EFFECTS OF COTININE AND NICOTINE ON CHROMAFFIN CELL SIGNALLING

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Academic dissertation

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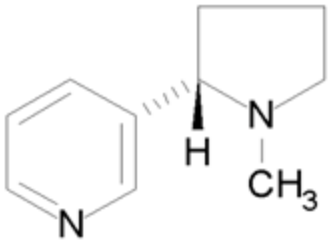

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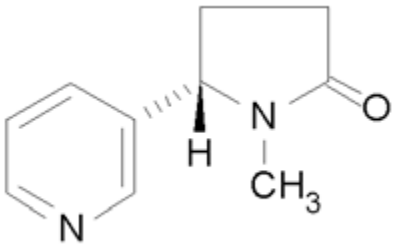

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (I – VI):

I Vainio PJ, Somerharju P, Jaakkola T, Tuominen RK. Receptor-stimulated phospholipase D activity in bovine adrenal chromaffin cells. Neurosci Res Commun 1999, 24: 179-185.

II Vainio PJ, Tuominen RK. Nicotine-evoked exocytosis from bovine chromaffin cells is independent of phospholipase D activation. Neurosci Res Commun 2000, 26: 93-101.

III Vainio PJ, Viluksela M, Tuominen RK. Inhibition of nicotinic responses by cotinine in bovine adrenal chromaffin cells. Pharmacol Toxicol 1998, 83: 188–193.

IV Vainio PJ, Viluksela M, Tuominen RK. Nicotine-like effects of cotinine on protein kinase C activity and noradrenaline release in bovine adrenal chromaffin cells. J Auton Pharmacol 1998, 18: 245–250.

V Vainio PJ, Törnquist K, Tuominen RK. Cotinine and nicotine inhibit each other's calcium responses in chromaffin cells. Toxicol Appl Pharmacol 2000, 163: 183-187.

VI Vainio PJ, Tuominen RK. Cotinine binding to nicotinic acetylcholine receptors in bovine chromaffin cell and rat brain membranes. A submitted manuscript.

ABBREVIATIONS

$[Ca^{++}]_i$	intracellular free Ca^{++} concentration
DMPP	1, 1-dimethyl-4-phenylpiperazinium
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PKC	protein kinase C
-cPKC	conventional PKC isoenzyme; Ca^{++}- and diacylglycerol-dependent
-nPKC	novel PKC isoenzyme; diacylglycerol-dependent, Ca^{++}-independent
-aPKC	atypical PKC isoenzyme; Ca^{++}- and diacylglycerol-independent
PLA_2	phospholipase A_2
PLC	phospholipase C
-PC-PLC	phosphatidylcholine-specific PLC
-PI-PLC	phosphatidylinositol-specific PLC
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate (12-tetradecanoylphorbol-13-acetate)

INTRODUCTION

Tobacco smoking has been an important social habit in American cultures since the prehistoric era. In the later half of this millennium, tobacco use has spread to nearly all communities. In addition to smoking, tobacco has been used as chewing tobacco, and as dry nasal and wetted oral snuff. Among the thousands of chemicals contained in fermented tobacco products, nicotine is the substance causing addiction (see Hoffmann and Wynder, 1986).

Besides being a drug of abuse, nicotine is a frequently used model compound in studies involving cholinergic neurotransmission. The nicotine effects are mediated through nicotinic acetylcholine receptors that are found both in the periphery and the central nervous system. Upon binding to its receptor, nicotine opens an integral non-selective cation channel. This leads to cell depolarisation and induces various responses. Acutely, for example, nicotine evokes transmitter release, while a sustained nicotine exposure upregulates enzymes involved in synthesis of catecholamine transmitters (Stachowiak *et al*., 1990).

Nicotine itself is not a major health hazard, but consumption of tobacco, especially smoking, has often been regarded as the single most important preventable cause of death and serious diseases (Hoffmann and Wynder, 1986). Thus, alternative ways of nicotine administration have been developed for smoking cessation. These include nicotine gum, patches, nasal spray, vapour inhaler, sublingual tablets and lozenges (Leischow and Cook, 1999).

No matter how nicotine is used, it is metabolised in the body. Cotinine is the major metabolite of nicotine. It is eliminated much slower than the parent compound, and in chronic nicotine administration its concentrations are much higher than those of nicotine (Benowitz and Jacob, 1999). Cotinine has frequently been considered biologically inactive (Benowitz, 1996), although some of its effects have been known for nearly four decades (Borzelleca *et al*., 1962). Depending on the model, cotinine has caused effects both similar to and opposite to those of nicotine. Cotinine passes the blood-brain barrier, and thus can participate in the central effects of nicotine administration (Paulson and Olson, 1995). Only few data on the effects of cotinine at the cellular level have been published.

Sustained changes in protein phosphorylation status and protein kinase activities can participate in various events, such as transmitter synthesis (Stachowiak *et al*., 1990) and memory formation (Izquierdo, 1994; Micheau and Riedel, 1999). In bovine adrenal chromaffin cells, nicotine causes a prolonged increase in diacylglycerol levels and protein kinase C (PKC) activity (Tuominen *et al*., 1992). The primary catalyst of diglyceride production, phosphatidylinositol-specific phospholipase C (PI-PLC), participates only in acute responses due to a rapid negative feedback. Thus, the enzymes more likely responsible for prolonged diacylglycerol production are phosphatidylcholine-specific phospholipase C (PC-PLC) and phospholipase D (PLD) (Liscovitch, 1992; Nishizuka, 1995). The existence of the former is uncertain. PLD has also been claimed to be lacking from bovine chromaffin cells (Purkiss *et al*., 1991).

In this study, I have used bovine adrenal chromaffin cells as a model of neural cells. The research focus has been on the role of PLD in nicotinic pharmacology and on the effects of cotinine on nicotinic responses and cell signalling.

REVIEW OF THE LITERATURE

PHARMACOKINETICS OF NICOTINE AND COTININE

The rewarding effect of nicotine depends on liberation and absorption pharmacokinetics of the dosage form. The more rapidly the brain, or arterial, concentration peaks, the more intense is the pharmacological response. At physiological pH, approximately one quarter of nicotine is present in uncharged form and is thus prone to pass through lipid membranes (Anonymous, 1991; Budavari *et al*., 1996). Since nicotine is a base, alkaline tobacco products, such as snuff, chewing tobacco and cigars, favour its absorption, which has been demonstrated for cigar smoke as compared with cigarette smoke (Armitage and Turner, 1970).

With cigarette smoking, nicotine reaches the brain in less than 20 seconds, and the blood nicotine concentration increases for the duration of the smoking period. Use of oral snuff or chewing tobacco yields peak blood levels comparable with those achieved by smoking. However, they take approximately half an hour to occur due to thicker barriers at the site of nicotine absorption (Benowitz *et al*., 1988). Immediately after smoking a cigarette, the arterial nicotine concentration is over two-fold higher than the venous concentration. Nicotine plasma levels in smokers are typically around 200 nM and they never exceed 1 μM (Benowitz and Jacob, 1984; Moreyra *et al*., 1992; Gourlay and Benowitz, 1997).

Pharmaceutical preparations used in smoking cessation, including chewing gum, transdermal patches, sublingual tablets, lozenges and vapour inhalers, release nicotine slower than cigarette smoking, thereby avoiding rapid peaks. Nasal spray pharmacokinetics are somewhat more comparable with smoking. However, no alternative route of nicotine administration yields as high nicotine peaks as quickly as cigarette smoking (Benowitz *et al*., 1988; Mulligan *et al*., 1990; Hurt *et al*., 1993, 1998; Gourlay and Benowitz, 1997; Schuh *et al*., 1997).

Nicotine is rapidly and widely distributed in the body. It accumulates in the stomach, bladder, kidneys and salivary glands. Nicotine is eliminated mainly by hepatic metabolism, with a typical elimination half-life of 2 h. Approximately 80 % of nicotine is C-oxidised, mainly by cytochrome P450 2A6, followed by aldehyde oxidase, yielding cotinine. In addition, nicotine may undergo pyrrolidine N-demethylation and N-oxidation, as well as pyridine N-methylation and N-glucuronidation (Figure 1). One tenth of the nicotine is excreted as such by the kidneys. As a weak base, nicotine can be trapped in an acidic environment, and its renal clearance can be enhanced five-fold by urine acidification (Benowitz *et al*., 1983, 1994; Anonymous, 1991; Yamazaki *et al*., 1999).

Cotinine is less lipophilic than nicotine. It distributes to the total body water. Upon nicotine infusion to rats, the highest cotinine concentrations are present in the kidneys, followed by the lung and the liver. In the brain, the cotinine level somewhat exceeds the arterial concentration. Interestingly, when cotinine is infused, the tissue cotinine concentration reaches the arterial concentration only in the kidneys (Gabrielsson and Bondesson, 1987). Cotinine has been detected in the brain of experimental animals and in the cerebrospinal fluid of smokers (Paulson and Olson, 1995; Crooks *et al*., 1997). However, in a positron emission tomography study, cotinine penetration through the blood-brain barrier was not observed (Halldin *et al*., 1992). Yet, the brain cotinine is probably of peripheral origin, since no evidence for nicotine C-oxidation in the brain has been found (Hansson and Schmitterlöw, 1965; Crooks *et al*., 1997).

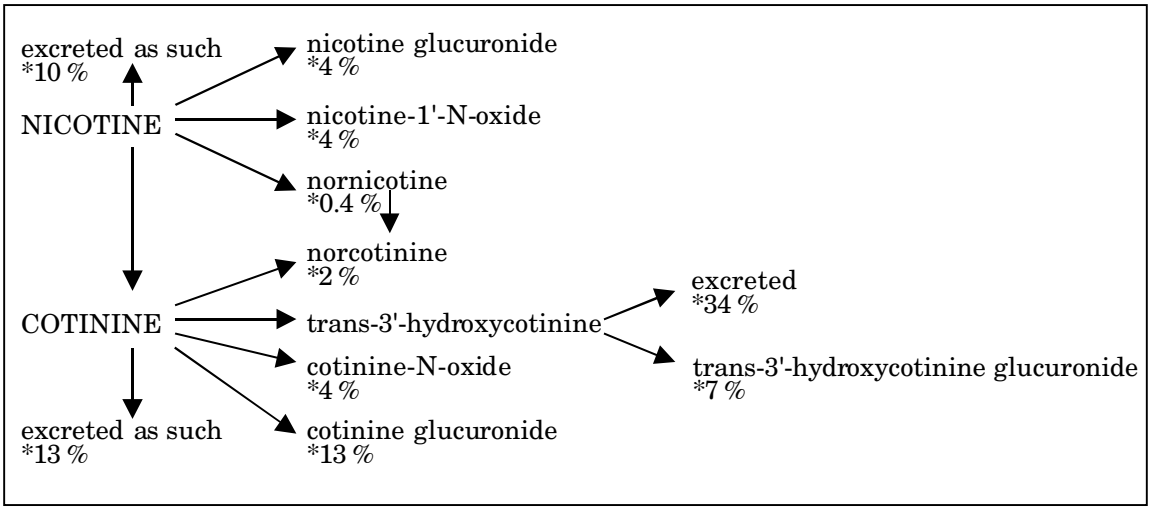

FIGURE 1. The main metabolic pathways of nicotine. The relative amount of the metabolites in the urine is displayed as percentages.

