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MODULATION OF DESENSITIZATION OF NICOTINIC ACETYLCHOLINE RECEPTORS (nAChRs) IN ENTERIC NEURONS

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MODULATION OF DESENSITIZATION OF NICOTINIC ACETYLCHOLINE RECEPTORS (nAChRs) IN ENTERIC NEURONS

By

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ABSTRACT

MODULATION OF DESENSITIZATION OF NICOTINIC ACETYLCHOLINE RECEPTORS (nAChRs) IN ENTERIC NEURONS

By

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The enteric nervous system (ENS) is the division of the autonomic nervous system (ANS) that controls gut functions. Acetylcholine (ACh), acting at nAChRs and muscarinic acetylcholine receptors (mAChRs), is a neurotransmitter in the ENS. NAChRs and mAChRs are expressed by the same neurons, but if these receptors interact is unclear. The hypothesis of this study is that nAChR desensitization is modulated by intracellular signaling mechanisms. Specifically, mAChR activation accelerates nAChR desensitization in the ENS. Whole-cell patch clamp methods were used to record nAChR mediated inward currents in guinea pig myenteric neurons maintained in primary culture. Drugs were used to activate and/or inhibit intracellular signaling pathways modulating nAChR desensitization. The major findings of this research were that: desensitization rate is influenced by intracellular ATP and GTP; desensitization and recovery are influenced by mAChR activation; ACh and nicotine cross-desensitize suggesting activation of the same nAChR; protein kinase C but not cyclic AMP-dependent pathways modulate nAChR desensitization. These results suggest that simultaneous activation of nAChRs and mAChRs coupled to PKC-linked signaling pathways modulate nAChR desensitization. This may be important in regulating enteric neurotransmission.

DEDICATION

I would like to dedicate this thesis to my parents, George and Carolyn Brown, Sr., and to my brother, George E. Brown, Jr. They have supported me throughout my graduate career, and they continue to do so through emotional, financial, and spiritual support.

Thank you Mom, Dad, and George Jr.

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4-α-PMA: 4-α-phorbol 12-myristate 13-acetate

5-HT: 5-hydroxytryptamine

ACh: acetylcholine

AH: after-hyperpolarization

ANS: autonomic nervous system

ChAT: choline acetyltransferase

CNS: central nervous system

DDF: 1, 9-dideoxy-forskolin

ENS: enteric nervous system

fEPSPs: fast excitatory postsynaptic potentials

GABA: γ-aminobutyric acid

GI: gastrointestinal

I_{7s}: current at seven seconds

I_{peak}: peak current

mAChRs: muscarinic acetylcholine receptors

nAChRs: nicotinic acetylcholine receptors

NE: norepinephrine

NO: nitric oxide

NOS: nitric oxide synthase

NPY: neuropeptide Y

PDBu: phorbol 12, 13-dibutyrate

PI: phosphatidyl inositol

PKA: protein kinase A

PKC: protein kinase C

sEPSPs: slow excitatory postsynaptic potentials

SP: substance P

TMDs: transmembrane domains

VIP: vasoactive intestinal peptide

INTRODUCTION

ENTERIC NERVOUS SYSTEM (ENS)

Development of the ENS

The ENS is the division of the autonomic nervous system (ANS) that is localized in the wall of the gastrointestinal (GI) tract and is responsible for controlling GI function. GI functions controlled by the ENS include motility, blood flow, exocrine and endocrine secretions, and regulation of immune and inflammatory processes **(Goyal and Hirano, 1996)**. The ENS differs from the sympathetic and parasympathetic divisions of the ANS in its embryonic origin. The ENS arises from precursor cells from the vagal neural crest. Precursor cells migrate to the gut beginning at the proximal portion of the GI tract and spreading to the distal portion of the GI tract **(Goyal and Hirano, 1996)**.

Structure of the ENS

The ENS is able to function without connections with the central nervous system (CNS). The ENS is capable of mediating coordinated reflexes such as intestinal peristaltic contractions through the activity of intramural sensory neurons, interneurons and motor neurons **(Goyal and Hirano, 1996; Costa et al, 1986)**. The ENS contains approximately 100 million neurons and the function of individual neurons and neurochemistry are similar to neurons in the CNS. Communication

between the CNS and ENS occurs via efferent neurons of the sympathetic and parasympathetic nervous system, and also via sensory afferent neurons from dorsal root ganglia and vagal afferent fibers. This communication is known as the central autonomic neural network which contributes to the neuronal control of GI functions **(Goyal and Hirano, 1996)**.

In the ENS, nerve-cell bodies are grouped together to form ganglia. Ganglia in the ENS are connected via interconnecting nerve strands which contain nerve axonal processes and form two ganglionated plexuses called the myenteric (Auerbach's) and the submucosal (Meissner's) plexuses **(Goyal and Hirano, 1996; Costa et al, 1986)**. The myenteric plexus resides between the longitudinal and circular smooth muscle layers and it controls motor function of the GI tract. The myenteric plexus contains intrinsic sensory neurons, interneurons, and excitatory and inhibitory motor neurons. The submucosal plexus resides in the connective tissue between the circular muscle and mucosal layer where it controls secretomotor function of the mucosal epithelium and local blood flow. The submucosal plexus also contains sensory neurons, interneurons, and motorneurons **(Goyal and Hirano,1996, Costa, Furness, and Gibbins, 1986)**.

Myenteric and submucosal ganglia contain tightly packed nerve cell bodies, terminal bundles of nerve fibers, and glial cells **(Goyal and Hirano, 1996)**. There are more glial cells in the ENS than neurons and enteric glia are similar to astrocytes found in the CNS **(Gershon and Rothman, 1991)**. Glial cells have laminar processes that are extend over the surface of enteric neurons **(Gershon**

and Rothman, 1991). In response to cytokine stimulation, glial cells are able to express interleukins and MHC class II antigens which suggests a role for glia in regulating inflammation in the GI tract **(Goyal and Hirano, 1996)**.

Neurotransmitters in the ENS

There are more than 20 neurotransmitters released by myenteric and submucosal neurons. These neurotransmitters include: norepinephrine (NE), 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA), ATP, nitric oxide (NO), neuropeptide Y (NPY), opioids, somatostatin, substance P (SP), and vasoactive intestinal peptide (VIP). The major excitatory neurotransmitter is ACT **(Costa et al, 1986, Gayal and Hirano, 1996, Kunze and Furness, 1999)**. Therefore, cholinergic neurotransmission plays a critical role in controlling normal GI function.

Morphology of enteric neurons

The morphological types of enteric neurons found in the ENS are Dogiel Type I, Dogiel Type II, and Dogiel Type III **(Hirst et al, 1974; Nishi and North, 1973; Furness and Costa, 1987; Costa et al, 1986; Brookes et al, 1990; Song et al, 1996)**. Dogiel Type I neurons have flat cell bodies, numerous short lamellar dendritic processes, and one long axon **(Brookes et al, 1990)**. These neurons are motor neurons and interneurons. Dogiel Type I neurons are "S" neurons classified using electrophysiological criteria **(Hirst et al, 1974; Nishi and North, 1973; Furness and Costa, 1987; Brookes et al, 1990)**.

Stimulation of interconnecting nerve strands produces fast excitatory postsynaptic potentials (fEPSPs). FEPSPs last less than 20 milliseconds and are associated with increased membrane conductance and reversal potentials near zero millivolts. ACT released from nerve terminals acts at nAChRs on S neurons to mediate fEPSPs **(Hirst et al, 1974; Nishi and North, 1973; North et al, 1980; Furness and Costa, 1987)**.

Dogiel Type II neurons are multipolar neurons that have large oval smooth cell bodies and several long axonal processes **(Furness et al, 1998 and Song et al, 1996)**. Axonal processes arise from cell bodies to project either to the mucosal layer or project circumferentially to other myenteric ganglia **(Furness et al, 1998)**. Dogiel Type II neurons are electrophysiologically characterized as AH (after-hyperpolarization) neurons and they are sensory neurons **(Hirst et al, 1974; Nishi and North, 1973; Furness et al, 1998; Kunze and Furness, 1999)**.

AH neurons receive synaptic input via slow excitatory postsynaptic potentials (sEPSPs). A prominent electrophysiological property of AH neurons is the action potential and hyperploarization that is slow in onset that lasts up to twenty seconds **(Nishi and North, 1973; Hirst et al, 1974)**. The long-lasting after-hyperpolarization results from an increase in calcium dependent potassium **(Hirst and Spence, 1973; North, 1973; Morita et al, 1982; Hirst et al, 1985; North and Nishi, 1974)**.

Filamentous type neurons have smooth ovoid cell bodies, single long axons and several filamentous dendrites **(Song et al, 1996)**. These neurons project anally in the myenteric plexus **(Song et al, 1996)**. There function is not known.

Functional classes of enteric neurons in the ENS

There are three functional types of enteric neurons. Excitatory and inhibitory motor neurons including secretomotor, interneurons, or intrinsic sensory neurons **(Kunze and Furness, 1999)**. Excitatory and inhibitory motor neurons have cell bodies that are localized along the wall of the GI tract. Motor neurons in the stomach are activated by the stimulation of the vagus nerve, but vagus nerve stimulation has less effect in the small intestine and colon **(Gayal and Hirano, 1996; Kunze and Furness, 1999)**. Excitatory motor neurons project to circular muscle and run circumferentially in the direction of muscle cells **(Gabella, 1972; Kunze and Furness, 1999)**. The primary neurotransmitter is ACT as cholinergic transmission can be largely blocked pharmacologically by muscarinic cholinergic antagonists. With the addition of tachykinin receptor antagonists, neuromuscular transmission is completely blocked **(Costa et al 1996; Kunze and Furness, 1999)**. Excitatory motor neurons function is to cause contractions. Inhibitory motor neurons project to muscle close and anal to their cell bodies **(Furness et al, 1995; Kunze and Furness, 1999)**. The transmitter released from inhibitory motor neurons is not clear but it is likely that NO, ATP, VIP, and pituitary adenyl cyclase-activating peptide participate in inhibitory neuromuscular transmission **(Stark and Szurszewski, 1992; Kunze and Furness, 1999)**. Inhibitory motor neurons function to cause smooth muscle relaxation.

Interneurons are localized in myenteric and submucosal ganglia, and they have orally directed or anally directed projections **(Costa et al, 1996; Kunze and**

Furness, 1999). There are four classes of interneurons consisting of one ascending interneuron class and three descending interneuron classes. The one ascending interneuron class is cholinergic and is immunoreactive for the calcium binding protein, calretinin **(Costa et al, 1996; Kunze and Furness, 1999)**. Descending interneuron classes are cholinergic but not purely as they also release other neurotransmitters. It has been found that with the addition of PPADS, a purinergic antagonist can inhibit fast excitatory transmission from some descending interneurons **(Lepard et al, 1997)**. The three descending interneuron classes consist of interneurons immunoreactive for choline acetyltransferase (ChAT)/ somatostatin, ChAT/ NOS/ VIP, andChAT/5-HT **(Costa et al, 1996; Kunze and Furness, 1999)**. From further studies, it has been found that ChAT/NOS interneurons are likely to be involved in inhibitory responses of the GI tract **(Young et al, 1995; Yuan et al, 1995; Mann et al, 1997; Kunze and Furness, 1999)**.

Intrinsic sensory neurons have cell bodies localized in the wall of the intestine and project axons to other nerve cells and to the mucosa **(Furness et al , 1997)**. They are the first neurons in the intrinsic nerve circuitry of the GI tract to be activated by sensory stimuli such as acid, short-chain neutralized fatty acid, and 5-HT. 5-HT is important because of its intermediate action in reflexes initiated from the mucosa **(Kunze et al, 1995; Bertrand et al, 1997; Kirchgessner et al, 1992; Kunze and Furness, 1999)**.

Synaptic transmission of the ENS

AH/Dogiel Type II neurons are intrinsic sensory neurons. When their synaptic inputs are activated by chemical stimuli or distention (stretch), they produce sEPSPs. Rarely, fEPSPs are recorded from AH neurons on those that are recorded, and recordings are usually low in amplitude **(Iyer et al, 1988; Kunze and Furness, 1999)**. Intrinsic sensory neurons give rise to varicose terminals which synapse with other neurons in adjacent. Experiments have also shown that sEPSPs from intrinsic sensory neurons originate from the synaptic input from other intrinsic sensory neurons. These observations suggest that intrinsic sensory neurons are interconnected networks and excitatory and therefore self-reinforcing **(Kunze and Furness,1999)**.

Interneurons project orally and anally along the intestine. Ascending interneurons connect with other ascending interneurons, and they also receive synaptic input from intrinsic sensory neurons. In return, they process this input to provide synaptic output to motor neurons orally along the intestine. Of the three classes of descending interneurons, the choline acetyltransferase (ChAT) and nitric oxide synthase (NOS) containing classes have been found to be involved in descending reflexes because they make connections with the inhibitory motor neurons **(Yuan et al, 1995; Kunze and Furness, 1999)**. Pharmacological studies have shown that the ChAT and 5-HT containing classes are not involved in descending reflexes due to the lack of connections with inhibitory motor neurons, and other studies have shown that the ChAT and somatostatin containing classes receive majority of their synaptic inputs from other chat neurons and few from

intrinsic sensory neurons suggesting that they are auxiliary descending interneurons involved in descending reflexes **(Yuan et al, 1994; Kunze and Furness, 1999)**.

The circular muscle cells are electrically coupled together and receive input from motor neurons. The motor neuron axonal processes branch within the circular muscle **(Kunze and Furness, 1999)**. Excitatory motor neurons have axons projecting short distances along the wall of the intestine, where majority of the them synapse adjacent to the muscle at 2 mm and few synapse anally at 8 mm, to cause contraction **(Brookes et al, 1991; Kunze and Furness, 1999)**. Inhibitory motor neurons project about 2-14mm anally along the muscle to cause relaxation. From this, muscle along the intestine undergo contractions and relaxations, and the strength of each depends on the quantity and frequency of neurons activated **(Brookes et al, 1991; Kunze and Furness, 1999)**.

Diseases and Disorders of the ENS

There are many disorders and diseases that cause a disruption of GI motility, secretory, and inflammatory responses of the GI tract. Motility disorders include achalasia, gastric stasis and outlet obstruction, acute intestinal ileus and chronic intestinal pseudo-obstruction, megacolon, and other general motility disorders. Achalasia is caused by an inability of the lower esophageal sphincter to relax. This is due to a dysfunction of inhibitory neurons that release VIP and NO **(Gayal and Hirano, 1996)**. Gastric stasis and outlet obstruction result from a congenital

disorder called infantile hypertrophic pyloric stenosis in which the neurons innervating the circular muscle do not release NO due to the lack of NOS **(Gayal and Hirano, 1996)**. Acute intestinal ileus and chronic intestinal pseudo-obstruction result from a loss of motor activity in the intestine. Megacolon results from a congenital disease called Hirschsprung's disease which is identified by the lack of enteric neurons in the colon **(Gayal and Hirano, 1996)**. Other general disorders are characterized by the involvement of more than one segment of the GI tract. This consists of either of two groups, the hyperganglionosis, cited as neuronal dysplasia or ganglioneuromatosis, or hypoganglionosis, resulting from acquired diseases leading to enteric neuron destruction **(Gayal and Hirano, 1996)**.

Secretory dysfunctions can result in diarrhea. Diarrhea can be caused by chemicals and toxins exposed to the lumen of the intestine. Such substances are ethanol, bile salts, heat-stable toxins of *Escherichia coli*, and cholera toxin. These substances act by stimulating mucosal receptors on the luminal layer of the intestine to cause intestinal secretomotor reflexes **(Gayal and Hirano, 1996)**.

Inflammatory and immunologic responses can cause bowel diseases. Such diseases are caused by a known toxin called *Clostidium difficile* toxin A (*C. difficile* toxin A) which result in necro-inflammatory responses that involve intrinsic neural pathways. Activation leads to mast-cell degranulation and release of inflammatory mediators such as prostaglandins, histamine, and 5-HT which stimulate secretomotor reflexes. Pharmacological experiments suggest that *C. difficile* toxin A acts at extrinsic primary afferent neurons containing SP. This conclusion is

based on studies in which these neurons were destroyed with capsaicin resulting in the inhibition of secretory and inflammatory responses to *C. difficile* toxin A. Another cause of bowel disease could be due to psychological stress suggesting an interaction between the CNS and the ENS **(Gayal and Hirano, 1996)**.

Summary of the ENS

The ENS plays an important physiological and pathophysiological role of the ANS by being responsible for controlling the function of the GI tract with and/or without connections to CNS. One of the reasons for studying the ENS is because it is a system of its own in which neuronal network patterns, neuronal receptors and its neurotransmitters, and the functions of motility are still being studied and analyzed. A second reason is to compare and contrast the differences and similarities between the CNS and ENS because as mentioned above the ENS is a system of its own and can function without CNS input. From this, scientists can obtain knowledge on how to treat GI diseases.

BACKGROUND OF mAChRs AND nAChRs

MAChRs and nAChRs

The first characterization of mAChRs and nAChRs was the demonstration that mAChRs are selectively activated by muscarine and blocked by atropine **(Hulme et al 1990)**. NAChRs were identified as being selectively activated by nicotine and

blocked by curare **(Hulme et al 1990)**. Subsequently, molecular biological studies would show that there are multiple genes whose products make up the mAChRs and nAChRs, therefore, making these receptors more complex than originally defined **(Hulme et al, 1990)**. Even though mAChRs and nAChRs belong to different gene superfamilies, they share ACT as their neurotransmitter for activation **(Hulme et al, 1990)**.

This holds true for the ENS because even though the ENS has many neurotransmitters responsible for the regulation of GI function, the major neurotransmitter is ACT **(Gayal and Hirano,1996, Galligan, 1993)**. In enteric ganglia, ACT acts at mAChRs and nAChRs to excite enteric neurons **(Bertrand and Galligan, 1995)**.

Structure of mAChRs

MAChRs belong to the family of G-protein coupled receptors that link to one or more intracellular signaling pathways. There are approximately 20 members of the G-protein coupled superfamily which includes the catecholamine receptors, serotonin receptors, peptide receptors, *mas* oncogen, rhodopsin family, and several other receptors of unknown specificity **(Hulme et al, 1990)**. Of these members, the mAChRs have the greatest homology with adrenergic receptors **(Hulme et al, 1990)**. MAChRs transverse the membrane seven times creating an extracellular NH_3^+ terminus and an intracellular CO_2^- terminus and extracellular and intracellular loops connecting seven transmembrane domains (TMD I-VII). There

is overall sequence variation in the NH_3^+ terminus, third intracellular loop, and in the CO_2^- terminus, but there are conserved subsections of the third intracellular loop and CO_2^- terminus. Also, there is great homology between TMD II-VII. Seven residues of the CO_2^- terminus of third intracellular loop are recognized as a determinant for G-protein coupled specificity. The third intracellular loop has substrate sites that are recognized by kinases and also influences mAChR expression. With other G-protein coupled receptors, the CO_2^- terminus influences the activation of effector systems, but this is yet to be observed with the mAChR CO_2^- terminus **(Hulme et al, 1990)**. For mAChR ligand binding, the TMD III has aspartate amino acid residues that participate in binding **(Hulme et al, 1990)**. MAChR subtypes have been recognized thus far to be either coupled via a pertussis toxin (PTX) insensitive G-protein that activates PI hydrolysis (G_q) or via a PTX sensitive G-protein that inhibits adenylate cyclase (G_i) **(Hulme et al, 1990)**.

Subtypes of mAChRs

There are five subtypes of the mAChR,1-5. The definition and nomenclature of the five subtypes have been partially characterized by binding, pharmacological, mechanistic, and molecular studies **(Hulme et al, 1990)**. Pharmacologically, the subtypes are named as M_1, M_2, M_3, M_4, and M_5, and structurally, the subtypes are defined as m1-m5 based upon their amino acid sequences **(Hulme et al, 1990)**. To provide a source for the receptor clones of the mAChR subtypes, cDNA libraries were obtained, and from this, the m1 gene has been cloned from the human, rat,

pig, and mouse, the m2 and m3 genes have been cloned from the human , rat, and pig, and the m4 and m5 genes have been cloned from the human and rat **(Hulme et al, 1990)**. From this, questions arose of whether if there are other muscarinic receptor genes present and this brought up the analysis of a DNA hybridization of a partial m1 cDNA which resulted in the production of up to ten hybridizing bands from rat and up to six hybridizing band from human **(Hulme et al, 1990)**. In northern blot studies, the m1 and m3 genes are localized in the brain and exocrine glands, the m2 gene is found in cardiac and smooth muscle, the m4 gene is localized mainly in neural tissue, and the m5 gene is found in the brain **(Hulme 1990)**.

Co-expression of mAChR gene subtypes

From *in situ* hybridization studies, m1, m2, m3, and m4 genes are localized in autonomic ganglionic neurons. But by studying the signal transduction pathway of the mAChR subtypes, observations show that the m1 and m3 receptors subtypes couple to the activation of PI hydrolysis, whereas the m2 and m4 receptor subtypes couple to the inhibition of adenylate cyclase **(Hulme et al, 1990)**. These coupling systems are only observed in heterologous expression systems, therefore, questions arise of why this is not observed in native cells. These question include: (1) Do effector systems *in vivo* couple differently than when expressed *in vitro*? This question can only be answered in cells expressing native mAChRs in studies using pharmacological agonists and antagonists that are able to act specifically to

identify function of the mAChR subtypes. (2) Can a specific effector system be coupled to different receptor subtypes but be regulated by different signals? This would allow cell flexibility to a signal modifying a response. (3) Are different subtypes localized on different cellular domains, that is, on presynaptic nerve terminals or postsynaptically? These factors would determine the different interactions between the subtypes on a cell **(Hulme et al, 1990)**.

