride current, a marker for increased $[Ca^{2+}]_i$ in *Xenopus* oocytes. Other G-protein coupled receptors that have been discovered to couple simultaneously to at least two intracellular second-messenger signaling cascades include the D_1 Dopamine receptor (Liu et al., 1992) and other members of the same receptor family as PACAP such as the parathyroid stimulating hormone, glucagon and secretin receptors (in Gudermann et al., 1996). Northern blotting of rat tissue mRNA identified the existence of the type I receptor throughout the brain, including the cortex, cerebellum, hippocampus and pituitary. No signal was detected in mRNA from lung, liver, pancreas, kidney or skeletal muscle (in Hashimoto et al., 1993). Recently a novel variant of the type I PACAP receptor that differs in TM2 and TM4 has been isolated from a rat cerebellar cDNA library. Interestingly, this receptor, which has the pharmacological binding characteristics of a type I PACAP receptor, activated L-type VDCCs but not AC or PLC (Chatterjee et al., 1996).

When the type II receptor was cloned (Ishihara et al., 1992) it was originally designated a VIP receptor, but subsequent analysis revealed that it had the binding properties expected of a PACAP type II binding site - nearly equal and high affinity for both PACAP isoforms and VIP (Shivers et al., 1991). As of yet, no specific binding site

has been identified for VIP (Suda et al., 1992). The type II receptor couples only to adenylate cyclase with the potencies of the three peptides mirroring the order of their affinities (reviewed in Christophe, 1993). In contrast to the distribution of the type I receptor, binding studies and Northern analysis have demonstrated the low abundance of these receptors in the brain and their relatively high abundance in lung, liver, spleen and intestine (reviewed in Gozes and Brenneman, 1989; Ishihara et al., 1992). The type III receptor, also known as the type II VIP receptor, also binds all three peptides with equal and high affinity. However, PACAP38, PACAP27 and VIP, are also equipotent in stimulating cAMP and IP production via this receptor. The distribution of the type III receptor complements that of the other two, being present, unlike them, in the heart, stomach and pancreatic islets (Inagaki et al., 1994). The type III receptor is thought to be involved in the amplification of glucose-induced insulin release by PACAP (Inagaki et al., 1994) but the signaling pathway involved is still unclear, although it may involve a wortmannin-sensitive step (Straub and Sharp, 1996). Recently, this receptor has been found to be involved in interleukin-6 production in rat bone marrow stromal cells (Cai et al., 1997).

Modulation of neuronal AChRs by PACAP

VIP, at a concentration of 1μM, has recently been demonstrated to increase the sensitivity of ciliary ganglion neurons to ACh (see above; Gurantz et al., 1994). This concentration is unusually high for the expected high affinity interaction between a peptide and its receptor (in Alberts, 1994; Deutsch and Sun, 1992). Moreover, a PACAP38 specific antibody that does not cross-react with VIP demonstrated the presence of a PACAP-like immunoreactivity in the ganglion (Margiotta and Pardi, 1995).

Being that PACAP-selective type I receptors have been shown to be more abundant than the non-selective type II receptors in the nervous system (Christophe, 1993) and that PACAP had been observed to be 1000-fold more potent than VIP in many systems previously shown to respond to VIP, we postulated that PACAP may also have a more potent effect on ciliary ganglion neurons. The importance of PACAP in many cell processes has become increasingly realized. In particular, several pieces of evidence implicate PACAP as an important neurotropic factor, where it plays a part in normal neuronal growth and development (Deutsch et al., 1993; Deutsch and Sun, 1992; Morio et al., 1996b) as well as in neuronal survival (Tanaka et al., 1996; Uchida et al., 1996; Morio et al., 1996a; Villalba and Journot, 1997). Furthermore, the gene encoding the human PACAP precursor has been localized to the short arm of chromosome18, a region associated with hereditary holoprosencephaly (Basille et al., 1993). In addition, a role for PACAP in memory formation is suggested by recent reports of a Drosophila mutant, amnesiac, that has no difficulty learning but is unable to retain what it has learned. Interestingly, the defect is located in the gene that encodes a neuropeptide that is highly homologous to mammalian PACAP (Feany and Quinn, 1995). Furthermore, high levels of PACAP type I receptors have been identified in the hippocampus (Hashimoto et al., 1993), a region of the brain thought to be involved in memory formation and which receives cholinergic afferents, (Christophe, 1993), implicating a possible cooperative role of ACh and PACAP in memory and learning. Since α7-AChRs can increase intracellular calcium to levels sufficient to have physiologic effects and both the AChRs and PACAP seem to play roles in similar, important cell functions, we endeavored to study the modulation of the α7-AChRs by PACAP.

Utilizing competition and cAMP assays we determined that ciliary ganglion neurons possess a high affinity binding site for PACAP with the pharmacological properties of the type I PACAP receptor. In addition, incubation with low concentrations of PACAP increased IP and intracellular calcium levels. Furthermore, the two classes of AChRs present on these neurons are differentially affected by PACAP depending on which signaling pathway is dominant. In this regard, incubation of neurons with PACAP alone, increases the current amplitude on subsequent agonist application. In neurons pre-exposed to 2'-5'-dideoxyadenosine (ddA), an adenylate cyclase inhibitor, incubation with PACAP selectively decreases the current produced by α7-AChRs without decreasing that produced by α3*-AChRs.

CHAPTER 2:

Methods

Neuron Isolation. *For use in the experiments described in Chapter 3.* Ciliary ganglia were removed from embryonic day 13-14 chicks and neurons were dissociated via treatment with collagenase A. This was followed by mechanical trituration and plating onto coated glass coverslips or wells as described in detail elsewhere (Margiotta and Gurantz, 1989). Neurons were plated onto 15mm-diameter glass coverslips or 16mm-diameter plastic wells coated with poly-lysine at 2 or 2-6 ganglia equivalents, respectively. Neurons were permitted to recover for 2-4 hours at 37 deg C in recording saline (in mM: 145 NaCl, 5.3 KCl, 5.4 $CaCl_2$, 0.8 $MgCl_2$, 5.6 glucose, and 5.0 HEPES, pH 7.4) supplemented with 10% horse serum.

For use in electrophysiological experiments involving 'fast-perfusion' (Chapter 4). Acutely dissociated ciliary ganglion neurons were prepared as follows. Embryonic day 13 and 14 (E13, E14) chick embryos were decapitated and the ciliary ganglia removed, cleaned and diced. Ganglionic chunks were subjected to gentle enzymatic treatment for 20 minutes at 37 deg C in 0.3mg/ml of collagenase A in DF0 (see solutions). This was followed by mechanical dissociation via trituration and plating onto 12-mm coated glass coverslips at the equivalent of one ganglion/coverslip. Acid-washed coverslips were coated daily at room temperature for one minute with a 1:1000 dilution of a 1mg/ml stock of poly-D-lysine (Sigma# P-0899). This allowed for easy lifting of cells from coverslips for exposure to agonist during experiments. Cells remained on coverslips at room temperature for 20 minutes before receiving DF++ (see solutions) and were allowed to recover at 37 deg C in a humidified atmosphere for at least one hour prior to use in experiments.

For use in Calcium Imaging experiments (Chapter 4). Neurons were isolated as above except they were plated for 40 minutes on glass coverslips coated overnight at 4 deg C with a 1mg/ml solution of higher molecular weight poly-lysine (Sigma#P-1024).

For use in IP Measurements (Chapter 4). Ciliary ganglia were removed from E12 chick embryos similarly as for other experiments except the isolations were performed under aseptic conditions. Neurons were plated into the wells of 12-well plates (Falcon) at about 2-6 ganglion equivalents/well in MEM supplemented with glutamine (2mM), Penicillin/Streptomycin (1:100v/v) and 10% Heat Inactivated Horse Serum. Wells were coated the night before with a high molecular weight poly-lysine (Sigma # P-1024). Neurons recovered at 37 deg C in a humidified atmosphere supplemented with 5% CO2 for one hour before receiving ^{3}H-myo-inositol (2μCi/ml). Plates were returned to 37 deg C for 24-36 hours prior to use in experiments.

Competition Assays. Determination of high affinity binding sites for PACAP was performed using competition binding assays (see Pisegna and Wank, 1993; Gottschall et al., 1990). Neurons were plated into 16mm wells at 2-3 ganglia equivalents/well and treated for one hour at room temperature (RT) with recording medium (see above) supplemented with 10% horse serum which contained ^{125}I-PACAP27 (NEN/Dupont; 50pM, 2200 Ci/mmol). PACAP38, PACAP27, VIP, secretin or glucagon were added to duplicate wells for each of the indicated concentrations from frozen stocks in dilutions which maintained the final volume at 250μl. Following this incubation, wells were washed four times with fresh saline without ^{125}I-PACAP27 at 4 deg C, to remove unbound tracer. Cells were then scraped in 500μl of 0.6N NaOH and binding determined from the amount of radioactivity remaining as assessed by counting in a Beckmann

Gamma 4000 γ counter. Nonspecific binding was assessed for each experiment by the inclusion of excess (1μM) PACAP27 in two wells, and typically accounted for less than 15% of the total binding. The counts acquired from control wells, representing nonspecific binding, were subtracted from the total counts obtained from the experimental wells. The concentration of each peptide required to compete off 50% of the tracer (IC50) was determined by fitting the displacement curves with the Hill equation; $y = [peptide]^n/[EC50]^n + [peptide]^n$, where n represents the Hill coefficient.

cAMP detection assays. cAMP accumulation was determined using a commercial ^{125}I-cAMP radioimmunoassay kit (NEN/Dupont). Neurons were plated as in competition assays at 1-6 ganglia/equivalents per duplicate well. Following a 10 minute preincubation at 37 deg C in recording saline plus 10% horse serum and 100μM of the phosphodiesterase inhibitor, IBMX, neurons were challenged for an additional 2-60 minutes with test peptides. Following the incubation, the bathing solution was removed, 200μl of ice cold 70% ethanol was added to each well and the plates placed at 4 deg C for 12-16 hours. Wells were then scraped and the extracted cAMP transferred to 1.5ml eppendorf tubes and centrifuged at 13,500g for 10 minutes at 4 deg C. Supernatant was transferred to a fresh tube, 100μl more of 70% ethanol was added to the original tube and centrifuged identically. Finally, the supernatants were combined and vacuum dried for 2-2.5 hours. The dried extracts were reconstituted in 50μl of RIA buffer supplied with the kit. Standard curves were constructed in parallel using known amounts of cAMP (from 25 to 1600 fmol/tube) and then the amount of cAMP in each test-tube was determined accordingly. The concentration of peptide required to increase the cAMP level to 50% of

its maximum (EC50) was determined by fitting the dose-response curves with the Hill equation for each peptide (see above).

Inositol Phosphate Release Assay. In order to determine whether activation of the type I PACAP receptor would lead to increased inositol phosphate we incubated ciliary ganglion neurons in the presence of various peptides and, utilizing a combination of the methods described in Rathouz et al. (1995) and Slivka and Insel (1987), studied whether total IP production would be stimulated. Following a 36 hour incubation in MEM supplemented with 10% heat-inactivated horse serum and 2μCi/ml of ^{3}H-myo-inositol (NEN/Dupont, SA~19mCi/mmol), wells were washed three times in MEM containing 50mM LiCl. Neurons remained in the final wash solution and incubated another 20 minutes at 37 deg C. Various test peptides (PACAP38, PACAP27, VIP or glucagon) or vehicle were added to duplicate or quadruplicate wells from frozen stocks (100-200μM) and were incubated another 50-60 minutes at 37 deg C. In some experiments, neurons were pre-incubated with the indicated concentration of inhibitor (see results) for 20 minutes before addition of peptide. Reactions were stopped by the placement of plates on ice followed by addition of ice-cold methanol (0.5V) to each well. Cells were scraped for one minute, the lipids extracted with chloroform and water (0.5:0.4, v/v), and an aliquot of the chloroform phase taken to determine the extent of incorporation of label into inositol phosphates. The aqueous layer was transferred to a tube containing 300mg of AG 1X8 Resin converted to the formate form. Samples were vortexed briefly followed by incubation at room temperature for 20 minutes. After rapid centrifugation, the liquid phase was removed and an aliquot counted in a scintillation counter to determine the amount of label remaining as free inositol. The resin was rinsed 3X with water and then

incubated for 30 minutes with 1ml ice-cold 2M Ammonium Formate/0.1M Formic Acid, the tubes centrifuged briefly and the eluate transferred to 10ml of ScintiVerse for scintillation counting. Counts obtained (minus that of the blank) from each individual sample well in a given condition (either two or four) were averaged. The change in total inositol phosphates was determined as the average of the counts in the presence of peptides minus the average of those in the absence (basal). The concentration of peptide required to increase IP levels to 50% of their maximum (EC50) was determined by fitting the dose-response curves with the Hill Equation; $y = [peptide]^n/[EC50]^n + [peptide]^n$, where n represents the Hill coefficient.

Calcium Imaging. Following a one-hour incubation at 37 deg C, cells were loaded with the calcium indicator dye Fluo-3AM (Kao et al., 1989) in DF+ (see solutions) for 45 minutes at room temperature in the dark. Cells were then washed 3X with DF++ and kept in the dark in final wash solution until use. Neurons were placed on the stage of an Olympus BX-50 upright microscope (Olympus, Inc.) and examined with an epifluorescence 40X LUMPFL immersion objective. A mercury lamp within a universal reflected light illuminator was used as the light source. The light beam was directed through the excitation filter onto a dichroic mirror. Light of 490nm was sent to the sample to excite the fluorescent probe, which then emitted light at a wavelength of 530nm. Cells were exposed to the excitation beam by manual opening of the shutter just prior to recording of calcium changes by IP lab spectrum (Scanalytics, Reading, PA) and maintained in that position until recording stopped (60-90 seconds). The bath was continuously perfused by gravity flow at a rate of 2ml/min and solutions were switched manually between control and test solutions (i.e. solution containing the various peptides

at the indicated concentrations) just after opening of the shutter. In some cases, prior to exposure to test peptides, neurons were approached by a micropipette filled with internal solution containing 200μM ddA with or without other test compounds (see results) and a high resistance seal was formed followed by attainment of the whole-cell configuration. Changes in fluorescence intensity were recorded every 0.5 seconds by a PVCam camera attached to a trinocular tube at the top of the microscope. Images were captured by IP Lab Spectrum and stored on a Macintosh for later analysis. Fields contained 1-5 neurons per experiment. For analysis, data were converted to Byte form and the fluorescence intensity measured at times zero (basal intensity) and 50 ms for each cell and the latter divided by the former, was multiplied by 100 and taken to represent the change in fluorescence in response to peptide or vehicle. Only those neurons responding with a 15% or greater increase in fluorescence intensity were considered to have responded positively. Statistical significance was predetermined at $p<0.05$. The number of neurons required to show a significant difference from 85% (i.e. the percent of neurons responding to 100nM PACAP alone) was decided for each test condition using the formula for determining the sample size for two independent binomial proportions ($\alpha = 0.05$; $\beta = 0.8$).

Electrophysiology. The electrophysiology experiments described in chapter 3 were performed by Dr. Joseph Margiotta and, therefore, will not be described in detail here. Rather, those interested are referred to Margiotta and Pardi, 1995. Briefly, ciliary ganglion neurons were dissociated and plated onto glass coverslips as described previously (Gurantz et al., 1994). The methods of preparation of patch pipettes and solutions are similar to those described for experiments utilizing 'fast perfusion' (see

below). Whole-cell ACh responses were recorded from neurons at room temperature in 10μM IBMX (with or without test peptides or AC inhibitors). Neurons were held at –70mV while ACh was applied by rapid pressure (5-10psi) microperfusion from patch pipettes 2-5μm in diameter. Current recordings obtained in this way were converted to values of conductance (assuming a reversal potential of –11mV) as described elsewhere (Gurantz et al., 1994). The conductance at time zero (G_0) was extrapolated and desensitization constants obtained by fitting conductance records with the sum of two exponentials corresponding to the two desensitization processes; with time constants of τ_f and τ_s. The method of perfusion allowed detection of currents with desensitization rates $\geq$ 50 msec. Conductance values were normalized (G_n) to cell size by dividing the value obtained for each neuron by its membrane capacitance ($G_n=G_0/C_m$; nS/pF). These normalized values provided a measure of sensitivity to agonist since they represent the peak agonist-induced response due to activation of all functional AChRs present on the cell membrane. The effects of test peptides are presented as the percent change (mean $\pm$ standard error) from the mean conductance of the controls acquired during each respective experiment. Significance was predetermined as $p<0.05$ and was determined using the formula for Student's unpaired *t* test.

Recording of Nicotine-Induced Currents using 'fast-perfusion' (Chapter 4). Whole-cell currents were recorded from acutely dissociated neurons loosely attached to glass coverslips and placed in a series-20 perfusion chamber held by an aluminum platform (Warner Instruments Corporation, Hamden, CT) as follows. A high resistance seal was formed (seals generally exceeded 10GΩ), and the cell was gently nudged and lifted from the coverslip. Once free from the coverslip, the whole-cell configuration was attained.