Cotinine is eliminated relatively slowly with a typical half-life of 15 – 20 h (Benowitz *et al*., 1983; Curvall *et al*., 1990). In the venous blood of smokers, the steady-state cotinine concentration is higher than the peak nicotine concentration, typically 3 – 4 μM (Benowitz *et al*., 1983; Benowitz and Jacob, 1994). Cotinine is not a metabolic end product (Figure 1). One sixth is excreted in the urine as such, and the rest is metabolised in the liver via both the phase I functionalisation reactions and the phase II conjugation reactions. At least pyridine N-methylation, N-glucuronidation and N-oxidation, hydroxylation of the pyrrolidine ring at two different sites, and pyrrolidine N-demethylation are known to occur (Byrd *et al*., 1992; Benowitz *et al*., 1994). Due to its relatively slow elimination and tendency to accumulate, cotinine could affect biological systems if it possessed even minor affinity for nicotinic receptors.

NICOTINIC ACETYLCHOLINE RECEPTORS AND THEIR LIGANDS

RECEPTORS

Studies of nicotine effects on muscle led John Newport Langley to suggest the existence of a receptive substance nearly one hundred years ago (Langley, 1905). Later, the nicotinic receptor was the first to be sequenced, cloned and gene-sequenced (Noda *et al*., 1982, 1983a, 1983b; Claudio *et al*., 1983). Assistance in reaching these milestones was provided by the electric eel *Electrophorus* and the electric ray *Torpedo* species, whose electric organs express a large number of nicotinic acetylcholine receptors. Furthermore, the electric organ nicotinic receptor protein was relatively easy to purify by affinity chromatography utilising α-bungarotoxin (see Lindstrom, 1999).

The nicotinic acetylcholine receptors are pentameric ion channels. Each of the subunits spans the membrane four times and all subunits contribute to channel formation. To date, genes encoding 16 different subunits of the vertebrate nicotinic receptors have been cloned. These subunits are identified as α1 – α9, β1 – β4, γ, δ and ε. The subunits present in adult neuromuscular junction are α1, β1, ε and δ. In the foetal muscle, γ subunit replaces ε, as is also the case in electric organs. Outside the neuromuscular junction, α2 – α6 and β2 – β4 are thought to form the heteropentameric receptors with both α and β subunits. Subunits α7 – α9 form the usually homomeric non-β receptors. The heteropentameric neuronal receptors are frequently presented as

consisting of two α and three β subunits. However, neither the subunit stoichiometry nor the arrangement of most of the receptors are known with certainty. Furthermore, it is unclear whether all subunits are naturally expressed in functional combinations (see Sargent, 1993; McGehee and Role, 1995; Lukas *et al*., 1999). Besides the central and the peripheral nervous systems and sensory organs, neuronal nicotinic receptors are reportedly expressed in lymphocytes, granulocytes, fibroblasts, chondrocytes, spermatozoa, placenta and pulmonary neuroendocrine cells (see Lukas *et al*., 1999).

The nomenclature of nicotinic acetylcholine receptors has not been established. The historical classification of nicotinic receptors to neuronal (ganglionic-type, C6, N_N, N_1) and muscle (C10, N_M, N_2) receptors (Watson and Girdlestone, 1993) ceased nearly a decade ago. After that, various terms have been used to identify receptor types (Watson and Girdlestone, 1995; Watling, 1998; Alexander and Peters, 1999). Reflecting the difficulty of creating a satisfying nomenclature, the IUPHAR compendium of receptor classification provides no information on nicotinic acetylcholine receptor (IUPHAR Committee on Receptor Nomenclature and Drug Classification, 1998). Recently, however, the subcommittee on nicotinic acetylcholine receptors has recommended a classification based on the subunits forming the receptors (Lukas *et al*., 1999). The subunit types and their stoichiometry are expressed if known. If only some of the subunits forming the receptor in question are known, the identified subunits are enumerated and an asterisk added to indicate that additional subunit types may exist.

According to the recommended classification, the adult muscle nicotinic acetylcholine receptor type is $(\alpha1)_2\beta1\varepsilon\delta$, and the foetal muscle and electric organ receptor type is $(\alpha1)_2\beta1\gamma\delta$. Autonomic ganglion receptors may be called α3α5β2β4, α3α5β4, α3β4, α3β4*, α3* or α7* receptors, depending on their composition. In the central nervous system, the major combination is $(\alpha4)_2(\beta2)_3$, and other receptors expressed include α4α5β2, α7* and α9* receptors. In the cerebellum, α3β2β3β4 and α3β2β4β5 combinations have also been demonstrated. The α8 subunit is expressed in avian retinas, and hitherto has only been observed in chicks.

AGONISTS

The natural ligand of nicotinic receptors is acetylcholine. Other agonists include nicotine, 1,1-dimethyl-4-phenylpiperazinium (DMPP), cytisine, epibatidine and numerous novel ligands developed by the pharmaceutical industry. However, many of the compounds regarded as agonists are partial agonists in certain models. Indeed, despite being called nicotinic receptors, many of the natural receptors respond to nicotine as to a partial agonist. Properties of selected nicotinic agonists are presented in Table 1.

The clinical use of nicotine has been limited to treating withdrawal symptoms upon smoking cessation. In the near future, nicotinic agonists may get new clinical indications. Thus far, they have proven effective in preclinical or early clinical studies for relieving pain and cognitive disorders, as well as symptoms of parkinsonism, Tourette's syndrome and schizophrenia (see Decker and Arneric, 1999; Donnelly-Roberts and Brioni, 1999; Flores and Hargreaves, 1999; Leonard *et al*., 1999; Levin *et al*., 1999; Menzaghi *et al*., 1999).

In receptor-binding studies utilising various nicotinic receptor agonists, both apparent one-site binding and apparent two-site binding have been reported. The high-affinity binding is considered to represent ligand binding to the desensitised receptors, while the low-affinity binding occurs at the ground-state receptors. Nevertheless, the differ-

ent binding affinities may also represent different receptor populations. Mathematical modelling of the binding data is further complicated by the fact that opening of a cholinergic receptor channel requires binding of two agonist molecules to the pentamer and that the agonist sites may cooperate (see Haylett, 1996; Gotti *et al*., 1997).

TABLE 1. Characteristics of nicotinic acetylcholine receptor agonists

Ligand (source)	**Affinity for receptors**[a]	**References**[b]
A-85380	rat forebrain: K_i 97 pM	Xiao *et al*., 1998
ABT-418	brain heteropentameric: K_i 3 nM	Arneric *et al*., 1994
acetylcholine (endogenous)	K_i: α2β2: 2 nM, α3β2 & α4β2: 30 nM, α4β4: 70 nM, α2β4 100 nM, α3β4: 560 nM	Parker *et al*., 1998
anabaseine (*Aphaenogaster sp.* ant; *Paranemertes sp.* worm)	K_i: α7: 32 nM, α4β2: 32 nM, fish muscle: 210 nM	Kem *et al*., 1997
anabasine (tobacco)	K_i: α2β2: 14 nM, α3β2: 57 nM, α7: 58 nM, α4β2: 76–260 nM, α4β4: 370 nM, α2β4: 450 nM, α3β4: 2.1 μM, fish muscle: 7.2 μM	Kem *et al*., 1997; Parker *et al*., 1998
anatoxin-a (cyanobacterium *Anabaena flos-aquae*)	K_i: rat forebrain 6.5 nM, α3β4: 53 nM	Thomas *et al*., 1993; Xiao *et al*., 1998
carbachol	K_i: rat forebrain 428 nM, α3β4: 3.8 μM; resistant to cholinesterase	Xiao *et al*., 1998
choline (endogenous)	α4β2:K_i 110 μM but induces no current; α7: full agonist with 2.3 mM EC_{50} (current)	Papke *et al*., 1996; Alkondon *et al*., 2000
cytisine (e.g. *Laburnum anagyroides*)	K_i: α2β2: 380 pM, α4β2: 990 pM, α4β4: 1.2 nM, α2β4: 4.9 nM, α3β2: 14 nM, α3β4: 56–200 nM; weak at bovine β4	Campos-Caro *et al*., 1997; Parker *et al*., 1998; Xiao *et al*., 1998
dimethylaminocinnamylidine	K_i: α7: 34 nM, α4β2: 350 nM	de Fiebre *et al*., 1995
DMPP	K_i: β2-containing: 10–50 nM, β4-containing: ca. 1 μM; guanethidine-like activity	Birmingham and Wilson, 1965; Parker *et al*., 1998
epibatidine (frog *Epipedobates tricolor)*	K_i: neuronal heteropentameric: picomolar, muscle & α7: 1–10 nM	Badio and Daly, 1994; Gerzanich *et al*., 1995
GTS-21	α7-selective partial agonist: K_i 310 nM, EC_{50} ca. 100 μM (current); has an active metabolite	Meyer *et al*., 1998
lobeline (tobacco)	K_i: α2β2: 2.6 nM; α4β2: 4 nM; α3β2: 13 nM; α4β4: 49 nM; α2β4: 220 nM; α3β4: 480 nM	Damaj *et al*., 1997; Parker *et al*., 1998
nicotine (tobacco)	K_i: α2β2: 800 pM; α4β2: 4–5 nM; α3β2: 16 nM; α4β4: 26 nM; α2β4: 70 nM; α3β4: 300–500 nM; α7: 400 nM; fish muscle: 1.4 μM	Kem *et al*., 1997; Parker *et al*., 1998; Xiao *et al*., 1998
RJR-2403	K_i: α4β2: 26 nM, α7: 36 μM	Bencherif *et al*., 1996
SIB-1508Y	neuronal, weak at α3β4, inactive at α7; rat brain estimated K_i 3–10 nM	Cosford *et al*., 1996
SIB-1553A	prefers β4; in rat brain estimated K_i 0.3–1 μM	Vernier *et al*., 1999
SIB-1765F	α4β2-selective; K_i: α4β2: 7.5 nM, α4β4: 235 nM	Sacaan *et al*., 1997
tetramethylammonium	K_i: α4β2: 260–480 nM	Papke *et al*., 1996; Whiteaker *et al*., 1998

[a]EC_{50}: concentration causing half-maximal stimulation (studied parameter in parentheses)
[b]General references: (McGehee and Role, 1995; Vizi *et al*., 1995; Adams and Swanson, 1996; Budavari *et al*., 1996; Gotti *et al*., 1997; Alexander and Peters, 1999)

ANTAGONISTS

As with most receptors and receptor families, several antagonists recognise closely related receptors with different affinities. However, most of the nicotinic acetylcholine receptor subtypes lack highly selective antagonists. Nicotinic antagonists may block the acetylcholine binding site, they may affect the agonist affinity state of the receptor, or they may block the integral ion channel itself (Alexander and Peters, 1999).