Function and G-protein pathways of mAChR subtypes

In the ENS, mAChRs mediate slow synaptic responses and long lasting changes in neuronal excitability **(Morita et al, 1982, Galligan, 1998)**. MAChR agonists that are able to mimic slow synaptic potentials are bethanecol, carbachol, methylcholine, muscarine, and oxotremorine **(Morita et al, 1982, Cassell and McLachlan, 1987, Hulme et al, 1990)**, and at this time there is no agonists that are able to act at mAChR subtypes specifically. MAChRs can be blocked nonspecifically by atropine, hyoscine, and scopolamine **(North 1982, Morita et al, 1982, and Wessler et al, 1987)**.

The M_1-mAChR subtype elicits slow excitatory responses and can be found in autonomic ganglia and in the CNS. This subtype is thought to act through a G_q protein that is coupled to PI hydrolysis. Responses can be blocked by pirenzepine **(Hulme et al, 1990)**. The M_2 subtype exhibits slow inhibitory responses and can be found in the CNS and peripherally. It is thought to act through a G_i protein and can be blocked by AF-DX116 **(Hulme et al, 1990)**. The M_3 subtype elicits slow

excitatory responses and can be found in secretory glands and smooth muscle and in the CNS. It is thought to act through a G_q protein that is coupled to PI hydrolysis and can be blocked by hexahydrosiladifenidol **(Hulme et al, 1990)**. The M_4 subtype elicits slow inhibitory responses and can be found in the brain and some smooth muscle. It is thought to act through a G_i protein and can be blocked by himbacine **(Hulme et al, 1990)**. The m_5 subtype was detected by RNA hybridization from to tissue collected from the rat brain, therefore, this mAChR subtype is localized in the brain **(Hulme et al, 1990)**. The physiological function and pathway of this receptor subtype is unknown. From this, a hypothesis can be formed that M_1 activation is responsible for mediating muscarinic effects in the ENS.

MAChRs in the ENS

MAChRs in the ENS mediate sEPSPs in myenteric S neurons **(North et al, 1982; Galligan, 1998)**. Although the specific signaling pathways involved in mediating sEPSPs are not known, there is evidence that PKC and adenylate cyclase activators are able to mimic sEPSPs on myenteric ganglia **(Bertrand and Galligan, 1995; Palmer et al, 1987; Galligan, 1998)**.

Summary of mAChRs

MAChRs are G-protein coupled receptors that mediate slow synaptic responses through either of the four subtypes to cause neuronal excitability and muscle contraction and relaxation responses. These responses are regulated by

intracellular signaling pathways which are able to cause modulation of receptors, mAChRs and other receptor subtypes, and also influence the expression of receptors. Pharmacologically, mAChRs can be activated by agonists such as ACT, bethanecol, carbachol, methylcholine, muscarine, and oxotremorine, and they are able to be blocked nonspecifically by atropine, hyoscine, and scopolamine, and specifically by pirenzepine, AF-DX116, hexahydrosiladifenidol, and himbacine. In the ENS, mAChRs mediate sEPSPs, but the intracellular signaling involved regulating these responses are not known. Therefore, future studies should focus on how mAChR activation modulates GI function through specific intracellular signaling pathways.

Structure of nAChRs

NAChRs belong to a superfamily of membrane bound ligand-gated ion channels that mediate cell to cell communication by transforming neurotransmitter signals into an ion flux at the postsynaptic neuron or muscle **(Corringer et al, 2000)**. Other members of this superfamily include receptors for glutamate, GABA, 5-HT, glycine, and ATP. The nAChR structure, based on the number of times it transverses the membrane (four TMDs), closely resembles the $GABA_A$, $GABA_C$, $5\text{-}HT_3$, and glycine receptors **(Corringer et al, 2000)**. NAChRs are one of the largest members and among this group. They contribute to cell to cell communication by being distributed at presynaptic and/or postsynaptic sites **(Corringer et al, 2000)**. NAChRs are found throughout the CNS and PNS, neuromuscular junctions, and adrenal glands.

The nAChR is composed of a pentameric structure that protrudes out extracellularly and intracellularly to form a five fold axis that is perpendicular to the membrane **(Corringer et al, 2000)**. This structure forms a pore that becomes narrower within the transmembrane **(Corringer et al, 2000)**. As a whole structure, the nAChR consists of an NH_3^+ and a CO_2^- terminus localized synaptically, three TMDs with short extracellular and intracellular loops, a large variable third intracellular loop, and a fourth TMD **(Corringer et al, 2000)**.

There are five subunits that make up the nAChR oligomer, and they fall into two main classes. These classes include the α subunit class and the non-α subunit class. The α subunit class consists of nine subtypes, $\alpha1$-$\alpha9$. This class has two adjacent cysteines present which are responsible for agonist binding **(Corringer et al, 2000)**. The non-α class consists of the β,γ,ε, and δ subunits which the β subunit has four subtypes, $\beta1$-$\beta4$. This class does not bind agonists **(Corringer et al, 2000)**.

Subtypes of nAChRs

There are two subtypes of nAChRs, the muscle nAChR and the neuronal nAChR. Four homologous subunits make up the muscle subtype of nAChR, and the subunit composition consists of $\alpha,\beta,\gamma,\delta$, or ε. These subunits assemble into a heteropentamer with a stoichiometry of $\alpha1_2,\beta1,\gamma$, and δ, or sometimes ε **(Rangwala et al, 1997; Corringer et al, 2000)**. The ion permeability ratio of Na^+, K^+, Ca^{2+} is 1:1:0.2 **(Adams et al, 1980; Vernino et al, 1992)**. Neurons express a varied range

of nAChR subtypes which makes characterizing the neuronal subtype difficult **(Rangwala et al, 1997)**. The subunit composition is composed of α and β with the α subunit consisting of eight subtypes, α2-α9, and the β subunit consisting of three subtypes, β2-β4. The neuronal subtype is expressed in the brain, sensory end organs, and peripheral ganglia, where the α and β subunits provide functional and pharmacological diversity **(Rangwala et al, 1997)**. Neuronal nAChRs have best been described in brain tissue by high affinity nicotine binding in which the nAChR is composed of α4 and β2 subunits with a stoichiometry of $\alpha4_2\beta2_3$, and it has also been characterized by the high affinity α-bungarotoxin receptor which is composed of homopentamer of α7 subunits **(Rangwala et al, 1997; Chen and Patrick, 1997)**. The ion permeability ratio of Na^+, K^+, Ca^{2+} is 1:1:5 **(Nutter and Adams, 1995)**.

Parts of TMD I and II of all the subunits make up the lining of the channel. For both types of nAChRs, the α subunit is organized around the channel so that the two agonist binding sites are at the interfaces between the α subunit and the non-α subunit **(Gerzanich et al, 1998)**. Pharmacologically, muscle nAChRs can be activated by such agonist as ACT **(Dionne and Stevens, 1975)** and can be blocked by tubocurarine **(Ascher et al, 1978)**. For the neuronal types, nAChRs can be activated by ACh, choline, cytisine, DMPP, (-) epibatidine, and (-) nicotine **(Albuquerque et al, 1997)**. These receptors can also be blocked nonspecifically by hexamethonium and decamethonium **(Ascher et al, 1979)**, and specifically , they can be blocked by α-bungarotoxin and methyllycaconitine (α7-nAChRs), dihydro-b-

erythroidine ($\alpha4\beta2$-nAChR composition), and mecamylamine ($\alpha3\beta4$-nAChR composition) **(Albuquerque et al, 1997)**.

Function of nAChRs

NAChRs mediate fEPSPs in the peripheral nervous system and the neuromuscular junction. These fast excitatory responses cause muscle to contract and neurons to become excitable. NAChRs depolarize neurons via their ion channels to permit the flow of cations across the membrane. NAChRs regulate neurotransmitter release presynaptically, and they cause synaptic currents from synaptic and presynaptic locations on postsynaptic sites **(Conroy and Berg 1997)**. The neuronal nAChRs can become functional with the $\alpha3\beta2$ combination $\alpha3\beta2$ or $\alpha3\beta4$ combinations, or with the $\alpha7$ combination **(Gerzanich et al, 1998; Blumenthal et al 1999)**. Also, the $\alpha5$ subunit has been found in the $\alpha3\beta4$ or $\alpha3\beta2$ combination but the exact stoichiometry of this combination has not been determined. The $\alpha5$ subunit has been found not to form functional nAChRs and is organized along the channel so that it does not interface with the sides that are involved in forming agonist binding sites **(Gerzanich et al, 1998)**.

One prominent property of nAChRs is that they desensitize rapidly and the rate of desensitization and recovery from desensitization can be altered by receptor modification. Desensitization was first described at the neuromuscular junction **(Katz and Thesleff, 1957)**. Agonist binding to the receptor activated the channel and thus, ion passage through the channel **(Katz and Thesleff, 1957; Reitstetter**

et al, 1999). Prolonged exposure to the agonist can lead to a desensitized, inactivated state where the ion passage through the channel decreases **(Reitstetter et al, 1999)**. Recovery from desensitization occurs when agonist is removed **(Reitstetter et al, 1999)**. In the neuronal nAChRs, desensitization has a role in synaptic plasticity and nicotine dependence in smokers **(Reitstetter et al, 1999)**. Although neurotransmission at nAChRs is an important mechanism in the ENS, there have been few studies of desensitization of these receptors in the ENS or how desensitization is modulated by simultaneous activation of other neurotransmitter receptors expressed by the same neurons.

Desensitization of the nAChR is thought to be modulated by phosphorylation when intracellular signaling pathways are activated during neurotransmission. It has been determined by using heterologously expressed nAChR in *Xenopus* oocytes that the α and β subunit composition influences desensitization of nAChRs **(Fenster et al, 1997)**. It was found that nAChRs containing α4 subunits were more sensitive to nicotine activation than nAChRs containing α3 subunits, and also that nAChRs containing β2 subunits reach maximal desensitization at lower concentrations of nicotine compared to nAChRs containing β4 subunits. It was also shown that the α subunit influences the rate of desensitization and the rate of recovery from desensitization **(Fenster et al, 1997)**. In another study using rat striatal synaptosomes, it was found that prolonged treatment with nicotine results in incomplete recovery from desensitization suggesting that high concentrations of nicotine can produce long-lasting inactivation of the nAChR **(Rowell and Duggan,**

1998). Using nAChRs expressed in *Xenopus* oocytes, it was found that the α5 subunit increases desensitization and calcium permeability of α3β2 containing nAChRs **(Gerzanich et al, 1998)**. Studies have also shown, using rat chromaffin cells, that changes in intracellular calcium following activation intracellular signaling pathways **(Khiroug et al, 1998)**. For example, nAChR desensitization is accelerated by PKA mediated phosphorylation, and PKA activation keeps the nAChR in a long-term desensitized state **(Paradiso and Brehm, 1998)**. It has also been shown that PKC and PKA mediated phosphorylation of nAChRs increases the rate of desensitization **(Nishizaki and Sumaikawa, 1998)**. Therefore, the properties of desensitization of the nAChR are dependent on subunit composition, changes in intracellular calcium, and activation of PKA and PKC dependent pathways.

Desensitization state of nAChRs

Subunit composition development seems to play a part in the desensitization state of the nAChR. In muscle, stabilization of the subunit composition on nAChR desensitization plays a part in the kinetics of gating modes during development because in *Xenopus* oocytes, the embryonic state (αβδψ) tended to interchange between short and long open time gating modes, but in the adult (αβδε), nAChRs have a short open time gating mode **(Naranjo and Brehm, 1993)**. The decay after ACT- induced maximal response at the nAChR is described by the sum of two exponential components and the recovery rate from the desensitized state is also

the sum of two exponential functions, a fast and slow time constant **(Naranjo and Brehm, 1993)**. This suggests that a fast onset to desensitization can lead to a fast recovery when agonist is removed from nAChR binding sites.

A proposed model of nAChR desensitization is based on two ACT molecules binding to the nAChR to cause a high probability of the channel to become open. At this stage, the receptor is considered to be in an unliganded closed state (A_2R) **(Naranjo and Brehm, 1993)**. From linear relationship analysis, the closed state undergoes a conformational change to either the open state (A_2R^*) or the desensitized state (A_2D) **(Naranjo and Brehm, 1993)**. From this study, the authors conclude that desensitization rates of specific agonists may depend on the destabilization of a particular gating mode. Also, the gating modes may be due to a limited number of protein conformation sets in which each gating mode is modulated. And they also proposed that modulation may be due to a short-term post-translational modification **(Naranjo and Brehm, 1993)**.

In another study, the authors wanted to test what a high agonist concentration would have on intrinsic channel kinetics **(Jones and Westbrook, 1996)**. A high concentration results in receptor saturation and minimizes the unbound state of the nAChR so that the nAChR current produced reflects only the bound to open transition, and the decay phase reflects the bound to desensitization state **(Jones and Westbrook, 1996)**. They concluded that nAChRs, under normal physiological conditions, are more likely to unbind than to desensitize with short pulses of agonist **(Jones and Westbrook, 1996)**.

A study on neuronal development has found that there are increases in ACT sensitivity and receptor number, but there are also decreases in the rate of receptor desensitization and apparent affinity for ACT **(Margiotta and Gurantz, 1989)**. Also, the cAMP-dependent mechanism develops to enhance the ACT response. These changes were due to a shift in the small and large conductances and also by a change in the large conductance kinetics **(Margiotta and Gurantz, 1989)**. The authors concluded that neuronal development plays a role in controlling receptor regulation and expression of different nAChR subtypes **(Margiotta and Gurantz, 1989)**.

There are studies that suggest that nAChRs are phosphoproteins **(Gordon et al, 1977; Teichberg et al,1977; Huganir and Greengard, 1983; Huganir et al, 1984; Miles et al, 1987)**. Studies using the *Torpedo* electric organ indicate that cAMP-dependent PKA, PKC and tyrosine-specific protein kinase target phosphorylation sites on nAChRs **(Huganir et al, 1984)**. Rat muscle cell studies have shown that phosphorylation by PKA increases the nAChR desensitization rate, and also that, forskolin and its analogs activate cAMP-dependent pathways to increase nAChR desensitization by phosphorylation of the receptor **(Albuquerque et al, 1986; Middleton et al, 1986)**. In another study, the authors wanted to test nAChR modulation on the single channel level, and they found that forskolin increased agonist-induced desensitization without changing the properties of single channel currents **(Middleton et al, 1988)**. The authors concluded that forskolin acts by promoting phosphorylation of nAChRs by PKA, and also that at low

concentration of forskolin, there is no direct affect of nAChR function **(Middleton et al, 1988)**.

In neuronal nAChRs, research studies have found that PKC an cAMP-dependent pathways regulate nAChR function by phosporylation. In a study using ACT- induced currents in voltage clamped sympathetic ganglion neurons, the authors tested the effect of PKC mediated phosphorylation on the regulation of neuronal nAChR desensitization **(Downing and Role, 1987)**. This study found that the desensitization rate is enhanced by PKC, and the study concluded that modulation of the nAChR function involves PKC mediated phosphorylation changes **(Downing and Role, 1987)**. In other studies using chick ciliary ganglion neurons, effects of cAMP enhances ACT response of the neurons by a mechanism involving an increase in number of functional nAChRs, which does not require protein synthesis nor an increase in total number of nAChRs **(Margiotta et al, 1987; Vijayaraghavan et al, 1990)**. Results from these studies show that cAMP mediates the recruitment of functional receptors from intracellular receptors or from nonfunctional receptors on the cell surface and causes a two to three fold increase in ACh response **(Margiotta et al, 1987; Vijayaraghavan et al, 1990)**. These studies concluded that this regulation provides a reversible mechanism of cell to cell interaction to modulate synaptic transmission **(Margiotta et al, 1987; Vijayaraghavan et al, 1990)**.

Phosphorylation of neuronal nAChRs differs from muscle type in that neuronal nAChRs are phosphorylated on the α subunits rather than the non-ligand

binding subunits. Two studies using α4 nAChRs purified from rat brains and the other using rat and chick α7 nAChRs expresses in *E. Coli* have shown that these subunits have intracellular domains containing serine residues that are phosphorylated by cAMP-dependent pathways **(Nakayama et al, 1993; Moss et al, 1996)**. These studies conclude that PKA plays a role in protein phosphorylation which mediates cellular regulation of neuronal nAChR function **(Nakayama et al, 1993; Moss et al, 1996)**.

Summary of nAChRs

NAChRs are ligand-gated ion channels that form a pentamer shape that transverses the membrane forming a central pore that is aligned by TMDs 1 and II. These receptors when activated by agonists cause muscle to contract and neurons to become excitable. These responses are regulated by desensitization following prolonged agonist exposure. The functional basis of activation and desensitization of nAChR can be caused by the subunit combination composition of the nAChR. Because nAChRs make up a diverse group of ligand-gated ion channels, it makes them difficult to study and analyze. Understanding the nAChRs in the CNS via there function and subunit composition is becoming clearer with pharmacological, molecular and desensitization studies, but similar studies of nAChRs in the ENS have not been done. Therefore, in future studies, it would be important to identify how subunit composition and nAChR modulation plays a role in GI tract function.

Functional significance of desensitization

There are multiple neurotransmitters and neurotransmitter receptors in the ENS. Drugs which either mimic or block the action of these neurotransmitters can alter gastrointestinal function *in vivo* and *in vitro*. Most previous studies have examined neurotransmission occurring during activation of only a single receptor. A question that has not been addressed is: Are there functional interactions between different receptors activated simultaneously during enteric neurotransmission? One way receptors can interact functionally is by modulation of desensitization. Activation of nAChRs excites enteric neurons and increases intracellular Ca^{2+}. Activation of M_1 receptors also excites enteric neurons and increases intracellular Ca^{2+}. Acceleration of nAChRs desensitization following M_1 activation may provide a negative feedback inhibition on nAChRs to minimize Ca^{2+} entry and neuronal excitation during bursts of high synaptic activity.

Overall significance

Because ACT is the principal excitatory transmitter in the ENS, cholinergic neurotransmission plays a critical role in controlling normal gastrointestinal function. Changes in cholinergic neurotransmission may be associated with gastrointestinal motility disorders such a diabetic gastropareisis, intestinal pseudo-obstruction, and constipation-irritable bowel syndrome. Therefore, it is relevant to study desensitization of nAChRs which is modulated by activation G-protein coupled receptors that activate intracellular signaling mechanisms.

HYPOTHESIS

Desensitization of the nAChR in the ENS is modulated by simultaneous activation of membrane bound G-protein receptors that activate intracellular signaling mechanisms. These intracellular signaling mechanisms alter the functional properties of nAChRs. **Specifically**, mAChR activation accelerates desensitization of nAChRs in the ENS.

RESEARCH DESIGN AND METHODS

PROTOCOL

Myenteric neurons maintained in primary culture and whole cell patch clamp methods were used to record inward currents caused by nAChR agonists. Neurons were voltage clamped at -60mV (resting membrane potential for myenteric neurons) **(Hodgkiss and Lees, 1983; Furness and Costa, 1987)** and agonists were applied by gravity fed flow tubes positioned within 100 μm of the neurons. Desensitization caused by ACh and nicotine was studied. The agonist concentration was 1mM based on previous dose-response curves performed in the laboratory and in the literature showing that the maximal response occurs at 1mM concentration for the two agonists **(Zhou and Galligan 1998, and Fenster et al 1997)**. Agonists were applied for 7 seconds to ensure complete desensitization of nAChRs. Peak desensitization was measured as the current remaining at 7 seconds (I_{7s}) as a fraction of peak current (I_{peak}). Desensitization rate was measured using an exponential standard fit and the Levenberg-Marquardt least square minimizing algorithm to determine the time course of decay of the agonist current during continuous application. Recovery rate was measured as the time required for complete recovery of agonist induced current after desensitization.

EXPERIMENTS

MAChR activation modulates nAChR desensitization

To test that desensitization of nAChRs is modulated by mAChR activation, the first study tested that ATP and GTP is needed to mediate the function of nAChRs. In this study, ACh was applied and ATP and GTP were eliminated from pipette solution. Peak desensitization, desensitization rate, and recovery rate were measured. The results were compared to those obtained under control conditions in which the standard pipette solution contains 1mM ATP and 0.25mM GTP.

The second study tested the affects of the mAChR antagonist scopolamine (1μM) on ACh and nicotine-induced desensitization of nAChRs. In this study, scopolamine was added to the extracellular solution and the drug-containing flow tubes. Peak desensitization, rate and recovery rate were measured and the results were compared to obtained with scopolamine free extracellular solution.

Cross-Desensitization

This study tested the hypothesis that ACh and nicotine act at the same set of nAChRs by attempting to demonstrate that they cross desensitize. In this study, nicotine was applied for 2 seconds to obtain control peak responses. Then ACh was applied for 7 seconds to desensitize the nAChR and then nicotine was re-applied for 2 seconds. The complementary experiment was also done, where ACh was applied for 2 seconds, then nicotine was applied for 7 seconds to desensitize the nAChR and then ACh was re-tested. The amplitude of the test

response (after desensitization) was compared to the initial control response for each agonist and this ratio was taken as an index of cross-desensitization.

Forskolin induced modulation of nAChR desensitization

This tested the effects of forskolin application on myenteric neurons. This study examined the possible role that cAMP-dependent activation has in modulating nAChR desensitization. Scopolamine (1μM) was present throughout these studies. In this study, ACh (1mM) application was applied for 7 seconds to myenteric neurons with additions of varied concentrations of forskolin (100nM, 1μM, and 10μM). The complementary experiment was also done in which nicotine (1mM, 7 seconds) was applied in the presence of varied concentrations of forskolin (10nM, 100nM, 1μM, and 10μM). To test that forskolin was not acting nonspecifically to alter nAChR function, 1,9-dideoxy-forskolin (DDF), an inactive analog of forskolin, was applied. Concentrations of DDF used were 10nM, 100nM, 1μM, and 10μM.