Depending on the experiment (see results), neurons were either raised immediately into a stream of recording saline (see below) or kept just below the level of this stream for several minutes. The latter approach was taken to allow for dialysis of the cell interior with the contents of the recording pipette which has been demonstrated to occur rapidly under conditions similar to those used here (Marty and Neher, 1983; Rawlings et al., 1994b). Saline streams, either with or without dissolved agonist, were delivered from theta tubing pulled to a diameter of ~100μm on a Model P-97 Micropipette Puller (Sutter Instrument Co). Switching between solutions was accomplished by an ultrafast solution switching system (Burleigh Instruments, Inc., Model# LSS-3100) driven by an amplifier/driver (Burleigh Instruments Inc., Model# PZ-150M) that was controlled by the recording software. A key press moved the theta tubing such that the stream containing agonist would then bathe the neuron within 1 msec (J. Margiotta, personal communication) for three seconds and then switch back to the saline stream. The bath was bathed continuously by gravity flow at a rate of 2ml/min from two, 30-ml syringes containing recording saline alone or recording saline plus peptide, connected by stopcocks to a single in-flow line. The bathing solution was switched manually between the two via the opening and closing of a stopcock. The time for complete exchange of the bath solutions was approximately one minute. In some experiments, bath solutions also contained 60nM αBgt. The whole-cell configuration was maintained for one minute prior to switching of the bath solution in some cases, (e.g. during the microperfusion of agents into cells). All recordings were performed at room temperature.

Recording electrodes were pulled from Corning 8161 Borosilicate Glass Capillary Tubes on a Model P-97 Micropipette Puller (Sutter Instrument Co) and were back filled

with internal solution alone or that containing various test reagents (see results), and had resistances ranging between 1 and 2 MΩ.

Nicotine-induced currents were collected using an Axopatch model 1B patch clamp amplifier and P-Clamp version 6.2.3 (Axon Instruments, Foster City, CA). Currents were filtered at 1 kHz and digitized at 2kHz, as described in Zhang et al., 1994. Measurement of current amplitudes and analysis of the desensitization rates were performed by use of the Clampfit program of the PClamp6 software. Nicotine-induced currents appeared similar to those observed by others and could be fit by the sum of three exponentials, previously attributed to activation of the two different classes of AChRs known to be present on ciliary ganglion neurons (Zhang et al.,1994; Vijayaraghavan et al.,1995). The rapidly activating and desensitizing component has been fit previously with a fast desensitization time constant (τf) of about 13 ms and is thought to be due to activation of the α7-containing, αBgt-sensitive class of AChRs (Zhang et al.,1994). In our experiments, τf was taken to be the rate desensitization of this fast component (I_f) and estimated from the best fit to an exponential function between the point of maximum current and a point 20-30 ms later. To determine the current amplitude of I_f, the contribution of the slow component (I_s) was assessed first and the result subtracted from the peak current (I_p). Consequently, slow currents were fit between 2900ms and a point at least 50 ms later than that determined as the time of occurrence of the peak and then extrapolated to time zero. In this way, slow components were usually fit best (σ of fit <50) by the sum of two exponentials; one that desensitizes very slowly and another that desensitizes with a rate intermediate (τi) that of the slowest component and the fast component (τf). These are characteristics expected of currents derived from activation of

the α3-containing class of AChR (Margiotta et al., 1987). The amplitude of the current at time zero was taken to represent the maximum of the entire slow component and designated as I_s. Thus, except for the rate of desensitization of the intermediate, the characteristics of the two components making up the slow were not analyzed further. Values were typified by those obtained for the top trace in Figure 4.1A: Cm=15.3 pF, I_s=-1370 pA, I_p=-6108.4 pA and rates of desensitization for the fast and intermediate components: τ_f= 7ms and τ_i =93ms. Current components (I_p, I_f and I_s) were normalized to cell size by dividing the current amplitude by the cell membrane capacitance (pA/pF) as estimated by the dials on the patch clamp amplifier. This value has previously been defined as a measure of the whole-cell sensitivity to agonist (Margiotta et al., 1987; Margiotta and Pardi, 1995). The percent decrease in agonist sensitivity of individual neurons in the test condition was determined by dividing the normalized current amplitude by the average amplitude of neurons in the control condition obtained during the same experiment. The percent differences were averaged for each condition and the differences between conditions statistically analyzed. Statistical significance was taken as a $p < 0.05$ determined using the formula for the Student's *t* test. Data are presented either as the percent decrease in current amplitude compared to that of control or as the average normalized current (pA/pF) for each condition.

Microperfusion of Compounds into Cells during Calcium Imaging Experiments (Chapter 4). The program, Pulse version 8.0 (Heka Electronics), was used to assess electrode and seal resistances as well as to establish and maintain the whole-cell configuration and holding potential (-70mV) while dialysis between the cell and micropipette took place.

Solutions. DF0: (in mM) 145 NaCl, 5.3 KCl, 5.4 Hepes, 5 glucose, and 0.8 $MgCl_2$. DF+ or recording saline: DF0 + 5.4mM $CaCl_2$. DF++: DF+ supplemented with 10% Heat Inactivated Horse Serum. Internal Solution: (in mM) 145.6 $CsCl_2$, 1.2 $CaCl_2$, 2 EGTA, 5 NaHepes, 15.4 Glucose, 1 ATP. All drugs used for microperfusion were diluted to the appropriate concentration in internal solution from stock solutions.

Materials. Fertilized white leghorn chicken eggs were obtained from a local farm (Hertzfeld Poultry Farm, Waterville, OH). PACAP38, PACAP27 and VIP were obtained from The American Peptide Company (San Diego, Ca). Glucagon and Secretin were obtained from Peninsula Laboratories (Belmont, Ca). Heat-Inactivated Horse Serum was acquired from Gibco-BRL (Grand Island, NY). 2'-5'-dideoxyadenosine (ddA), D-myo-Inositol 1,4,5-Triphosphate, 3-deoxy-Hexasodium salt (3-deoxy-IP3), D-myo-Inositol 1,4,5-Triphosphate, 3-deoxy-3-flouro-Hexasodium salt (La-IP3), H89, H7, Ruthenium Red and αBgt were all purchased from Calbiochem (La Jolla, Ca). Unless otherwise specified, all other reagents were obtained from Sigma.

CHAPTER 3:

Pituitary Adenylate Cyclase-activating Polypeptide (PACAP) type I binding sites are present on chick ciliary ganglion neurons: modulation of AChRs by PACAP.

RESULTS

High affinity PACAP binding sites are present on ciliary ganglion neurons. The presence of PACAP-selective high affinity binding sites was determined using ^{125}I-PACAP27 competition binding assays. ^{125}I-PACAP27 was used rather than iodinated PACAP38 because it reportedly exhibits less nonspecific binding (Gottschall et al., 1990). Both PACAP38 (IC50=0.5 $\pm$ 0.3nM, n=4) and PACAP27 (IC50=1.1 $\pm$ 0.6nM, n=3) potently displaced the tracer (Fig 3.1). While PACAP38 appears more potent than PACAP27, the difference is only apparent and was found to be insignificant in pairwise comparison ($p>0.1$). VIP, however, was much less potent ($p<0.05$, Student's *t* test) than either PACAP at displacing the iodinated tracer (IC50=1028 $\pm$ 552nM, n=3). Neither glucagon nor secretin competed significantly with ^{125}I-PACAP27 at any of the concentrations tested. Hill coefficients (mean $\pm$ standard error) for PACAP38, PACAP27 and VIP were approximately one (-1.87 $\pm$ 0.58, n=4; -0.65 $\pm$ 0.16, n=3; -1.12 $\pm$ 0.81, n=3, respectively). The pharmacological profile expected of a native type I PACAP receptor (Shivers et al., 1991) is exemplified by these results.

PACAP stimulates cAMP formation. Whether the high affinity binding sites described above would stimulate the synthesis of cAMP was evaluated using a commercial cAMP RIA kit. The ability of PACAP and its related peptides to increase cAMP mirrored the order of their affinity for the receptor (Fig 3.2), as expected for type I PACAP receptors. Again, PACAP38 appeared to be slightly more potent than PACAP27 (EC50=0.38 $\pm$ 0.05nM, n=6 vs. 0.62 $\pm$ 0.12nM, n=4). VIP, however, was about 600 fold less potent at stimulating cAMP production (EC50=306 $\pm$ 42nM, n=5, $p<0.05$). Glucagon and secretin were both unable to increase cAMP at any of the concentrations tested. Maximum cAMP

levels were taken to be those levels achieved with a maximum concentration of peptide (10μM) minus the basal levels occurring in the absence of peptide (see Table 1 in Margiotta and Pardi, 1995). That PACAP increased cAMP through activation of AC via interaction with a PACAP receptor, was evidenced by the much decreased cAMP level in the presence of peptide and 200μM of the adenylate cyclase inhibitor, ddA (Margiotta and Pardi, 1995). ddA significantly reduced the maximum cAMP levels induced by PACAP38, PACAP27 and VIP (78% ± 9 decrease, n=6; 60% ± 7, n=3 and 77% ± 2, n=4, respectively). In other experiments, 200μM ddA also suppressed, to a similar degree, the ability of forskolin, a direct AC activator, to increase cAMP production in ciliary ganglion neurons (Margiotta and Pardi, 1995). Both PACAP and VIP increased cAMP in a concentration-dependent manner, suggesting a specific interaction of peptide with the binding sites. The potencies of these peptides in increasing cAMP are in good agreement with reports on other cell systems thought to naturally express type I PACAP receptors (Rawlings, 1994a) and on artificially expressed type I PACAP receptors (Spengler et al., 1993; Journot et al., 1995). Taken together, the results of the binding and cAMP studies suggest the presence of PACAP-specific, type I PACAP receptors on the neurons of the ciliary ganglion.

PACAP can increase the sensitivity to ACh. A recent report (Gurantz et al., 1994) describes an increase in sensitivity (described as the maximal conductance response normalized per unit membrane of capacitance or $G_n=G_\infty/Cm$; Margiotta and Pardi, 1995) of ciliary ganglion neurons to applications of 500μM ACh when the neurons were preincubated for short periods with 1μM VIP. This increase in sensitivity was correlated with the ability of VIP to increase cAMP in the neurons. Since PACAP not only increases

cAMP in ciliary ganglion neurons, but also does so with far greater potency than does VIP, we determined whether PACAP would increase the sensitivity of the neurons to ACh (Fig 3.3). As expected, 1μM VIP increased the sensitivity to ACh by ~50% (52 ± 11%). Not only did PACAP38 and PACAP27 increase the sensitivity of the neurons to ACh by a similar percentage ($p>0.05$, by Student's *t* test) but the concentration required to do so was 100 fold less (10nM). Both PACAP38 and PACAP27 significantly ($p<0.05$) increased the sensitivity by 53 ± 15% (n=10) and 52 ± 23% (n=10), respectively. The response to 10nM VIP, however, was not significantly different from control. None of the other components (e.g. desensitization rates) of the ACh-induced current were significantly affected by any of the peptides (Margiotta and Pardi, 1995). Thus, the potencies of the peptides in raising cAMP correlated well with their potencies in raising the ACh sensitivity of the neurons.

In the process of defining the role of adenylate cyclase activation by the PACAP receptor in the observed increase in ACh sensitivity we exposed neurons to 200μM ddA prior to peptide challenge. Similar to its lack of effect on basal cAMP levels, ddA had no effect on ACh sensitivity on its own (Margiotta and Pardi, 1995). However, it completely prevented the increase in ACh sensitivity normally observed when neurons are incubated with 10nM PACAP or 1μM VIP alone (Fig 3.3). Furthermore, not only was the increased sensitivity abrogated by ddA, but a ~50% decrease in ACh sensitivity ($p<0.05$, by Student's *t* test) was observed with all three peptides compared to neurons treated with ddA alone. As noted in the absence of ddA, none of the other components of the ACh-induced current were affected (Margiotta and Pardi, 1995). The reversal of the PACAP-induced increases in ACh sensitivity observed when AC activity is suppressed

argues that the peptides increase the ACh sensitivity through increases in cAMP. The binding data, cAMP assays, and ACh sensitivity results confirm the existence of type I PACAP receptors on ciliary ganglion neurons and support the contention that the enhanced-ACh sensitivity seen on exposure to PACAP38, PACAP27 and VIP occurs through a cAMP-dependent mechanism.

DISCUSSION

We have demonstrated the existence of a high affinity binding site for PACAP on ciliary ganglion neurons of the chick. As determined by competition assays utilizing ^{125}I-PACAP27, these sites exhibit the characteristic pharmacology expected of a type I PACAP binding site which binds PACAP with about 1000 fold greater affinity than VIP (Tatsuno et al., 1990; Shivers et al., 1991; Christophe, 1993). In our experiments, PACAP38 and PACAP27 reduced ^{125}I-PACAP27 to 50% of its maximum binding at concentrations of 0.5nM and 1.1nM, respectively (fig 3.1). In contrast the concentration of VIP required to displace 50% of the tracer was about 1μM. As expected of a type I PACAP receptor, both PACAP38 and PACAP27 potently increased cAMP levels whereas VIP was 600 times less potent (EC50s=0.38, 0.62 and 306nM, respectively). Importantly, not only did PACAP increase cAMP but it also did so in a concentration-dependent manner (fig 3.2), lending support to the idea that these binding sites are specific for PACAP. This is further supported by the lack of other related peptides, such as glucagon and secretin, to elicit similar responses. These results are in good agreement with several published reports on the potency of PACAP to increase cAMP levels in other neuronal cell systems via activation of a type I PACAP receptor (Miyata et al., 1989; Robberecht et al., 1992; Deutsch and Sun, 1992). The order of potency is also in agreement with that published for type I receptors that have been heterologously expressed in *Xenopus* oocytes (Spengler et al., 1993). Therefore, we conclude that the high affinity binding site located on the membranes of ciliary ganglion neurons is a type I PACAP receptor.

The potencies of the peptides in raising cAMP correlated well with their potencies in raising the sensitivity of the neurons to application of ACh (fig 3.3). 1μM VIP increased the ACh sensitivity by ~50% over that of controls. PACAP38 and PACAP27 also increased the sensitivity to ACh by a similar percentage, however the concentration required to achieve this increase was only 10nM. The response to 10nM VIP was not significantly different from that of controls. An increase in ACh sensitivity has been correlated with increased cAMP levels as incubation with the membrane permeant cAMP analog, 8-Br-cAMP, also increases neuronal sensitivity to ACh by ~200% (Margiotta et al., 1987). Furthermore, addition of cAMP, but not cGMP, in the recording electrode also increased the ACh sensitivity. A later study, testing the effects of VIP using a cAMP-sensitive fluorescent probe, demonstrated that VIP could rapidly increase cAMP in ciliary ganglion neurons. Additionally, a 10-minute pre-incubation with 1μM of the peptide could increase ACh sensitivity by ~50% (Gurantz et al., 1994), results corroborated in the present report. It has been suggested that the number of functional receptors present on the membrane doubles in the presence of cAMP (Margiotta et al., 1987). This has lead to the conclusion that the neurons have the ability to recruit functional receptors from a pool of non-functional receptors through some type of post-translational modification, possibly a phosphorylation of the receptor itself (Margiotta et al., 1987). These results are consistent with later reports showing that these neurons maintain receptor numbers in much larger quantities than their mRNA transcripts. Moreover, culture conditions that change functional receptor density do not change the levels of mRNA, suggesting that these neurons regulate their receptors through post-translational modifications (Corriveau and Berg, 1994). Another report demonstrated

that the α3 subunit can be phosphorylated by the catalytic subunit of PKA and that it is phosphorylated when incubated with 8-Br-cAMP (Vijayaraghavan et al.,1990).

In the process of defining the role of AC activation and elevated cAMP levels due to activation of the type I PACAP receptor, we employed the adenosine analog, ddA, to block AC (Johnson et al., 1989; Margiotta and Pardi, 1995). ddA significantly reduced the maximum cAMP levels induced by incubation with PACAP38, PACAP27 and VIP while leaving basal levels unaffected (Margiotta and Pardi, 1995). In keeping with its lack of an effect on basal cAMP levels, ddA itself had no effect on the sensitivity of the neurons to ACh. However, on incubation with ddA, neither PACAP nor VIP resulted in an increase in ACh sensitivity. Of even greater interest, there was a reversal in the affects of the peptides in the presence of ddA; now a decreased sensitivity of ~50% was observed (fig 3.3). These results clearly indicate that PACAP can increase the sensitivity of ciliary ganglion neurons to ACh and that it does so through the activation of the type I PACAP receptor and subsequent elevations in cAMP.

The native type I PACAP receptor is known to activate a second signaling cascade in parallel with cAMP in several neuronal cell systems. This second signaling pathway involves the activation of a PLCβ (Basille et al., 1995; Hezareh et al., 1996) followed by increases in 1,4,5-IP3 and intracellular calcium (Rawlings et al., 1994b) due to release from intracellular calcium stores via the action of IP3 at its receptor (Tatsuno et al., 1992; Canny et al., 1992). The type I PACAP receptor has also been shown to activate both signaling pathways in parallel when heterologously expressed in *Xenopus* oocytes (Spengler et al., 1993). Therefore it is possible that, as in other neuronal cell systems, both signaling cascades are being activated, but while the AC path is suppressed

by ddA, the effects of activation of the other pathway are now unmasked. Preliminary results suggest that PACAP does indeed increase IP levels in ciliary ganglion neurons and thus can activate two signaling pathways simultaneously. Results of experiments designed to further explore the mechanism behind the apparent decrease in ACh sensitivity in response to PACAP and ddA are detailed in chapter 4.