Since development of various antihypertensive agents by the pharmaceutical industry, neuronal nicotinic antagonists have not been used much in clinical practice. However, promising results have been obtained in relieving Tourette's syndrome with mecamylamine (Sanberg *et al*., 1998), and muscle nicotinic receptor antagonists such as rocuronium, vekuronium and mivacurium, are used in anaesthetic practice to sustain skeletal muscle relaxation. Despite their limited used in humans, neuronal nicotinic receptor antagonists are important tools in pharmacological and toxicological research. Properties of selected nicotinic antagonists are listed in Table 2.

TABLE 2. Characteristics of nicotinic antagonists

Ligand (source)	**Affinity for receptors**[a]	**References**[b]
atropine (*Atropa belladonna*)	α9: IC_{50} 1.3 μM (10 μM acetylcholine)	Elgoyhen *et al*., 1994
α-bungarotoxin (krait *Bungarus multicinctus*)	IC_{50}: muscle, α7, α8, α9: 0.5 – 10 nM; inactive at neuronal heteromers	Motomura *et al*., 1992; López *et al*., 1998
κ-bungarotoxin (*B. multicinctus*)	K_i: α3β2: 10 nM; muscle, α3β2: low micromolar	Luetje *et al*., 1990
chlorisondamine (synthetic)	neuronal channel blocker: micromolar	El-Bizri and Clarke, 1994
α-conotoxins GI, GIA, GII, MI, SI, SIA, SII (snails *Conus spp.*)	muscle: IC_{50} 10 – 100 nM (100 and 500 nM acetylcholine)	Luetje *et al*., 1990; Johnson *et al*., 1995
α-conotoxin IMI (*C. imperialis*)	IC_{50}: α7: 0.22 μM (500 μM acetylcholine); α9: 1.8 μM (100 μM acetylcholine)	Johnson *et al*., 1995
α-conotoxin MII (*C. magus*)	IC_{50}: α3β2 0.5 nM (300 μM acetylcholine)	Cartier *et al*., 1996
decamethonium (synthetic)	IC_{50} micromolar, weak partial agonist	
dihydro-β-erythroidine (seeds of *Erythrina spp.*)	K_i: α3β4: 220 μM; rat brain: 29 μM	Xiao *et al*., 1998
gallamine (synthetic)	brain: K_i ca. 100 μM; muscle IC_{50} 8 μM (electrical stimulation)	Törocsik *et al*., 1989; Fletcher and Steinbach, 1996
hexamethonium (synthetic)	neuronal channel blocker; α3β4: IC_{50} ca. 200 μM (100 μM nicotine)	Xiao *et al*., 1998
histrionicotoxin (frog *Dendrobates histrionicus*)	IC_{50}: α3β4*: 3 μM (0.3 mM carbachol); muscle: micromolar	Wada *et al*., 1989
lophotoxin (corals *Lophogorgia spp*.)	K_i: muscle, β2-containing receptors: ca. 10 μM; irreversible	Luetje *et al*., 1990
mecamylamine (synthetic)	β4-containing receptors IC_{50} ca. 1 μM (20 – 200 μM nicotine); channel blocker	Stauderman *et al*., 1998; Xiao *et al*., 1998
methyllycaconitine (*Delphinium brownii* seeds)	K_i: α7: ca. 1 nM; neuronal: 4–6 μM	Ward *et al*., 1990; Whiteaker *et al*., 1998
muscarine (mushrooms *Clitocybe spp., Inocybe spp.*)	α9: IC_{50} 75 μM (10 μM acetylcholine)	Elgoyhen *et al*., 1994
neosurugatoxin (Japanese ivory mollusc *Babylonia japonica*)	IC_{50}: α3β4*: 27 nM (0.3 mM carbachol); β2-containing receptors: low nanomolar; muscle: ca. 100 nM (100 nM ACh)	Wada *et al*., 1989; Luetje *et al*., 1990
nereistoxin (annelid *Lumbriconereis heteropoda*)	muscle, neuronal; chick retina: IC_{50} 3.5 μM (0.3 mM DMPP)	Xie *et al*., 1993; Xie *et al*., 1996
nicotine (tobacco)	IC_{50}: α9: 30 μM (10 μM acetylcholine)	Elgoyhen *et al*., 1994
strychnine (nut *Strychnos nux-vomica*)	IC_{50}: α9: 20 nM (10 μM acetylcholine); muscle,α2β2, α2β4: micromolar	Elgoyhen *et al*., 1994; García-Colunga and Miledi, 1999
tubocurarine (*Chondodendron tomentosum* stems)	muscle, neuronal: K_i micromolar	Xiao *et al*., 1998

[a]IC_{50}: concentration causing half-maximal inhibition (stimulus in parentheses)
[b]General references: (Deneris *et al*., 1991; McGehee and Role, 1995; Vizi *et al*., 1995; Adams and Swanson, 1996; Budavari *et al*., 1996; Gotti *et al*., 1997; Alexander and Peters, 1999)

NICOTINE SIGNALLING

Nicotinic acetylcholine receptors are ligand-gated cation channels. Binding of two agonist molecules on the pentamer stabilises the channel open-state. The activated receptors non-selectively conduct cations. The overall conductance as well as the relative conductances of various ions depend on the subunit composition of the receptor (see Lindstrom *et al*., 1995; McGehee and Role, 1995; Ramirez-Latorre *et al*., 1999). Receptor-carried Ca^{++} alone may increase the intracellular free Ca^{++} concentration ($[Ca^{++}]_i$) sufficiently to evoke responses (Zhou and Neher, 1993). However, usually membrane depolarisation and a secondary Ca^{++} influx through voltage-operated channels follow nicotinic activation.

A rapid increase in $[Ca^{++}]_i$ activates various intracellular processes, such as transmitter and neuropeptide release and activation of phospholipase A_2 (PLA_2). The events are not unique to nicotinic stimulation, but occur upon membrane depolarisation in general (Livett *et al*., 1981; Holz *et al*., 1982; Frye and Holz, 1984). The rapid increase in $[Ca^{++}]_i$ also activates phospholipase C (PLC), generating diacylglycerol which, in turn, activates PKC (TerBush and Holz, 1986; Eberhard and Holz, 1987; TerBush *et al*., 1988). Despite generally being accompanied and modulated by kinase activation, transmitter exocytosis can also occur in its absence (TerBush and Holz, 1990).

The agonists act not only by stabilising the receptor open-state thus causing activation, but also by evoking desensitisation. Desensitisation, by definition, refers to a situation, where the decline in response, either fade or tachyphylaxis, involves the receptor itself or is a direct consequence of receptor activation (Jenkinson *et al*., 1998). Although nicotinic receptors may be in several un-, mono- and diliganded conformations, relatively simple models on activation-desensitisation cycles can be presented due to the low probability of certain conformational changes (Katz and Thesleff, 1957; Auerbach and Akk, 1998). The desensitisation is mainly thought to occur in diliganded state, and the recovery through ligand dissociation (Figure 2).

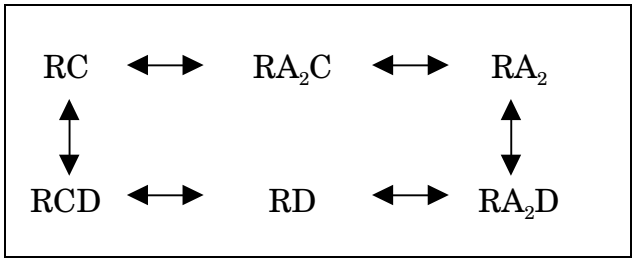

FIGURE 2. A two-gate model of nicotinic receptor activation and desensitisation (Auerbach and Akk, 1998). R represents the receptor and A_2 the two bound agonists. C and D represent the closed states of the putative activation and desensitisation gates. Thus, the ion channel is conductive only in conformation RA_2. No monoliganded states are presented because of their low probability of undergoing desensitisation or recovering from it.

Desensitisation also seems to occur without a preceding activation, not in full agreement with the simplified models. The receptor function is frequently desensitised at a lower concentration than that required for activation, in particular during prolonged exposures. Thus, whether a nicotinic agonist evokes or inhibits cholinergic responses depends not only on the subunit composition of the receptor in question, but also on the length of the exposure and the concentration of the agonist (Riker, 1968; Marks *et al*., 1996; Marks, 1999). Indeed, the nicotine concentrations reached while smoking more closely resemble receptor-desensitising concentrations than activating concentrations. Thus, it is tempting to believe that at least some of the common nicotine effects would be mediated by receptor desensitisation instead of activation. This is supported by the ability of mecamylamine to relieve symptoms of Tourette's syndrome.

SIGNALLING THROUGH PHOSPHOLIPASE C AND PHOSPHOLIPASE D

Phospholipases are enzymes that hydrolyse ester bonds of various phospholipids. The mammalian phospholipases are PLA_2, PLC and PLD, which are named after the linkages they cleave (Figure 3A). The primary glycerol-based products of PLC and PLD, diacylglycerol and phosphatidic acid, can be interconverted by diacylglycerol kinase and phosphatidic acid phosphohydrolase (Figure 3B).