Phorbol 12, 13-dibutyrate (PDBu) induced nAChR desensitization

PDBu was used to examine the possible role PKC activation and PI hydrolysis pathway may have on modulating nAChR desensitization. Scopolamine (1mM) was present throughout these studies. This was tested using ACh (1mM, 7 seconds) application with varied concentrations of PDBu, 10nM, 30nM, 100nM, and 300nM. The complementary experiment was also done in which nicotine (1mM, 7 seconds) was applied to the neurons with the same concentrations of PDBu. To test that PDBu was not acting nonspecifically to alter nAChR function,

4-α-Phorbol 12-myristate 13-acetate (4-α-PMA), and inactive phorbol ester analog, was applied in the presence of ACh (1mM, 7 seconds) and nicotine (1mM, 7 seconds). The concentrations of 4-α-PMA used were 10nM, 30nM, 100nM, and 300nM.

Bethanecol-induced nAChR desensitization

This tested the effects of bethanecol, a muscarinic receptor agonist, application on nAChR desensitization. Bethanecol was used to examine the possible role muscarinic receptor activation may have on modulating the nAChR desensitization. In this study, 1mM-7 second nicotine application was applied to neurons which was followed by an application of nicotine plus bethanecol after a 5 minute recovery period in which the neurons were bathed in Krebs' solution. The concentrations of bethanecol used were 1μM, 10μM, 100μM, and 1mM.

GENERAL METHODS

The specific hypothesis proposed was tested by using whole cell patch clamp methods to make electrophysiological recordings from myenteric neurons maintained in primary culture. This method allows adjustments to the intracellular environment by using a patch-clamp pipette, and it also allows the transmitter ACh and other drugs to be applied to the cell via computer - controlled, solenoid-gated flow tubes. Drugs are used to activate or block receptors and intracellular signaling pathways. Any changes in nAChR desensitization are measured after drug treatment.

PRIMARY TISSUE CULTURE OF MYENTERIC NEURONS (Zhou and Galligan 1998)

The procedures used in these studies are similar to previously published methods using similar preparations. Newborn (1 to 2 days old) guinea pigs were anesthetized by halothane inhalation, stunned, and exsanguinated by severing major neck blood vessels. The ileum (20cm) were placed in 4°C Krebs bicarbonate buffer of the following composition (mM): NaCl, 117; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11. The longitudinal muscle myenteric plexus was stripped free using a moist cotton swab and then cut into 5mm pieces. Dissected tissues were divided into two equal aliquots and each aliquot was transferred to 1mL of sterile-filtered Krebs solution containing 1600 U of trypsin (Sigma Chemical Co., St. Louis, MO) for 30 minutes at 37°C. Following trypsin incubation, tissues were triturated 30 times through a fire-polished Pasteur pipette and centrifuged at medium speed for 10 minutes using a bench-top centrifuge. The supernatant was discarded and the pellet resuspended in 1mL Krebs solutions containing 4000 U crab hepatopancreas collagenase (Calbiochem-Novabiochem, Corp., La Jolla, CA). The low protease activity of this collagenase preparation allows incubation in the presence of Ca^{2+} and Mg^{2+}. The presence of Ca^{2+} in the incubation medium preserves the viability of neurons to a greater extent than obtained when incubation is done in the Ca^{2+}/Mg^{2+} - free media. The suspension was triturated using fire-polished Pasteur pipette and the centrifuged for 10 minutes. The pellet was resuspended

in Easgle's minimum essential medium (MEM) containing 10% fetal bovine serum, gentamycin (10mM/mL), penicillin (100U/mL) and streptomycin (50mg/mL) (all from Sigma). Aliquots (200mL) was plated onto sterile, poly-L-lysine (MW 30-70K, Sigma) - coated 35 mm plastic culture dishes containing 3mL of MEM. After two days of incubation, cytosine arabanoside (10mM) was added to the MEM to limit smooth muscle and fibroblast proliferation. Cultures were maintained at 37°C in a tissue culture incubator containing a 5% CO_2 atmosphere. Cultures were maintained up to 2 weeks after plating with medium replacement every 2 days.

WHOLE-CELL PATCH CLAMP RECORDINGS

These studies focused on the characterization of the agonist-induced desensitization of the nAChR in cultured neurons. The extracellular solution was the standard Krebs solution described above and was maintained at 37°C and superfused through the culture dish at a flow rate of 4 mL/min. Patch electrodes had a tip resistance of 3-7 MΩ. Electrode liquid junction potentials and series resistance was compensated. The standard intracellular (pipette) solution for patch clamp recording is as follows (mM): CsCl, 160; MgCl2, 2.9; EGTA, 10; HEPES, 10; ATP, 0.5; GTP, 0.25; pH was adjusted to 7.4 using CsOH. The $CaCl_2$/EGTA ratio yielded a resting level of free [Ca2+] of less than 100 nM. Experiments were performed using an Axopatch 200B patch clamp amplifier, a Digidata 1200 A/D converter and pCLAMP 6.01 programs for acquisition, storage

and analysis of data (Axon Instruments, Burlingame, CA). Data was filtered at 2 kHz using a 4 pole Bessel filter (Warner Instruments, New Haven, CT) and were digitized at a rate of 5 kHz and stored on the computer hard drive. Studies were performed on myenteric neurons.

DRUG APPLICATION

Drugs were applied via flow tubes gated by computer controlled solenoid valves. Four of these flow through tubes were glued together at the tip and each were connected via polyethylene tubing to a reservoir (10 mL) syringe containing a known concentration of drug. The reservoir platform was positioned above the recording chamber so that drugs were gravity fed through the flow tubes. Platform height was adjusted to provide a flow rate of 0.1 mL/min. Patch electrodes were mounted on a micromanipulator and drugs were applied by positioning the flow tube over the neuron. This method permitted rapid adjustments between control and drug-containing solution (equilibrium<100 ms).

STATISTICAL PROCEDURES

For each of the experiments described in this study, a minimum of 5 myenteric neurons were used for each treatment. Data were expressed as mean±S.E.M., Student's t-test for paired and unpaired data, and one-way ANOVA for data containing three or more treatment groups were used for comparisons.

RESULTS

MUSCARINIC ACTIVATION COUPLED ACh INDUCED nAChR DESENSITIZATION

ACh, acting at nAChRs and mAChRs are the predominant mechanisms of excitatory transmission in the ENS. These receptors are co-expressed by many myenteric neurons and when activated may cause an alteration in nAChR function. Therefore, these studies were conducted to characterize some of the properties of nAChRs and to study the effects of muscarinic receptor blockade on nAChR desensitization in myenteric neurons. Whole cell patch-clamp recordings (inward currents were measured) were obtained from myenteric neurons maintained in primary culture, and a maximum concentration of ACh (1mM) was applied via gravity fed flow tubes. In this study, a standard intracellular pipette solution containing ATP (1mM) and GTP (0.25) was used. The results from this study show that at seven seconds application of ACh, there was a maximum of 81% (ACh I_{7s} / ACh I_{peak} = 0.19±0.02, n=26) desensitization. NAChRs recovered completely from desensitization in five minutes (ACh $Imax_{5min}$ / ACh $Imax_{initial}$ = 0.93±0.04, n=5). When recordings were obtained using ATP/GTP-free pipette solutions, a seven ACh application caused a maximum desensitization of 60% (ACh I_{7s} / ACh I_{peak} = 0.4±0.04, n=18, $P<0.05$) and recovery from desensitization was complete in 5 minutes ($Imax_{5min}$ / ACh $Imax_{initial}$ = 1.11±0.12, n=8, $P>0.05$). These data suggest that ATP and /or GTP-dependent mechanisms modulate desensitization of nAChRs

in myenteric neurons (Figure 1).

Also in this study, the time course of desensitization was determined. From curve fitting analysis, a sum of a two exponential fit was the best model to describe ACh-induced nAChR desensitization at seven seconds application of ACh. The time constants for desensitization were 1.9±0.3 seconds for the slow time constant and 0.6±0.3 seconds for the fast time constant.

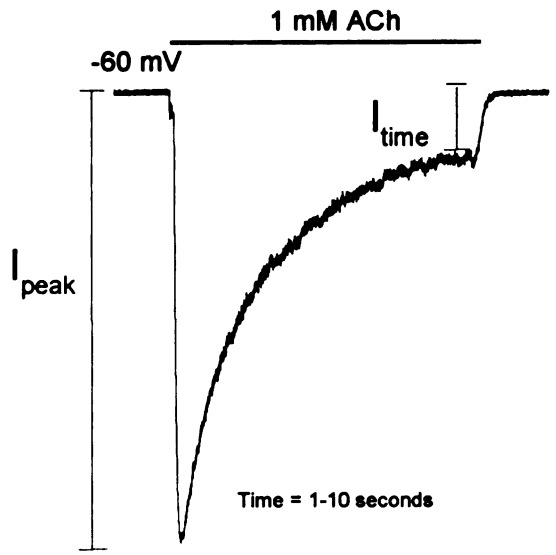
ACh acts at nAChRs and mAChRs. MAChRs couple to intracellular signaling that use ATP and/or GTP as substrates. Therefore, the possibility that mAChR activation altered nAChR desensitization was tested. Scopolamine (1μM), a muscarinic antagonist, was added to the extracellular solution. The agonist application protocol described above was used. It was found in the presence of scopolamine that ACh induced a maximum desensitization of 57% (ACh I_{7s} / ACh I_{peak} = 0.43±0.04, n=16, $P \leq 0.0001$) compared to control. Recovery from desensitization was complete in 30 seconds. Therefore, this suggests that simultaneous activation of mAChRs can modulate nAChR desensitization (Figure 2 and Figure 3).

Lastly, to show that scopolamine did not act nonspecifically to alter nAChR function, nicotine, which does not activate muscarinic receptors, was applied in the presence of scopolamine. It was found that scopolamine did not affect nAChR desensitization caused by nicotine (Nic I_{7s} / I_{peak} = 0.34±0.04, n=7; $Nic_{scopolamine}$ I_{7s} / I_{peak} = 0.33±0.07, n=6, $P \geq 0.9$). Recovery was complete in 5 minutes (Figure 4). Also in this study, the time course of desensitization was calculated using an

Figure 1. Desensitization rate of the nAChR. **(A)** Trace of an inward current caused by ACh. I_{peak} is the maximum current flowing through the nAChR and I_{time} is the current flowing through the nAChR where time of application is 1 to 10 seconds. **(B)** Plot of I_{time} / I_{peak} vs. Time application. ACh with ATP and GTP compared to ACh without ATP and GTP in pipette solution and ACh in the presence of scopolamine with ATP and GTP in pipette solution desensitizes faster with the nAChR approximately 80% desensitized at 7 seconds. (*P value<0.05)

Desensitization Rate of the nAChR

A



B

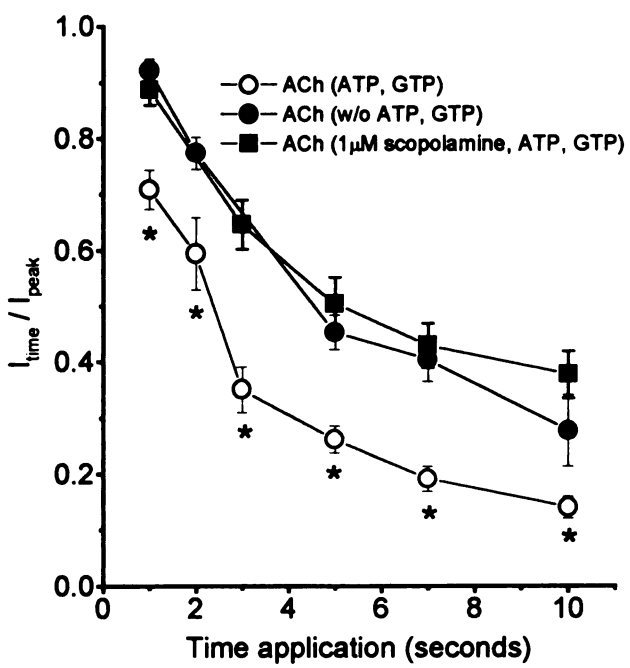
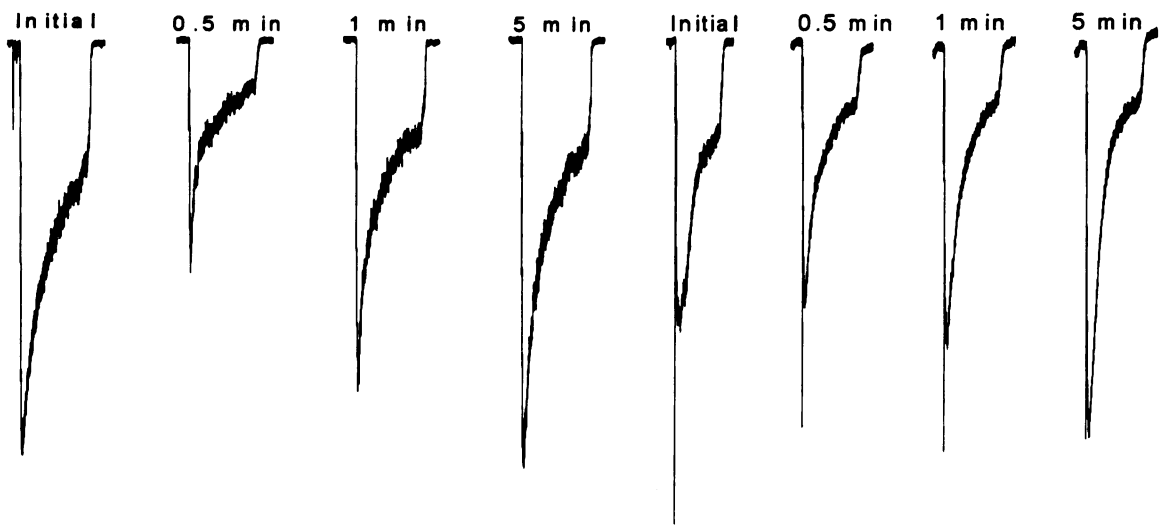


Figure 2. Recovery rate of the nAChR from desensitization. (A) Initial current peak vs. 0.5,1,5 minute recovery period between ACh application in presence of ATP and GTP in the pipette solution (Control); nAChR recovery takes 5 minutes. **(B)** Initial current peak vs. 0.5, 1 , 5 minute recovery period between ACh application without ATP and GTP present in intracellular solution. **(C)** Initial current peak vs. 0.5, 1, 5 minute recovery between ACh application in presence of scopolamine. Traces show that nAChR recovery in presence of scopolamine takes approximately 30 seconds.

Traces of Recovery Rate of the nAChR from Desensitization

A. ACh with ATP, GTP

B. ACh without ATP, GTP



C. ACh with scopolamine, ATP, GTP

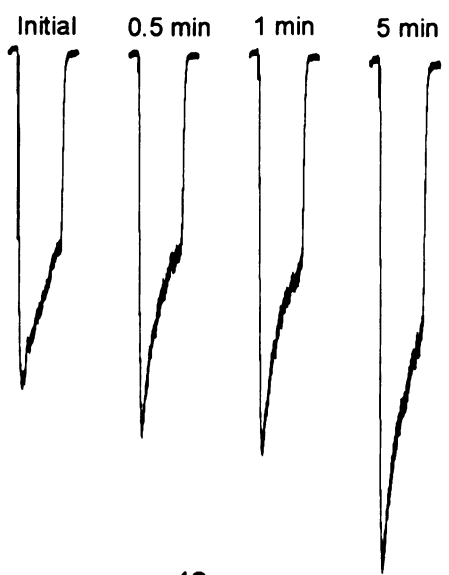


Figure 3. Recovery rate of nAChR from desensitization. Recovery plot of I_{time} / $I_{initial}$ vs. Recovery period where time equals 0.017 to 10 minutes. Data suggest that recovery rate is affected by muscarinic receptor activation. *P value<0.05 compared to ACh with ATP and GTP and #P value<0.05 compared to ACh with scopolamine, ATP, and GTP.

Recovery Rate of nAChR from Desensitization

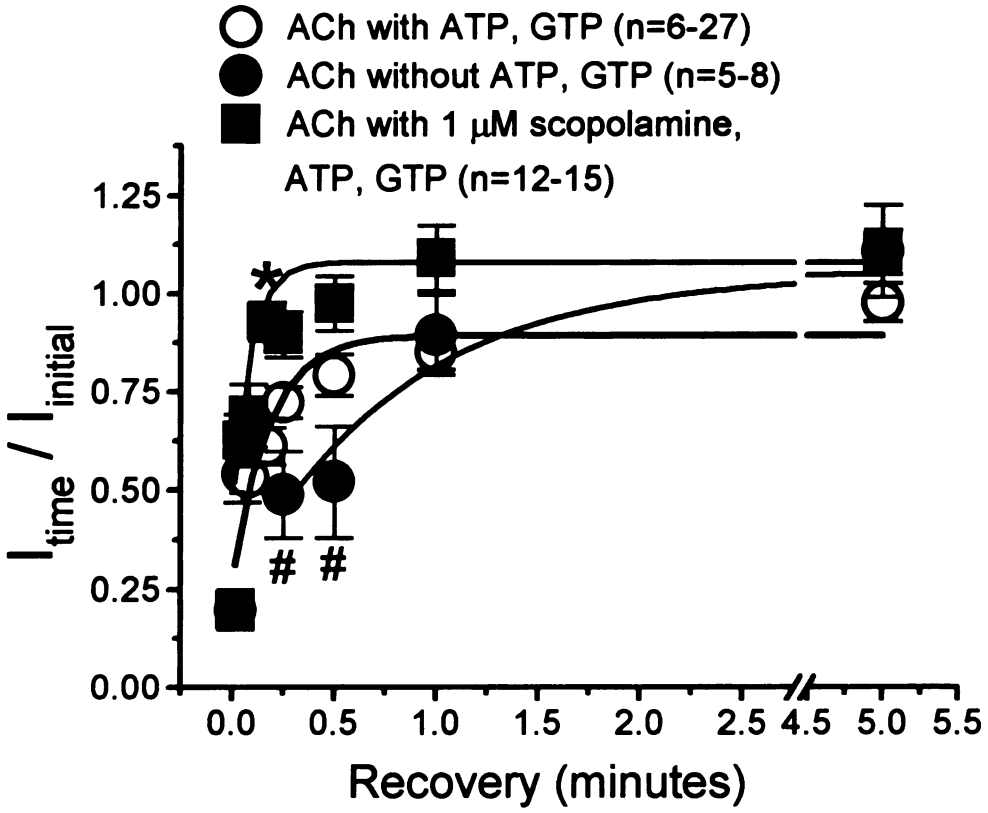
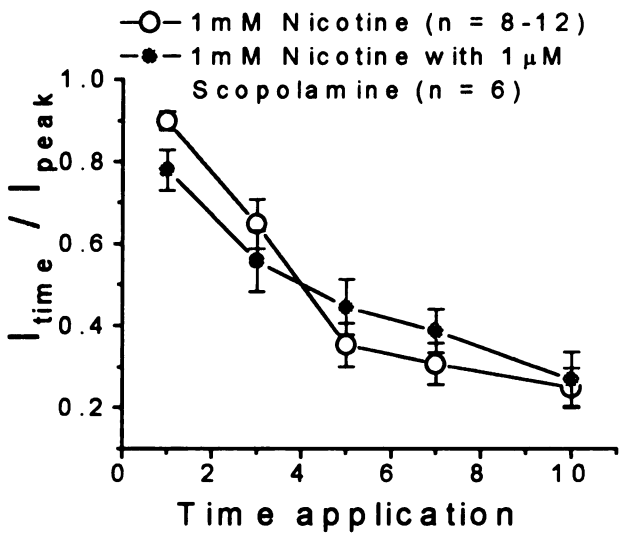


Figure 4. Nicotine effects on nAChR desensitization and recovery in presence of scopolamine. **(A)** Desensitization rate plot shows that scopolamine does not affect nicotine induced nAChR desensitization. **(B)** Scopolamine does not affect recovery rate from nicotine induced nAChR desensitization.