A VIP-like immunoreactivity has been localized to the preganglionic terminals on ciliary neurons in the chick (Reiner, 1987). This determination was made prior to the discovery of PACAP in 1989. Given the high homology, (68%), between VIP and PACAP and results of differential binding studies comparing the distribution of ^{125}I-PACAP and ^{125}I-VIP, (which indicate that the levels of VIP are 8-17 fold lower than that of PACAP throughout the nervous system (Suda et al., 1992)), antibody cross-reactivity is a concern in the early studies. Taking all of the above into account, along with the results of the binding studies and cAMP assays reported here, it can be argued that the actual in-vivo peptide present in the terminals and acting on the ciliary ganglion neurons, either alone or in conjunction with VIP, is PACAP.

Figure 3.1 PACAP receptors on ciliary ganglion neurons show binding specificity. Dissociated neurons were incubated with ^{125}I-PACAP27 in the presence of unlabeled PACAP38 (filled diamonds), PACAP27 (empty squares), VIP (filled triangles), glucagon (empty circles), or secretin (filled circles) at the indicated concentrations. For each concentration of peptide, the ability to displace 50pM ^{125}I-PACAP27 is expressed as a percentage of ^{125}I-PACAP27 bound in the absence of competing peptide. The binding assays were performed in duplicate, and the percentage displacements (mean $\pm$ standard error) for three or four experiments using PACAP38, PACAP27, or VIP as competitor are plotted; results are plotted from two experiments using secretin or glucagon as competitor. IC50 values are presented in the text and were determined as described in methods. Both the figure and caption were printed with permission from Margiotta and Pardi, 1995.

Figure 3.1

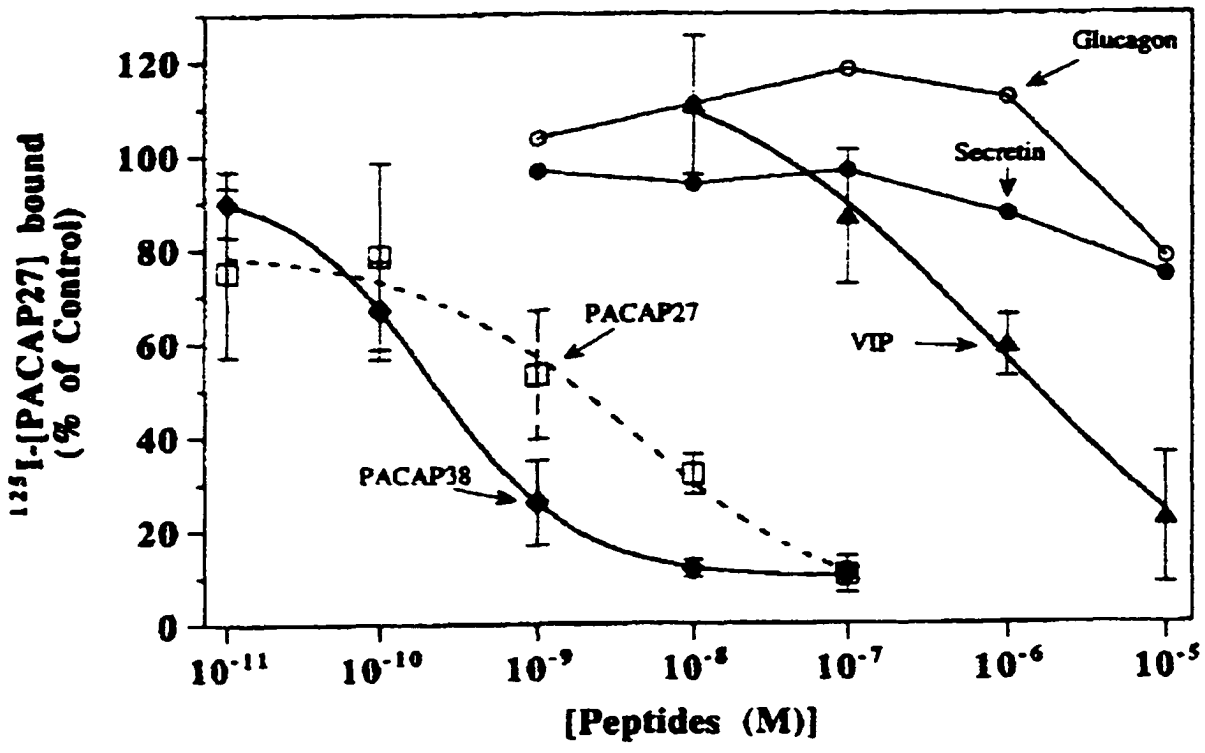


Figure 3.2 PACAP receptors on ciliary ganglion neurons are coupled to rapid efficient cAMP accumulation. The dose-response relationship for various PACAP-related peptides is shown. Dissociated neurons were incubated for 15 min with the indicated concentrations of PACAP38 (filled diamonds), PACAP27 (empty squares), VIP (filled triangles), glucagon (empty circles), or secretin (filled circles). Stimulation of cAMP accumulation was quantified as a percentage of the maximum levels (mean $\pm$ standard error), as described in the text. The assays were performed in duplicate, and the average percentages were plotted for four to six experiments in the case of PACAP38, PACAP27, and VIP and for two duplicate-well assays in the case of glucagon and secretin. EC50 and $cAMP_{max}$ values were determined for each peptide as described, and the means presented in the text. Both the figure and caption were printed with permission from Margiotta and Pardi, 1995.

Figure 3.2

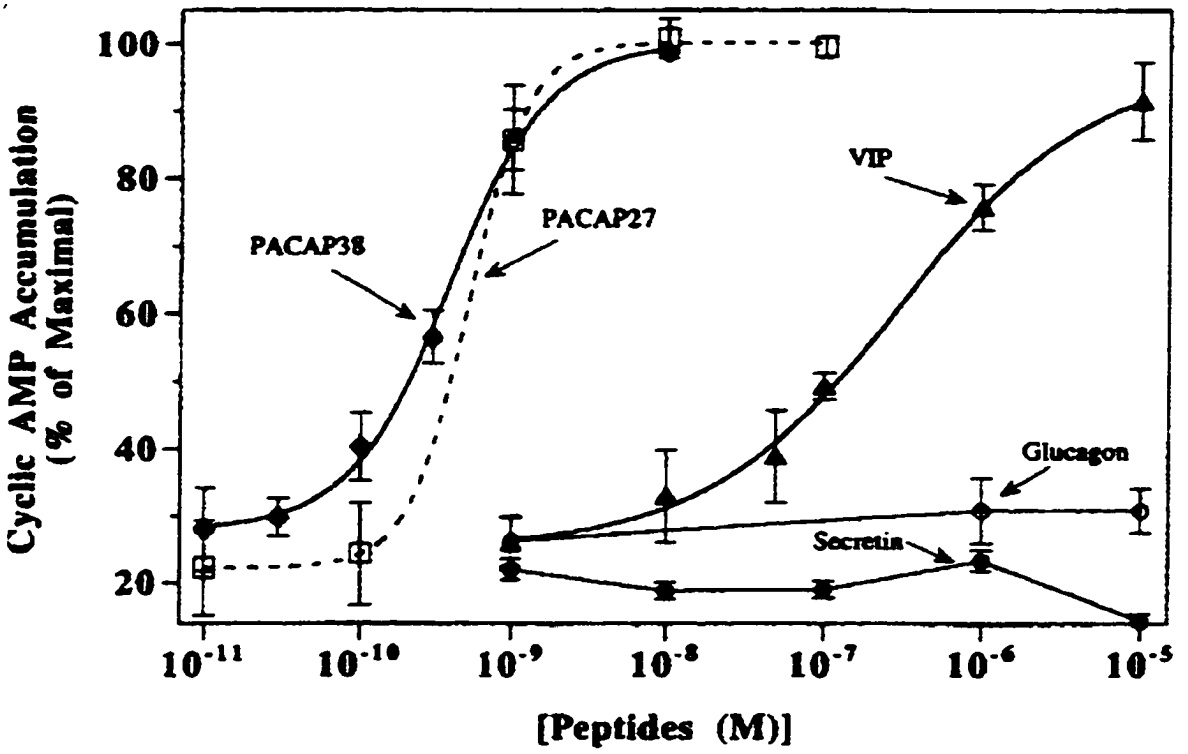
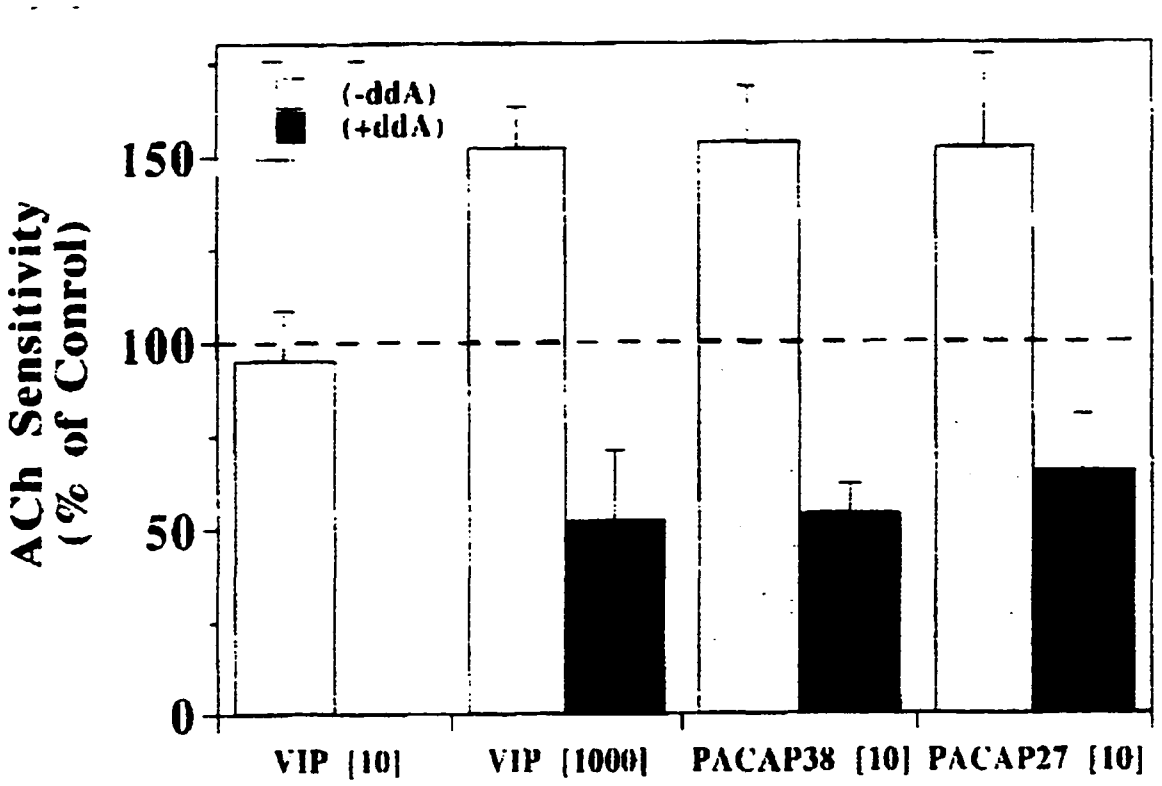


Figure 3.3 PACAP38, PACAP27 and VIP increase the ACh sensitivity of ciliary ganglion neurons by a cAMP-dependent mechanism. Size normalized maximal conductance values ($G_n=G_o/C_m$) obtained for each cell, expressed as a percentage of responses in the same experiment. Control neurons were subjected to the same treatment regimen without added peptide. For the ddA experiments, ACh responses of neurons treated with ddA alone (without added peptides) were used as controls. Such values were indistinguishable from those for untreated naïve cells tested in the same experiments. Each measurement represents the mean $\pm$ standard error of G_n values from 10-55 neurons. Except for 10nM VIP, all of the treatments revealed a significant difference in mean G_n, compared with control neurons not treated with peptides (dashed line). In all cases, neurons were preincubated for 1hr at 37 deg C in recording solution supplemented with the 10% horse serum, with or without the AC inhibitor ddA (200μM). Test neurons were next incubated for 15 min in the same solution with VIP, PACAP38 or PACAP27 at the indicated concentrations and then examined at room temperature for peak conductance responses to 500μM ACh, as described (Margiotta and Pardi, 1995). Both the figure and caption were printed with permission from Margiotta and Pardi, 1995.

Figure 3.3



CHAPTER 4:

Differential inhibition of α7-AChRs by Pituitary Adenylate Cyclase-activating Polypeptide.

RESULTS

PACAP differentially regulates α7- and α3*-AChRs. PACAP (100nM) decreases the amplitude of the fast component of the current evoked by fast perfusion with 20μM nicotine while leaving the slow component unaffected. Previously, we reported that PACAP could either increase or decrease the ACh sensitivity of ciliary ganglion neurons (Margiotta and Pardi, 1995), depending on whether neurons were first treated with the adenylate cylcase inhibitor, ddA. In an effort to identify the AChR class(es) affected by PACAP, we employed the method of fast perfusion which allows us to distinguish between the two major receptor classes present on ciliary ganglion neurons - the α7-containing and α3-containing AChRs (Zhang et al.,1994; Blumenthal et al., 1998). Fast perfusion of a neuron with 20μM nicotine in a typical experiment (Figure 4.1A, top left) resulted in an inward current composed of an initial fast component (I_f) that activated and desensitized rapidly (τ_f= 7ms) and a second component (I_s) that decayed ~10 times more slowly (τ_i = 93ms; see methods). Rapid activation and desensitization kinetics such as those observed here for I_f, are properties expected of the αBgt-sensitive, α7-containing AChRs (Zhang et al.,1994; Horch and Sargent, 1995). Indeed, incubation of neurons with 60nM αBgt (Figure 4.1A, top right) abolished I_f completely. On the other hand I_s is likely to be due to activation of α3-containing receptors since the resistance to αBgt, of this receptor class on ciliary ganglion neurons, has previously been established (Zhang et al.,1994; Conroy and Berg, 1995). Nicotinic responses and αBgt sensitivities were identical in neurons not pretreated with ddA (data not shown). Following a 2-minute pretreatment with ddA, when neurons were exposed to 100nM PACAP for 5-8 minutes to maximally activate PACAP receptors (Margiotta and Pardi, 1995), subsequent nicotine

exposure resulted in a ~50% decrease in I_f, without affecting I_s (compare Fig 4.1A, top left and bottom).

The type I PACAP receptor, thought to be present on ciliary ganglion neurons (Margiotta and Pardi, 1995), exhibits a particular selectivity for PACAP versus the highly homologous (68%) VIP, a member of the same peptide family as PACAP (Christophe, 1993). This receptor binds to and is activated by both PACAP species with equal and high affinity, while about 1000X greater concentrations of VIP are required to bind to the receptor and to produce the same effects (Rawlings, 1994a; Gourlet et al., 1996). Thus, we determined whether the efficacies of PACAP and VIP in producing the decrease in nicotine-induced current would correlate with the known affinities of the peptides at the type I receptor. A summary of the abilities of PACAP and VIP to decrease the two current components induced by nicotine is depicted in Figure 4.1B. 100nM of PACAP38 or PACAP27 decreased I_f, the component due to activation of α7-containing AChRs, to a similar degree (58 ± 0.05 and 41 ± 0.09 %, respectively; $p<0.05$ for both). VIP, however, failed to result in a significant decrease in I_f at either 100nM or 1μM. In contrast, neither PACAP nor VIP significantly decreased I_s, the α3*-AChR current component. PACAP did not significantly affect any of the other attributes of the current evoked by nicotine (e.g. desensitization kinetics). These data indicate that while adenylate cyclase activity is suppressed, PACAP, via activation of the type I receptor, leads to a decrease in the nicotine-induced current that preferentially affects the component produced by α7-containing receptors.

The same approach was used to examine the ability of PACAP to regulate α7- and α3*-AChR currents when the ddA pre-treatment was omitted, allowing AC activity to remain intact and PACAP receptor activation to maximally stimulate cAMP production (Margiotta and Pardi, 1995). Under such conditions, and in accord with previous observations (Margiotta and Pardi, 1995; Margiotta et al., 1987), PACAP38 treatment (100nM, 5-8 min) significantly increased α7- and α3*-AChR current components of the subsequent response to 20μM nicotine. The increases were 134 ± 41% (n=8; $p<0.05$) for I_f, and 81 ± 29% (n=8; $p<0.05$) for I_s relative to control neurons not exposed to PACAP (n=7). Thus, PACAP enhances the function of both the α7- and α3*-AChR when AC is active and selectively inhibits α7-AChR function when AC is suppressed. We next focused on the signaling pathway utilized by PACAP in inhibiting α7-AChRs.