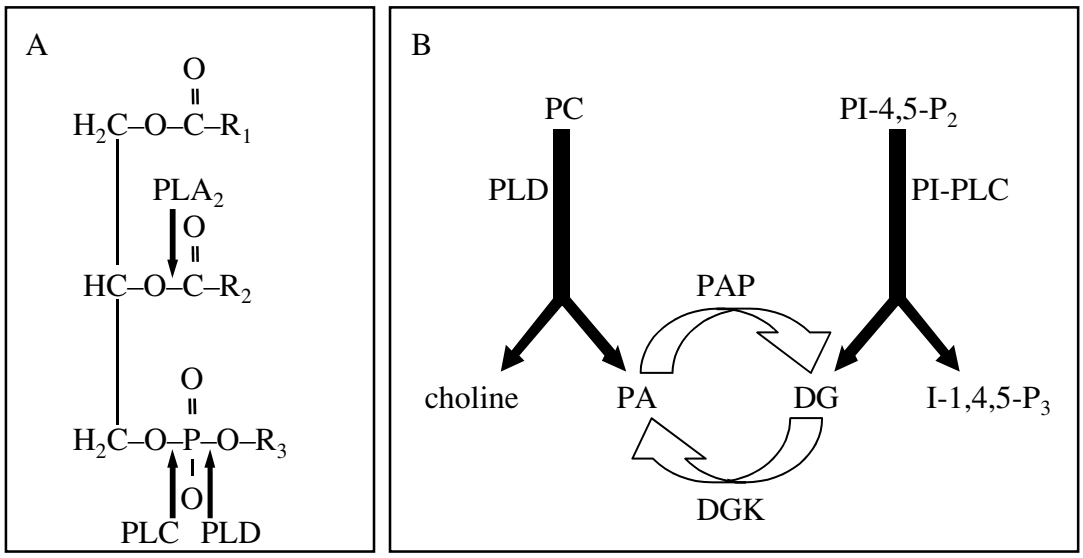

FIGURE 3. A: Bonds hydrolysed by phospholipases A_2 (PLA_2), C (PLC) and D (PLD). R_1 and R_2 represent esterified fatty acids, *e.g.* oleic, palmitic, or arachidonic acid. R3 represents the polar head of the phospholipid, *e.g.* choline or inositol 4,5-bisphosphate. B: Interconversion of phosphatidic acid (PA), and diacylglycerol (DG), generated by PLD and phosphatidylinositol-specific PLC (PI-PLC). DGK: diacylglycerol kinase; PAP: phosphatidic acid phosphohydrolase; PC: phosphatidylcholine; PI-4,5-P_2: phosphatidylinositol 4,5-bisphosphate; I-1,4,5-P_3: insitol-1,4,5-trisphosphate.

PHOSPHOLIPASE C

PLC can be divided into two major categories according to substrate specificity: PI-PLC and PC-PLC. PI-PLC is divided into PLCβ, PLCγ and PLCδ subclasses, all of which have isoenzymes identified by Arabic numerals (see Singer *et al*., 1997; Bairoch and Apweiler, 1999). Although mammalian PC-PLC has not been cloned, an abundance of data suggests its existence (Cook and Wakelam, 1992; Nofer *et al*., 1997).

PLCβ and PLCδ enzymes are regulated by heterotrimeric G proteins. In the classical activation pathway, PLCβ interacts with the α subunits of G_q proteins (Smrcka *et al*., 1991; Hepler *et al*., 1993). In addition, the G protein βγ subunits stimulate PLCβ, albeit not as effectively as the α subunits (Hepler *et al*., 1993; Gudermann *et al*., 1996). PLCδ is stimulated by the α subunits of G_h proteins (Feng *et al*., 1996), whereas PLCγ is activated by growth factors probably via tyrosine phosphorylation (see Singer *et al*., 1997; Lymn and Hughes, 2000).

Inositol lipid hydrolysis can be stimulated by increasing $[Ca^{++}]_i$, without prior activation of G-protein-coupled or tyrosine kinase receptors (Eberhard and Holz, 1987, 1991). Although inositol lipids are required for Ca^{++}-stimulated exocytosis, PI-PLC activity and hydrolysis are not necessary (Eberhard *et al*., 1990).

In addition to diacylglycerol, which activates the conventional and novel PKC isoenzymes (see below), PI-PLC activity yields inositol 1,4,5-trisphosphate. It acts on specific endoplasmic reticular receptors, thus evoking Ca^{++} release. Ca^{++}, in turn, is an

important intracellular messenger affecting transmitter release and the recovery of nicotinic receptors from desensitisation, as well as the activity of various other ion channels and enzymes (see Berridge, 1993; Fenster *et al*., 1999).

Stimulation of PLCβ-linked receptors leads to a biphasic diglyceride production. While the early phase is accompanied by inositol trisphosphate formation, the later phase is not, suggesting that PI-PLC activation is transient (Griendling *et al*., 1986; Wright *et al*., 1988; Fukami and Takenawa, 1989). Sustained diglyceride formation can be catalysed by a sequential action of PLD and phosphatidic acid phosphohydrolase (Martinson *et al*., 1990), or by a putative PC-PLC (Cook and Wakelam, 1992). Furthermore, a direct PKC activation by phorbol esters or synthetic diglycerides inhibits the early phase of diglyceride formation by PLCβ, without affecting the later phase (Griendling *et al*., 1986; Portilla *et al*., 1988; Tilly *et al*., 1990).

PHOSPHOLIPASE D

PLD activity was initially observed in plants more than fifty years ago. Nearly thirty years later, it was detected in mammalian tissues, first in the rat brain (Saito and Kanfer, 1975). Thereafter, PLD has been demonstrated in virtually all mammalian cells and tissues studied (Meier *et al*., 1999). Bovine adrenal chromaffin cells have been claimed to be an exception, and whether they possess PLD activity is under debate (Purkiss *et al*., 1991; García *et al*., 1992; Caumont *et al*., 1998; Glenn *et al*., 1998).

The first mammalian PLD was cloned and characterised relatively late (Hammond *et al*., 1995). The enzyme exists in two alternatively spliced forms, PLD1a and PLD1b (Hammond *et al*., 1997). A distinct PLD isoenzyme, PLD2, has also been cloned. It is constitutively active and appears to be regulated mainly by inhibition (Colley *et al*., 1997; Kodaki and Yamashita, 1997). There is also evidence suggesting that at least one more type, dependent on oleic acid but independent of phosphoinositides, remains to be cloned (Massenburg *et al*., 1994; Okamura and Yamashita, 1994; Frohman *et al*., 1999). Some structural and regulatory properties of cloned PLDs and oleate-dependent PLD activity are summarised in Table 3.

TABLE 3. Characteristics of phospholipase D enzymes and activities

	PLD1a, PLD1b	**PLD2**	**Oleate-dependent PLD**
Size	124 kDa (a), 119 kDa (b)	106 kDa	190 kDa[a]
Preferred membranes	Golgi membranes, microsomes, endosomes	plasma membrane	plasma membrane, nucleus, microsomes
Cytosolic	probably	possibly	no
Basal activity	low	high	low
Activation by			
*lipids	PIP2, PIP3[b]	PIP2, PIP3	oleate, arachidonate
*GTPases	Arf, RhoA, Rac, Cdc42	minor[c]	no
*kinases	cPKCα, β; nPKCε	no	no
Inhibitory proteins	synaptojanin, fodrin, AP3	synaptojanin, synuclein	not known
Other inhibitors	oleate, primary alcohols, ceramides	oleate, primary alcohols	primary alcohols

References: (Okamura and Yamashita, 1994; Colley *et al*., 1997; Exton, 1997; Hammond *et al*., 1997; Kodaki and Yamashita, 1997; Morris *et al*., 1997b; Brown *et al*., 1998; Katayama *et al*., 1998; Lopez *et al*., 1998; Frohman *et al*., 1999; Houle and Bourgoin, 1999)

[a]size approximated from electrophoresis gels
[b]*PIP2: phosphatidylinositol 4,5-bisphosphate, PIP3: phosphatidylinositol 3,4,5-trisphosphate*
[c]*Arf reportedly activates a truncated PLD2*

A full activation of PLD1 requires the presence of Arf, RhoA, active PKC and inositol phospholipids. However, neither phosphorylation nor phosphoinositidase activity is necessary. Since the effects of the above-mentioned activators are synergistic, it is probable that each has a separate regulatory binding site on the enzyme (see Singer *et al*., 1996, 1997; Morris *et al*., 1997b).

A number of extracellular signals that act through tyrosine kinase receptors or G_q family proteins and PKC, increase PLD activity. Examples include angiotensin II, bradykinin, choline esters, insulin, and phorbol esters (Qian and Drewes, 1989; Horwitz, 1991; Purkiss *et al*., 1991; Horwitz and Ricanati, 1992; Kanoh *et al*., 1992; Standaert *et al*., 1996; Jung *et al*., 1998). Effects of nicotinic agonists and cell depolarisation by high extracellular potassium on PLD activity are less clear (Caumont *et al*., 1998; Glenn *et al*., 1998; Singh *et al*., 1998).

PLD and PLD-produced phosphatidic acid have been suggested to have a role in various phenomena. Clearly, PLD alters membrane composition and physical properties by hydrolysing phosphatidylcholine to phosphatidic acid. The released choline is not known to serve intracellular signalling functions, but can be used as a substrate in acetylcholine synthesis. PLD has a role in exocytosis for example from neutrophils and mast cells, but it does not appear to be necessary for the process in all cell types (Glenn *et al*., 1998; Jones *et al*., 1999). Importantly, diglyceride generation from phosphatidic acid by phosphatidic acid phosphohydrolase may in part be responsible for sustained PKC activation (Liscovitch, 1992; Nishizuka, 1995). PLD and phosphatidic acid have a mitogenic effect on multiplying cells (Boarder, 1994; Venable and Obeid, 1999). Phosphatidic acid may also directly participate in PKC activation, and may enhance the receptor-mediated PLC activation (see English *et al*., 1996). Furthermore, it selectively activates a putative dual specificity protein kinase (McPhail *et al*., 1999). Phosphatidic acid can be converted to lysophosphatidic acid by PLA_2 to stimulate cell proliferation, tissue regeneration and transmitter release via G_q- and G_i-coupled receptors and to enhance PKC activation (see Moolenaar, 1995; Nishizuka, 1995).

CALCIUM SIGNALLING

In resting cells, the $[Ca^{++}]_i$ is usually around or below 100 nM. Plasma membrane and smooth endoplasmic reticulum Ca^{++} pumps, plasma membrane Na^+/Ca^{++} antiporter as well as mitochondrial Ca^{++} carriers extrude Ca^{++} from the cytosol and hence participate in sustaining the low $[Ca^{++}]_i$ and terminating Ca^{++} signals (Alberts *et al*., 1994).