Nicotine Effects on nAChR Desensitization and Recovery in Presence of Scopolamine

A



B

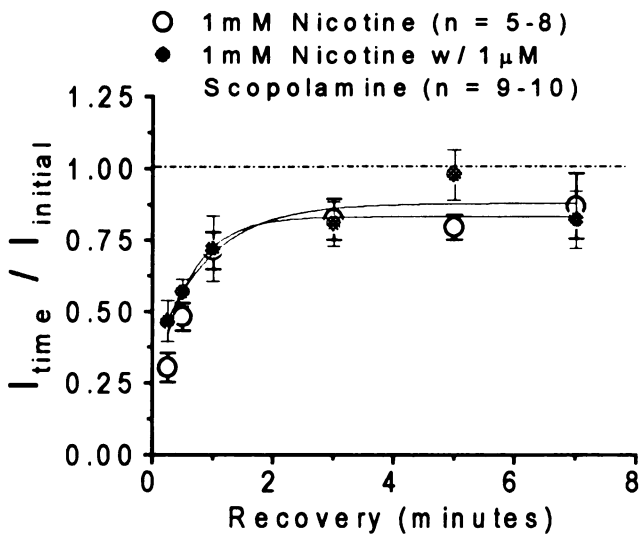
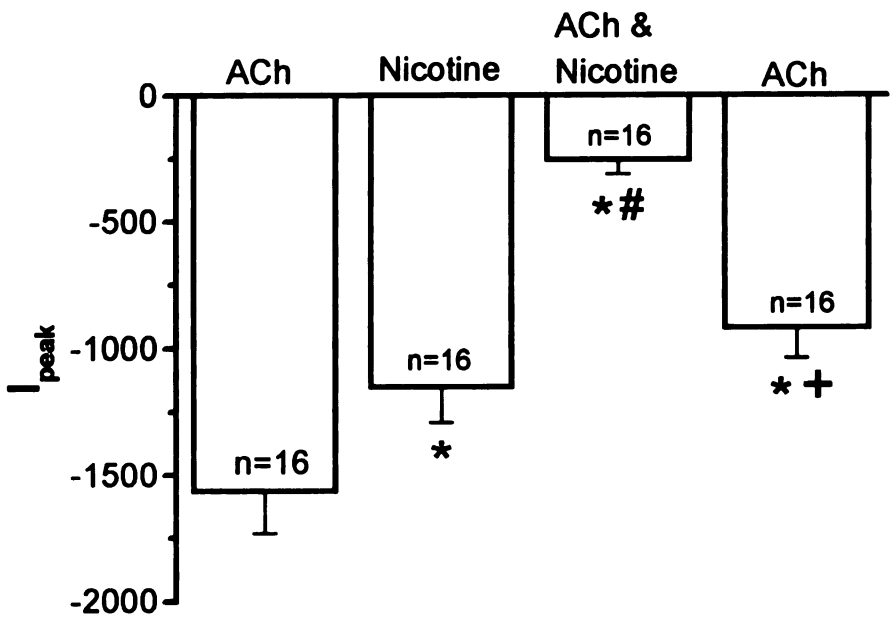


Figure 5. Cross-desensitization effects of the nAChR. Plot of 1mM agonist application vs. I_{peak}. Data suggest that ACh and nicotine act at the same nAChR. *P value<0.05 when compared to initial ACh, #P value<0.05 when compare to nicotine, and +P value<0.05 when compare to ACh and nicotine mixture.

Cross-Desensitization Effects of the nAChR



exponential standard equation for the function and a Levenberg-Marquardt for the search method to obtain the best model to fit nAChR decay during a seven second application of nicotine. From this curve fitting analysis, a sum of a two exponential fit was the best model for nAChR decay at seven seconds application of nicotine. The time constants were 2.2±0.4 seconds for the slow time constant and 0.2±0.1 seconds for the fast time constant. These were not significantly different from ACh time constant parameters ($P>0.05$).

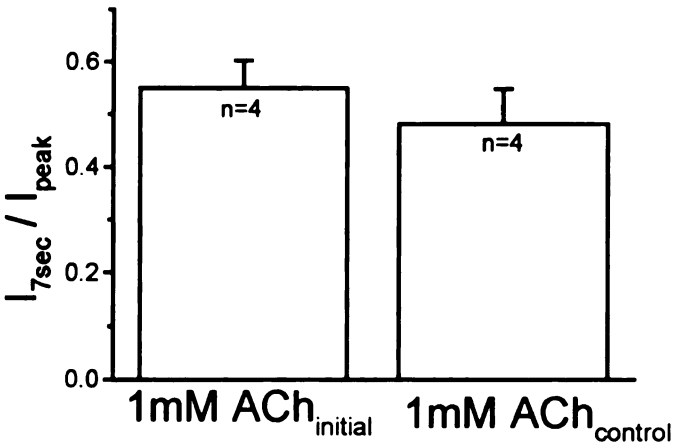
CROSS-DESENSITIZATION OF nAChRs IN MYENTERIC NEURONS

ACh and nicotine are the two nAChR agonists that were used in these experiments to induce nAChR responses. From this a question arose as to whether there were more than one population of nAChRs present on myenteric neurons, and if there were more than one, then does co-application of ACh and nicotine act at the same nACHR subunit subtype composition to result in a cross-desensitized (inactivated) nAChR. In this study, ACh was applied for two seconds, to obtain a peak response, which was followed by five minutes of recovery. Then ACh was applied for seven seconds to desensitize the nAChR followed by two seconds of nicotine application. The results indicate that application of ACh and nicotine mixture following ACh and nicotine application reduces I_{peak} to -258±58 pA ($P<0.05$) compared to the initial ACh I_{peak} of -1565±168 pA and to nicotine I_{peak} of -1150±144 pA ($P<0.05$), and the recovery of the nAChR had a partial I_{peak} recovery of -923±116 pA ($P<0.05$). Therefore, these data suggest that ACh and nicotine act at the same nAChR in

Figure 6. Time control effects of ACh-induced nAChR desensitization. (A) Histogram of I_{7sec} / I_{peak} vs. $ACh_{initial}$ (1mM) and $ACh_{control}$ (1mM). $ACh_{initial}$ and $ACh_{control}$ were not significantly different, and therefore, this indicates that repeated ACh application in presence of scopolamine (1μM) recovers in five minutes. **(B)** Histogram of I_{7sec} / I_{peak} vs. $ACh_{initial}$ (1mM) and ACh (1mM) plus 0.05% EtOH. $ACh_{initial}$ and ACh plus 0.05% EtOH were not significantly different and indicated that the 0.05%EtOH vehicle does not alter nAChR desensitization. **(C)** Histogram of I_{7sec} / I_{peak} vs. $ACh_{initial}$ (1mM) and ACh (1mM) plus 0.005% EtOH. $ACh_{initial}$ and ACh plus 0.005% EtOH were not significantly different and indicated that the 0.005%EtOH vehicle does not alter nAChR desensitization.

Time Control Effects of ACh-induced nAChR desensitization

A



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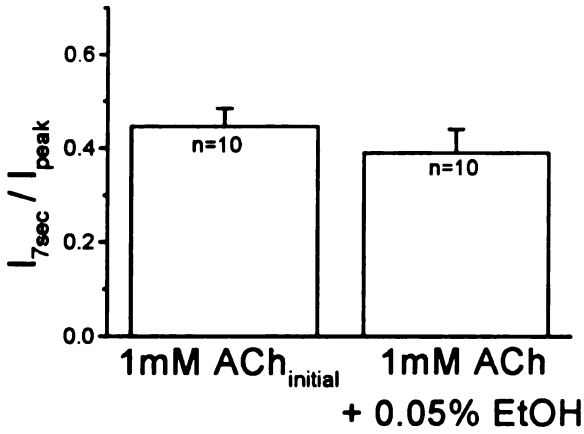

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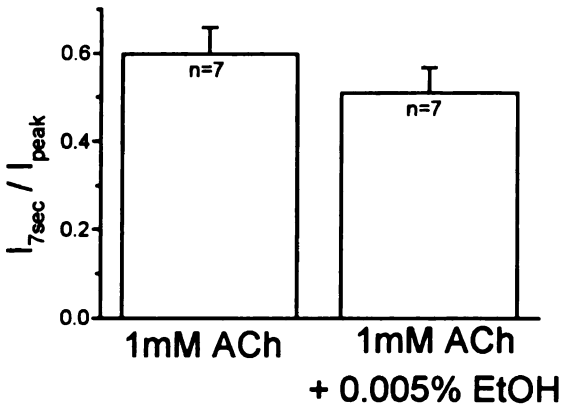

Figure 7. Time control effects of nicotine-induced nAChR desensitization. (A) Histogram of I_{7sec} / I_{peak} vs. $Nicotine_{initial}$ (1mM) and $Nicotine_{control}$ (1mM). $Nicotine_{initial}$ and $Nicotine_{control}$ were not significantly different, and therefore, this indicates that repeated Nicotine application recovers in five minutes. **(B)** Histogram of I_{7sec} / I_{peak} vs. $Nicotine_{initial}$ (1mM) and Nicotine (1mM) plus 0.05% EtOH. $Nicotine_{initial}$ and Nicotine plus 0.05% EtOH were not significantly different and indicated that the 0.05%EtOH vehicle does not alter nAChR desensitization. **(C)** Histogram of I_{7sec} / I_{peak} vs. $Nicotine_{initial}$ (1mM) and Nicotine (1mM) plus 0.005% EtOH. $Nicotine_{initial}$ and Nicotine plus 0.005% EtOH were not significantly different and indicated that the 0.005%EtOH vehicle does not alter nAChR desensitization.

Time Control Effects of Nicotine-induced nAChR desensitization

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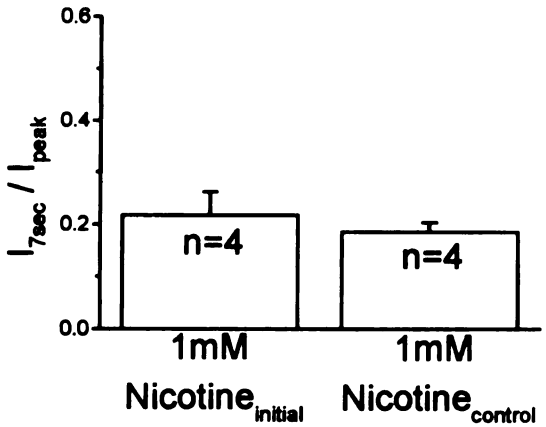

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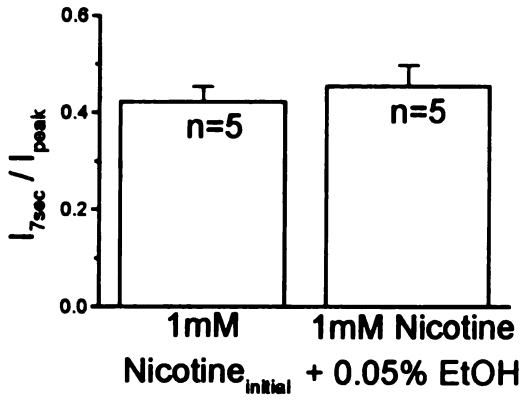

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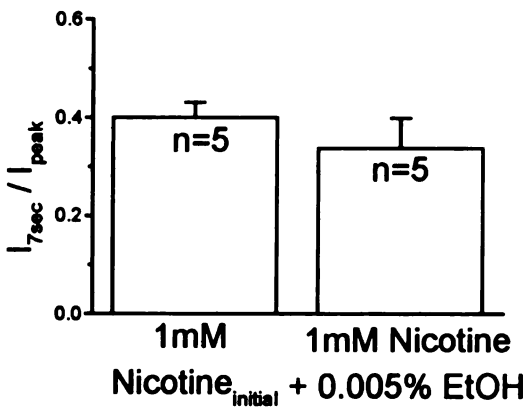


myenteric neurons (Figure 5).

TIME CONTROL OF ACh AND NICOTINE

A time control for nAChR inward current recordings was examined using a seven second application of 1mM ACh (1μM scopolamine present) and 1mM nicotine, and because 0.05 and 0.005% concentrations of ethanol (EtOH) were used as the vehicle for forskolin, DDF, PDBu, and 4-α-PMA solubility. EtOH was added to the control drug applications. This experiment was performed to show that desensitization of nAChRs was reproducible and that run-down of ACh or nicotine responses does not occur during the time course of these studies. Therefore, in the presence of ACh-scopolamine, the 0.05% EtOH time control had an initial ACh desensitization response of 55.5±4% (n=10) and a ACh-0.05% EtOH desensitization response of 61±5% (n=10,.$P \geq 0.05$). The 0.005% EtOH time control had an initial ACh desensitization response of 40.2±6% (n=7) and a ACh-0.005% EtOH desensitization response of 49±6% (n=7, $P \geq 0.0.05$) (Figure 6).

In the presence of nicotine, the 0.05% EtOH time control had an initial nicotine desensitization response of 57.8±3% (n=5) and a nicotine-0.05% EtOH desensitization response of 54.6±4% (n=5, $P \geq 0.05$). The 0.005% EtOH time control had an initial nicotine desensitization response of 60±3% (n=5) and a nicotine-0.005% EtOH desensitization response of 66.2±6% (n=5, $P \geq 0.05$). These data indicate that recovery from 7second-1mM ACh and nicotine - induced nAChR desensitization is complete in 5 minutes, and that the vehicle does not alter nAChR

desensitization (Figure 7).

PDBu INDUCED MODULATION OF nAChR DESENSITIZATION

Previous studies have shown that mAChR activation couples to G_q protein which activates phosphatidyl inositol (PI) intracellular signaling pathway **(Hulme1990)**. Therefore, the effects of PDBu, a PKC activator was examined to test for a possible role of PKC activation in modulating nAChR desensitization. ACh (1mM) was applied for seven seconds in the presence of varied concentrations (10nM, 30nM 100nM, and 300nM) of PDBu. The data suggest that PKC activation and the PI hydrolysis pathway are involved in modulating the nAChR because at 10nM, 30nM, 100nM and 300 concentrations of PDBu, there was a significant increase in nAChR desensitization of 45% (I_{7s} / I_{peak} = 0.55±0.07, n=5, $P \leq 0.05$), 71% (I_{7s} / I_{peak} = 0.29±0.05, n=7, $P \leq 0.05$), 56% (I_{7s} / I_{peak} = 0.44±0.04, n=5, $P \leq 0.05$), and 62% (I_{7s} / I_{peak} = 0.38±0.04, n=6, $P \leq 0.05$), respectively, compared to the initial ACh desensitization response of 41% (I_{7s} / I_{peak} = 0.59±0.07, n=5), 51% (I_{7s} / I_{peak} = 0.49±0.04, n=7), 35% (I_{7s} / I_{peak} = 0.65±0.07, n=5), and 51% (I_{7s} / I_{peak} = 0.49±0.02, n=6), respectively (Figure 8 and Figure 9).

To test that PDBu was not acting nonspecifically to alter nAChR function, 4-α-Phorbol 12-myristate 13-acetate (4-α-PMA), an inactive phorbol ester analog, was applied in the presence of ACh (1mM) applied for seven seconds. The data show that there was no change in nAChR desensitization at any 4-α-PMA concentration (10nM I_{7s} / I_{peak} = 0.48±0.05, n=5, $P \geq 0.8$; 30nM I_{7s} / I_{peak} = 0.6±0.06,

Figure 8. Traces of PDBu and 4-α-PMA effects on ACh-induced nAChR desensitization. (A) Traces of an inward current caused by $ACh_{initial}$ (1mM) and ACh (1mM) plus 30 nM PDBu in the presence of 1μM scopolamine. $ACh_{initial}$ and ACh plus 30 nM PDBu were significantly different. **(B)** Traces of an inward current caused by $ACh_{initial}$ (1mM) and ACh (1mM) plus 30 nM 4-α-PMA in the presence of 1mM scopolamine. $ACh_{initial}$ and ACh plus 30 nM 4-α-PMA were not significantly different, and therefore, this indicates that PDBu acts specifically to modulate nAChR desensitization.

Traces of PDBu and 4-α-PMA Effects on ACh-induced nAChR Desensitization

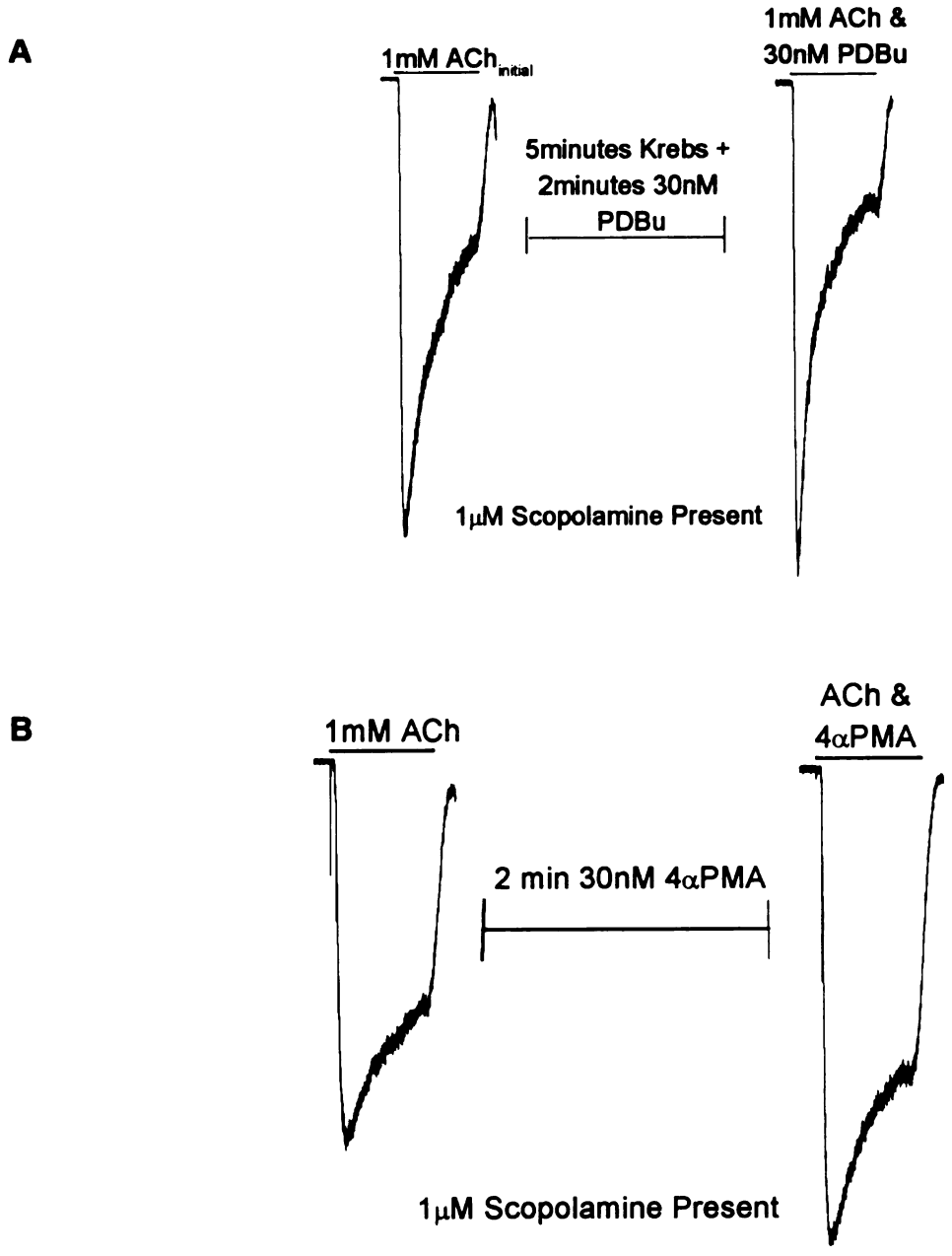


Figure 9. PDBu effects on ACh-induced nAChR desensitization. Histograms A, B, C, and D show that ACh pretreated with 10 nM, 30 nM, 100 nM, and 300 nM concentrations of PDBu, respectively, significantly increases nAChR desensitization of 45%, 71%, 56%, and 62%, respectively. This was compared to the initial ACh desensitization response of 41%, 51%, 35%, and 51%, respectively. (*P value<0.05)

PDBu Effects on ACh-induced nAChR Desensitization

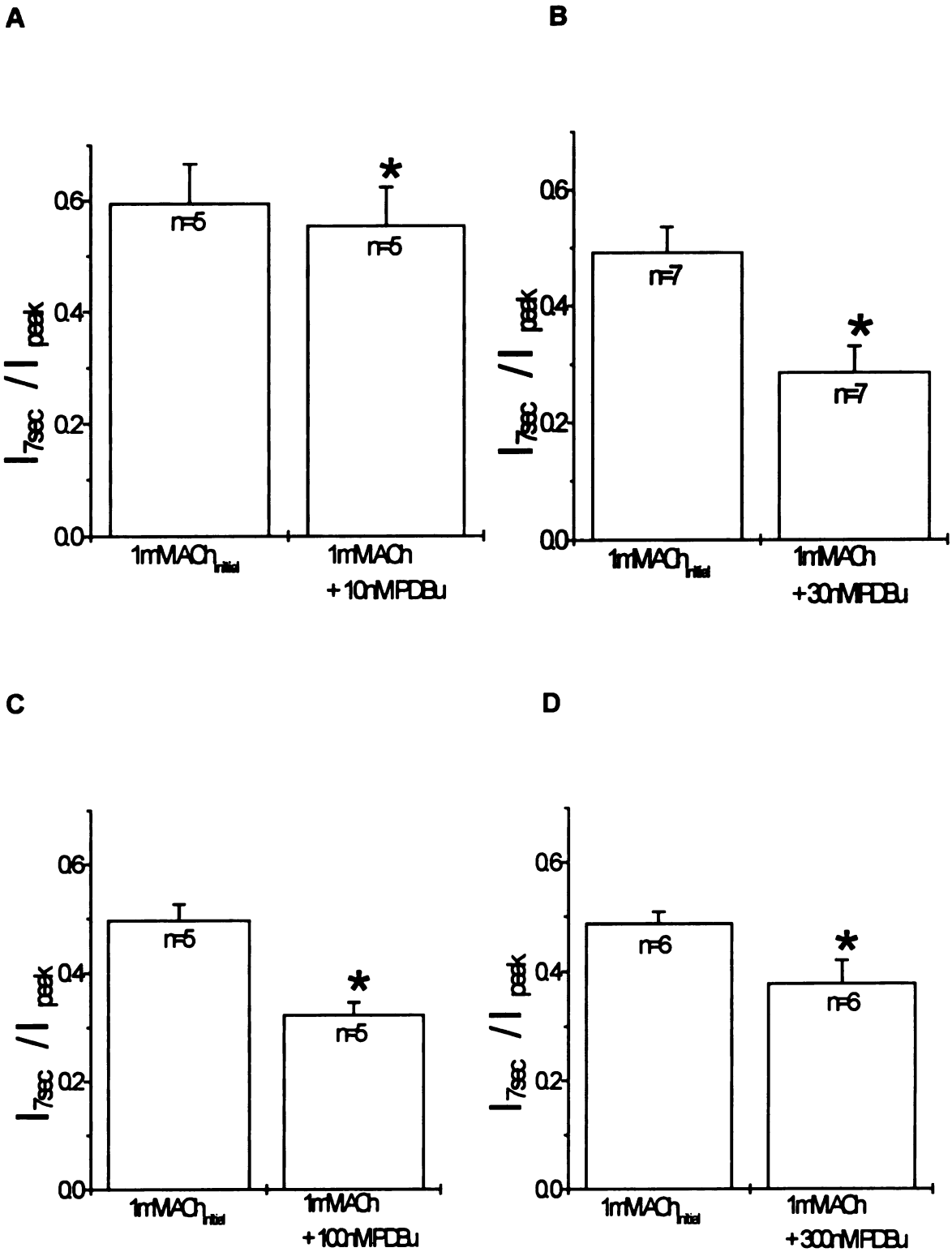


Figure 10. 4-α-PMA effects on ACh-induced nAChR desensitization. Histograms A, B, C, and D show that ACh pretreated with 10 nM, 30 nM, 100 nM, and 300 nM 4-α-PMA, respectively, do not significantly increase nAChR desensitization of 52%, 40%, 46%, and 36%, respectively. This was compared to the initial ACh desensitization response of 50%, 43%, and 41%, respectively. These data suggest that activation of PKC by PDBu acts specifically to modulate nAChR desensitization. (P value>0.05)