The ability of PACAP to inhibit α7-AChR function requires intracellular calcium. A recent study suggested that an inhibition of the αBgt-sensitive component of the nicotine-induced current required an increase in intracellular $[Ca^{2+}]$ (Vijayaraghavan et al.,1995). To examine whether an increase in $[Ca^{2+}]_i$ is involved in the ability of PACAP to decrease I_f, we added 10mM BAPTA to the solution in the patch pipette to chelate $[Ca^{2+}]i$ before and during the 5-8 minute incubation with 100nM PACAP38. BAPTA, introduced into cells in this way, did not affect nicotine-induced currents of control neurons (data not shown; Zhang et al.,1994; McGehee et al., 1995) but did significantly prevent PACAP-induced increases in $[Ca^{2+}]_i$ during calcium imaging experiments using Fluo-3AM (Table 4.1; see below). Likewise, in physiology experiments, chelation of $[Ca^{2+}]_i$ with BAPTA, completely abolished the ability of PACAP to reduce I_f (Figure

4.2A and B). As in Figure 4.1, treatment with 100nM PACAP38 reduced the α7-AChR response to fast perfusion with 20μM nicotine (I_f= -76.34 ± 13.88 pA/pF) compared to control neurons (I_f= -216.95 ± 16.57 pA/pF; $p<0.05$). In contrast, when the patch pipette contained 10mM BAPTA, α7-AChR responses were not detectably different from control neurons (I_f= –218.8 ± 15.98 pA/pF; $p>0.05$ for both). These results support the contention that an increase in $[Ca^{2+}]_i$ by PACAP is required to decrease I_f.

PACAP increases PIP_2 turnover in a concentration dependent manner. One method that cells utilize to raise $[Ca^{2+}]_i$ is through generation of inositol-1,4,5-trisphosphate which then acts on the IP3 receptor (IP3R) on internal calcium stores thereby stimulating calcium release into the cytoplasm (Berridge, 1997). Activation of G-protein coupled peptide receptors that activate PLC is a common way for cells, including neurons, to generate IP3 (Exton, 1996). Given that an increase in $[Ca^{2+}]_i$ appeared to be required for the ability of PACAP to inhibit the fast component of the nicotine-induced current, and that activation of the PACAP type I receptor has been shown to increase IPs in many neuronal systems (Rawlings, 1994a; Chatterjee, 1997), we examined whether PACAP would increase IP levels in ciliary ganglion neurons. In a typical experiment, a one hour incubation with either PACAP38 or PACAP27 resulted in elevated IP levels that increased along with the concentration in the range of 10^{-10} M and 10^{-6} M compared to neurons not exposed to PACAP (Figure 4.3).

In addition, as evidenced by the concentrations required to reach 50% of their maximum IP production (EC50) both PACAP38 and PACAP27 were potent in generating IPs (EC50= 0.38 ± 0.15 nM, n=10 and 3.64 ± 0.92nM, n=6, respectively). Maximal IP levels were achieved after incubation for 1 hour with 10^{-7}M of PACAP38 or

PACAP27. In our cells, as in others (Deutsch and Sun, 1992), PACAP38 was more potent than PACAP27 in elevating IP levels ($p<0.05$). Unexpectedly, in comparison to the effects of PACAP38 and PACAP27 on cAMP content (Margiotta and Pardi, 1995), PACAP38 was equally potent at stimulating IP production whereas PACAP27 was ~ 10 fold less potent. In contrast, VIP began to increase IPs over basal only at 1μM (19.9 ± 19%; $p>0.05$), while glucagon, a peptide belonging to the same peptide family as PACAP, did not have any effect at 1μM. The Hill coefficients for PACAP38 and PACAP27 (0.72 ± 0.09 and 0.70 ± 0.22, respectively) were not significantly different from each other nor from 1. These results indicate that incubation of ciliary ganglion neurons with PACAP can increase IPs over control levels. Furthermore, the similar potencies of PACAP38 and PACAP27 along with the ineffectiveness of VIP in generating IPs, implicates involvement of the type I PACAP receptor in this response.

PACAP stimulates Phospholipase C activity. Many G-protein coupled receptors generate IP3 by activating PLC-dependent hydrolysis of phosphotidylinositol (Exton, 1996). Previously, PACAP, acting through the type I receptor, has been demonstrated to increase IP levels and this increase was suppressed by prior incubation with the PLC inhibitor, U73122 (Basille et al., 1995; Barnhart et al., 1997). Thus, we examined whether prior exposure to1μM U73122 would block the PACAP-induced increase in IPs (Figure 4.4). While a 20 minute preincubation with 1μM U73122 had no demonstrable effects on basal IP levels, when neurons were then challenged with 1μM PACAP38, neurons produced ~60% less IPs than neurons exposed to 1μM PACAP38 alone (60 ± 7.1%; $p<0.05$). In contrast, prior incubation with the adenylate cyclase inhibitor ddA (200μM), neither affected basal nor PACAP-induced IP levels significantly (9 ± 7.38%;

$p>0.1$). These results, taken along with the differential potencies of PACAP and VIP for stimulating IP production, suggest that PACAP increases IP levels via activation of the type I PACAP receptor with subsequent stimulation of PLC.

PACAP raises $[Ca^{2+}]_i$ via release from intracellular stores. An elevation in IP3 would be expected to stimulate the release of calcium from intracellular stores by the action of IP3 at its receptor and thereby increasing $[Ca^{2+}]_i$ (Berridge, 1997). In many cases in which an elevation in IPs by PACAP has been demonstrated, a subsequent rise in $[Ca^{2+}]_i$ has also been observed (Spengler et al., 1993; Journot; 1995) and a release from intracellular stores has been implicated as the source (Rawlings et al., 1994b; Barnhart et al., 1997). To examine the effects of PACAP on intracellular calcium in ciliary ganglion neurons, we loaded cells with the calcium indicator dye, Fluo-3AM and challenged cells with PACAP under a variety of extracellular and intracellular conditions (Figure 4.5 and Table 4.1). In a typical experiment, a neuron would be sealed by a recording pipette containing 200μM ddA with or without other drugs (see methods and Table 4.1) and access to the whole cell would be established. A representative neuron, exposed for 90 seconds to 100nM PACAP38 dissolved in bath solution, responded with a gradual rise in calcium as indicated by the increase in fluorescence (Figure 4.5A). Panel 1 in figure 4.5A depicts the neuron under consideration just after achieving the whole cell configuration. Prior to peptide exposure (Panel 2) the test neuron exhibits very little fluorescence (basal calcium levels). During exposure to 100nM PACAP however, an increase in fluorescence is observed as indicated by an increase in brightness of the cell (compare panels 2, 3 and 4). The increase shown here corresponded to a 52% increase in fluorescence intensity over that of basal. That the two neurons photographed along with

the test cell also responded to PACAP exposure with comparable increases in fluorescence intensity, suggests that ddA is not necessary for the observed increase in intracellular calcium. Other neurons exposed to bathing medium without peptide did not respond detectably (data not shown). To determine whether the increase in $[Ca^{2+}]_i$ required extracellular calcium, the bathing solution was replaced with one containing100nM PACAP38 dissolved in medium without added calcium (Figure 4.5B). In this experiment, unclamped neurons again responded to a 90 second exposure to PACAP with a gradual increase in fluorescence intensity as compared to basal. Thus, ciliary ganglion neurons respond to 100nM PACAP38, the same concentration of peptide shown to result in a decrease in the α7-AChR component of the nicotine-induced current, with an increase in intracellular calcium as determined by the calcium indicator dye Fluo-3AM. This increase does not require external calcium and occurs in the presence or absence of 200μM ddA. Interestingly, only 57 out of 67 neurons (85%) exposed to 100nM PACAP38, with or without ddA, responded with an increase in fluorescence (Table 4.1). These results are in accord with other accounts of the ability of PACAP to increase intracellular calcium using the same peptide concentration (Rawlings et al., 1994b).

When neurons were treated with inhibitors of IP3 formation or IP3 action, such as 100nM of the PLC inhibitor U73122, or blockers of IP3 at its receptor, such as 300μM heparin (Rawlings et al., 1994b; Barnhart et al., 1997), prior to and during peptide challenge, the percentage of neurons responding with an increase in fluorescence decreased significantly (25 and 11%, respectively; Table 4.1). Neither compound however, prevented the calcium increase induced by 20mM caffeine (data not shown), a

compound known to stimulate release from the ryanodine-sensitive internal calcium store (Berridge, 1997). However, when neurons were exposed to 1μM ruthenium red (RR), a compound known to block release from this internal calcium store (Yu, 1995), the percentage of neurons responding to a challenge with 100nM PACAP38 did not differ significantly (86%; $p>0.05$; Table 4.1) from control cells. In separate experiments, this same concentration of RR prevented the increase in fluorescence due to 20mM caffeine (data not shown). These results, along with those showing the ability of PACAP to potently increase IP levels in a concentration-dependent manner, indicate that PACAP, acting through the type I PACAP receptor, stimulates the release of intracellular calcium from an IP3-sensitive store.

The release of calcium from an IP3-sensitive store is required for the inhibition of I_f by PACAP. Given that an increase in intracellular calcium appeared to be important in the inhibition of I_f by PACAP, and that inhibitors of calcium release from the IP3-sensitive store prevented the PACAP-induced increase in $[Ca^{2+}]_i$, we tested whether these same inhibitors would also block the decrease in I_f due to PACAP (Figure 4.6). To examine the role of PLC, we added 100nM U73122, the same concentration shown to prevent the PACAP-induced rise in $[Ca^{2+}]_i$ (Table 4.1), to the solution in the patch pipette to inhibit PLC before and during the 5-8 minute exposure to 100nM PACAP38. At this concentration, U73122, when introduced into neurons along with 200μM ddA, did not significantly affect I_f or I_s compared to those neurons exposed to ddA alone (data not shown). However, U73122 did prevent the PACAP-induced decrease in I_f (Figure 4.6A). While treatment with 100nM PACAP38 reduced I_f ($I_f = -82.25 \pm 14.03$ pA/pF) compared to control neurons ($I_f = -192.54 \pm 20.57$ pA/pF; $p<0.05$), the presence of

100nM U73122 resulted in α7-AChR currents that were not detectably different from control neurons not exposed to PACAP (I_f = -195.99 ± 19.1 pA/pF; $p>0.05$ for both). Likewise, while 300μM heparin had no affect on basal nicotinic currents (data not shown), it abolished the ability of PACAP to decrease I_f (Figure 4.6C; I_f = -137.57 ± 29.2 for PACAP plus heparin versus -79.55 ± 7.55 pA/pF for PACAP alone; $p<0.05$ for the difference). Moreover, the addition of 2μM 3-deoxy-IP3 mimicked the inhibition of I_f observed with PACAP (I_f = -106.65 ± 19.98 pA/pF for IP3 versus –251.54 pA/pF for controls, $p<0.05$) while 2μM of the weak IP3 receptor agonist 2,3,6-trideoxy-IP3 (Figure 4.6B; I_f = -188.77 ± 22.37 pA/pF for LA-IP3 versus –202.01 ± 23.72 pA/pF for controls, $p>0.05$) did not. Taken together, these results indicate that release of intracellular calcium from an IP3-sensitive store is involved in the ability of PACAP to decrease the α7-AChR current due to fast perfusion with 20μM nicotine.

DISCUSSION

We have shown that treatment of ciliary ganglion neurons with 100nM PACAP38 or PACAP27 in the presence of the adenylate cyclase inhibitor, ddA, resulted in ~ 50% decrease in the fast component (I_f) of the current evoked by fast perfusion with 20μM nicotine (Fig 4.1). However, the amplitude of the slow component (I_s) was unaffected. In contrast to the effect of 100nM PACAP, VIP at 1μM but not at 100nM decreased I_f. This decrease required an increase in intracellular calcium, as it was completely prevented by the addition of BAPTA in the recording pipette. BAPTA also prevented the PACAP-induced increase in $[Ca^{2+}]_i$ in neurons loaded with the calcium indicator dye, Fluo3-AM (Table 4.1).

As in other neuronal systems (Christophe, 1993) PACAP increased total IP levels. The low concentrations of PACAP required (EC50 = 0.38nM for PACAP38 and 3.64nM for PACAP27) for this increase and the concentration-dependent manner in which it occurred suggests a specific interaction of PACAP with a peptide receptor (in Alberts, 1994; Deutsch and Sun, 1992). Moreover, the Hill coefficients for PACAP38 and PACAP27 did not differ significantly from one, (0.72 and 0.70, respectively), nor from each other, suggesting a specific interaction with a single binding site. In this regard, the order of potencies of the peptides in raising IP levels are comparable to those reported by others as that of the peptides in activating the type I PACAP receptor and increasing IP levels in other cell systems (Rawlings, 1994a). Furthermore, preincubation with the PLC inhibitor, U73122, decreased the PACAP-induced elevation of IPs by ~60% indicating that PACAP raises IP levels via activation of a PLC (Fig 4.4). U73122 has been used to demonstrate the activation of PLC by PACAP in several other cell systems (Basille et al.,

1995; Barnhart; 1997). More specifically, PACAP has been shown to activate a PLCβ in rat gonadotropes (Hezareh et al., 1996). The ability of PACAP to raise IPs was unaffected by ddA, implying that the cAMP pathway was not involved in the response. This result was not unexpected, as previous reports have also ruled out a role for cAMP or other components of the cAMP signaling cascade in IP turnover in response to PACAP (Rawlings et al., 1994b; Tatsuno et al., 1992). While PACAP38 appeared to be equally potent at stimulating IP turnover as for elevating cAMP, the extent to which both PACAP isoforms increased IP levels was lower than that observed by others (Deutsch and Sun, 1992). Such findings have previously been interpreted as an indication of a decreased efficiency of coupling to this signaling pathway (Delporte et al., 1993: Christophe, 1993; Zhu et al., 1994).

Increased IP levels are expected to result in increased $[Ca^{2+}]_i$ levels via an IP3-dependent release of calcium from internal stores (Berridge, 1997). In neurons loaded with the calcium indicator dye Fluo-3AM, incubation with 100nM PACAP38 resulted in an increase in fluorescence intensity thus indicating a rise in $[Ca^{2+}]_i$ levels (Fig 4.5). This increase occurred irrespective of extracellular calcium. PACAP has been shown to increase intracellular calcium levels through the activation of voltage-dependent calcium channels in GH3 cells. This was discovered to be brought about by depolarization of the membrane via voltage-activated sodium channels (Koshimuro et al., 1997). Given that the increase in intracellular calcium we observed here did not depend on entry of calcium from the external medium and that PACAP, when applied directly to neurons did not affect the resting conductance (Margiotta and Pardi, 1995), it is unlikely that a similar mechanism was involved here. Similarly to their effects on IPs, both 100nM PACAP27

and 1μM VIP increased $[Ca^{2+}]_i$ while 100nM VIP and 1μM glucagon did not (data not shown). Furthermore, the rise in $[Ca^{2+}]_i$, just as the rise in IPs, was prevented by the PLC inhibitor U73122 (Table 4.1) suggesting that the increase in calcium was due to activation of PLC and formation of IP3. Although we, like others (Deutsch and Sun, 1992; Chatterjee et al., 1996), did not specifically assay for IP3, the results presented here suggest that at least a portion of the observed increase in IPs stimulated by PACAP included IP3.

The order of potencies required for elevating IP levels and intracellular calcium concentrations resemble the potencies of the three peptides in decreasing I_f (PACAP38>PACAP27>VIP). Moreover, U73122, the same compound that prevented the increase in total IPs and intracellular calcium, also prevented the PACAP-evoked decrease in I_f(Fig 4.6). In addition, heparin, an inhibitor of the action of IP3 at its receptor on internal calcium stores (Takahashi et al., 1994) that has also been shown to prevent the PACAP-induced increase in $[Ca^{2+}]_i$ in other cell systems (Rawlings et al., 1994b; Barnhart et al., 1997), prevented both the PACAP-induced increase in intracellular calcium (Table 4.1) and the decrease in I_f(Fig 4.6). The same concentration of heparin used in these experiments had no effect on the calcium increase induced by 20mM caffeine (data not shown) suggesting that a release of calcium from a ryanodine-sensitive internal store, was not involved (Yu, 1995).

85% of the neurons exposed to 100nM PACAP38 responded with an increase in $[Ca^{2+}]_i$ (Table 4.1). These results are in accord with other accounts of the ability of PACAP to increase intracellular calcium using this same concentration of PACAP (Rawlings et al., 1994b). That a similar percentage of neurons responded to 100nM

PACAP38 with an increase in $[Ca^{2+}]_i$ as responded with a decrease in I_f (87.5%; experimental observations) provides further support for a connection between the PACAP-induced calcium increase and the PACAP-induced decrease in I_f.