Various mechanisms are involved in the entry of extracellular Ca^{++} into the cytosol. Ca^{++} may enter a cell through Ca^{++}-carrying transmitter receptors, such as nicotinic acetylcholine receptors and NMDA glutamate receptors. Store-operated Ca^{++} channels are opened in response to emptied intracellular Ca^{++} stores, and they are responsible for capacitative calcium entry. The mechanism is unknown, but protein-protein interactions may be involved (Berridge *et al*., 1998). Voltage-operated Ca^{++} channels are opened upon membrane depolarisation. They are classified according to activation threshold, kinetics, conductivity and pharmacology to L-, T-, N-, P-, Q- and R-type channels. The L- and T-type channels are found in several cell types while the rest of the channel types appear neuronal (see Trist and Spedding, 1998; Watling, 1998).

Ca^{++} may be released to the cytosol also from endoplasmic reticular stores. Two receptor-operated release pathways are known. Inositol 1,4,5-trisphosphate receptors are

activated by PLC-generated inositol 1,4,5-trisphosphate. Ryanodine receptors are activated by caffeine, ryanodine and their putative physiological agonist cyclic adenosine diphosphate ribose. These endoplasmic reticular receptors are sensitised by Ca^{++}, and they mediate calcium-induced Ca^{++} release (Berridge, 1998).

Changes in the $[Ca^{++}]_i$ are detected by various proteins including calmodulin, troponin C, annexins, cPKCs and ion channels. Hence, it regulates a multitude of cell events from fertilisation to cell death. It is a crucial relay pathway controlling cytoskeletal organisation, contraction, secretion including transmitter release, many metabolic functions, proliferation and apoptosis (see Berridge *et al*., 1998).

PROTEIN KINASE C

REGULATION

PKC was originally identified as a proteolytically activated protein kinase with no obvious function in signal transduction (Inoue *et al*., 1977; Takai *et al*., 1977). Soon it was realised that it was a calcium-activated enzyme with absolute phospholipid dependence and great diacylglycerol sensitivity (for early reviews, see Nishizuka, 1984, 1986). After less than a decade, however, it became evident that PKC is not a single entity, but rather a family of serine-threonine kinases (Coussens *et al*., 1986; Parker *et al*., 1986). Moreover, calcium- and diglyceride-independent isoenzymes were detected (Ohno *et al*., 1988; Ono *et al*., 1988, 1989; Schaap *et al*., 1989). Indeed, the differences in activation requirements have led to confusions in nomenclature. Upon uncovering the structure and basic biochemistry of mouse PKCμ, it was first named protein kinase D (Valverde *et al*., 1994), and even today both of these names are used.

In mammals, eleven protein kinase C isoenzymes have been identified (Bairoch and Apweiler, 1999). The conventional isoenzymes (cPKCα, -β1, -β2 and -γ) require Ca^{++} and diacylglycerol for activation. The novel isoenzymes (nPKCδ, -ε, -η, -θ and -μ) are Ca^{++}-independent but still diacylglycerol-dependent. The atypical isoenzymes (aPKCζ and -ι) need neither diglyceride nor Ca^{++} for activation. Conventional PKCβ1 and -β2 are alternative splicing products of a single gene, while distinct genes code the rest of the isoenzymes (Mellor and Parker, 1998; Ron and Kazanietz, 1999).

In addition to the conventional lipid regulators of PKC, namely phosphatidylserine and diacylglycerols, free *cis*-unsaturated fatty acids and some phosphatidylcholine species have an activity-enhancing effect on cPKC isoenzymes (Shinomura *et al*., 1991; Burns and Bell, 1992; Ron and Kazanietz, 1999). Phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate activate the n- and aPKCs (Nakanishi *et al*., 1993; Singh *et al*., 1993). Phosphatidic acid and ceramide can also activate aPKCζ (English *et al*., 1996; Galve-Roperh *et al*., 1997; Wang *et al*., 1999).

In the resting state, PKC is cytosolic and a pseudosubstrate domain occupies the catalytic site. Upon enzyme activation, Ca^{++} permits cPKC isoenzymes to associate with membranes. The membrane-associated PKC is then inserted into the membrane, the pseudosubstrate leaves the catalytic site and the kinase is activated. Diacylglycerol is required for membrane insertion of c- and nPKCs. Activation of aPKCs is less well understood (Burns and Bell, 1992; Keranen and Newton, 1997; Ron and Kazanietz, 1999).

A great number of stimuli, especially those acting through receptors coupled to PLC and phosphatidylinositol bisphosphate hydrolysis, transiently activate PKC. PKC is

also the major receptor for tumour-promoting phorbol esters which bind to the diglyceride site of the enzyme (see Parker, 1992). A prolonged increase in the membrane-bound, *i.e.* the active, PKC accompanied with an increased level of diglycerides can be evoked by phorbol esters and by the cell membrane receptor agonists nicotine and angiotensin II (Tuominen *et al*., 1991; Tuominen *et al*., 1992; Tuominen *et al*., 1993).

Phorbol esters not only activate PKC, but upon prolonged exposure, they cause down-regulation of PKC protein and activity (Rodriguez-Pena and Rozengurt, 1984). This is due to increased proteolysis of the membrane-associated enzyme by proteases, such as calpain. Initially, a constitutive kinase activity is generated as the regulatory lipid-binding domain is separated from the catalytic one. However, the final net effect is a reduced PKC activity. This phenomenon also provides a widely used tool for studying PKC-dependence of signal transduction (see Parker, 1992; Rasmussen *et al*., 1992).

FUNCTION

Similar to various other protein kinases, PKC plays a role in signal amplification from cell surface through kinase cascades to the nucleus. It also participates in the fine-tuning of cellular events (Figure 4). In addition to other kinases, PKC phosphorylation targets include other enzymes, structural proteins, ion channels and receptors (Aderem and Seyrkora, 1992; Borner and Fabbro, 1992; Jaken, 1992; Knox and Kaczmarek, 1992). PKC can directly interact with a variety of proteins, not all of them PKC substrates, an important example being PLD1 (see Ron and Kazanietz, 1999).

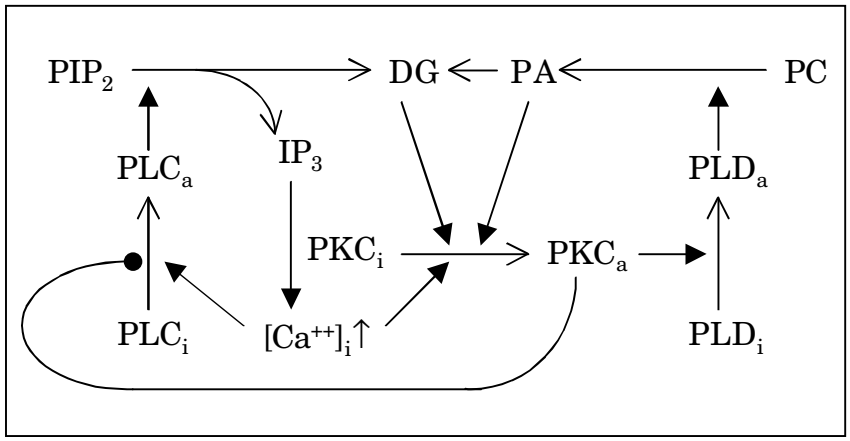

FIGURE 4. A simplified model of the interplay of phospholipases C (PLC) and D (PLD), protein kinase C (PKC), and free intracellular Ca^{++} ($[Ca^{++}]_i$). PC: phosphatidylcholine, PA: phosphatidic acid, PIP_2: phosphatidylinositol 4,5-bisphosphate, DG: diglyceride, IP_3: inositol 1,4,5-trisphosphate, subscripts i: inactive, a: active. Closed head arrow indicates an activating effect and closed circle an inhibitory effect on adjacent signalling pathways.

PKC-catalysed phosphorylation of nicotinic acetylcholine receptors has been reported to enhance the receptor recovery from desensitisation in rat chromaffin cells and in *Xenopus* oocytes expressing α4β2 receptors, as well as to reduce the deepness of desensitisation of the α4β2 receptors (Khiroug *et al*., 1998; Fenster *et al*., 1999). However, in voltage-clamped chick sympathetic ganglion neurons, PKC activators enhance nicotinic receptor current decay (Downing and Role, 1987). Moreover, an increased number of human α4β2 receptors in a cell line have been noted in response to a prolonged exposure to PMA but not to PKC inhibitors (Gopalakrishnan *et al*., 1997), suggesting PKC-enhanced desensitisation and thus concurring with previous views (Huganir and Greengard, 1990; Swope *et al*., 1992). Yet, dephosphorylated nicotinic receptors have been suggested to be a signal for inhibiting receptor turnover (Peng *et al*., 1994).

BOVINE ADRENAL CHROMAFFIN CELLS

Adrenal medulla originates from the embryonic neural crest and functionally resembles a sympathetic ganglion. In the 1960's, a method for isolating and culturing gerbil adrenal chromaffin cells was established (Douglas *et al*., 1967a, 1967b). In the 1970's, bovine chromaffin cell cultures were developed (Schneider *et al*., 1977), and within a decade several laboratories adopted and further elaborated these methods (Kilpatrick *et al*., 1980; Unsicker and Müller, 1981; Waymire *et al*., 1983; Wilson, 1987). During this period, chromaffin cells, especially of bovine origin, became a widely used tool in neurochemical and neurobiological studies (see Livett, 1984).

Bovine adrenal chromaffin cells synthesise noradrenaline and adrenaline. The cells also have a high-affinity uptake mechanism for catecholamines (Kenigsberg and Trifaró, 1980). Upon nicotinic stimulation and increased $[Ca^{++}]_i$, the chromaffin cells quantally secrete not only catecholamines but also peptides, such as enkephalin (Livett *et al*., 1981). Bovine adrenal chromaffin cells retain their neural properties in culture for prolonged periods, whether or not they were allowed to attach to the culture dish (Kilpatrick *et al*., 1980; Waymire *et al*., 1983).

Although the muscarinic receptors of cultured bovine chromaffin cells are coupled to phosphoinositide hydrolysis (Eberhard and Holz, 1987; Nakaki *et al*., 1988), their activation evokes only weak if any secretion of catecholamines (Ballesta *et al*., 1989; Cheek *et al*., 1989; McKay *et al*., 1991; Yamagami *et al*., 1991). Thus, cholinergic effects on these cells are almost purely nicotinic, in contrast to porcine (Nassar-Gentina *et al*., 1997) and feline chromaffin cells (Ballesta *et al*., 1989).