4-α-PMA Effects on ACh-induced nAChR Desensitization

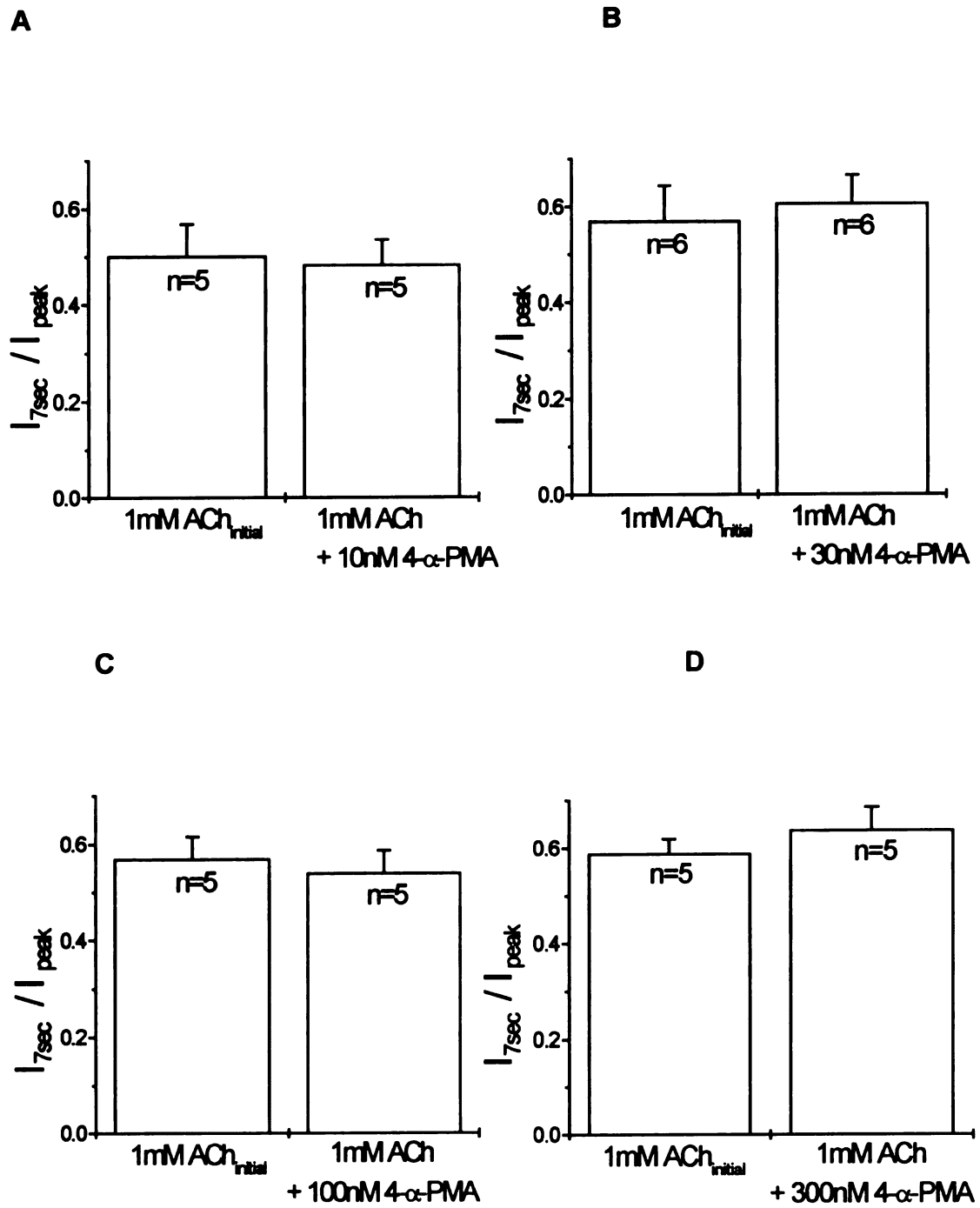
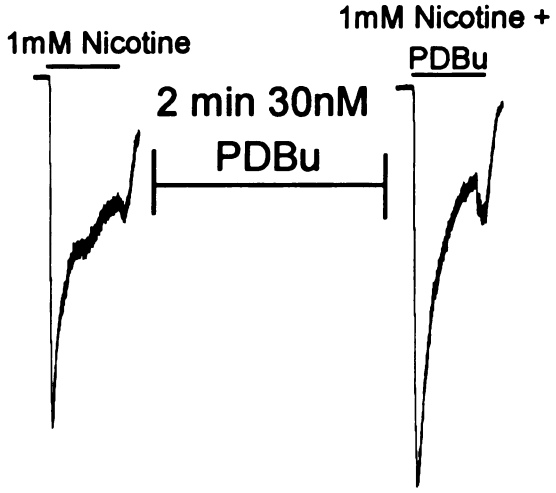


Figure 11. Traces of PDBu and 4-α-PMA effects on nicotine-induced nAChR desensitization. (A) Traces of an inward current caused by $Nicotine_{initial}$ (1mM) and Nicotine (1mM) plus 30 nM PDBu. $Nicotine_{initial}$ and Nicotine plus 30 nM PDBu were significantly different. **(B)** Traces of an inward current caused by $Nicotine_{initial}$ (1mM) and Nicotine (1mM) plus 30 nM 4-α-PMA. $Nicotine_{initial}$ and Nicotine plus 30 nM 4-α-PMA were not significantly different, and therefore, this indicates that PDBu acts specifically to modulate nAChR desensitization.

Traces of PDBu and 4-α-PMA Effects on Nicotine-induced nAChR Desensitization

A



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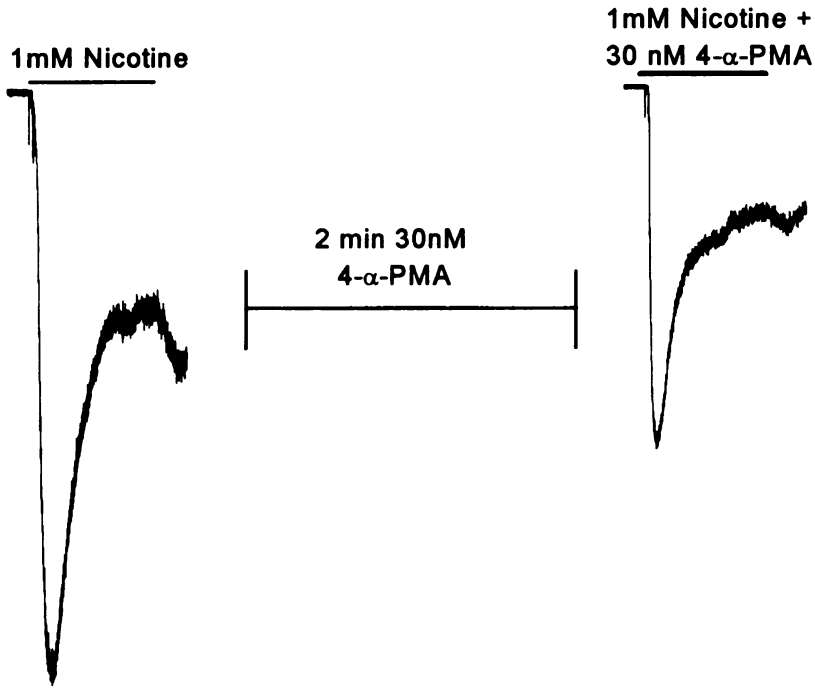


Figure 12. PDBu effects on nicotine-induced nAChR desensitization. Histograms A, B, C, and D show that nicotine pretreatment with 10 nM, 30 nM, 100 nM, and 300 nM concentrations of PDBu, respectively, significantly increases nAChR desensitization of 64%, 72%, 77%, and 74%, respectively. This was compared to the initial nicotine desensitization response of 53%, 65%, 64%, and 61%, respectively. (*P value<0.05)

PDBu Effects on Nicotine-induced nAChR Desensitization

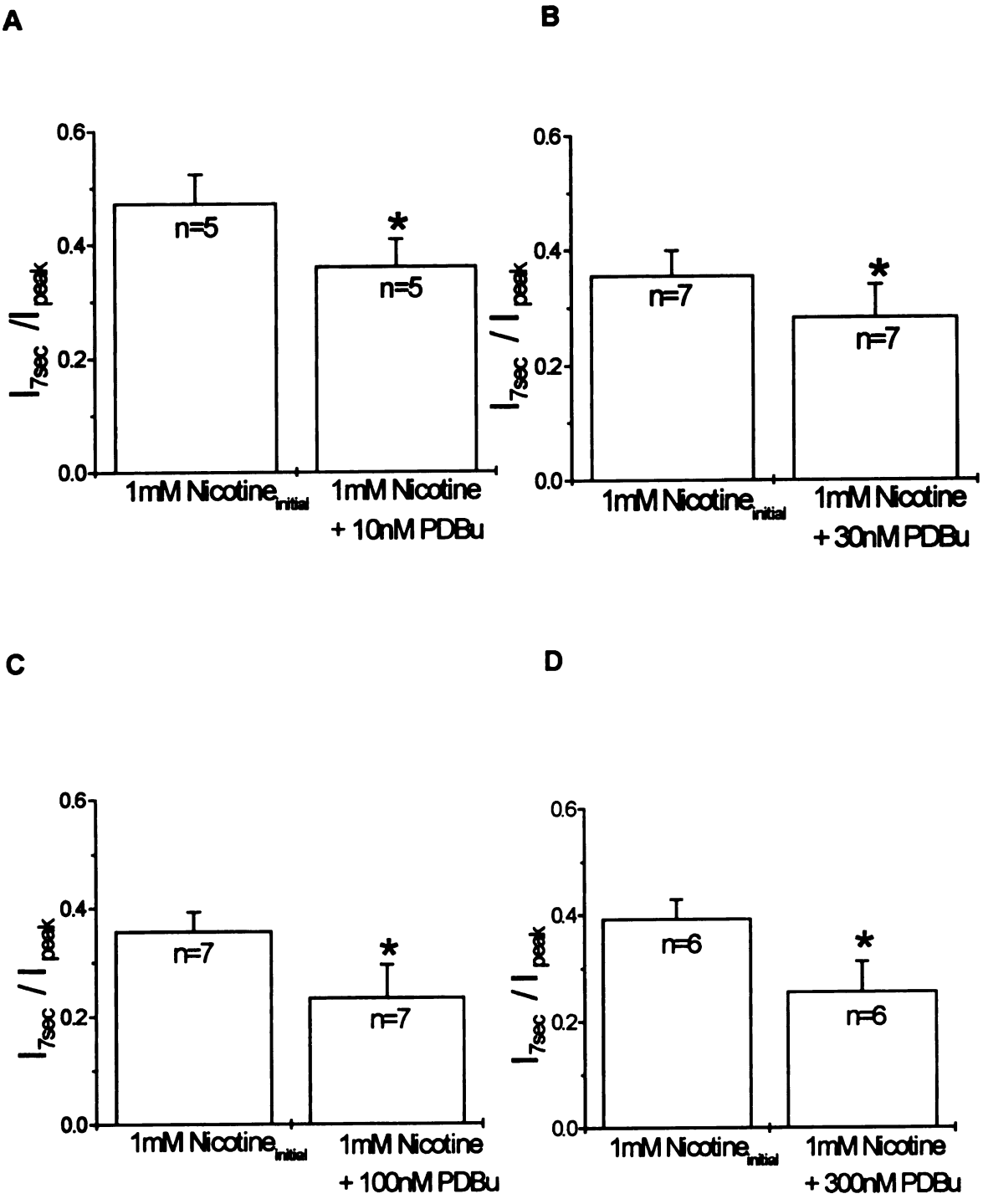
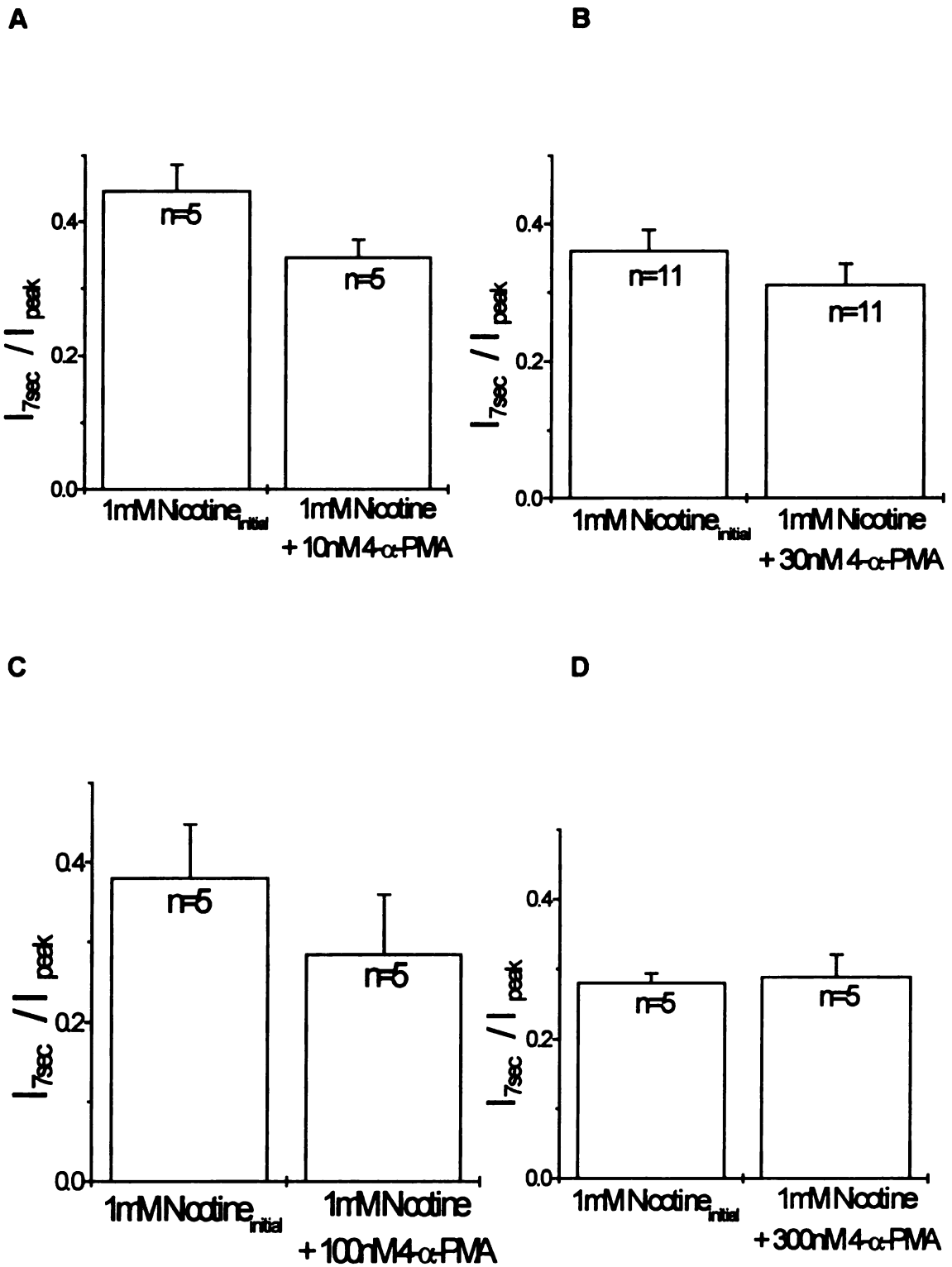


Figure 13. 4-α-PMA effects on nicotine-induced nAChR desensitization. Histograms A, B, C, and D show that nicotine pretreatment with 10 nM, 30 nM, 100 nM, and 300 nM concentrations of 4-α-PMA, respectively, do not significantly increase nAChR desensitization of 65%, 69%, 72%, and 71%, respectively. This was compared to the initial nicotine desensitization response of 60%, 64%, 62%, and 72%, respectively. These data suggest that PKC activation by PDBu act specifically to modulate nAChR desensitization. (P value>0.05)

4–α-PMA Effects on Nicotine-induced nAChR Desensitization



n=6, P≥0.7; 100nM I_{7s} / I_{peak} = 0.54±0.05, n=5, P≥0.6; 300nM I_{7s} / I_{peak} = 0.64±0.05, n=5, P≥0.05) compared to the initial ACh desensitization response (I_{7s} / I_{peak} = 0.5±0.07, n=5; I_{7s} / I_{peak} = 0.57±0.07, n=6; I_{7s} / I_{peak} = 0.57±0.05, n=5;I_{7s} / I_{peak} = 0.59±0.03, n=5). Therefore, this suggests that activation of PKC, by PDBu, acts specifically to modulate nAChR desensitization (Figure 8 and Figure 10).

In the next study, nicotine (1mM) was applied for seven seconds in the presence of varied concentrations of PDBu (10nM, 30nM, 100nM, and 300nM). The data suggest that PKC and PI hydrolysis pathway activation are involved in nicotine - induced nAChR desensitization because at 10nM , 30nM, 100nM, and 300nM concentrations of PDBu, there was a significant increase in nAChR desensitization 64% (I_{7s} / I_{peak} = 0.36±0.05, n=5, P≤0.05), 72% (I_{7s} / I_{peak} = 0.28±0.06, n=7, P≤0.05), 77% (I_{7s} / I_{peak} = 0.23±0.06, n=7, P≤0.05), and 74% (I_{7s} / I_{peak} = 0.26±0.06, n=6, P≤0.05), respectively, compared to the initial nicotine desensitization response of 53% (I_{7s} / I_{peak} = 0.47±0.05, n=5), 65% (I_{7s} / I_{peak} = 0.35±0.05, n=7), 64% (I_{7s} / I_{peak} = 0.36±0.04, n=7), and 61% (I_{7s} / I_{peak} = 0.39±0.04, n=6) (Figure 11 and Figure 12).

4-α-PMA was applied to the neurons to examine nonspecificity towards the alteration of nAChR functions. In the presence of nicotine (1mM) applied for seven seconds in addition to varied concentrations of 4-α-PMA, 10nM, 30nM, 100nM, and 300nM were used to compare to the nicotine-induced nAChR desensitization. The data show that there was not a significant effect on nAChR desensitization at 10nM, 30nM, 100nM, and 300nM concentrations of 4-α-PMA (10nM I_{7s} / I_{peak} = .35±.02,

n=8, P≥0.05; 30nM I_{7s} / I_{peak} = 0.31±0.03, n=11, P≥0.05; 100nM I_{7s} / I_{peak} = 0.28±0.08, n=5, P≥0.05; 300nM I_{7s} / I_{peak} = 0.29±0.03, n=5, P≥0.05) compared to initial nicotine desensitization response (I_{7s} / I_{peak} = 0.40±0.04, n=8; I_{7s} / I_{peak} = 0.36±0.03, n=11; I_{7s} / I_{peak} = 0.38±0.07, n=5; I_{7s} / I_{peak} = 0.28±0.01, n=5). Therefore, these data indicate that activation of PKC by phorbol esters, PDBu, act specifically to cause modulation of nAChR desensitization (Figure 11 and Figure 13).

FORSKOLIN INDUCED MODULATION OF nAChR DESENSITIZATION

Forskolin is an activator of adenylate cyclase. The effects of forskolin application on nAChR desensitization in myenteric neurons were assessed to examine the possible role of cAMP - dependent pathway activation on nAChR desensitization. In this study, ACh (1mM) was applied for seven seconds to the neurons in the presence of forskolin (100 nM, 1 μM, and10 μM). At 1μM and 10μM concentrations of forskolin, there was a significant increase in nAChR desensitization of 64% (ACh I_{7s} / I_{peak} = 0.36±0.03, n=7, P≤0.05) and 70% (ACh I_{7s} / I_{peak} = 0.30±0.06, n=7, P≤0.05), respectively, compared to the initial ACh (ACh - scopolamine) desensitization response of 49% (ACh I_{7s} / I_{peak} = 0.51±0.05, n=7) and 55% (ACh I_{7s} / I_{peak} = 0.45±0.07, n=7), respectively (Figure 14 and Figure 15).