The ability of U73122 to block the PACAP-dependent rise in IPs and intracellular calcium in ciliary ganglion neurons implicates PLC activation by PACAP as the mechanism involved in formation of these second messengers. The order of potency of the PACAPs, VIP and glucagon in stimulating an increased IP and $[Ca^{2+}]_i$ are similar to that described for activation of the type I PACAP receptor elsewhere (Rawlings, 1994a). This potency was mirrored in the ability of the peptides to decrease I_f (Fig 4.1B). Furthermore, that the same PLC inhibitor also blocked the PACAP-dependent decrease in I_f provides strong evidence in support of the hypothesis that the effect of PACAP is due to activation of this second signaling pathway by the type I PACAP receptor. The ability of an IP3 analog to mimic PACAP in producing the decrease in I_f (Fig 4.6), as well as in causing release of calcium from intracellular stores (data not shown), lends further support to the involvement of this pathway in the response. Although a direct inhibition of the channel by IP3 is possible, that heparin, an antagonist of IP3 at its receptor, prevented the decrease in I_f due to PACAP argues against a direct effect of IP3 on the AChR.

How could an increase in $[Ca^{2+}]_i$ translate into a decrease in the nicotine-induced current produced by α7-AChRs? At present, a direct action of calcium on the channel protein itself cannot be ruled out. However, this mechanism seems unlikely. Several factors have contributed to this conclusion. Many studies, in the process of examining other characteristics of the αBgt-sensitive class of AChRs, have employed a double

nicotine pulse protocol. In control neurons, it was shown that a second pulse of nicotine, following at the most two minutes after the first, produced currents that were no different than those produced during the first pulse (Khiroug et al., 1998). αBgt-sensitive receptors can increase intracellular calcium to levels high enough to produce physiological effects (Messi et al., 1997; Delbono et al., 1997; Wonnacott, 1997). Thus, it would be expected that if calcium were inhibiting the channels directly, then the intracellular calcium increase caused by AChR channel opening would result in a decrease in the amplitude of the currents produced during the second pulse with nicotine. Direct inhibition of an ion channel, (the potassium M channel), by intracellular calcium has been observed (Selyanko and Brown, 1996). However, this response occurred within seconds. In contrast, the inhibition of I_f by PACAP requires at least two minutes to occur and can last up to 10 minutes (experimental observations).

An inhibition of AChRs by VIP is thought to occur via a membrane-delimited pathway (Cuevas and Adams, 1996) hypothesized to involve the βγ subunits released on activation of a G-protein. Not only does this response occur much quicker (within 30 milliseconds) than the inhibition of I_f observed in our experiments ($\geq$2 minutes), but 100μM ACh was used as the agonist. This concentration of ACh would be unlikely to activate the αBgt-sensitive class of receptors (Zhang et al.,1994), the class of receptors thought to be inhibited by PACAP in our study. Moreover, that inhibitors of PLC and the IP3-dependent release of calcium prevented the ability of PACAP to decrease I_f, provides further evidence against a direct interaction of βγ with the channel. Likewise, such arguments also tend to rule out a direct block of the channel by peptide. However, whether the PACAP receptor activates dual second messenger pathways via stimulation

of one G-protein, where the α subunit stimulates one cascade and the βγ subunits stimulate the other (Birnbaumer, 1992) or via two different G-proteins, is still a subject of some debate.

A calcium-dependent inhibition of the fast component of the nicotine-induced current has recently been demonstrated (Vijayaraghavan et al.,1995). In this study, application of nicotine to ciliary ganglion neurons caused release of ^{3}H-Arachidonic Acid (AA) into the medium. The release was calcium dependent as it also occurred on incubation of neurons in medium high in potassium (25mM KCl) which stimulates the activation of voltage-dependent calcium channels, and was completely abolished when neurons were incubated in medium containing zero calcium. Additionally, application of low concentrations of AA inhibited the current evoked by subsequent application of nicotine. Of considerable interest, the inhibition of the AChRs by low concentrations of AA was selective for the rapidly desensitizing, α7-containing, αBgt-sensitive receptor class (α7-AChRs), 20-fold higher concentrations being required to inhibit the α3-containing, αBgt-insensitive class (α3*-AChRs). This was a direct effect of AA as other fatty acids, particularly linolenic acid which most closely resembles AA, were also effective. Other ligand-gated ion channels such as the NMDA receptor (Dumuis et al., 1988; Miller et al., 1992) have been demonstrated to activate the AA cascade, inducing AA release, which then had subsequent effects on an ion channel. Since the enzyme that would stimulate AA formation, phospholipase A2 (PLA2), requires an increase in intracellular calcium (Mayer and Marshall, 1993) it is possible that the calcium increase induced by PACAP could activate PLA2 and stimulate release of AA. In this regard, a recent study (Stella and Magistretti, 1996) indicated that PACAP could potentiate the

release of AA induced by glutamate from mouse cortical neurons. Other examples of peptide hormones inducing AA synthesis and release, with subsequent modulation of an ion channel by the released AA, have been described in the literature. For example, in cardiac myocytes, endothelin has been shown to stimulate AA synthesis via activation of PLC. The released AA then leads to inhibition of a cardiac potassium channel (Damron et al., 1993). While, it is possible that PACAP could activate PLA2 directly, we believe that this scenario is unlikely since inhibitors of PLC and IP3-dependent calcium release prevented the decrease of I_M by PACAP. Further studies will be required to clarify this issue.

The results of several studies have pointed to the idea that populations of functional and non-functional AChRs coexist on the membranes of ciliary ganglion neurons (Margiotta et al., 1987) and that neurons can shift between the two populations via phosphorylation and dephosphorylation of the receptors themselves. In this case, it is believed that the phosphorylated form of receptor is the active species. That cells maintain such a balance is not a novel idea. It has long been recognized that blood glucose concentrations are regulated by the delicate balance between phosphorylation and dephosphorylation of particular hepatic enzymes (Stryer, 1988). Furthermore, regulation of ion channels by phosphorylation and dephosphorylation reactions has also been demonstrated (Pederzani et al., 1998; Fischer et al., 1998). Previous studies on ciliary neurons (Margiotta et al., 1987; Gurantz et al., 1994) have concluded that elevations of cAMP in these neurons increase the sensitivity of the neurons to subsequent ACh exposure, and this increased sensitivity is due to an increase in the number of functional receptors, possibly a result of a phosphorylation of the receptor itself. These results are

consistent with later reports showing that these neurons maintain receptor numbers in much larger quantities than their mRNA transcripts. Furthermore, conditions, which change the receptor numbers, do not change the levels of mRNA, suggesting that these neurons regulate their receptors through post-translational modifications (Corriveau and Berg, 1994). In this regard, the catalytic subunit of protein kinase A, but not that of the calcium-dependent protein kinase C nor the calcium calmodulin-dependent protein kinase II, has recently been demonstrated to phosphorylate the α7 subunit of both rat and chick on a conserved serine residue (Moss et al., 1996). Another report demonstrates that the α3 subunit, a subunit contained in the other major class of AChRs present on these neurons (α3*-AChRs), can also be phosphorylated by the catalytic subunit of PKA and that this subunit is phosphorylated when incubated with the cAMP analog, 8-Br-cAMP (Vijayaraghavan et al.,1990). It is true that there are basal levels of cAMP and therefore basal activities of PKA. Hence, as has been observed for voltage-activated sodium channels (Li et al., 1993), it is expected that there would be a basal level of phosphorylated and thus functional, channels. This balance is shifted when cAMP levels become elevated and PKA activity increases (Margiotta et al., 1987).

Modulation of ion channels through the phosphorylation by protein kinases has long been realized as an important component in their function (Swope et al., 1992). However, the role of protein phosphatases has been less well studied. If there exists a balance between functional and non-functional AChRs on ciliary ganglion neurons and phosphorylation can positively disrupt that balance then it is not unreasonable to hypothesize that a dephosphorylation can negatively disrupt the balance. A similar conclusion was reached by the authors of a study demonstrating the effects of addition of

phosphatase and kinase inhibitors on a calcium-activated potassium current in pyramidal neurons (Pederzani et al., 1998). In another study, the calcium-dependent protein phosphatase 2B (PP2B), also known as calcineurin, was demonstrated to be responsible for tempering of CFTR activity. In the presence of a PP2B inhibitor, detamethrin and forskolin, a direct activator of AC, CFTR activity increased 6-fold over the activity of those channels not exposed to detamethrin (Fischer et al., 1998). In our experiments, when neurons were incubated with PACAP in the presence of ddA, a decrease in the fast component of the nicotine-induced current was observed (Figure 4.1). Since the PACAP type I receptor can activate a PLC-dependent pathway, we hypothesized that the downstream effects of the second pathway were unmasked while the AC pathway was suppressed by ddA. As an increase in intracellular calcium is required for the observed decrease in I_f, it is possible that the calcium rise induced by PACAP could activate a PP2B-like calcium-dependent protein phosphatase. In this way, receptors would become dephosphorylated, consequently decreasing the proportion of functional receptors present on the cell membrane. This would then be observed as a decrease in the whole-cell current since there would now be a decrease in the number of receptors carrying ions through the membrane. Activation of PP2B through a PLC-IP3-dependent increase in $[Ca^{2+}]_i$ is thought to result in a decrease of potassium current in Guard Cells (Assmann and Wu, 1994). Whether it is via an activation of a PP2B-like protein phosphatase, stimulation of the release of AA or both, or whether an entirely different pathway is the mechanism by which an increase in $[Ca^{2+}]_i$ would cause a decrease in the current produced by nicotine stimulation of α7-AChRs, remains to be seen. Further studies will be required to ferret out the actual underlying mechanism(s) involved.

It is unlikely that a molecule similar to ddA exists *in vivo*; therefore, one has to consider how activation of a dual signaling pathway by a neuropeptide hormone might affect the actual cell. In this regard, it has been observed that neuropeptides often act as fine tuners of fast synaptic signaling (Alberts, 1994) and that cholinergic terminals will increase their release of neuropeptides under high frequency stimulation (Agoston et al., 1988) and chronic depolarization (Brandenburg et al., 1997). Therefore, it is reasonable to postulate that PACAP may act as a neuromodulator and that the amount of PACAP released during synaptic signaling may vary depending on the frequency of stimulation of the presynaptic terminal. Furthermore, in preliminary experiments that have confirmed and extended our previous findings (Margiotta and Pardi, 1995), incubation of neurons with PACAP in the absence of ddA resulted in a 2-fold increase in the amplitude of the fast and slow components of the nicotine-induced current (data not shown). That PACAP would result in an increase in the amplitude of the current produced by α7-AChRs was not completely unexpected since incubations with 8-Bromo-cAMP results in increased receptor responsiveness in cells stably transfected with the rat α7 cDNA (Quik et al., 1997).

Thus, within this hypothetical framework, it would be expected that under relatively low frequency stimulation, low levels of PACAP would be released. This would lead to elevations in both cAMP and calcium in the postsynaptic neuron. However, it has been observed that the PACAP receptor couples less well to the PLC pathway (Delporte et al., 1993; Christophe, 1993; our results). This suggests that at low concentrations of PACAP, the cAMP pathway would predominate, leading to an increase in sensitivity of the postsynaptic neurons to further stimulation by nicotine. Under these

conditions, however, some AA would be released or some receptors would become dephosphorylated. Since α7-AChRs are far more plentiful on these neurons (~90% of the AChR population) a small decrease in the proportion of functional receptors of this class may not be obvious. As the frequency of impulses increases, the concentration of PACAP in the synaptic cleft would increase, and therefore, the effects of maximally elevated IP and intracellular calcium levels, such as an increase in [AA] or dephosphorylated receptors, would become increasingly evident. In this regard, one of the best known substrates for PP2B is inhibitor 1, which becomes inactivated on dephosphorylation. Inhibitor 1 prevents the activity of protein phosphatase 1, which dephosphorylates proteins phosphorylated by PKA (Yakel, 1997). It is in this way that an increase in intracellular calcium can directly antagonize elevations in cAMP (Kurosawa, 1994). Therefore, PACAP, in essence, would act to temper its own effects on agonist sensitivity of the neurons, a result of activation of a PP2B also observed for CFTR channel activity (Fischer et al., 1998). At the beginning of synaptic activity, PACAP would act to increase the proportion of AChRs that are phosphorylated (and therefore active), thus increasing the sensitivity of the postsynaptic neuron to agonist. However, as activity at the synapse increases and maximal IP and $[Ca^{2+}]_i$ levels are attained, PACAP would then result in a decrease in the rate of phosphorylation of the receptors (or increase the proportion inhibited by AA) thereby preventing further increases in agonist sensitivity, akin to a negative feedback loop.

A ddA-like increase in the contribution of the second messengers formed on activation of PLC could also occur as a result of 'coincidence detection'. Coincidence detection most often refers to the interaction of the second messenger pathways activated

by two different G-protein coupled receptors on the same cell (Anholt, 1994). In addition to type I PACAP receptors, ciliary ganglion neurons also possess type 3 muscarinic AChRs (m_3AChR). These receptors are coupled to PLC and have been demonstrated to result in elevations in IPs and $[Ca^{2+}]_i$ in ciliary ganglion neurons (Rathouz et al., 1995). On superior cervical ganglion neurons, elevations in IP levels due to activation of mAChRs and vasopressin receptors are additive. This is thought to result from the selective localization of the two receptors to different sites on the neuron and thus the use of two different pools of phospholipid substrates (Horwitz et al., 1986). Thus, it is possible that the simultaneous activation of PACAP type I receptors and m_3AChRs on ciliary ganglion neurons might also result in additive effects on IP levels and subsequent increases in $[Ca^{2+}]_i$. Interestingly, muscarine has been demonstrated to promote synaptic fatigue during high frequency synaptic signaling in the ciliary ganglion (Bowers, 1992). Another example of coincidence detection is observed for the interaction of the thyrotropin receptor and an isoform of the adenosine receptor that is coupled to G_i. In this case, similar to the type I PACAP receptor, the thyrotropin receptor activates both AC and PLC pathways simultaneously. However, when adenosine activates its receptor, the activation of AC by the thyrotropin receptor is antagonized, therefore leading to an amplification of the downstream effects of PLC-dependent cascade activated by the thyrotropin receptor (Tomura et al., 1997; reviewed in Bygrave and Roberts, 1995). While ciliary ganglion neurons do not express adenosine receptors, two other peptides that have been localized to the terminals ending on the neurons, substance P and enkephalin, may have roles in modulating synaptic signaling there (Reiner, 1987). Whether there are receptors that recognize these peptides and to which intracellular

signaling pathways they couple is unknown at present. Characterization of the receptors for these peptides and studies of the intracellular signaling cascades to which they couple has been described in other cell systems. Interestingly, the μ-opiod receptor which binds enkephalin appears to be coupled to G_i (Chieng and Christie, 1994; Meneray et al., 1998) and if expressed on ciliary ganglion neurons and simultaneously activated with PACAP type I receptors, could have similar consequences on PACAP signaling as observed for adenosine and the thyrotropin receptor.

While we have demonstrated a decrease of I_f, I_s was not decreased when neurons were incubated with PACAP in the presence of ddA. However, when neurons are incubated with PACAP in the absence of ddA, the amplitudes of both the fast and slow components of the nicotinic current are increased. Thus there is a differential effect of PACAP on the two classes of AChR present on ciliary ganglion neurons. Being that high [AA] were required to inhibit α3*-AChRs while low concentrations were required to inhibit α7-AChRs (Vijayaraghavan et al.,1995), a PACAP-dependent release of AA would be expected to result in a selective decrease of α7-AChR current.

The mechanism by which one receptor class could be dephosphorylated while the other is not, is less obvious. One study suggests that the protein phosphatase PP2B requires a particular sub-cellular location or environment in order to be active (Fischer et al., 1998). Interestingly, the two classes of AChRs have been shown to have different distributions on the cell surface with α3-containing receptors associated with postsynaptic membranes and the α7-containing receptors associated with extrasynaptic membranes (Jacob and Berg, 1982). Hence, it is possible that a PP2B-like protein

phosphatase may reside in the vicinity of the α7-containing receptors and, therefore, have more effects on this class of AChRs.

Thus, the activation of dual signaling cascades by PACAP would serve to fine-tune synaptic transmission within the ganglion-increasing agonist sensitivity of the postsynaptic neuron by way of both the α3*-AChRs and the α7-AChRs during early stages of synaptic activity and then dampening the response of the α7-AChRs at later stages. Since α7-AChRs are far more plentiful and have the highest calcium permeability of any ligand-gated ion channel (McGehee and Role, 1995), and given that intracellular calcium in high levels is known to be cytotoxic (Choi, 1987), a selective inhibition of these receptors at times of high synaptic activity, would be advantageous.