Similar to sympathetic ganglion cells in general, bovine chromaffin cells express nicotinic acetylcholine receptors. In these cells, genes encoding the α3, α5, α7 and β4, but not the β2, nicotinic acetylcholine receptor subunits are expressed (Criado *et al*., 1992; García-Guzmán *et al*., 1995; Campos-Caro *et al*., 1997; Wenger *et al*., 1997). Hence, the possible subunit combinations are α3β4* with or without an α5 subunit, and α7.

Stimulation of nicotinic acetylcholine receptors on bovine adrenal chromaffin cells leads to Na^+ influx and depolarisation of the plasma membrane. Then $[Ca^{++}]_i$ is increased, PLA_2 is activated, and transmitters are released (Holz *et al*., 1982; Frye and Holz, 1984). Although the secretory events are accompanied by inositol phospholipid breakdown and PKC activation (TerBush and Holz, 1986; Eberhard and Holz, 1987; TerBush *et al*., 1988), and direct PKC activation by phorbol esters can evoke exocytosis, changes in PI-PLC and PKC activity are not necessary for catecholamine secretion (TerBush and Holz, 1990; Loneragan *et al*., 1996).

COTININE PHARMACOLOGY

Cotinine structure and synthesis have been known for more than one-hundred years (Pinner, 1893). Cotinine has mainly been considered a pharmacologically inert metabolite of its psychoactive parent compound (Jaffe, 1990; Anonymous, 1991; Benowitz, 1996; Rang *et al*., 1999). However, already in the early sixties, the first evidence of possible cotinine activity was published. The compound was found to be hypotensive and to abolish the nicotine-induced vasopressor response in dogs (Borzelleca *et al*., 1962). Cotinine was also found to have effects opposite to nicotine, first as a rabbit and rat intestinal smooth muscle relaxant (Kim *et al*., 1968).

Cotinine was then reported to counteract the catecholamine decreases secondary to tyrosine hydroxylase inhibition in the rat (Eneroth *et al*., 1977). The same group further reported a nicotine-like reduction in 5-hydroxytryptamine turnover by cotinine in the rat brain. The effects of nicotine on the central nervous system were speculated to be in part mediated by cotinine, independently of nicotinic acetylcholine receptors (Fuxe *et al*., 1979). In mice, cotinine has been reported to mimic nicotine as an antinociceptive compound (Erenmemisoglu and Tekol, 1994) and in counteracting ethanol-induced incoordination (Dar *et al*., 1994). Recently, a cotinine-evoked dopamine release from rat striatal slices has been reported (Dwoskin *et al*., 1999). In humans, cotinine slightly worsens nicotine withdrawal symptoms (Keenan *et al*., 1994), and it eliminates the beneficial effects of a nicotine patch (Hatsukami *et al*., 1998).

Outside the nervous system, cotinine has been reported to affect eicosanoid release both in a nicotine-like manner (Saareks *et al*., 1993) and differently from nicotine (Chahine *et al*., 1990). Moreover, in steroid metabolism both nicotine-like (Patterson *et al*., 1990) and nicotine-unlike effects (Andersson *et al*., 1993) have been detected.

Although cotinine has been nearly or totally inert in receptor binding assays (Abood *et al*., 1983, 1985), it seemingly has pharmacological activity. The question arises as to whether biological activity of cotinine is mediated through nicotinic acetylcholine receptors; by activation, desensitisation or blockade.

AIMS OF THE STUDY

As outlined in the previous section, cotinine pharmacologically acts on neural systems, but its effects remain obscure. Since cotinine is structurally related to nicotine, its probable targets include nicotinic receptors, through which it can modify nicotinic responses. PLD, in turn, is a good candidate for catalysing the nicotine-evoked prolonged diglyceride production and sustaining the increased PKC activity. The finding – presented in a single report at the time this work was planned – that PLD is lacking from bovine chromaffin cells does not concur with this hypothesis. If PLD activity exists in chromaffin cells, it may well have a role in nicotinic signalling.

The aim of the present study was to clarify whether:

1 receptor-linked PLD activity exists in bovine adrenal chromaffin cells;

2 PLD participates in nicotine signalling, especially in sustained kinase activation;

3 cotinine modulates the effects of nicotine on intracellular (PKC activity, $[Ca^{++}]_i$) and intercellular (noradrenaline release) chromaffin cell signalling;

4 cotinine itself evokes chromaffin cell responses; and

5 cotinine effects are mediated by nicotinic acetylcholine receptors.

MATERIALS AND METHODS

CELL CULTURE

Bovine adrenal glands were obtained from local slaughterhouses. They were carried to the laboratory chilled in an ice bath. The medullae were digested by retrograde perfusion through the central vein with collagenase B (Roche Molecular Biochemicals / Boehringer Mannheim Biochemicals, Mannheim, Germany), and the medullary suspension was purified by centrifugation through a meglumine diatrizoate – sodium diatrizoate gradient (made in the laboratory, or available commercially as Urografin® from Schering AG, Berlin, Germany). Purification was continued by differential plating in tissue culture flasks for two to three hours. The cells were plated on appropriate dishes or plates (Wilson and Viveros, 1981; Waymire *et al*., 1983; Wilson, 1987; Tuominen *et al*., 1991), grown in standard medium (Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 with 10% foetal calf serum and antibiotics), and used within a week of preparing the culture. The cultures yielded 10 – 120 x 10^6 cells per gland. Despite differences in cell yield, cultures were consistently of ≥ 90% purity as estimated by neutral red staining.

PLD SAMPLE PREPARATION AND ASSAY

The cells were labelled with [^{3}H]oleic acid or [^{3}H]myristic acid (both from Amersham, Buckinghamshire, UK) for 24 h. They were then washed with isotonic saline and gently suspended in an appropriate buffer (pH 7.4 at 37°C): based on bicarbonate (5% CO_2 atmosphere) or on N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES, room air). The assay was based on a PLD-catalysed characteristic reaction, where primary alcohol replaces water in accepting the phosphatidyl moiety. The phosphatidylalcohol thus formed is more stable than phosphatidic acid and can be used as a measure of PLD activity (see Morris *et al*., 1997a). Hence, the cells were exposed to stimulants in the presence of 50 mM n-butanol (I) or 220 mM ethanol (II). In 1-h exposures, the alcohol was present for the last 15 min.

The lipids were extracted with chloroform : methanol : water (10 : 10 : 9, by volume), acidified with HCl (Bligh and Dyer, 1959). The chloroform phase was collected, the solvent was evaporated, and the lipid residue was redissolved in chloroform : methanol (9 : 1). The phospholipids were then separated by thin layer chromatography (LK6D plates from Whatman, Clifton, NJ, USA). The solvent used for phosphatidylbutanol separation was the upper phase of ethylacetate : isooctane : acetic acid : water (13 : 2 : 3 : 10) (Liscovitch and Amsterdam, 1989). When phosphatidylethanol was formed, oxalate-coated thin layer chromatography plates were used with a chloroform : methanol : acetic acid : water (75 : 45 : 3 : 1) mixture as the solvent (Horwitz, 1991; Caumont *et al*., 1998). Phosphatidylalcohol markers were run along with the samples. The phospholipids on chromatography plates were stained with iodine.

Phosphatidylethanol and phosphatidylbutanol standards were prepared from 1-palmitoyl-2-oleyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) by *Streptomyces sp.* PLD (Sigma, St. Louis, MO, USA), as previously described (Eibl and Kovatchev, 1981; Kobayashi and Kanfer, 1987; Comfurius *et al*., 1990). A two-phase system consisting of 50 ml l^{-1} n-butanol or ethanol, 450 ml l^{-1} chloroform, and 500 ml l^{-1} 185 mM Tris buffer (pH 7.4) with 185 mM $CaCl_2$, was used. The mixture was shaken for 2 – 4 h at 37°C. The lipids were extracted as above (Bligh and Dyer, 1959).

WESTERN BLOT

The cultured cells were washed and homogenised by sonication in the presence of protease inhibitors phenylmethylsulfonyl fluoride 1 mM and leupeptin 250 μg ml^{-1}. The protein extracts were prepared by high-acceleration centrifugation (100 000 x g, 45 – 60 min), first in the absence and then in the presence of 0.1% Triton X-100 to extract the soluble and membrane proteins, respectively. The extracts were denatured, and the protein samples were separated in 8% polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose membranes (Schleicher & Shüll, Dassel, Germany) by semi-dry electroblotting (SemiPhor™, Hoefer Pharmacia Biotech, San Fransisco, CA, USA). The membranes were blocked in 5% fat-free milk, exposed to antibodies, and the immunoreactive proteins were detected by enhanced chemiluminescence reaction (Durrant, 1990; Crisp and Dunn, 1994).

Rabbit anti-rat PKC antisera were a generous gift from Dr. William C. Wetsel (NIEHS, NC, USA). They were raised against carboxyterminal peptides of PKC isoenzymes as follows: SYVNPQFVHPILQSAV (α), SYTNPEFVINV (β1), SFVNSEFLKPEVKS (β2), DARSPTSPVPVPVM (γ), SFVNPKYEQFLE (δ), KGFSYFGEDLMP (ε), EYINPLLLSAEESV (ζ), and their preparation and properties have previously been described (Wetsel *et al*., 1992). Anti-PLD antibody, against sequence CIIGSANINERS, was a product of Upstate Biotechnology, Lake Placid, NY, USA. Goat anti-rabbit secondary antibody was purchased from Bio-Rad Laboratories, CA, USA.

PROTEIN ASSAY

The protein concentrations of the extracts were determined according to Bradford (1976). The dye reagent was either commercial (III, IV; Bio-Rad, Richmond, CA, USA), or it was prepared from Serva Blue G (I, VI; Serva, Heidelberg, Germany). The protein standards were prepared from bovine serum albumin concentrate (Pierce, Rockford, IL, USA).

[^{3}H]NORADRENALINE RELEASE

The experiments were run largely as previously described (McKay and Schneider, 1984), in saline buffered with HEPES and $NaHCO_3$ (pH 7.4 at 5% CO_2). Ascorbic acid 1 mg ml^{-1} was added to the medium to avoid oxidation of the catecholamines. To get a lower non-specific release, 1 mg ml^{-1} bovine serum albumin was added in some experiments (II, IV). The cell catecholamine pools were labelled with 500 nM [^{3}H]noradrenaline (Amersham, 22 kBq ml^{-1}) for 10 min (II, IV) or 60 min (III). Then, the cells were washed and exposed to stimulants as indicated. The treatments were terminated by pipetting the medium into scintillation vials or plates. The cells were disrupted in 0.1% Triton X-100 before transferring them to scintillation vials or plates. Results are expressed as percentages of total cellular [^{3}H]noradrenaline contents released.