To test that forskolin was not acting nonspecifically to alter nAChR function, 1,9 - dideoxy - forskolin (DDF), an inactive analog of forskolin, was applied at concentrations of 10nM, 100nM, 1μM, and 10μM. The data show that there was not a significant effect of nAChR desensitization at 10nM (I_{7s} / I_{peak} = 0.36±0.06, n=5,

Figure 14. Traces of forskolin effects on ACh-induced nAChR desensitization. **(A)** Traces of an inward current caused by $ACh_{initial}$ (1mM) and ACh (1mM) plus 1 μM forskolin in the presence of 1μM scopolamine. $ACh_{initial}$ and ACh plus 1 μM forskolin were significantly different. **(B)** Traces of an inward current caused by $ACh_{initial}$ (1mM) and ACh (1mM) plus 1 μM DDF in the presence of 1mM scopolamine. $ACh_{initial}$ and ACh plus 1 μM DDF were significantly different, and therefore, this indicates that forskolin acts nonspecifically at the nAChR to modulate nAChR desensitization.

Traces of Forskolin and DDF Effects on ACh-induced nAChR Desensitization

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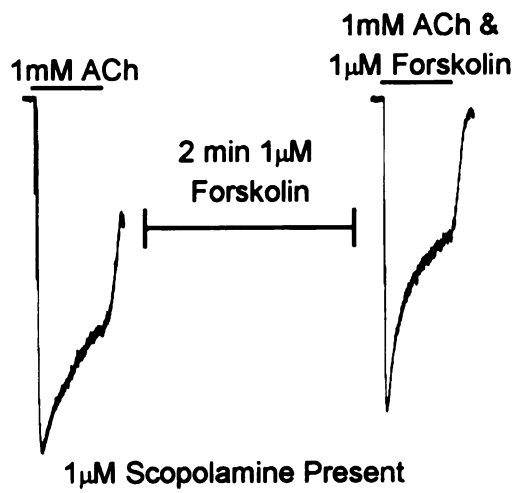

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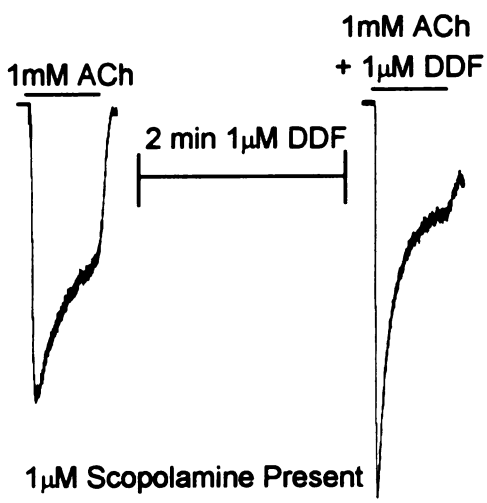

Figure 15. Forskolin effects on ACh-induced nAChR desensitization. Histogram A shows that ACh pretreatment with 100 nM forskolin does not significantly increase nAChR desensitization of 47%. This was compared to the initial ACh desensitization response of 41%. Histograms B and C show that ACh pretreatment with 1 μM and 10 μM forskolin significantly increases nAChR desensitizaiton of 64% and 70%, respectively. This was compared to the initial ACh desensitization response of 49% and 55%, respectively. (*P value<0.05)

Forskolin Effects on ACh-induced nAChR Desensitization

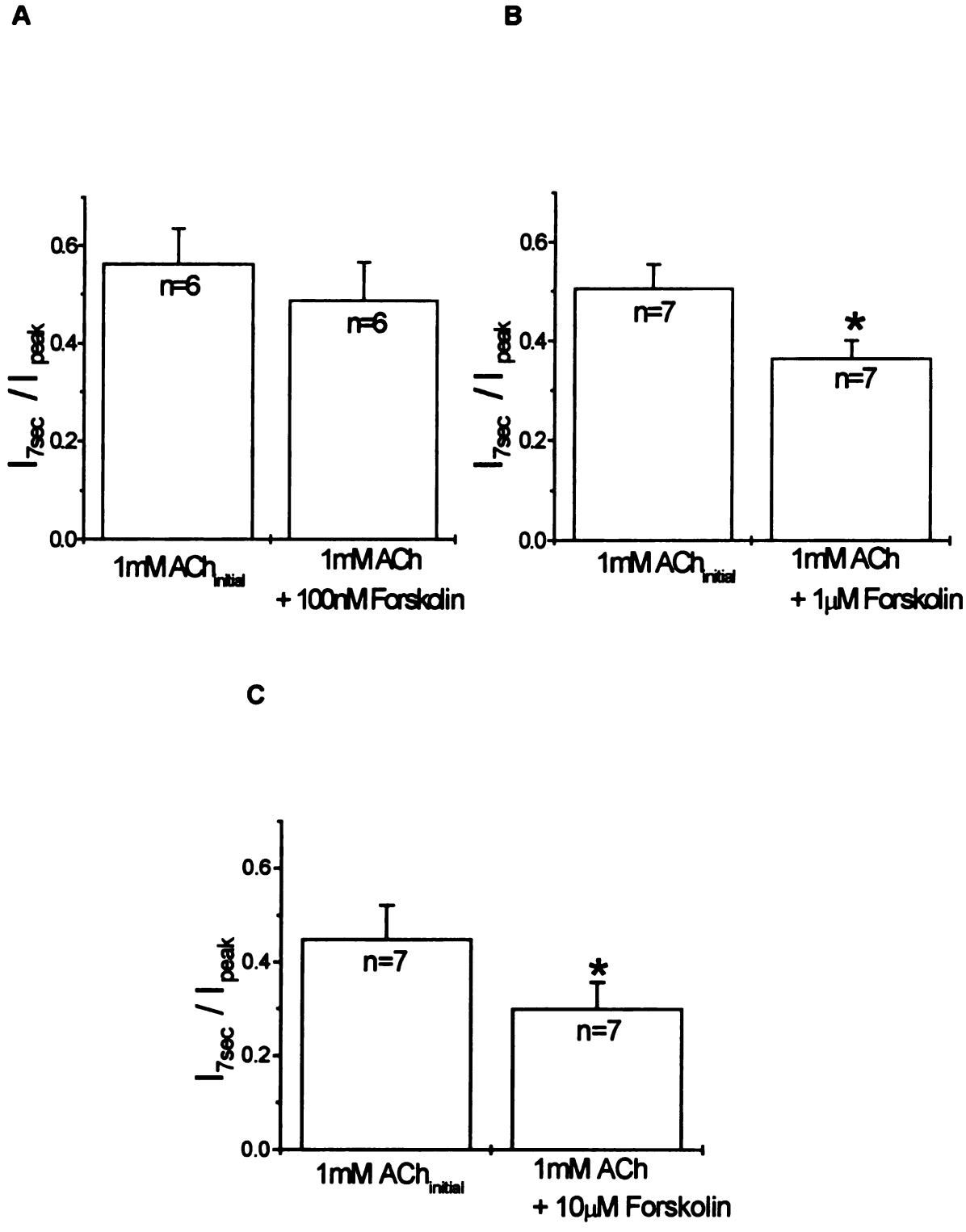

Figure 16. DDF effects on ACh-induced nAChR desensitization. Histograms A, B, and D show that ACh pretreatment with 10 nM, 100 nM, and 10μM concentration of DDF do not significantly increase nAChR desensitization of 64%, 67%, and 51%, respectively. This was compared to initial ACh desensitization response of 58%, 55%, and 51%, respectively. Histogram C shows that ACh pretreatment with 1μM DDF significantly increases nAChR desensitization of 72%. This was compared to initial ACh desensitization response of 53%. These data indicate that activation of cAMP dependent pathways by forskolin acts nonspecifically to modulate nAChR desensitization.

DDF Effects on ACh-induced nAChR Desensitization

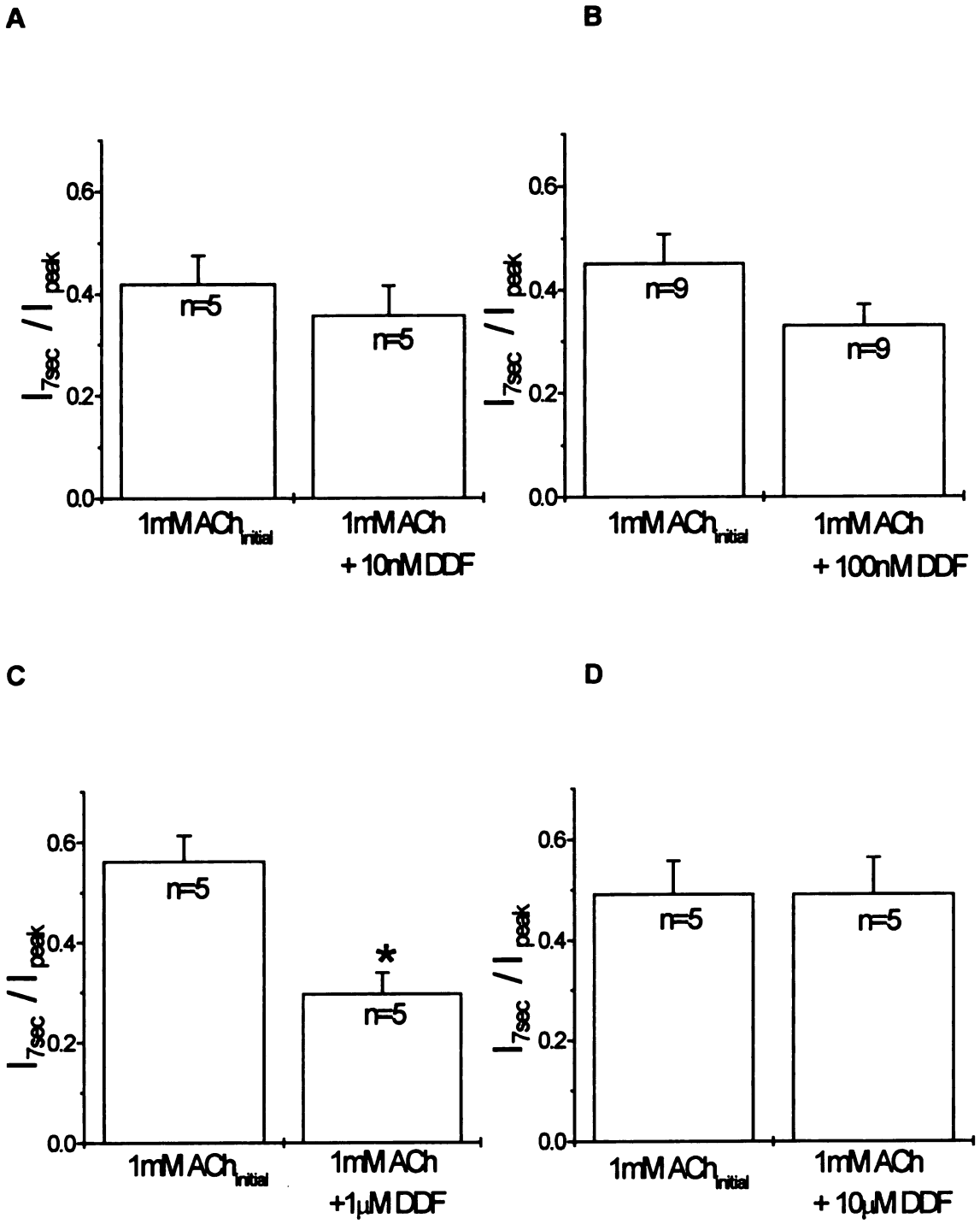

Figure 17. Traces of forskolin effects on nicotine-induced nAChR desensitization. (A) Traces of an inward current caused by $Nicotine_{initial}$ (1mM) and Nicotine (1mM) plus 1 μM forskolin. $Nicotine_{initial}$ and Nicotine plus 1 μM forskolin were significantly different. **(B)** Traces of an inward current caused by $Nicotine_{initial}$ (1mM) and Nicotine (1mM) plus 1 μM DDF. $Nicotine_{initial}$ and Nicotine plus 1 μM DDF were not significantly different but was significantly different at 10 mm DDF. Therefore, this indicates that forskolin acts nonspecifically at the nAChR to modulate nAChR desensitization.

Traces of Forskolin Effects on Nicotine-induced nAChR Desensitization

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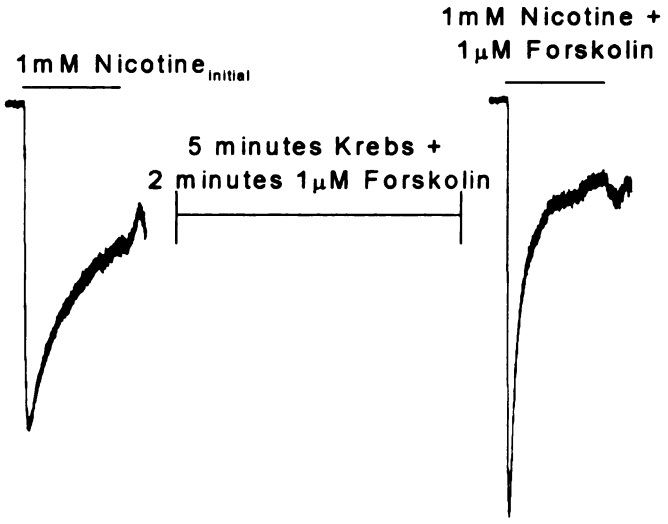

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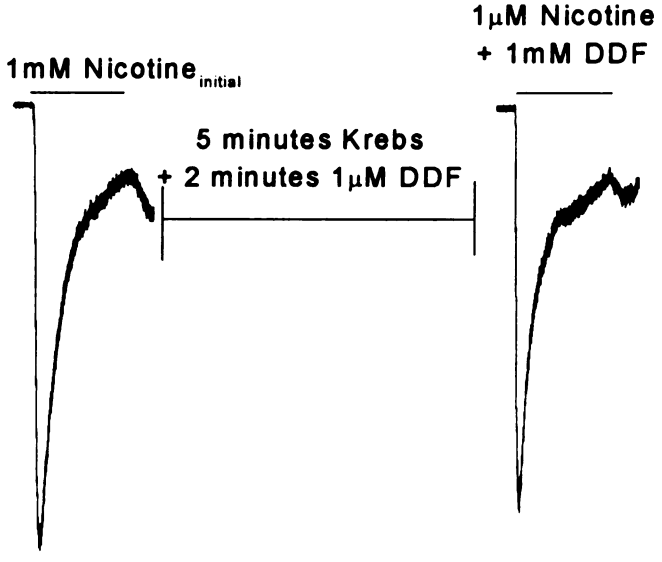


Figure 18. Forskolin effects on nicotine-induced nAChR desensitization. Histogram A shows that nicotine pretreatment with 10 nM forskolin does not significantly increase desensitization of 67% compared to the initial nicotine desensitization response of 60%. Histograms B, C, and D show that nicotine pretreatment with 100nM, 1μM, and 10μM forskolin significantly increases nAChR desensitization of 78%, 78%, and 79%, respectively, compared to the initial nicotine desensitization response of 69%, 66%, and 69%, respectively. (*P value<0.05)

Forskolin Effects on Nicotine-induced nAChR Desensitization

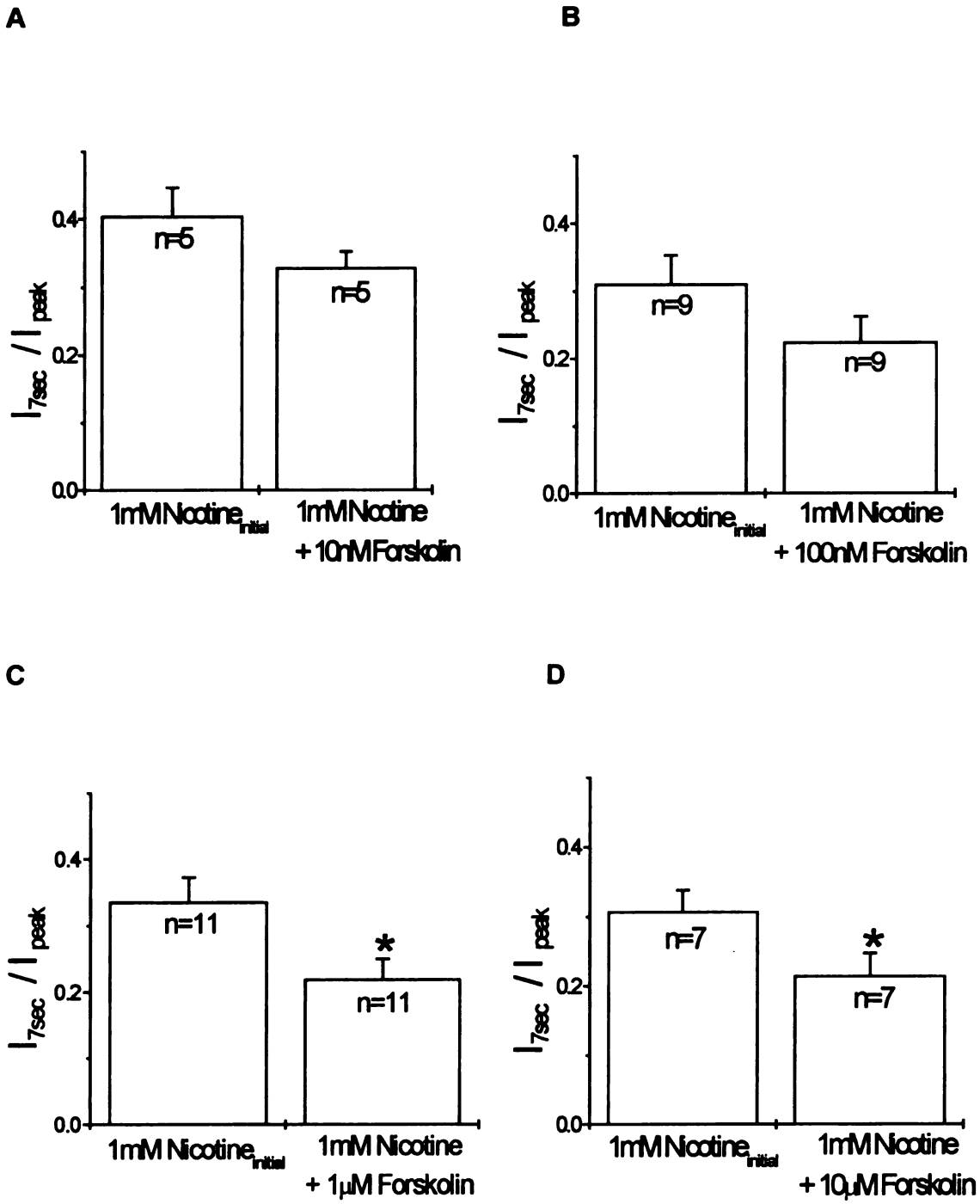
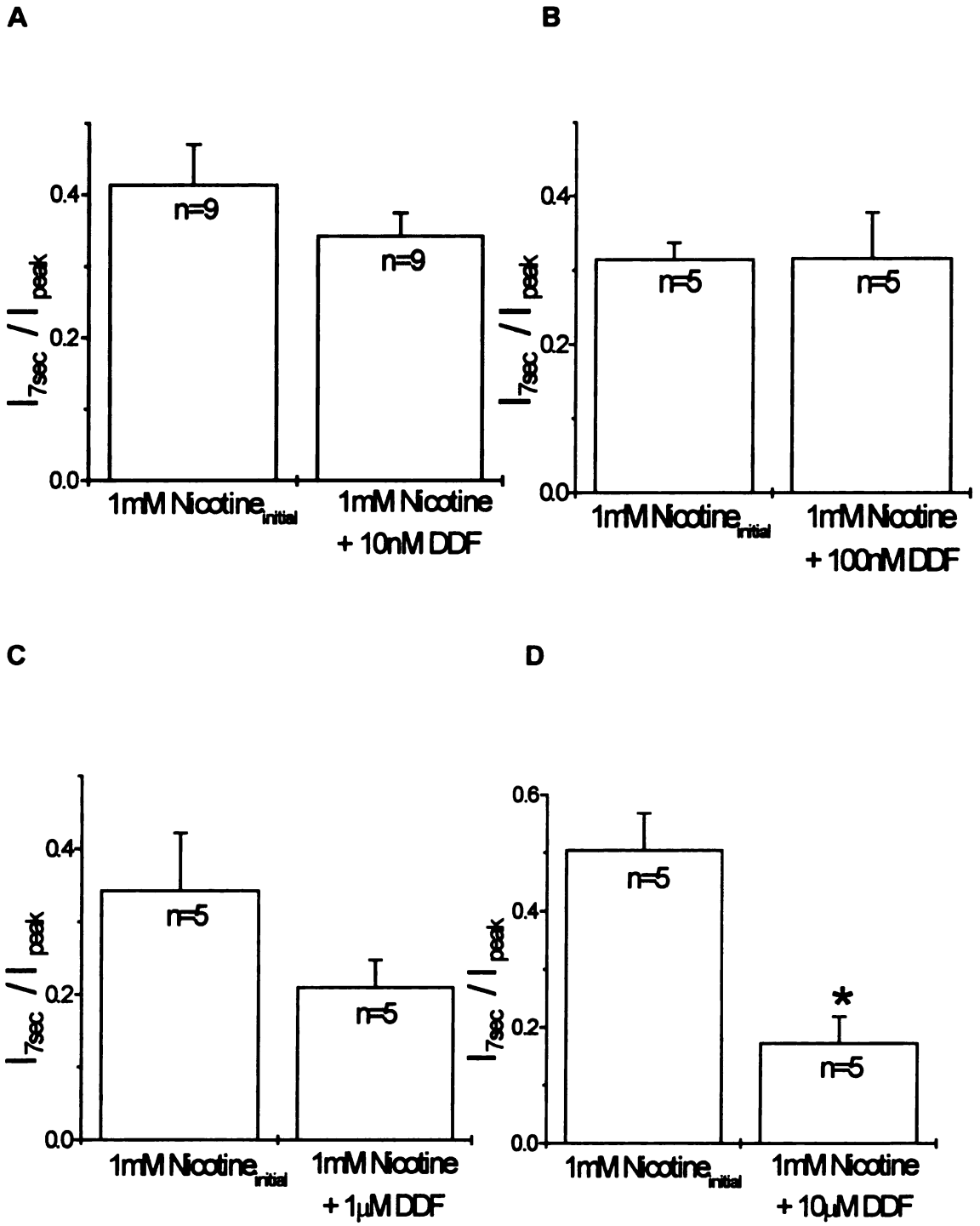


Figure 19. DDF effects on nicotine-induced nAChR desensitization. Histograms A, B, and C show that nicotine pretreatment with 10 nM, 100 nM, and 1 mm DDF do not significantly increase nAChR desensitization of 68%, 68%, and 79%, respectively, compared to initial nicotine desensitization responses of 59%, 69%, and 66%, respectively. Histogram D shows that nicotine pretreatment with 10μM DDF significantly increases nAChR desensitization of 83% compared to initial nicotine desensitization response of 50%. These data indicate that forskolin acts at the nAChR nonspecifically to modulate desensitization. (*P value<0.05)

DDF Effects on Nicotine-induced nAChR Desensitization



$P \geq 0.05$), 100nM (I_{7s} / I_{peak} = 0.33±0.04, n=9, $P \geq 0.05$), and 10μM (I_{7s} / I_{peak} = 0.49±0.07, n=5, $P \geq 0.05$) concentrations of DDF compared to initial ACh desensitization responses (I_{7s} / I_{peak} =0.42±0.06, n=5, I_{7s} / I_{peak} = 0.45±0.06, n=9, and I_{7s} / I_{peak} = 0.49±0.07, n=5, respectively), but at 1μM DDF there was a significant increase in nAChR desensitization of 72% (I_{7s} / I_{peak} = 0.28±0.03, n=8, $P \leq 0.05$) compared to initial ACh desensitization response of 53% (I_{7s} / I_{peak} = 0.47±0.05). Therefore, this suggests that forskolin has the potential to act nonspecifically at the nAChR to modulate nAChR desensitization (Figure 14 and Figure 16).