Additionally, in the hippocampus, where both presynaptic and postsynaptic "plastic changes" are thought to be important in memory formation (Kupferman, 1991), αBgt-sensitive AChRs are believed to be present on presynaptic terminals. Moreover, an αBgt-sensitive AChR has recently been implicated in a pre-synaptic increase in glutamate release at a central relay synapse (McGehee and Role, 1995). Glutamate is the major excitatory neurotransmitter in the central nervous system, and its action at the NMDA receptor has been shown to be required for the formation of long term potentiation (LTP) and long term depression (LTD) in the hippocampus, phenomena associated with memory and learning (Larkman and Jack, 1995). PACAP has also been linked to processes involved in memory formation and maintenance (Kandel and Abel, 1995) and has been demonstrated to potentiate transmission at the perforant path-granule cell synapse in the dentate gyrus of the hippocampus (Kondo et al., 1997). Thus, the modulation of the αBgt-sensitive, α7-AChRs by PACAP may represent an important

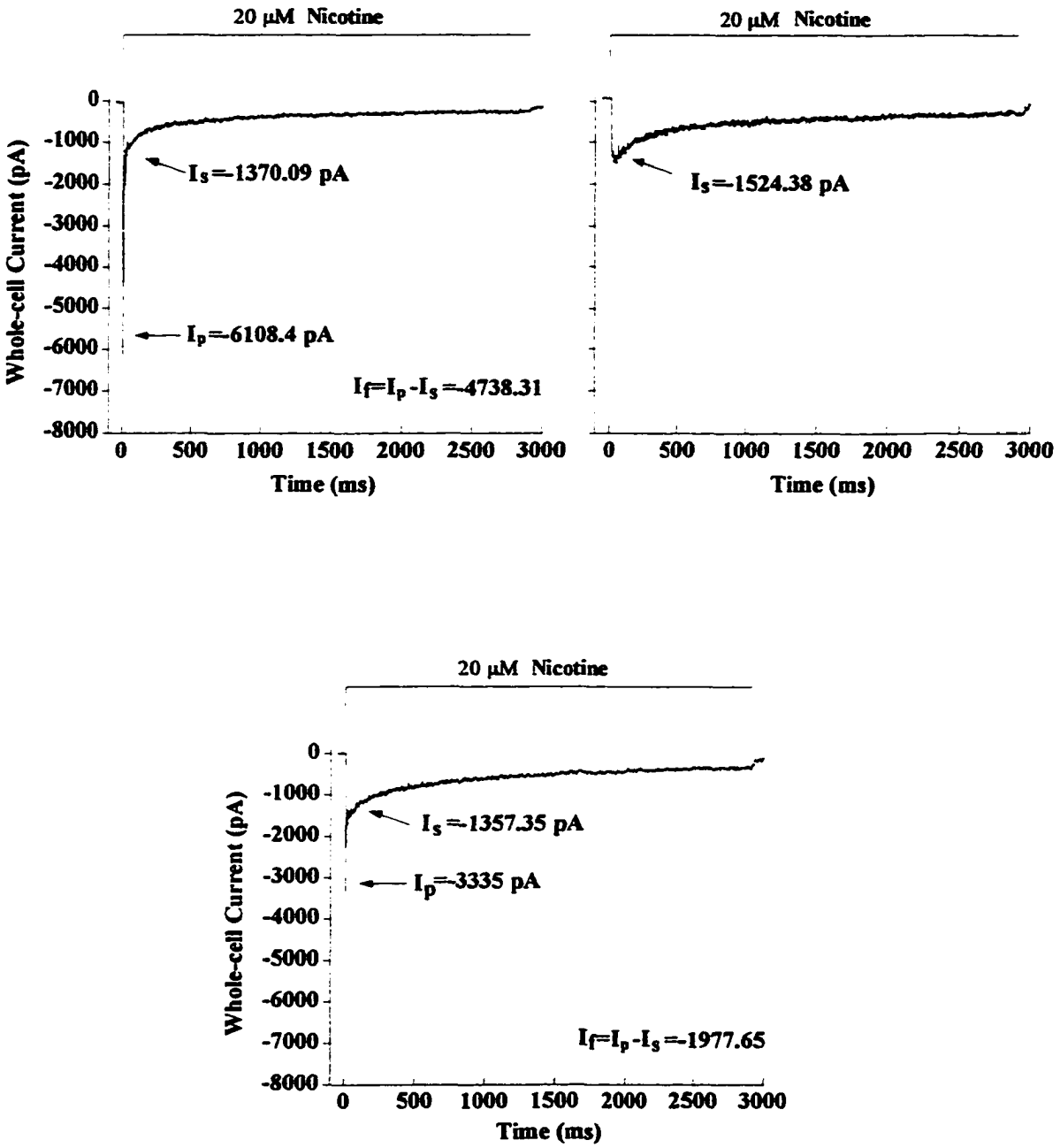
cooperation between ACh and PACAP in synaptic plasticity as well as in other cellular processes.

As stated above, uncovering the exact nature of the mechanism involved in the decrease of the fast component of the current induced by fast perfusion with nicotine in the presence of the adenylate cyclase inhibitor, ddA will require further exploration. The importance of studies designed to uncover the differential effects of compounds on the different classes of AChR cannot be overestimated as they would aid in the formulation of new subunit-specific and disease-specific drug therapies such as those utilized in the treatment of Alzheimers and Parkinson's diseases (Lloyd et al., 1998).

Figure 4.1 PACAP selectively inhibits α7-AChR currents induced by fast perfusion with nicotine when AC activity is suppressed. A, Representative whole-cell current records are displayed from separate E13, 14 ciliary ganglion neurons held at –70mV and exposed to 20μM nicotine for 3 sec, as indicated by the top bar. Values corresponding to the current amplitude of the peak (I_p), slow (I_s) and fast (I_f) current components were determined as described in Methods and are shown for each trace. As depicted in the figures, the value of I_f was obtained by subtraction of the current amplitude of the slow component (I_s) from the amplitude of the peak (I_p) current for each trace. All neurons were pretreated with 200μM ddA for 1-8 minutes to block AC as described in Methods. Top, left: Control nicotine response. Top, right: Nicotine response following preincubation with 60nM αBgt for 2h preceding the ddA treatment. Bottom: Nicotine response following treatment with 100nM PACAP38 for 6 min. B, Summary of the percent decrease (mean ± s.e.m.) in α7-AChR and α3*-AChR nicotine responses for different peptides. Each bar is based on responses like those in A where the individual current components (pA) are determined as described in Methods and normalized to cell size (estimated by measuring membrane capacitance, i.e. pA/pF) for each peptide versus controls treated with ddA alone. Bars representing very small decreases for both α7 and α3 in the presence of 100nM VIP and α3 in the presence of 1μM VIP are not real and are presented here for descriptive purposes only.

Figure 4.1

A



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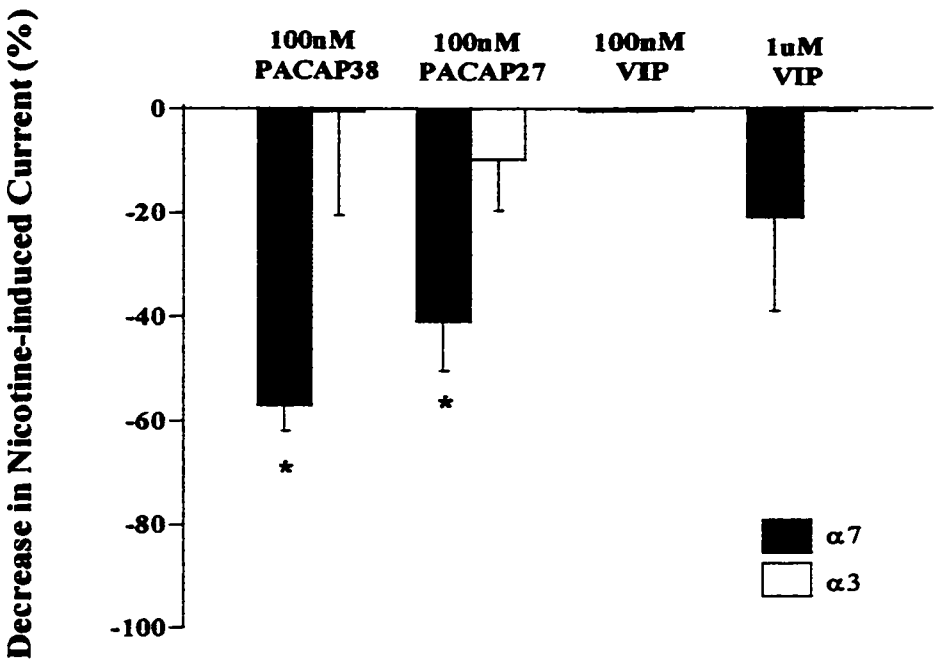
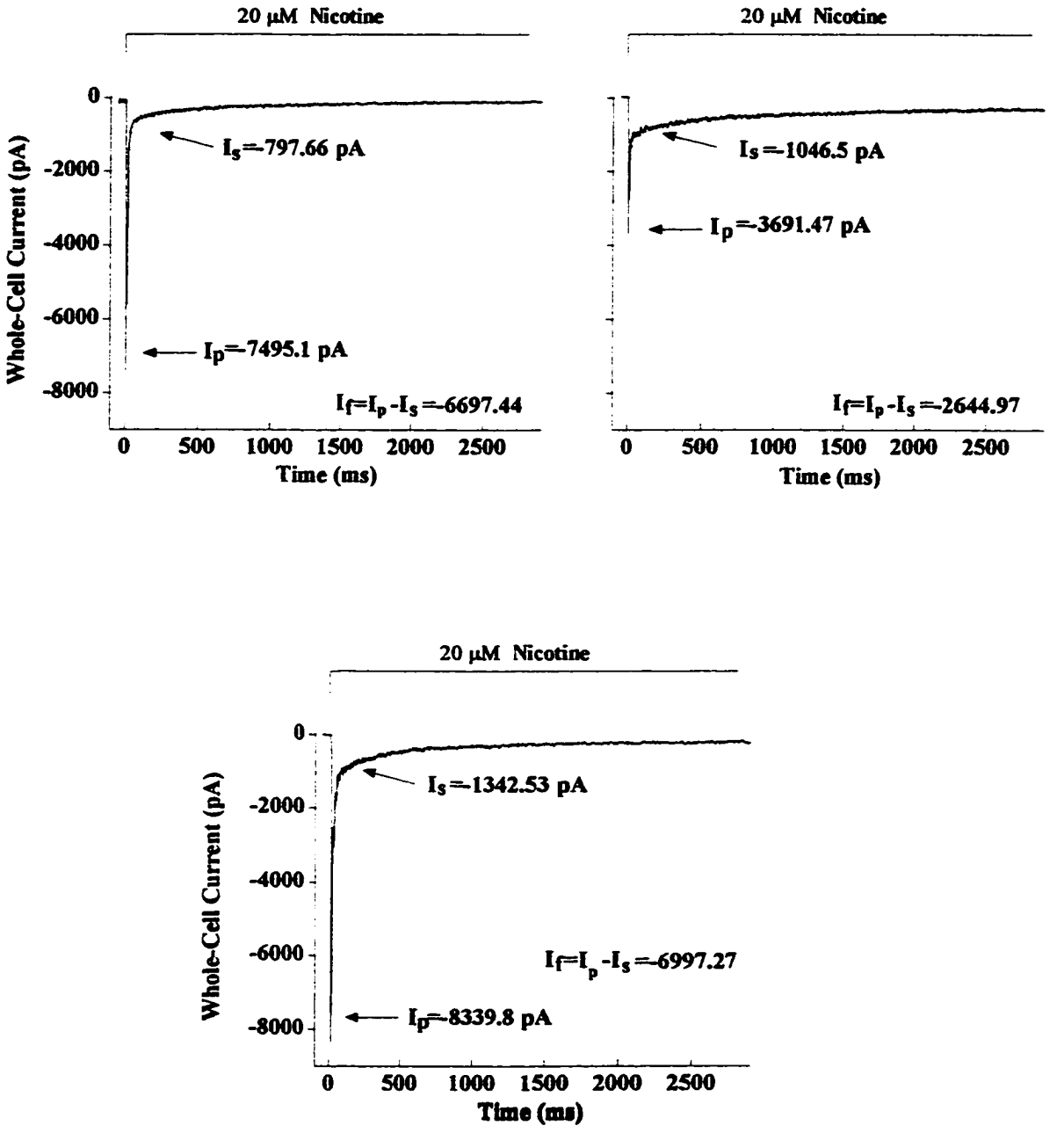


Figure 4.2 The decrease in I_f by PACAP requires intracellular calcium. A, Effects of PACAP plus 10mM BAPTA on the current induced by fast perfusion with nicotine. Representative whole-cell current records are displayed from separate E13, 14 ciliary ganglion neurons held at –70mV and exposed to 20μM nicotine for 3 sec, as indicated by the top bar. Values corresponding to the current amplitude of the peak (I_p), slow (I_s) and fast (I_f) current components were determined as described in Methods and are shown for each trace. As depicted in the figures, the value of I_f was obtained by subtraction of the current amplitude of the slow component (I_s) from the amplitude of the peak (I_p) current for each trace. All neurons were pretreated with 200μM ddA for 1-8 minutes to block AC as described in Methods. Top, left: Control nicotine response. Top, right: Nicotine response following treatment with 100nM PACAP38 for 6 min. Bottom: Nicotine response following introduction of 10mM BAPTA into the recording pipette with subsequent exposure to 100nM PACAP38. The neuron was pre-exposed to BAPTA for 1-2 min prior to peptide challenge as described in Methods. B, Summary of effects of 10mM BAPTA on the current produced by activation of α7-AChRs by 20μM nicotine. Bars represent the average size normalized current amplitudes (pA/pF) of the fast component (I_f, where $I_f = I_p - I_s$) of the nicotine-induced currents obtained from control, PACAP-exposed and BAPTA + PACAP-exposed neurons. 10mM BAPTA alone, had no effect on nicotine induced currents. Each measurement represents the mean ± standard error of the normalized currents (pA/pF) from six, four and six neurons, respectively. Significant differences ($p<0.05$) are represented by a star above the bars in the figure. All neurons were pretreated with 200μM ddA for 1-8 min.

Figure 4.2

A



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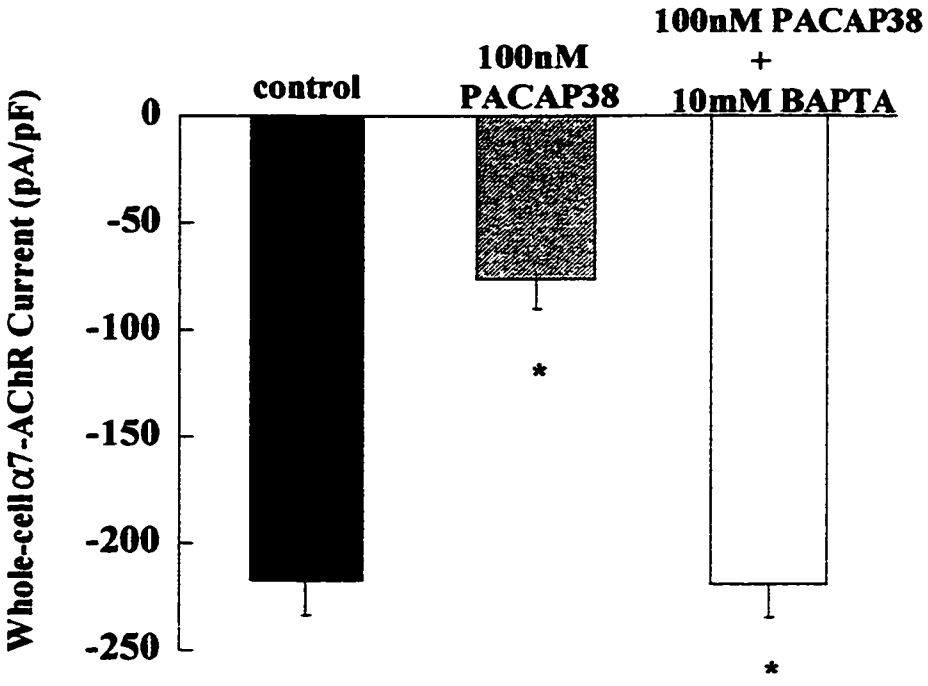


Figure 4.3 PACAP receptors on ciliary ganglion neurons can stimulate IP production. The concentration-response relationships for PACAP38 (filled circles), PACAP27 (empty circles), VIP (100nM,empty diamond; 1μM VIP, cross) and Glucagon (hatched box) are depicted. Dissociated neurons were incubated for 50-60 min at 37 deg C with the indicated concentration of peptide following overnight incubation with 2μCi/ml of ^{3}H-myo-inositol. IP production was calculated as the percentage increase over levels obtained from separate neurons from the same experiment not exposed to peptide. Assays were performed in duplicate or quadruplicate and the average percentages were plotted. Results of a single duplicate-assay experiment are shown for PACAP38 and PACAP27. The average percentages of two experiments are shown for 1μM VIP and the relevant value for the mean increase ± standard error is 19 ± 19%. Neither 100nM VIP nor 1μM Glucagon resulted in any significant increase in IP levels. EC50 values were determined from composite dose-response curves and results are presented in the text.