MEASUREMENT OF $[Ca^{++}]_i$

The cells were washed and gently resuspended in experiment buffer (NaCl 118 mM, KCl 5 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, HEPES 20 mM, glucose 10 mM, pH 7.4). The cells were labelled with 5 μM Fura 2-AM (Molecular Probes, Eugene, OR, USA) for the first 10 min, and then with 500 nM Fura 2-AM for an additional hour (McMillian *et al*., 1992). Then, the cells were washed, incubated for at least 10 min and washed again. All the incubations were performed in the dark to avoid photobleaching. For ex-

periments in a Ca^{++}-free environment, $CaCl_2$ was omitted from the experiment buffer and 100 μM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) was added. Measurements were performed at room temperature in a quartz cuvette (2 x 10^6 cells/ml), with a Hitachi F2000 fluorometer essentially as previously described (Törnquist *et al*., 1999). The excitation wavelengths were 340 nm and 380 nm, and the emission wavelength was 510 nm. $[Ca^{++}]_i$ was calculated as previously described (Grynkiewicz *et al*., 1985), using a software designed for the fluorometer. The K_d value for Fura 2 was 224 nM.

CYTOTOXICITY TEST

The cells were exposed to the test compounds for the desired time. In the 24-hour experiments, 0.5 mg ml^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) was added three hours before stopping the treatment. In short experiments, MTT was present throughout the incubation. For measuring toxicity, media were aspirated and replaced with dimethyl sulfoxide. After shaking for 5 min, the absorbances were read at 540 nm to estimate the MTT reduction. The extent of reduction was considered as a measure of mitochondrial function and cell survival (Supino, 1990).

PKC ASSAY

Standard methods (Kikkawa *et al*., 1983; Roskoski, 1983), with some modifications (Tuominen *et al*., 1991), were used. Treatments were stopped by washing the cells with ice-cold Ca^{++}-free medium (pH 7.5). Cells were disrupted by sonication. The suspension was centrifuged (100 000 x g, 60 min, 4°C), first without (soluble extract) and then with (particulate extract) 1 ml l^{-1} Triton X-100. In the assay, the protein extracts were allowed to catalyse histone (0.3 mg ml^{-1} calf thymus histone type III-S; Sigma) phosphorylation for 5 min at 30°C in the presence of adenosine γ-^{32}P-triphosphate (Amersham), both with and without the PKC activators: 1 mM $CaCl_2$, 40 μg ml^{-1} phosphatidylserine and 8 μg ml^{-1} 1,2-sn-dioctanoylglycerol. The reaction was terminated by spotting reaction mixture onto P81 phosphocellulose paper (Whatman, Maidstone, UK), and washing the papers in 75 mM phosphoric acid. The trapped radioactivity was counted after air-drying. PKC activity was calculated by subtracting the non-specific activity from total activity. Results are expressed as nmoles of inorganic phosphate incorporated into histone min^{-1} mg^{-1} of protein.

[^{3}H]PHORBOL-12,13-DIBUTYRATE BINDING

For short treatments, the growing medium was replaced by HEPES- and $NaHCO_3$-buffered saline (pH 7.4 at 5% CO_2), and the cells were allowed to stabilise for 60 min. [^{3}H]Phorbol-12,13-dibutyrate (PDBu, Amersham) with (non-specific binding) or without (total binding) 1 μM phorbol 12-myristate 13-acetate (PMA) was added into the wells. The 24-h experiments were run in the growing medium, and 15 min before stopping the treatment [^{3}H]PDBu with or without PMA was added. The final concentration of [^{3}H]PDBu was 10 nM. Treatments were terminated by washing the cells with isotonic saline. The bound radioactivity was measured, and results are expressed as specific binding.

ANIMALS

Adult female Wistar rats (HsdBrlHan:WIST) with free access to food and water were used. Brains were obtained after decapitation, and brain areas were dissected on ice. The study protocol was approved by the University of Helsinki ethics committee for animal experiments.

RECEPTOR BINDING

Tissues and cells were homogenised in a glass-teflon homogeniser in hypotonic Tris buffer containing protease inhibitors. The homogenates were spun twice at 30 000 x g for 15 min, and the pellets were resuspended in fresh buffer. [^{3}H]Epibatidine (NEN Life Science Products, Boston, MA, USA) was used as the nicotinic label. The experiments were performed in polypropene tubes in 50 mM Tris HCl buffer (pH 7.4) at 37°C. The binding time was 4 h. To determine non-specific binding, 300 μM nicotine was added to the incubations. In saturation binding experiments, the [^{3}H]epibatidine concentration was 14.5 pM – 6.75 nM (chromaffin cells) or 10 pM – 2.15 nM (brain). In competition experiments, single concentrations of 100 pM (chromaffin cells) and 250 pM (brain) were used. The incubations were terminated by using Brandell M-24R cell harvester. The bound ligand was trapped in Whatman GF/B filters presoaked for five hours in 5 mg ml^{-1} polyethyleneimine, and the filters were washed three times with cold Tris buffer. The filters were counted for radioactivity after overnight incubation in the scintillation liquid.

CHEMICALS

If not otherwise stated, the chemicals were obtained from standard commercial sources. Inorganic salts and solvents were of analysis grade and were supplied by Merck, Darmstadt, Germany and by J.T. Baker, Deventer, The Netherlands. The pharmacological agents and the cell culture media were mostly from Sigma, St. Louis, MO, USA. Other suppliers included Tocris Cookson, Bristol, UK; Alomone Labs, Jerusalem, Israel; and LC Services, Woburn, MA, USA.

DATA ANALYSIS

Arithmetic means, standard deviations and standard errors of means were calculated from the numeric data. Parametric tests were preferred whenever data distribution allowed. Angular transformation for percentage data was done if required to homogenise the variances (IV). In comparing two groups with a dependent variable, paired t-test was used (V).

Primarily, analysis of variance was used for estimating the existence of differences. Repeated measures tests were used where appropriate (II, V). *Post hoc* tests for multiple comparisons were Tukey's test (II – IV) or Dunnett's test (II, V). For non-parametric analyses, the Kruskall-Wallis test was used to evaluate whether a difference existed between any groups. Non-parametric *post hoc* tests were done using Mann-Whitney U-test, followed by Dunn-Sidak (I) or Bonferroni (III) procedure.

For receptor binding data (VI), nonlinear regression analyses were used. The data were fitted to one-site and two-site models, and the fits were compared by F-test. The analyses were performed with Prism 2.0b for Macintosh (GraphPad Software, San Diego, CA, USA).

RESULTS

PLD IN CHROMAFFIN CELL SIGNALLING

The anti-PLD antibody detected a protein of apparent molecular weight of 105 kDa in the particulate protein fraction of adrenal medullary chromaffin cell homogenates. In the soluble fraction, the antibody detected several proteins, all giving only a weak signal (I).

Upon stimulation by angiotensin II (200 nM), bradykinin (100 nM), or PMA (1 μM) in the presence of 50 mM n-butanol, a significant accumulation of phosphatidylbutanol was detected in 5- and 15-min treatments. When butanol was present for the last 15 min of 1-h drug exposure, only PMA was able to sustain a significant transphosphatidylation activity (I).

To assess whether nicotine increases transphosphatidylation activity, ethanol was used instead of *n*-butanol. Neither 10 μM nicotine nor cell depolarisation by 56 mM K^+ had any effect in 10-min exposures. However, 1 μM PMA greatly increased the accumulation of phosphatidylethanol (II). The stimulant effects on phosphatidylethanol accumulation remained unaffected by replacing [^{3}H]oleate with [^{3}H]myristate in phospholipid labelling.

If PLD and phosphatidic acid are crucial in catecholamine exocytosis, primary alcohols should efficiently inhibit secretion. However, ethanol only weakly inhibited nicotine- and KCl-evoked exocytosis, and no selectivity of the primary alcohols over *sec*- and *tert*-butanol, regarded as poor PLD substrates, in inhibition of catecholamine exocytosis was detected. Interestingly, *n*-butanol somewhat increased noradrenaline exocytosis in the absence of other stimulants (II).

Since the inhibitory effect of alcohols on catecholamine release appeared not to be secondary to trapping phosphatidic acid, I recorded their effect on chromaffin cell $[Ca^{++}]_i$. All the alcohols raised $[Ca^{++}]_i$, even in the absence of extracellular Ca^{++}. They also inhibited a secretagogue-evoked further increase in $[Ca^{++}]_i$ (Table 4; II). An increased fluorescence upon excitation with 340 nm light was always accompanied with a decreased fluorescence upon excitation with 380 nm light.

TABLE 4. Effect of 260 mM ethanol and 160 mM butyl alcohols on $[Ca^{++}]_i$ in chromaffin cells in buffer containing either 1 mM $CaCl_2$ or no Ca^{++} but 100 μM EGTA, and on the increase in $[Ca^{++}]_i$ evoked by sequentially added 10 μM nicotine and 56 mM KCl, or addition of 1 mM $CaCl_2$.

	1 mM $CaCl_2$			Ca^{++}-free	
	Alcohol	Nicotine	KCl	Alcohol	1 mM $CaCl_2$
control	-1.2 ± 1.0	380 ± 40	530 ± 42	-1.3 ± 0.60	120 ± 13
ethanol	63 ± 11*	290 ± 29*	420 ± 33**	23 ± 2.9	140 ± 16
n-butanol	380 ± 26**	-2.3 ± 1.3**	7.6 ± 5.6**	130 ± 14**	270 ± 32**
sec-butanol	230 ± 18**	12 ± 11**	100 ± 5.8**	120 ± 7.5**	180 ± 11*
tert-butanol	200 ± 18**	-3.3 ± 1.6**	210 ± 17**	52 ± 3.3**	180 ± 17*

Values reflect the change in $[Ca^{++}]_i$ in nM (mean ± standard error). Asterisks indicate statistically significant differences *vs*. corresponding controls by Dunnett's test (*$p < 0.05$, **$p < 0.01$).

The alcohol-evoked apparent increase in $[Ca^{++}]_i$ could be due to cytotoxicity. However, a 2-h exposure to the alcohols at the concentrations used in the noradrenaline release and $[Ca^{++}]_i$ experiments was not cytotoxic to chromaffin cells, as estimated by reduction of MTT (II).