The effects of forskolin on nicotine-induced nAChR desensitization was examined to determine its role in altering the function of nAChRs on myenteric neurons. In this study, nicotine (1mM) was applied for seven seconds to the neurons in the presence of forskolin (10 nM, 100 nM, 1 mm, and 10 mm). 100 nM, 1 mm, and 10 mm concentrations of forskolin, there was a significant increase in nAChR desensitization of 78% (nicotine I_{7s} / I_{peak} = 0.22±0.04, n=9, $P \leq 0.05$), 78% (nicotine I_{7s} / I_{peak} = 0.22±0.03, n=11, $P \leq 0.05$), and 79% (nicotine I_{7s} / I_{peak} = 0.21±0.03, n=7, $P \leq 0.05$), respectively, compared to the initial nicotine desensitization response of 69% (nicotine I_{7s} / I_{peak} = 0.31±0.04, n=9), 66% (nicotine I_{7s} / I_{peak} = 0.34±0.04, n=11), and 69% (nicotine I_{7s} / I_{peak} = 0.31±0.03, n=7), respectively (Figure 17 and Figure 18).

DDF was applied to the neurons to test for a nonspecific effect of forskolin on nicotine-induced nAChR desensitization. In the presence of nicotine (1mM) applied for seven seconds with the addition of 10nM, 100nM, 1μM, and 10μM

Figure 20. Traces of Bethanechol effects on nicotine-induced nAChR desensitization. Traces of an inward current caused by Nicotine (1mM) and Nicotine (1mM) plus 100 mm bethanechol. Nicotine and Nicotine plus 100 mm bethanechol were significantly different. This indicates that mAChR activation plays a role in nAChR modulation in myenteric neurons.

Traces of Bethanechol Effects on Nicotine-induced nAChR Desensitization

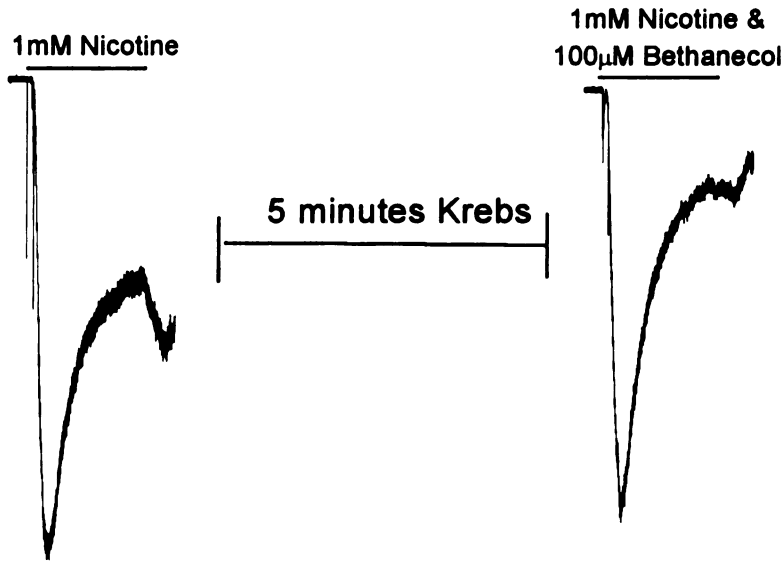
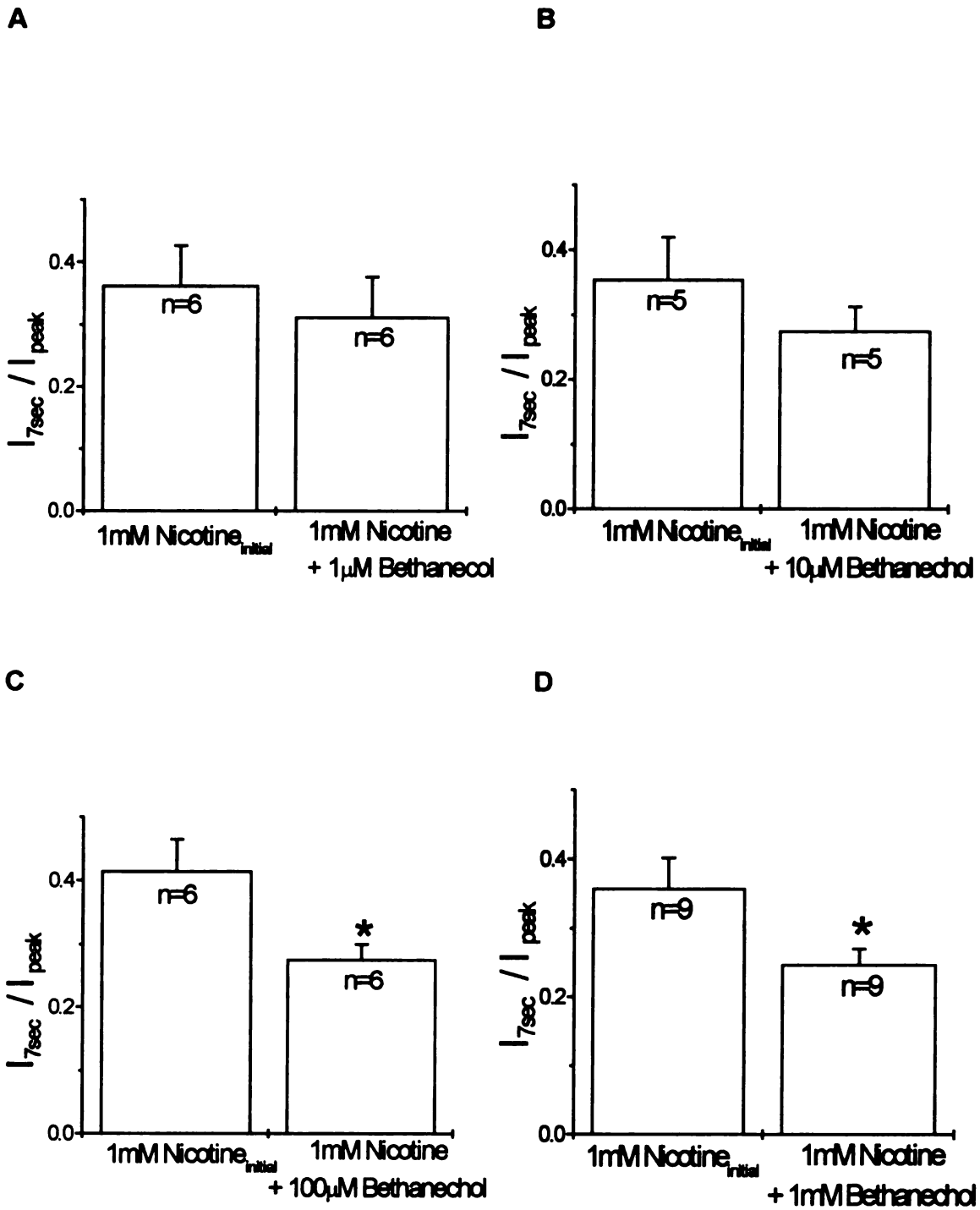


Figure 21. Bethanechol effects on nicotine-induced nAChR desensitization. Histograms A and B show that nicotine pretreatment with 1 mm and 10 mm bethanechol do not significantly increase nAChR desensitization compared to the initial nicotine response. Histograms C and D show that 100 mm and 1 mm concentrations of bethanechol significantly increases nAChR desensitization. These data suggest that mAChR activation is involved in modulating nAChR desensitization. (*P value<0.05)

Bethanechol Effects on Nicotine-induced nAChR Desensitization



concentrations of DDF were used to compare to the nicotine-induced nAChR desensitization. The data show that there was not a significant effect observed with 10nM (I_{7s} / I_{peak} = 0.32±0.03, n=9, P≥0.05), 100nM (I_{7s} / I_{peak} = 0.32±0.06, n=5, P≥0.05), and 1μM (I_{7s} / I_{peak} = 0.21±0.04, n=5, P≥0.05) concentrations of DDF compared to initial nicotine desensitization responses(I_{7s} / I_{peak} = 0.41±0.06, n=9, I_{7s} / I_{peak} = 0.31±0.02, n=5, and I_{7s} / I_{peak} = 0.34±0.08, n=5, respectively), but at 10μM DDF, there was a significant increase in nAChR desensitization of 83% (I_{7s} / I_{peak} = 0.17±0.05, n=5, P≤0.05) compared to initial nicotine desensitization of 50% (I_{7s} / I_{peak} = 0.50±0.06, n=5). Therefore, this further suggests, as with ACh and DDF, that forskolin acts at the nAChR nonspecifically to modulate desensitization (Figure 17 and Figure 19).

BETHANECHOL INDUCED NICOTINE - nAChR DESENSITIZATION

To further test that muscarinic receptor activation may modulate nAChR desensitization, bethanechol was applied to myenteric neurons. In this study, nicotine (1mM) was applied for seven seconds in addition to various concentration of bethanecol (1μM, 10μM, 100μM, and 1mM). The data suggest that mAChR activation plays a role in nAChR modulation of myenteric neurons because at 100μM and 1mM concentrations, there was a significant increase in nAChR desensitization of 72% (I_{7s} / I_{peak} = 0.28±0.02, n=6, P≤0.05) and 75% (I_{7s} / I_{peak} = 0.25±0.02, n=9, P≤0.05), respectively, compared to the initial nicotine desensitization response of 59% (I_{7s} / I_{peak} = 0.41±0.05, n=6) and 64% (I_{7s} / I_{peak} =

0.36±0.04, n=9) (Figure 20 and Figure 21).

DISCUSSION

The data presented suggest that intracellular signaling pathways are involved in the modulation of nAChR desensitization. This conclusion is supported by studies in which mAChRs were blocked and also those in which activation of the cAMP-dependent pathway and the PI pathway with forskolin and PDBu, changed nAChR desensitization. This was further implicated with direct activation of the muscarinic receptor with bethanechol application increased nAChR desensitization. Therefore, in the ENS, interactions occurring during simultaneous activation of nAChRs and mAChRs and/or other G-protein coupled receptors coupled to intracellular signaling pathways are responsible for modulating nAChR desensitization and also may be important in regulating enteric ganglionic neurotransmission.

INTRACELLULAR SIGNALING ACTIVATION ON nAChR DESENSITIZATION

Data presented show that nAChR desensitization is altered specifically by PKC activity which significantly increases nAChR desensitization at 10 to 300 nM concentrations of PDBu. NAChR desensitization was also shown to be altered nonspecifically by forskolin. This conclusion is supported by previous published data in muscle and neurons which have shown that agonist induced modulation of nAChR desensitization occurs following phosphorylation via activation of intracellular proteins such as PKA in muscle **(Huganir and Greengard, 1983:**

Huganir, 1986), PKC in muscle **(Safran et al, 1987)**, tyrosine specific protein kinases in muscle **(Huganir, 1984; Hopfield, 1988)**, and PKA and PKC in neurons **(Khirong et al, 1998; Fenster et al, 1998)**.

To further show that PKC activation is a regulator in modulating nAChR desensitization, bethanecol, a mAChR agonist, significantly increased nAChR desensitization at 100 μM and 1 mM concentrations. This is significant because published data suggest that mAChR activation couples to PI hydrolysis to lead to PKC activation **(Song et al, 1999; Hulme et al, 1990)**.

NON - ADENYLATE - CYCLASE SITE OF ACTION

The data show that by mimicking the activation of intracellular signaling pathways with forskolin and PDBu increases nAChR desensitization compared to that induced by ACh and nicotine alone. To show that these responses were specific, inactive analogs of forskolin and PDBu were used. The data show that there was specificity with intracellular activation of PKC and/or PI hydrolysis pathways by PDBu because 4-α-PMA did not modulate nAChR desensitization in the ENS. This was not the case for intracellular activation of cAMP dependent pathways by forskolin because of the DDF, an inactive analog of forskolin, caused a significant increase in nAChR desensitization. This has been shown in a previous experiment done by **Wagoner and Pallotta in 1988** who they showed that receptor desensitization mediated by forskolin was not due to cAMP-dependent phosphorylation but due to forskolin interaction with the hydrophobic moieties on or near the nAChR. This was further suggested by the effects of the inactive analog, DDF.

This nonspecific action of forskolin to alter nAChR activity is possibly due to the lipophilicity of forskolin which gives forskolin the ability to cross the neuronal membrane and interact with nAChRs. Nonspecificity was confirmed by using an inactive analog of forskolin, DDF, which significantly altered the nAChR activity at high concentrations of DDF at 1 μM for ACh induced nAChR desensitization and 10 μM for nicotine induced nAChR desensitization. For future reference, to confirm that cAMP - dependent pathways are not involved in modulating nAChR desensitization in the ENS, studies need to be performed to analyze the levels of cAMP when ACh or nicotine are exposed to the nAChR for seven seconds.

TIME CONTROL - EtOH VEHICLE ON nAChR DESENSITIZATION

These recordings involved an EtOH-Krebs solution vehicle. 50% EtOH in these experiments were used to make the analogs soluble in solution so that application of the analog was feasible. To show that this vehicle alone has no effect on nAChR desensitization or run-down, a time control was done using nAChR agonists, ACh and nicotine, as well as the protocol that was used for the forskolin, DDF, PDBu, and 4-α-PMA experiment showed that there was an insignificant effect on nAChR desensitization. Therefore, low EtOH concentrations do not affect nAChR desensitization.

MAChR EFFECTS ON nAChR

In these studies, the mAChR was blocked by scopolamine to show that nAChR desensitization induced by ACh is mediated by mAChR activation. This was shown to be true due to the significant decrease in nAChR desensitization. To show that

this was not a nonspecific interaction of scopolamine at the nAChR, nicotine, another nAChR agonist that acts specifically at nAChRs and not mAChRs, was applied to the neurons in addition to scopolamine being present. Scopolamine did not affect nicotine induced nAChR desensitization. Therefore, scopolamine has a specific effect on blocking the mAChR.

CROSS-DESENSITIZATION OF nAChR

The data presented from the cross-desensitization study with ACh and nicotine application showed that these two agonists when co-applied act at the same subtype of nAChRs localized on myenteric neurons. But what these results do not indicate are the number and subunit subtype compositions of nAChR populations. Preliminary data obtained from this laboratory (not presented) has shown that the subunits involved in the nAChR subtype composition are $\alpha 3$, $\alpha 5$, $\beta 2$, and $\beta 4$, but the positions of these subunits on the nAChR of myenteric neurons are not known.

During neurotransmission in the ENS, there are many neurotransmitters that are released and act postsynaptically at their receptors to excite enteric neurons. The activation of the receptors cause a cascade of intracellular mechanisms to become activated to mediate the activity of other receptors to modulate the excited state of enteric neurons. This process acts as a homeostatic feedback mechanism to keep the neurons from being over extended in their excited state.

DESENSITIZATION CHARACTERISTICS IN S-TYPE MYENTERIC NEURONS

From the exponential standard fit analysis of nAChR responses, the decay phase was calculated to have a sum of a two exponential fit for both ACh and nicotine,

which were not significant from one another. These results indicate that the decay phase, which represents the desensitized or inactivated state of the nAChR, has two phases, a fast and a slow phase. A fast phase represents a desensitized state that has been exposed to a short time to agonist application, and in this case, the results indicate that the time constant (τ) of approximately 600 milliseconds for ACh and approximately 200 milliseconds for nicotine were calculated for the time course between the maximum inward peak current and the first state of nAChR desensitization. The slow phase represents a desensitized state that has been exposed to a longer period of agonist application, and from the results, approximately two seconds for both ACh and nicotine were calculated as the time course between the peak response and the second state of nAChR desensitization.

From previous published data, the decay phase for muscle-nAChRs resulted in a sum of a two exponential fit meaning these nAChRs exhibited a fast and slow phase of nAChR desensitization **(Naranjo and Brehm, 1993)**. In ciliary ganglion neurons, the decay phase of nAChRs resulted in a sum of a two exponential fit **(Blumenthal et al, 1999)**, which means that the nAChRs exhibited a fast, slow, and a steady state phase because of an exponential fit from the peak response to the steady state response instead of an exponential fit from the peak response to the plateau response of the nAChR. Also, the seven second application of ACh and nicotine was used to guarantee that the nAChR was in a desensitized state. From this, the nAChR desensitized state was useful in determining the recovery rate of the nAChR at a seven second application.

NAChRs IN MYENTERIC NEURONS

As mentioned above the subunit subtype compositions of nAChRs in the ENS are not known. Also, the mechanism involved in modulating nAChR desensitization in the ENS is not known. In studies from neuronal nAChRs, results indicate PKC activation mediates phosphorylation on the nAChR to modulate nAChR desensitization **(Downing and Role, 1987)**. The results from the research studies presented in this thesis indicate that PKC activation specifically mediates nAChR modulation, and that also, activation of the mAChR plays a role in mediating nAChR desensitization possibly through a PI hydrolysis dependent pathway.

For nAChRs, subunit composition has been determined to have either a α7-homopentamer, $\alpha3\beta4$, $\alpha3\beta2$, $\alpha4\beta2$, and $\alpha4\beta4$ compositions. The desensitization rates of these subunit compositions have been observed and are continuously being studied. Also these subunit compositions have been mainly studied in the CNS and other cell preparations, such as *Xenopus* oocytes , but the subunit composition for nAChRs localized in the ENS has not been determined. From the data that has been published on subunit composition of nAChRs **(Fenster et al, 1997)**, the $\alpha3\alpha5\beta2$ subunit composition of the nAChR is closely mimicked by nAChRs localized in the ENS. Also the desensitization rate of $\alpha3\beta2$ subunit composition and the ENS nAChRs both have a two exponential fit. Because nAChR subunit composition in the ENS has not been studied extensively, the subunit composition or the number of subunit compositions of ENS nAChRs can not be determined. This raises the question of whether there are a number of different

subunit compositions of nAChRs localized in the ENS. If there are different subunit nAChR compositions in the ENS, then does subunit composition have an affect on how the nAChR desensitizes and does nAChR subunit composition have an affect on how desensitization of nAChRs in the ENS are modulated?