Figure 4.3

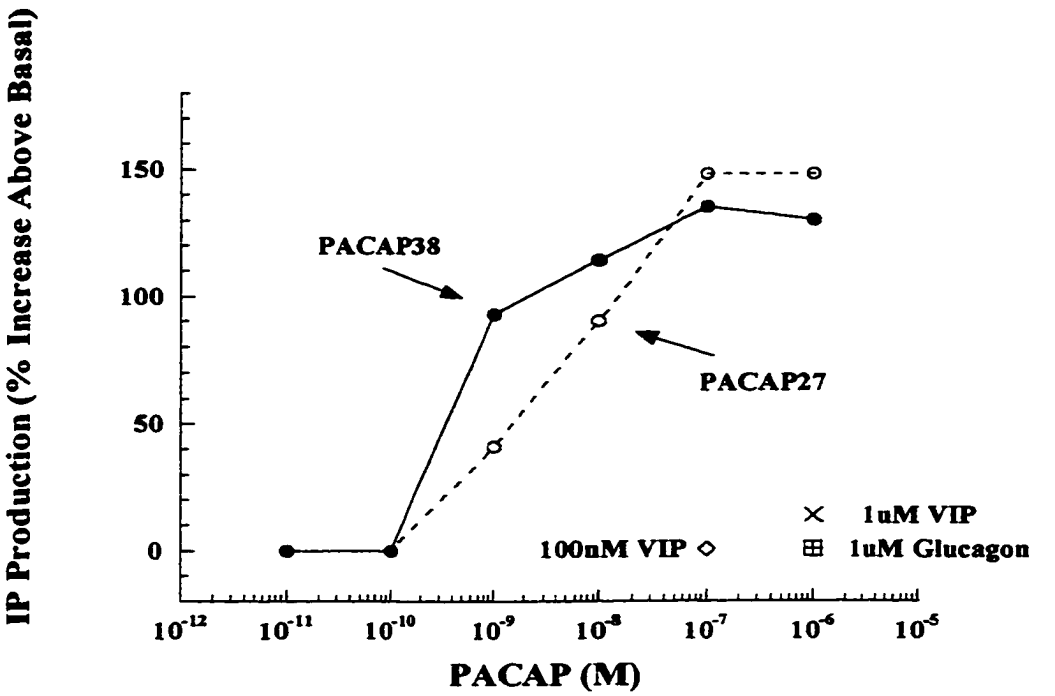


Figure 4.4 The PACAP receptor present on ciliary ganglion neurons increase IP levels via a pathway involving PLC but not AC. Following overnight incubation with 2μCi/ml of ^{3}H-myo-inositol, dissociated neurons were incubated for 20 minutes with 200μM ddA or 1μM U73122 prior to exposure to 1μM PACAP38 for 50-60 min. Assays were performed in duplicate or quadruplicate. The average percent increase (mean ± s.e.m.) of IP levels of neurons treated with ddA or U73122 and PACAP relative to the average IP increase observed from neurons exposed to 1μM PACAP38 alone, are presented. Percent increases in IP levels were determined as described in Methods. The average of four quadruplicate-well assays are presented for ddA and of two duplicate-well and two quadruplicate-well assays for U73122. Significant differences ($p<0.05$) are represented by a star above the bars in the figure.

Figure 4.4

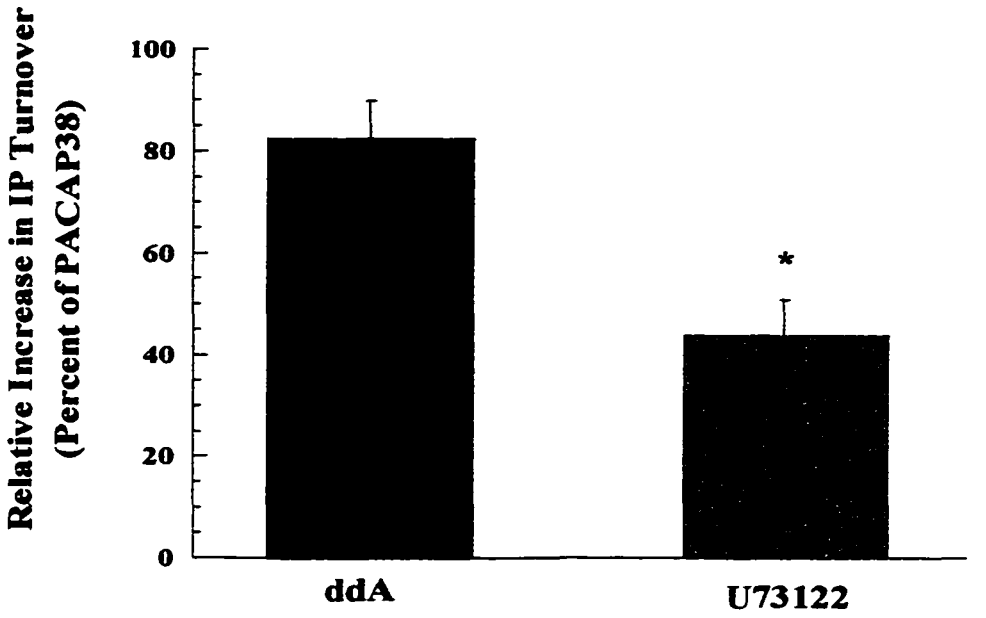
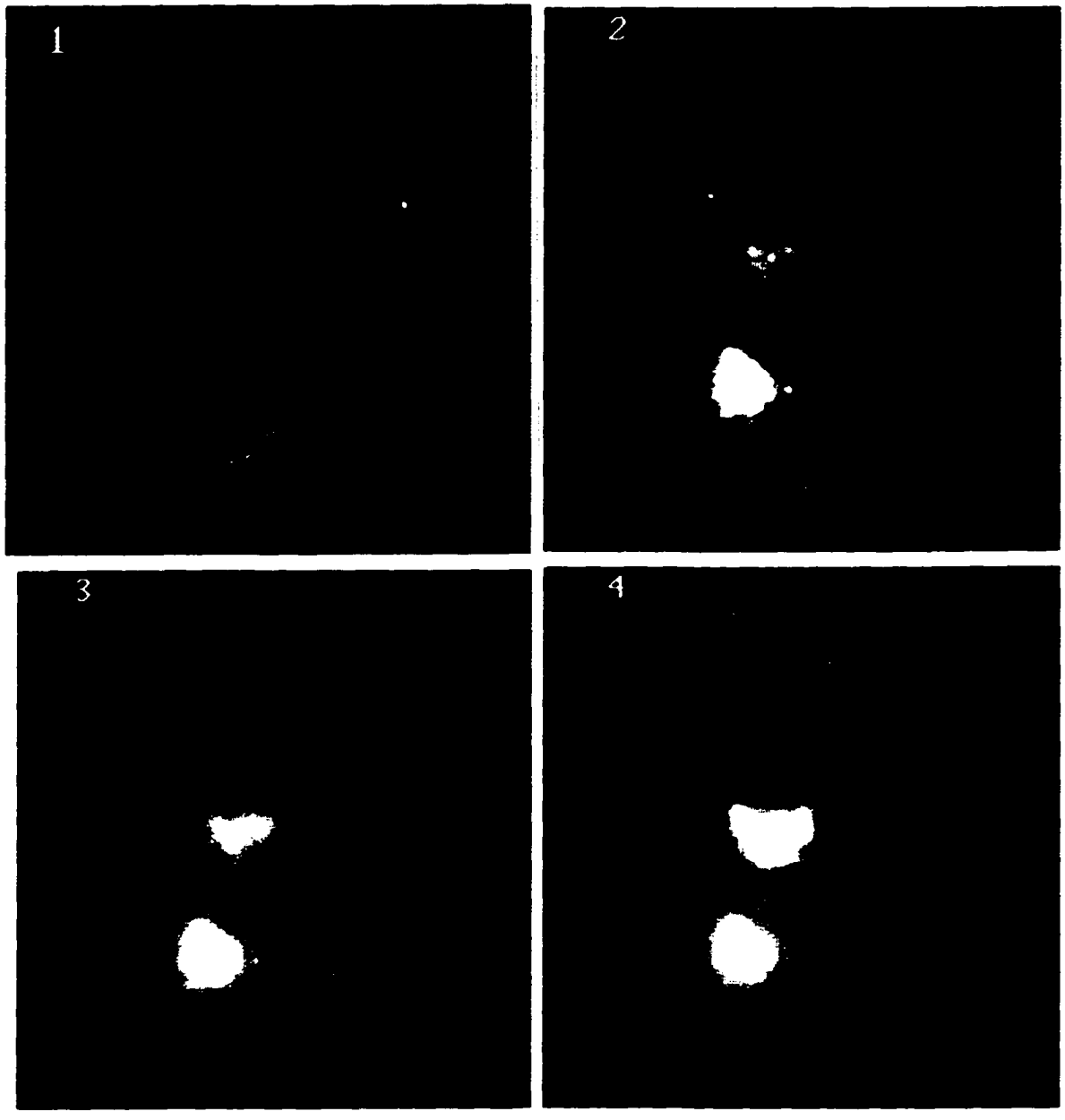


Figure 4.5 PACAP increase $[Ca^{2+}]_i$ in ciliary ganglion neurons. A, Fluorescence images of ciliary ganglion neurons exposed to 100nM PACAP38 for 0 (Panel 2), 50 (Panel 3) and 90 (Panel 4) sec. Panel 1: Phase contrast photograph of the same three neurons shown in Panels 2, 3 and 4, loaded with 2μM Fluo-3AM just prior to exposure to fluorescent light and peptide. A high resistance seal was formed on the center cell with a glass micropipette filled with internal solution and 200μM ddA and access to the whole cell was attained, as described in the text. Fluorescence responses from the same three neurons just prior to (Panel 2), and following a 50 sec (Panel 3) and a 90 sec (Panel 4) exposure to 100nM PACAP38 dissolved in recording solution. B, Fluorescence images from two different neurons exposed to 100nM PACAP38 dissolved in medium without calcium. Panel 1: Fluorescence responses of the two neurons just prior to challenge with PACAP in zero calcium. Panel 2: Fluorescence responses of the same neurons after a 90-sec exposure to 100nM PACAP38 in zero calcium. All neurons were loaded for 45 minutes in the dark with Fluo-3AM and maintained in the dark until use in experiments.

Figure 4.5

A



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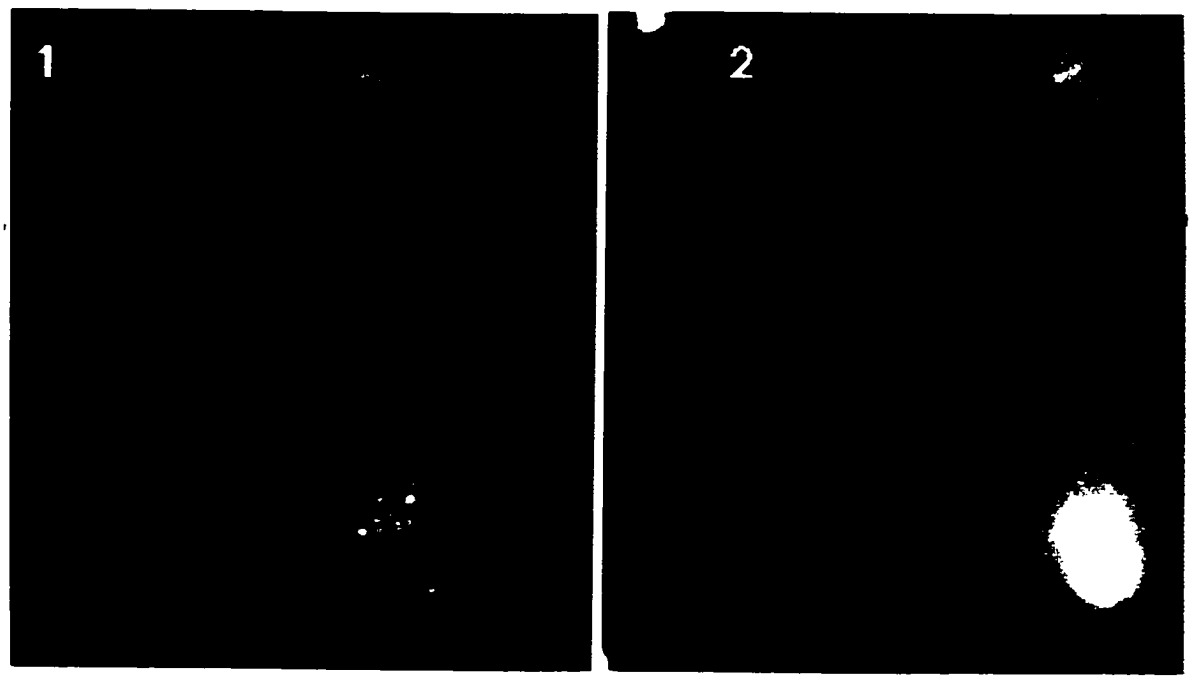


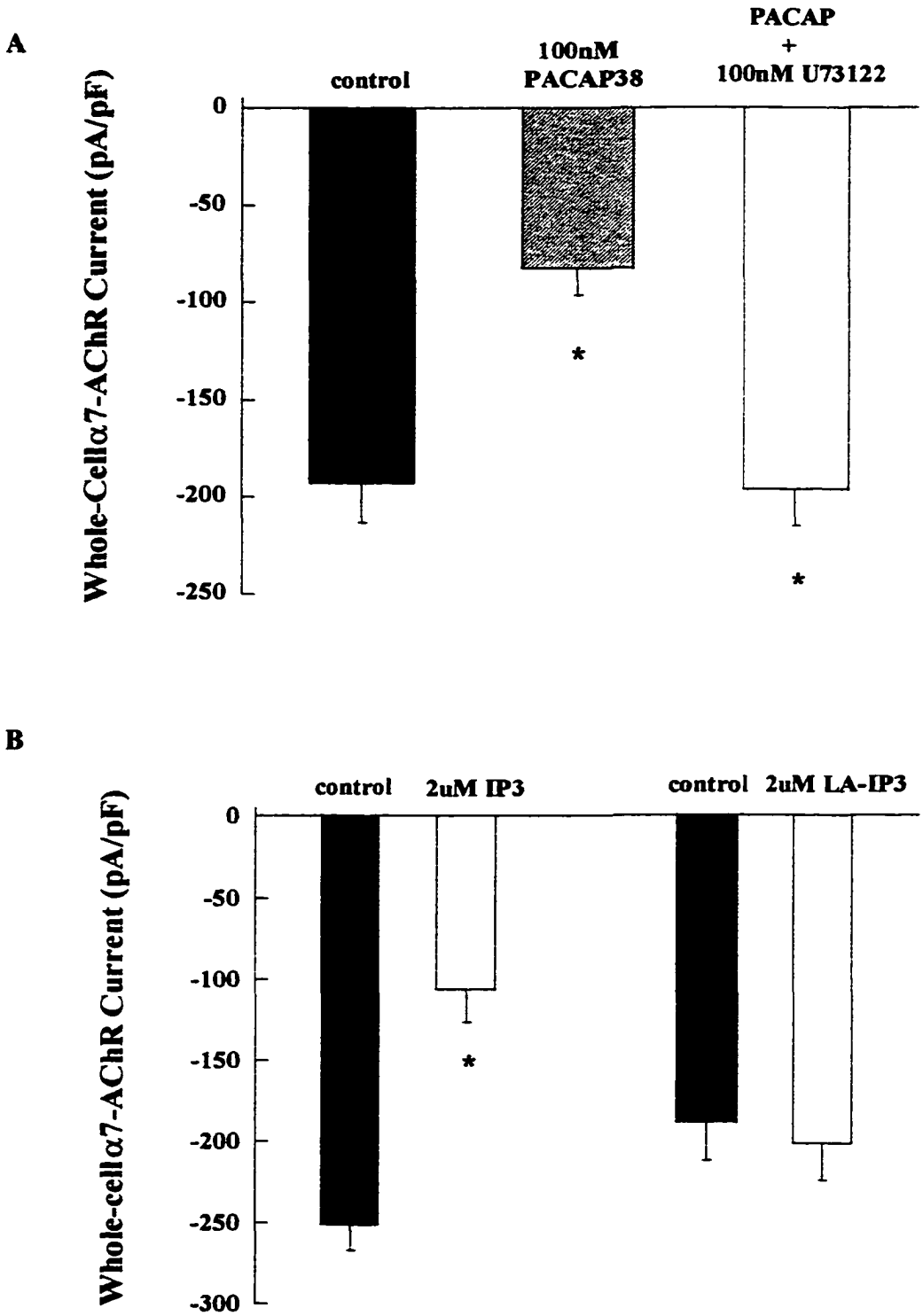
Table 4.1 Stimulation of intracellular calcium by PACAP under various intracellular conditions. Data are presented as the percent of individual neurons (n) of those exposed to intracellular solution alone ((-)ddA), 200μM ddA ((+)ddA), 10mM BAPTA, 100nM U73122, 300μM Heparin or 10μM Ruthenium Red (RR) that responded to 100nM PACAP38 with an increase in fluorescence intensity (see methods). n values were predetermined for the experiments involving BAPTA, U73122, and Heparin and were determined as that number required to show a significant difference ($p<0.05$) in the percent of neurons responding compared to 85% (the percent of 67 neurons responding to 100nM PACAP38 with or without ddA). Since there was no significant difference in the percent increase in fluorescence intensity regardless of the presence of ddA ($62 \pm 8.9\%$, (-)ddA vs. $81.75 \pm 18.9\%$, (+)ddA), the percent of neurons responding in the two conditions were pooled and the average used to represent control responses.

Table 4.1

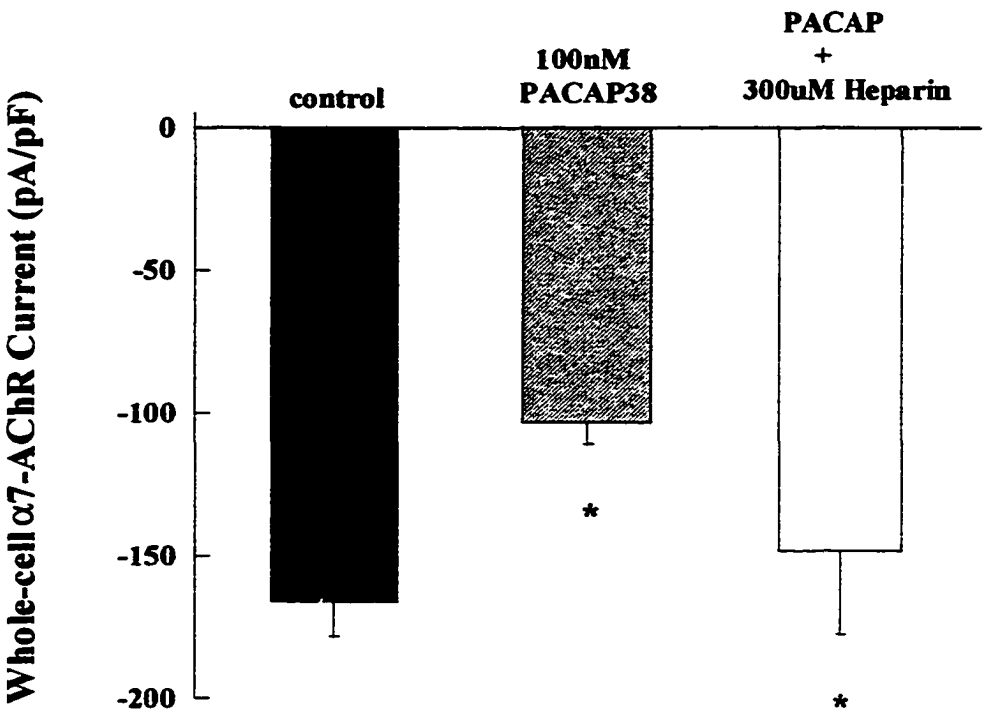
	% of cells responding to 100nM PACAP38	n
+/- ddA	85	67
10mM BAPTA	29	7
100nM U73122	25	8
300μM Heparin	11	9
10μM Ruthenium Red	86	7

Figure 4.6 The decrease in I_f by PACAP involves an IP3-dependent release of calcium from internal stores. Summary of effects of different compounds on the whole-cell current component produced by activation of α7-AChRs evoked by rapid application of 20μM nicotine to ciliary ganglion neurons also exposed to either 100nM PACAP38 (A and C) or not (B). In all cases, control neurons are those neurons from each respective experiment that were exposed only to ddA. All neurons were exposed to 200μM ddA for 1-8 min prior to application of nicotine for 3 sec. Bars represent the average ± standard error of current amplitudes of the fast component (I_f, where $I_f = I_p - I_s$) of the nicotinic response normalized to cell size (pA/pF). A and C, Normalized current amplitudes of I_f from neurons exposed to 100nM U73122 or 300μM Heparin for 1-2 min prior to peptide challenge, compared to those exposed only to peptide or not. B, Normalized current amplitudes of I_f from neurons exposed to 2μM of the IP3 analog, 3-deoxy-IP3 (IP3) or 2μM of the IP3 analog with low affinity (LA-IP3) for the IP3 receptor, 2,3,6-trideoxy-IP3 compared to neurons not exposed to these compounds. Significant differences ($p<0.05$) are indicated by a star over the bars in the figure.

Figure 4.6



C



CHAPTER 5:

Summary and Conclusions

Effects of PACAP on neuronal AChRs

PACAP binds to a family of G-protein coupled receptors that include those that are coupled to either activation of AC alone (type II PACAP receptor) or AC and phosholipase C (type I and type III PACAP receptor) (Spengler et al., 1993; Cai et al., 1997). Utilizing competition assays we detected the presence of the type I PACAP receptor on ciliary ganglion neurons and determined that incubation with low levels of PACAP can lead to elevations in cAMP and that this occurred in a concentration-dependent fashion. Furthermore, we demonstrated that activation of the type I PACAP receptor can enhance the current induced by puff-application of acetylcholine (Margiotta and Pardi, 1995). This phenomenon is described as an increased sensitivity for agonist and is thought to be associated with elevated cAMP levels (Margiotta et al., 1987; Gurantz et al., 1994). Interestingly, incubation of the neurons with PACAP along with the adenosine analog and adenylate cyclase inhibitor (Johnson et al., 1989) ddA, not only abolished the enhancement but instead reversed the effect and resulted in a ~50% decrease in sensitivity to agonist, while ddA alone had no significant effects. More specifically, a recent study described a calcium-dependent inhibition of the αBgt-sensitive, α7-containing class of AChRs (Vijayaraghavan et al.,1995). These receptors are one of the two classes of AChRs ascertained to be present on these neurons and whose activation has been shown to account for the rapidly activating and decaying current (fast component) seen on agonist application (Zhang et al.,1994; Conroy and Berg, 1995). Being that the type I PACAP receptor is known to couple to a second signaling pathway involving PLC, we determined whether incubation with PACAP would lead to elevations in inositol phosphates (IPs) and intracellular calcium. Indeed,

PACAP increased both IP turnover and intracellular calcium and the PLC inhibitor U73122 could block both increases. Moreover, we report a PACAP-dependent selective decrease in the fast component of the agonist-induced current and that this decrease is mimicked by a potent IP3 analog, prevented by the PLC inhibitor U73122 and by heparin, an antagonist of IP3 at its receptor on intracellular calcium stores. Thus, we conclude that activation of the type I PACAP receptor can either increase or decrease the sensitivity of the neurons to agonist depending on which pathway is dominant at the time.

PACAP, AChRs and memory

Second messenger systems elaborated by the action of peptides binding to their respective receptors have been known to have a role in memory formation since 1976 when Dudai and colleagues identified the first of several *Drosophila* mutants that exhibited defects in learning and memory (reviewed in Kandel and Abel, 1995). One mutant, termed *dunce*, which has a defect in its ability to be classically conditioned, is missing cAMP phosphodiesterase (PDE). Another, termed *rutabaga*, is defective in calcium/calmodulin-dependent adenylate cyclase. The defects in these two mutants suggest an association between memory and cAMP. Many neuropeptides, including PACAP, bind to receptors that couple to a cAMP signaling cascade. Interestingly, another *Drosophila* mutant, *amnesiac*, has recently been identified. This mutant has no difficulty learning but is subsequently unable to retain what it has learned. The defect is in a neuropeptide that is highly homologous to mammalian PACAP (Feany and Quinn, 1995), suggesting a role for PACAP in memory. Furthermore, high levels of type I PACAP receptors have been identified in the hippocampus (Hashimoto et al., 1993), a region of the brain known to be involved in memory formation and which receives

cholinergic afferents (Christophe, 1993), implicating a possible cooperative role of ACh and PACAP in memory. In this regard, experimental work designed to elucidate the role of nicotine in memory and learning have shown increased performance of smokers on associative learning tasks as well as on other tasks involving memory consolidation (Warburton, 1992). Memory formation is thought to involve "plastic changes" in neuronal connections through mechanisms of LTP and LTD (Kupferman, 1991). LTP at the cellular level is best studied in the hippocampus where both postsynaptic and presynaptic changes have been demonstrated. The increased sensitivity of the non-NMDA receptors present on the postsynaptic membranes is an example of a "plastic change". It is thought to occur through a mechanism that includes phosphorylation of the receptor by cAMKII which is activated by calcium influx through the NMDA receptor channel (Schulman, 1995). The phosphorylation of the non-NMDA receptors by cAMKII has been demonstrated to increase the peak current amplitude of these receptors. Presynaptic plastic changes are thought to result from the elaboration of a retrograde messenger from the postsynaptic cell that has actions at the presynaptic terminal (Larkman and Jack, 1995). These actions then lead to an increase in the probability of release of the excitatory neurotransmitter, glutamate. The action of glutamate at the NMDA receptor is necessary for the induction of LTP and LTD (Larkman and Jack, 1995; Kandel, 1991). Interestingly, in the hippocampus of the rat, both LTP and LTD can be altered by PACAP (Kondo et al., 1997). Furthermore, a recent report by McGehee and colleagues (1995) has revealed a role for nicotine and the αBgt-sensitive, α7-containing class of AChRs in the increased probability of release of neurotransmitter at a central glutamatergic and a peripheral cholinergic, synapse. A role for AChRs is

exemplified by other studies demonstrating the ability of ACh to induce the release of dopamine from striatal synaptosomes and serotonin in hypothalamic tissue slices (Morley and Kemp, 1981). More recent studies have demonstrated the nicotine-induced release of other more classic neurotransmitters such as noradrenaline, ACh, glutamate and GABA (Wonnacott, 1997) in the central nervous system.

Nicotine, AChRs, normal and abnormal growth and development

High affinity ACh, nicotine and αBgt binding sites are present throughout the brain of mammals (McGehee and Role, 1995; Role and Berg, 1997). Therefore, aberrations in AChRs may be expected to result in pathological states involving neuronal systems, such as Nocturnal Frontal Lobe Epilepsy (Steinlan et al., 1995), in which a missense mutation in the α4 subunit has been associated with an autosomal dominant form of the disease. In relatives of some schizophrenics, levels of the αBgt binding, α7 subunit mRNAs are decreased, and administration of (-)nicotine can restore normal function in these patients (Adler et al., 1992). Patients with Alzheimer's disease, a neurodegenerative disease involving the loss of cholinergic cell bodies from the Nucleus Basalis of Meynert, are unable to learn as well as to form new or retain old memories (Gallagher and Colombo, 1995).

The importance of AChRs in normal growth and development cannot be underscored. In PC12 cells, the induction of c-*fos* and -*jun*, two immediate early genes, is calcium dependent (Bartel et al., 1989). Both classes of AChRs present on ciliary ganglion neurons are permeable to calcium and can significantly raise intracellular calcium levels both independently and by activation of voltage activated calcium channels secondary to sufficient membrane depolarization (Rathouz and Berg, 1994;

Vijayaraghavan et al.,1992). Moreover, as stated earlier, the αBgt-sensitive class of AChR has been shown to have the largest calcium permeability of any ligand-gated ion channel (McGehee and Role, 1995). Calcium entering through these AChRs plays a role in neurite outgrowth and growth cone morphology in PC12 cells (Chan and Quick, 1993). More recently, nicotine has been demonstrated to protect rat cortical neurons from glutamate-induced cytotoxicity (Kaneko et al., 1997) and to promote the survival of rat motoneurons via activation of an α7-containing AChR (Messi et al., 1997). Another indication that the αBgt-sensitive class of AChR is important for normal cell development is the observation that incubation of neuronal cultures with αBgt prevents cell death (Meriney et al., 1987). This observation is especially significant since calcium is known to be important in the normal developmental attrition of neurons in the ciliary ganglion (Landmesser and Pilar, 1974). Additionally, AChRs may be regulated by their own calcium ion flux since chronic exposure to cholinergic agonists leads to receptor down regulation (Delorme and McGee, 1988).

PACAP is a neurotropic factor

PACAP can also act as a neurotropic factor. In the human neuroblastoma cell line, NB-OK-1, PACAP has been shown to lead to growth arrest followed by morphological differentiation (Hoshino et al., 1993), including the induction of process outgrowth (Deutsch et al., 1993). Process outgrowth induced by PACAP has also been demonstrated in PC12 cells (Deutsch and Sun, 1992). Recently, PACAP has been shown to protect growth factor-deprived dorsal root ganglion neurons from cell death (Lioudyno et al., 1998). Additionally, the gene encoding the human PACAP precursor has been localized to the short arm of chromosome 18, a region associated with hereditary

holoprosencephaly (Christophe, 1993). Moreover, PACAP receptors have been identified on immature cerebellar cells during development (Basille et al., 1993) and PACAP can protect these neurons from apoptosis through activation of the mitogen-activated protein kinase pathway (Villalba and Journot, 1997). Both of these findings implicate PACAP as an important factor in normal neuronal development. Furthermore, the sequence of PACAP has been remarkably conserved throughout evolution, suggesting a crucial physiological role (Schafer et al., 1991). The further study of the interplay between PACAP and the AChR would be invaluable to increasing our understanding of the process of normal and abnormal neuronal growth in health and disease as well as in increasing our understanding of the role of both in memory and learning.

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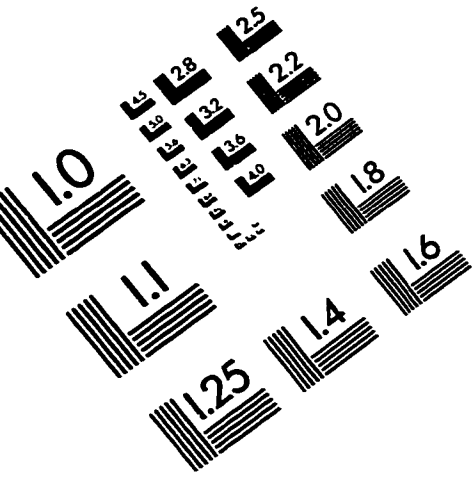
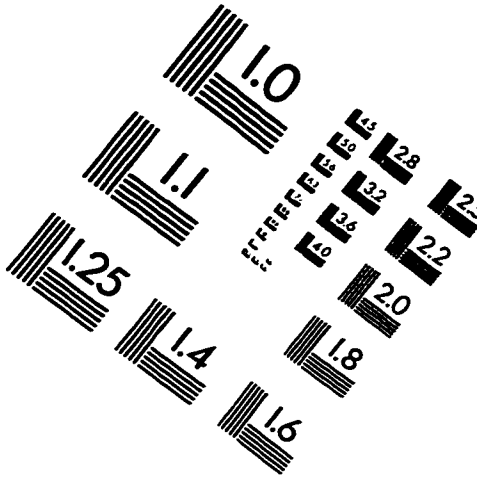
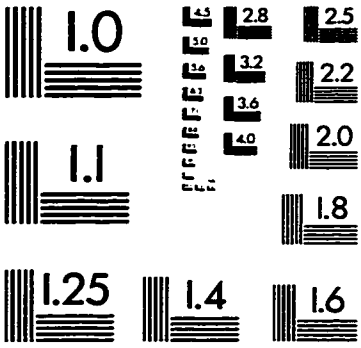
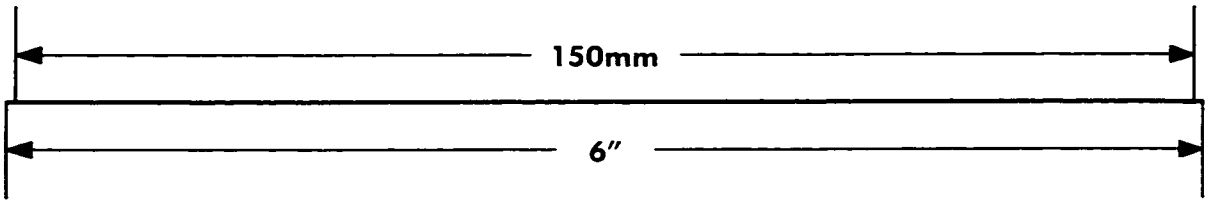
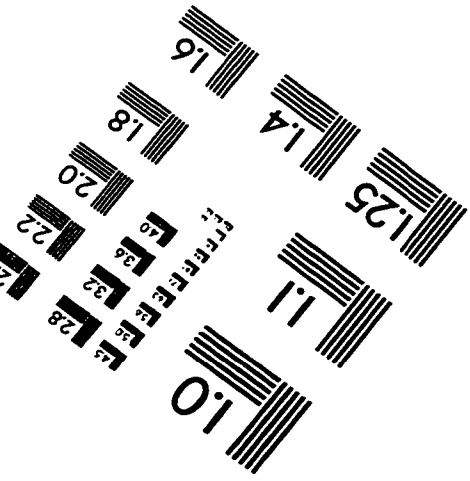
IMAGE EVALUATION TEST TARGET (QA-3)

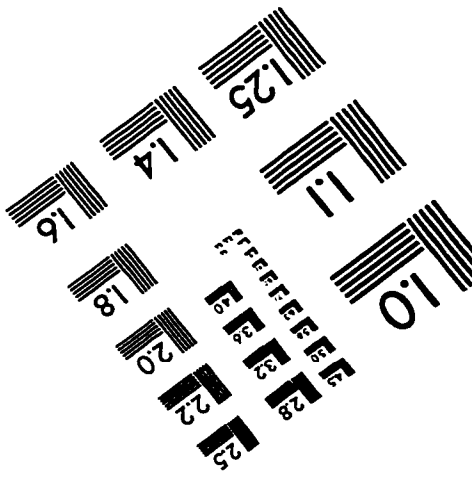












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