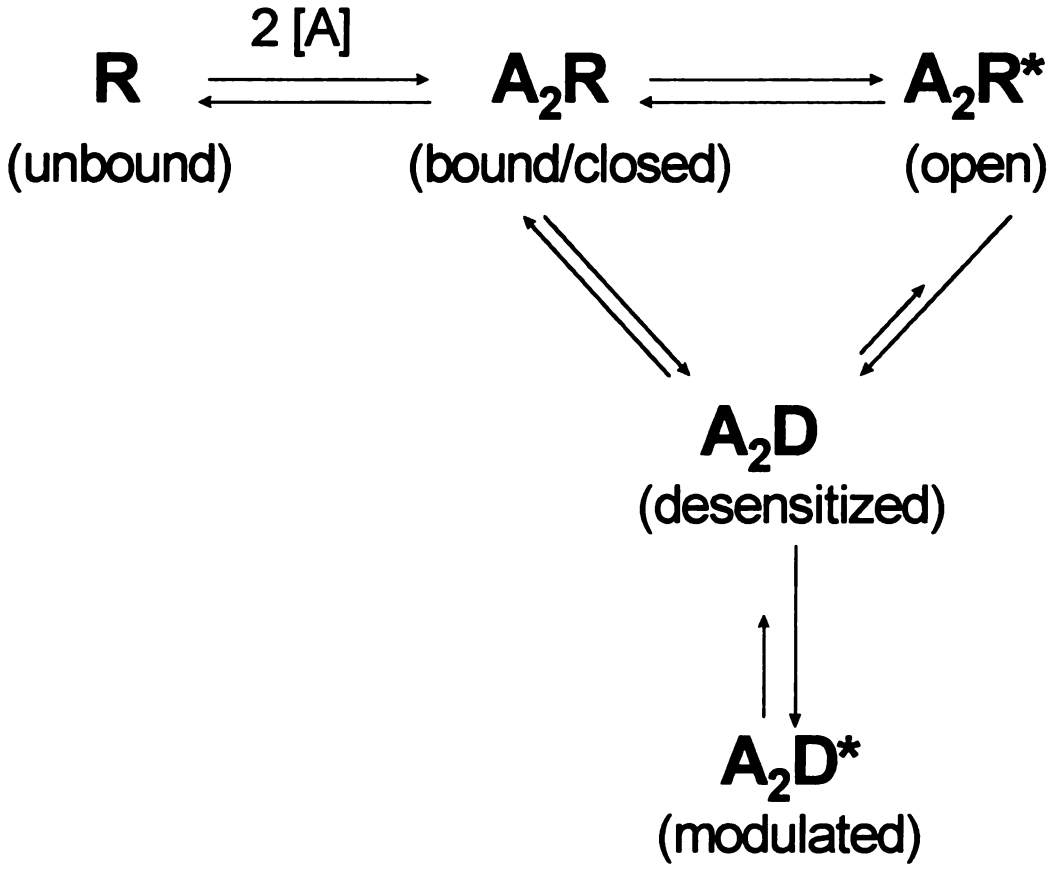
CONCLUSIONS

In summary, the results from these research studies indicate that the desensitization and recovery rate are influenced by intracellular ATP and GTP and muscarinic activation. During a seven second application of ACh and nicotine, the nAChR response decays exponentially with a fast and slow phase. ACh and nicotine co-application results in cross-desensitization of the nAChR which suggest that activation of the same nAChR. PKC activation results in a specific involvement in modulating the nAChR by ACh and nicotine in myenteric neurons. This is consistent with other published data which indicates that PKC activation plays a role in mediating phosphorylation of the nAChR to alter its function (Figure 22). Lastly, muscarinic activation modulates nAChR desensitization in myenteric neurons.

In conclusion, data presented in these studies show that interactions occurring during simultaneous activation of nAChRs, mAChRs, and intracellular signaling pathways modulate nAChR desensitization. Therefore, activation of these receptors and intracellular signaling pathways may be important in regulating enteric ganglionic neurotransmission.

Figure 22. Proposed model for nAChR desensitization in the ENS. Based on the time constants for the fast and slow phase of nAChR desensitization, this model proposes that at approximately 600 milliseconds for ACh and 200 milliseconds for nicotine that the nAChR has reached the peak of the first phase of desensitization (A_2D). For stability in function, the nAChR, during the first phase of desensitization prefers to stay in the desensitized state which is indicated in the model by the short and long arrow directions. The long arrow represents the direction the nAChR will tend to stabilize in the desensitized state versus the short arrow represents the less likelihood for the nAChR to reverse back to the open state. With continued exposure of the agonist, the nAChR has reached the second phase of desensitization (A_2D^*) at approximately two seconds for both ACh and nicotine. At the A_2D^*, the nAChR is indicated as being modulated. The long arrow represents the direction that the nAChR prefers to be in the modulated state, which forms a more stabilized nAChR, and the short arrow indicates the less likelihood for the nAChR to reverse back to the desensitized state. Overall, this model shows that a stabilized nAChR, when exposed to long periods of agonist, prefers to be in the modulated state. Therefore, the modulated state of the nAChR makes the recovery from desensitization more difficult, which leads to an increase time recovery for long periods exposure of agonists to the nAChR.

Proposed Model for nAChR Desensitization in the ENS



BIBLIOGRAPHY

Adams, D. J., Dwyer, T. M., and Hille, B. The permeability of endplate channels to mononvalent and divalent metal cations. *Journal of General Physiology* **75:** 493-510, 1980.

Albuquerque, E. X., Deshpande, S. S., Aracava, Y., Alkondon, M., and Daly, J. W. A possible involvement of cyclic AMP in the expression of desensitization of the nicotinic acetylcholine receptor. A study with forskolin and its anlogs. *FEBS Lett.* **199:** 113-120, 1986.

Albuquerque, E. X., Alkondon, M., Pereira, E. F. R., Castro, N. G., Schrattenholz, A., Barbosa, C. T. F., Bonfante-Cabaarcas, R., Aracava, Y., Eisenberg, H. M., and Maelicke, A. Properties of neuronal nicotinic acetylcholine receptors: pharmacological characterization and modulation of synaptic function. *The Journal of Pharmacology and Experimental Therapeutics* **280:** 1117-1136, 1997.

Ascher, P., Marty, A., and Neild, T. O. The mode of action of antagonists of the excitatory response to acetylcholine in *Aplysia* neurones. *Journal of Physiology* **278:** 207-235, 1978.

Ascher, P., Large, W. A., and Rang, H. P. Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells. *Journal of Physiology* **295:** 139-170, 1979.

Bertrand, P. P. and Galligan, J. J. Signal-transduction pathways causing slow synaptic excitation in guinea pig myenteric AH neurons. *American Journal of Physiology* **269:** G710-G720, 1995.

Bertrand, P. P., Kunze, W. A. A., Bornstein, J. C., Furness, J. B., and Smith, M. L. Analysis of the responses of myenteric neurons in the small intestine to chemical stimulation of the mucosa. American Journal of Physiology 273: G422-G435, 1997.

Blumenthal, E. M., Shoop, R. D., and Berg, D. K. Developmental changes in the nicotinic responses of clliary ganglion neurons. *Journal of Neurophysiology* **81:** 111-120, 1999.

Brookes, S. J. H., Steele, P. A., and Costa, M. Calretinin immunoreactivity in cholinergic motor neurones, interneurones and vasomotor neurones in the guinea-pig small intestine. *Cell and Tissue Research* **263:** 471-481, 1991.

Brookes, S. J. H., Song, Z. -M., Steele, P. A., and Costa, M. Identification of motor neurons to the longitudinal muscle of the guinea pig ileum. *Gastroenterology* **103:** 961-973, 1992.

Cassell, J. F. and McLachlan, E. M. Muscarinic agonists block five different potassium conductances in guinea-pig sympathetic neurones. *British Journal of Pharmacology* **91:** 259-261, 1987.

Chen, D. and Patrick, J. W. The α-bungarotoxin-binding nicotinic acetylcholine receptor from rat brain contains only the α7 subunit. *Journal of Biological Chemistry* **272:** 24024-24029, 1997.

Clerc, N., Furness, J. B., Bornstein, J. C., and Kunze, W. A. A. Correlation of electrophysiological and morphological characteristics of myenteric neurons of the duodenum in the guinea-pig. *Neuroscience* **82:** 899-914, 1998.

Conroy, W. G. and Berg, D. K. Nicotinic receptor subtypes in the developing chick brain: appearance of a species containing the α4, β2, and α5 gene products. *The American Society for Pharmacology and Experimental Therapeutics* **53:** 392-401, 1998.

Costa, M., Furness, J. B., and Gibbins, I. L. Chemical coding of enteric neurons. *Progress in Brain Research* **68:** 217-239, 1986.

Corringer, P. -J., Le Novére, N., and Changeux, J. P. Nicotinic receptors at the amino acid level. *Annual Reviews of Pharmacology and Toxicology* **40:** 431-458, 2000.

Dionne, V. E. and Stevens, C. F. Voltage dependence of agonist effectiveness at the frog neuromuscular junction: resolution of a paradox. *Journal of Physiology* **251:** 245-270, 1975.

Downing, J. E. G. and Role, L. W. Activators of protein kinase C enhance acetylcholine receptor desensitization in sympathetic ganglion neurons. *Proceedings of the National Academy of Sciences* **84:** 7739-7743, 1987.

Fenster, C. P., Rains, M. F., Noerager, B., Quick, M. W., and Lester, R. A. J. Influence of subunit composition on desensitization of neuronal acetylcholine receptors at low concentrations of nicotine. *The Journal of Neuroscience* **17:** 5747-5759, 1997.

Furness, J. B. and Costa, M. The Enteric Nervous System. pp. 1-290, New York: Churchill Livingstone.

Furness, J. B., Trussell, S. P., Borstein, J. C., and Smith, T. K. Calbindin neurons of the guinea-pig small intestine: quantitative analysis of their numbers and projections. *Cell and Tissue Research* **260:** 261-272, 1990.

Furness, J. B., Young, H. M., Pompolo, S., Bornstein, J. C., Kunze, W. A. A., and McConalogue, K. Plurichemical transmission and chemical coding of neurons in the digestive tract. Gastroenterology 108: 554-563, 1995.

Furness, J. B., Kunze, W. A. A., Bertrand, P.P., Clerc, N., and Bornstein, J. C. Intrinsic primary anferent neurons of the intestine. *Progress in Neurobiology* **54:** 1-18, 1998.

Gabella, G. Innervation of the intestinal muscular coat. *Journal of Neurocytology* **1:** 341-362, 1972.

Galligan, J. J. Gastrointestinal Regulatory Peptides. In Handbook of Experimental Pharmacology, ed. Brown, D. R., pp. 253-276. Berlin Heidelberg: Springer-Verlag, 1993.

Galligan, J. J. Mechanisms of excitatory synaptic transmission in the enteric nervous system. *Tokai Journal of Experimental and Clinical Medicine* **23:** 129-136, 1998.

Gershon, M. D. and Rothman, T. P. Enteric Glia. *Glia* **4:** 195-204, 1991.

Gerzanich, V., Wang, F., Kuryatov, A., and Lindstrom, J. α5 subunit alters desensitization, pharmacology, Ca^{++} permeability and Ca^{++} modulation of human neuronal α3 nicotinic receptors. *The Journal of Pharmacology and Experimental Therapeutics* **286:** 311-320, 1998.

Gordon, A. S., Davis, C. G., Mifay, D. D., and Diamond, I. Phosphorylation of acetylcholine receptor by endogenous membrane protein kinase in receptor-enriched membranes of *Torpedo californica*. *Nature* **267:** 539-540, 1977.

Goyal, R. K. and Hirano, I. The Enteric Nervous System. *The New England Journal of Medicine* **334:** 1106-1115, 1996.

Hirst, G. D. S. and Spence, I. Calcium action potentials in mammalian peripheral neurones. Nature 243: 54-56, 1973.

Hirst, G. D. S., Holman, M. E., and Spence, I. Two types of neurones in the myenteric plexus of duodenum in the guinea-pig. *Journal of Physiology* **236:** 303-326, 1974.

Hirst, G. D. S., Johnson, S. M., and van Helden, D. F. The slow calcium-dependent potassium current in a myenteric neurone of the guinea-pig ileum. *Journal of Physiology* **361:** 315-337, 1985.

Hodgkiss, J. P. and Lees, G. M. Morphological studies of electrophysiologically-identified myenteric plexus neurons of the guinea-pig ileum. *Neuroscience* **8:** 593-608, 1983.

Hopfield, J. F., Tank, D. W., Greengard, P., and Huganir, R. L. Functional modulation of the nicotinic acetylcholine receptor by tyrosine phosphorylation. *Nature* **336:** 677-680, 1988.

Huganir, R. L. and Greengard, P. CAMP-dependent protein kinase phosphorylates the nicotinic acetylcholine receptor. *Proceedings of the National Academy of Sciences* **80:** 1130-1134, 1983.

Huganir, R. L., Miles, K., and Greengard, P. Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase. *Proceedings of the National Academy of Sciences* **81:** 6968-6972, 1984.

Huganir, R. L., Delcour, A. H., Greengard, P., and Hess, G. P. Phosphorylation of the nicotinic actylcholine recpeotr regulates its rate of desensitization. *Nature* **321:** 774-776, 1986.

Hulme, E. C., Birdsall, N. J. M., and Buckley, N. J. Muscarinic receptor subtypes. *Annual Reviews in Pharmacology and Toxicology* **30:** 633-673, 1990.

Iyer, V., Bornstein, J. C., Costa, M., Furness, J. B., Takahashi, Y., and Iwanaga, T. Electrophysiology of guinea-pig myenteric neurons correlated with immunoreactivity for calcium binding proteins. *Journal of Autonomic Nervous System* 141-150, 1988.

Jones, M. V. and Westbrook, G. L. The impact of receptor desensitization on fast synaptic transmission. *Trends in Neuroscience* **19:** 96-101, 1996.

Katz, B. and Thesleff, S. A Study of the 'desensitization' produced by acetylcholine at the motor end-plate. *Journal of Physiology* **138:** 63-80, 1957.

Khiroug, L., Sokolova, E., Giniatullin, R., Afzalov, R. and Nistri, A. Recovery from desensitization of neuronal nicotinic acetylcholine receptors of rat chromaffin cells is modulated by intracellular calcium through distinct second messengers. *The Journal of Neuroscience* **18:** 2458-2466, 1998.

Kirchgessner, A. L., Tamir, H., and Gershon, M. D. Identificaiton and stimulation

by serotonin of intrinsic sensory neurons of the submucosal plexus of the guinea pig gut: activity-induced expression of Fos immunoreactivity. *The Journal of Neuroscience* **12:** 235-248, 1992.

Kunze, W. A. A., Bornstein, J. C., and Furness, J. B. Identification of sensory nerve cells in a peripheral organ (the intestine) of a mammal. *Neuroscience* **66:** 1-4, 1995.

Kunze, W. A. A. and Furness, J. B. The enteric Nervous system and regulation of intestinal motility. *Annual Reviews in Physiology* **61:** 117-142, 1999.

LePard, K. J., Messori, E., and Galligan, J. J. Purinergic fast excitatory postsynaptic potentials in myenteric neurons of guinea pig: distribution and pharmacology. *Gastroenterology* **113:** 1522-1534.

Mann, P. T., Southwell, B. R., Young, M. H., and Furness, J. B. Appositions made by axons of descending interneurons in the guinea-pig small intestine, investigated by confocal microscope. *Journal of Chemical Neuroanatomy* **12:** 151-164, 1997.

Margiotta, J. F., Berg, D. K., and Dionne, V. E. Cyclic AMP regulates the proportion of functional acetylcholine receptors on chicken ciliary ganglion neurons. *Proceedings of the National Academy of Sciences* **84:** 8155-8159, 1987.

Margiotta, J. F. and Gurantz, D. Changes in the number, function, and regulation of nicotinic acetylcholine receptors during neuronal development. *Developmental Biology* **135:** 326-339, 1989.

Middleton, P., Rubin, L. L., and Schuetze, S. M. Forskolin increases the rate of acetylcholine receptor desensitization at rat soleus endplates. *Proceedings of the National Academy of Sciences* **83:** 4967-4971, 1986.

Middleton, P., Rubin, L. L., and Schuetze, S. M. Desensitization of acetylcholine receptors in rat myotubes is enhanced by agents that elevate intracellular cAMP. *The Journal of Neuroscience* **8:** 3405-3412, 1988.

Miles, K., Anthony, D. T., Rubin, L. L., Greengard, P., and Huganir, R. L. Regulation of nicotinic acetylcholine receptor phosphorylation in rat myotubes by forskolin and cAMP. Proceedings of the National Academy of Sciences 84: 6591-6595, 1987.

Morita, K., North, R. A., and Tokimasa, T. The calcium-activated potassium conductance in guinea-pig myenteric neurones. *Journal of Physiology* **329:** 341-354, 1982.

Morita, K., North, R. A., and Tokimasa, T. Muscarinic agonists inactivate potassium conductance of guinea-pig myenteric neurones. *Journal of Physiology* **333:** 125-139, 1982.

Moss, S. J., McDonald, B. J., Rudhard, Y., and Schoepfer, R. Phosphorylation of the predicted major intracellular domains of the rat and chick neuronal nicotinic acetylcholine receptor α7 subunit by cAMP-dependent protein kinase. *Neuropharmacology* **35:** 1023-1028, 1996.

Nakayama, H., Okuda, H., and Nakashima, T. Phosphorylation of rat brain nicotinic acetylcholine receptor by cAMP-dependent protein kinase in vitro. *Molecular Brain Research* **20:** 171-177, 1993.

Naranjo, D. and Brehm, P. Modal shifts in acetylcholine receptor channel gating confer subunit-dependent desensitization. *Science* **260:** 1811-1814, 1993.

Nishi, S. and North, R. A. Intracellular recording from the myenteric plexus of the guinea-pig ileum. *Journal of Physiology* **231:** 471-491, 1973.

Nishizaki, T. and Sumikawa, K. Effects of PKC and PKA phosphorylation on desensitization of nicotinic acetylcholine receptors. *Brain Research* **812:** 242-245, 1998.

North, R. A. The calcium-dependent slow after-hyperpolarization in myenteric plexus neurones with tetrodotoxin-resistant actionn potentials. *British Journal of Pharmacology* **49:** 709-711, 1973.

North, R. A., Henderson, G., Katayama, Y., and Johnson, S. M. Presynaptic inhibition of acetylcholine release by 5-hydroxytryptamine in the enteric nervous system. *Neuroscience* **5:** 581-586, 1980.

North, R. A. and Tokimasa, T. Muscarinic synaptic potentials in guinea-pig myenteric plexus neurones. *Journal of Physiology* **333:** 151-156, 1982.

Nutter, T. J. and Adams, D. J. Monovalent and divalent cation permeability and block of neuronal nicotinic receptor channels in rat parasympathetic ganglia. *Journal of General Physiology* **105:** 701-723, 1995.

Palmer, J. M., Wood, J. D., and Zafirov, D. H. Elevation of adenosine 3'-5' phosphate mimics slow synaptic excitation in myenteric neurones of the guinea pig. *Journal of Physiology* **376:** 451-460, 1987.

Paradiso, K. and Brehm, P. Long-term desensitization of nicotinic acetylcholine receptors is regulated via protein kinase A-mediated phosphorylation. *The Journal of Neuroscience* **18:** 9227-9237, 1998.

Rangwala, F., Drisdel, R. C., Rakhilin, S., Ko, E., Atluri, P., Harkins, A. B., Fox, A. P., Salman, S. B., and Green, W. N. Neuronal α-bungarotoxin receptors differ structurally from other nicotinic acetylcholine receptors. *The Journal of Neuroscience* **17:** 8201-8212, 1997.

Reitstetter, R., Lukas, R. J., and Gruener, R. Dependence of nicotinic acetylcholine receptor recovery from desensitization on the duration of agonist exposure. *The Journal of Pharmacology and Experimental Therapeutics* **289:** 656-660, 1999.

Rowell, P. P. and Duggan, D. S. Long-lasting inactivation of nicotinic receptor function *in vitro* by treatment with high concentrations of nicotine. *Neuropharmacology* **37:** 103-111, 1998.

Safran, A., Sagi-Eisenberg, R., Neumann, D., and Fuchs, S. Phosphorylation of the acetylcholine receptor by protein kinase C and identification of the phosphorylation site within the receptor δ subunit. *Journal of Biological Chemistry* **262:** 10506-10510, 1987.

Song, Z. -M., Brookes, S. J. H., and Costa, M. Projections of specific morphological types of neurons within the myenteric plexus of the small intestine of the guinea-pig. *Cell and Tissue Research* **285:** 149-156, 1996.

Song, L., De Sarno, P., and Jope, R. S. Muscarinic receptor stimulation increases regulators of g-protein signaling 2 mRNA levels through a protein kinase C-dependent mechanism. *The Journal of Biological Chemistry* **274:** 29689-29693, 1999.

Stark, M. E. and Szurszewski, J. H. Role of nitric oxide in gastrointestinal and hepatic function and disease. *Gastroenterology* **103:** 1928-1949, 1992.

Teichberg, V. I., Sobel, A., and Changeux, J. -P. In vitro phosphorylation of the acetylcholine receptor. *Nature* **267:** 540-542, 1977.

Vernino, S., Amador, M., Luetje, C. W., Patrick, J., and Dani, J. A. Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. *Neuron* **8:** 127-134, 1992.

Vijayaraghavan, S., Schmid, H. A., Halvorsen, S. W., and Berg, D. K. Cyclic AMP-dependent phosphorylation of a neuronal acetylcholine receptor α-type subunit.

The Journal of Neuroscience **10:** 3255-3262, 1990.

Wagoner, P. K. and Pallotta, B. S. Modulation of acetylcholine receptor desensitization by forskolin is independent of cAMP. *Science* **240:** 1655-1657, 1988.

Wessler, I., Eschenbruch, V., Halin, S., and Kilbinger, H. Presynaptic effects of scopolamine, oxotremorine, noradrenaline and morphine on [^{3}H] acetylcholine release from the myenteric plexus at different stimulation frequencies and calcium concentrations. *Naunyn-Schmiedeberg's Archives of Pharmacology* **335:** 597-604, 1987.

Yuan, S. Y., Bornstein, J. C., and Furness, J. B. Investigation of the role of 5-HT$_3$ and 5-HT$_4$ receptors in ascending and descending reflexes to the circular muscle of guinea-pig small intestine. *British Journal of Pharmacology* **112:** 1095-1100, 1994.

Yuan, S. Y., Bornstein, J. C., and Furness, J. B. Pharmacological evidence that nitric oxide may be a retrograde messenger in the enteric nervous system. British Journal of Pharmacology 114: 428-432, 1995.

Zhou, X. and Galligan, J. J. Non-additive interaction between nicotinic cholinergic and P2X purine receptors in guinea-pig enteric neurons in culture. *Journal of Physiology* **513.3:** 685-697, 1998.