COTININE EFFECTS ON CHROMAFFIN CELLS

In agreement with previous knowledge (Tuominen *et al*., 1992), nicotine was observed to increase specific [^{3}H]phorbol dibutyrate binding, reflecting PKC activity, to chromaffin cells upon both 15-min and 24-h exposures. Cotinine alone also increased [^{3}H]phorbol dibutyrate binding upon 15-min but not 24-h exposure. Nonetheless, cotinine pretreatment for 10 min, at 1 mM and higher concentrations, and for 24 h, at 100 μM – 10 mM, resulted in a decrease in the nicotine-induced binding (III).

The altered [^{3}H]phorbol dibutyrate binding suggested redistribution of PKC. In enzyme assays, a 24-h incubation of the cells with 1 mM cotinine failed to increase the PKC kinetic activity in the particulate protein fraction. However, the nicotine-induced increase in membrane-bound PKC activity was markedly decreased by the same treatment (III). Cytotoxicity of cotinine could in part explain its inhibitory effect on nicotine-induced PKC activity. Nevertheless, significant inhibition in the MTT reduction was detected only at higher concentrations than those used in the PKC and phorbol-binding assays (III).

When the chromaffin cells were incubated in the absence of stimulants after a 60-min labelling period with [^{3}H]noradrenaline, the basal [^{3}H]noradrenaline release over a 20-min period was 7 – 8% of the cellular contents (III). Nicotine and DMPP, for the latter 10 min, dose-dependently increased the release as anticipated. A 10-min pre-incubation with 10 mM cotinine increased basal release slightly but significantly (III). To reduce non-specific noradrenaline release, 2 mg ml^{-1} bovine serum albumin was added to the incubation buffer. In addition, the labelling time with [^{3}H]noradrenaline was reduced to 10 min. These measures drastically reduced basal label release during incubations of 10 s – 20 min. Under these circumstances, cotinine concentration- and time-dependently stimulated [^{3}H]noradrenaline exocytosis (Table 5; IV).

TABLE 5. Time-dependence of cotinine-evoked [^{3}H]noradrenaline release as percentage of cellular label.

	10 s	60 s	10 min	20 min
control	0.35 ± 0.055	1.1 ± 0.092	1.4 ± 0.080	1.9 ± 0.32
peak effect (at [cotinine])	1.4 ± 0.13 (32 mM)	5.9 ± 0.17 (10 mM)	28 ± 0.27 (3.2 mM)	31 ± 0.98 (3.2 mM)
effect of 10 μM nicotine	0.58 ± 0.070	5.0 ± 0.21	33 ± 0.21	37 ± 0.87

Cotinine clearly and significantly inhibited the release caused by nicotine and DMPP. The effect of cotinine on nicotine-evoked release was dose-dependent. However, cotinine did not appear to be a general membrane-stabilising agent, as the exocytotic effects of high extracellular K^+ (56 mM) and veratrine (10 mg l^{-1}) were not reduced. Indeed, cotinine slightly but significantly increased the veratrine-induced release (III).

Cotinine, at concentrations of 10 – 32 mM, also significantly increased the binding of [^{3}H]phorbol dibutyrate to chromaffin cells in 15-min but not in 24-h incubations. This effect was inhibited by the nicotinic receptor channel blocker hexamethonium. In line with this, a 30-min exposure to 10 mM cotinine increased the membrane-associated PKC activity (IV). The PKC isoenzymes activated by cotinine appeared to be cPKCα and nPKCε, as indicated by Western blot. The cPKCβ1 and cPKCβ2 were not translocated, while the anti-rat PKC antibodies against γ, δ and ζ isoenzymes recognised no protein in the bovine chromaffin cell electrophoresis preparations (IV).

The cotinine-evoked effects on noradrenaline release could well be mediated by nicotinic acetylcholine receptor activation and increased $[Ca^{++}]_i$. Indeed, cotinine, at concentrations of 320 μM and higher, increased the $[Ca^{++}]_i$. The effect of cotinine on $[Ca^{++}]_i$

was inhibited by both competitive and non-competitive nicotinic receptor antagonists, as well as by omitting extracellular Ca^{++} (V). Inhibition occurred, in part, by blocking the voltage-dependent L-type calcium channels by 300 nM nimodipine. Emptying the endoplasmic reticular Ca^{++} stores with 1 μM thapsigargin, a Ca^{++}-ATPase inhibitor, increased the chromaffin cell $[Ca^{++}]_i$ but had no effect on the response to cotinine. Nor did the blockade of voltage-sensitive sodium channels with 10 μM tetrodotoxin affect the cotinine-induced increase in $[Ca^{++}]_i$ (V).

In addition to its own effect, cotinine dose-dependently inhibited the nicotine-induced rise in $[Ca^{++}]_i$. Reciprocally, nicotine pretreatment inhibited the cotinine-induced rise in $[Ca^{++}]_i$. Cotinine also inhibited the effect of administration of additional cotinine (V).

COTININE BINDING TO NICOTINIC ACETYLCHOLINE RECEPTORS

Finally, a nicotinic receptor binding assay for bovine chromaffin cell membrane homogenates was established. The radioligand, [^{3}H]epibatidine, bound to membranes in a saturable fashion. Upon analysis, the Rosenthal plots appeared curvilinear, and a non-linear regression analysis revealed a significantly better fit for two-site than for one-site binding. The apparent K_d values were 93 pM and 1 400 pM, with B_{max} values of 98 fmol mg^{-1} and 220 fmol mg^{-1}, respectively (VI).

Cotinine, as well as the competitive nicotinic receptor ligands nicotine, methyllycaconitine and dihydro-β-erythroidine displaced 100 pM and 1 nM [^{3}H]epibatidine from its chromaffin cell binding sites, fitting to a one-site binding model (VI). Nicotine was the most potent of the competitors tested (concentrations causing half-maximal inhibition 0.30 and 1.6 μM upon labelling with 100 pM and 1 nM [^{3}H]epibatidine, respectively), followed by methyllycaconitine (1.9 and 4.8 μM), dihydro-β-erythroidine (75 and 160 μM) and cotinine (130 and 310 μM). Alpha-bungarotoxin, over a concentration range of 100 pM – 10 μM, was unable to displace 100 pM [^{3}H]epibatidine. However, 10 nM – 10 μM α-bungarotoxin displaced 17 % of the bound radioligand upon labelling with 1 nM [^{3}H]epibatidine.

In rat frontal cortex and hippocampus, the [^{3}H]epibatidine saturation binding was consistent with a one-site model. The affinity was twice that observed for chromaffin cell high-affinity sites. In frontal cortex and hippocampus, nicotine affinity (K_i 8.6 – 13 nM and 12 – 22 nM, respectively) was ten times and cotinine affinity (K_i 2.3 – 3.8 μM and 3.0 – 4.8 μM) even more higher than in the chromaffin cell preparation (VI).

DISCUSSION

METHODOLOGICAL CONSIDERATIONS

To evaluate whether functional PLD activity in bovine chromaffin cells exists, the cells were exposed to primary alcohol together with the stimulants. In such a medium, PLD yields metabolically more stable phosphatidylbutanol instead of phosphatidic acid (Thompson *et al*., 1991; Singer *et al*., 1997). The phosphatidylalcohol was allowed to accumulate for 5 – 15 min, which was considered sufficiently long to answer the question of whether PLD activity in chromaffin cells could be stimulated in a receptor-mediated manner or by PMA, a universal activator of PLD1. In the first studies, *n*-butanol was used as the substrate alcohol. Since at the 50 mM concentration used in PLD assay, *n*-butanol inhibits nicotinic responses (Harper and Littleton, 1990) and voltage-activated channels (Elliott and Elliott, 1994), ethanol was used when nicotinic responses were studied.

In noradrenaline secretion experiments, the addition of albumin to the release buffer greatly reduced the basal release, as also previously recorded (Powis and O'Brien, 1991). Furthermore, adequate dose-response curves for cotinine were obtained only in the presence of albumin, while in its absence spontaneous release or overflow may have masked much of the effect, especially at lower concentration.

In histone kinase assay, some of the PKC activity remains undetected because histone is phosphorylated only by the conventional PKC isoenzymes, α, β and γ, requiring both diglycerides and Ca^{++} for their activation (Marais and Parker, 1989; Schaap *et al*., 1989; Olivier and Parker, 1991; Liyanage *et al*., 1992). Moreover, since a risk of PKC redistribution and proteolysis exists during sample preparation, methods, such as phorbol binding, avoiding major sample preparation steps are indicated.

[^{3}H]Phorbol-12,13-dibutyrate binding can be performed utilising intact cells. Therefore, sample preparation, with the associated risks therein, is not needed. In short-term incubations with substimulatory concentrations of the phorbol ester, the binding occurs at membrane-translocated PKC and reflects the *in situ* PKC activity (Trilivas and Brown, 1989; Rasmussen *et al*., 1992). In primary cultures of bovine adrenal medullary cells, prepared essentially as in the present work, the non-chromaffin cells represent only 7% of the total specific binding (Tuominen *et al*., 1991). Thus, phorbol binding can be reasoned to be a valid measure of PKC activity in the chromaffin cell model. However, other receptors for phorbol esters also exist (Ron and Kazanietz, 1999).

Although kinase assay using different subcellular extracts is a widely used method for demonstrating PKC translocation, Western blot has been claimed to be the only reliable way to demonstrate PKC translocation (Parker, 1992), and it is the only way of showing the isoenzyme-specific changes. Nevertheless, it is complicated by species-specific differences in protein sequences and immunogenic properties. Bovine-specific antibodies against PKC isoenzymes are not readily available, and the only sequenced bovine isoenzymes are conventional (Bairoch and Apweiler, 1999). In the present work, most of the PKC antibodies raised against rat carboxyterminal sequences (Wetsel *et al*., 1992) cross-reacted with bovine proteins. However, the anti-EYINPLLLSAEESV antibody failed to recognise the bovine aPKCζ, although the isoenzyme has previously been detected in bovine chromaffin cells with an apparent molecular weight of 80 000 (Pavlovic-Surjancev *et al*., 1993).

PHOSPHOLIPASE D IN CHROMAFFIN CELL NICOTINIC RESPONSES

PLD activity has previously been suggested to be lacking from bovine adrenal chromaffin cells, as no accumulation of [^{32}P]phosphatidylbutanol was observed in [^{32}P]$_i$ prelabelled cells treated with 50 mM butanol and either PMA or bradykinin (Purkiss *et al*., 1991). Contradictory results have also been obtained (García *et al*., 1992; Caumont *et al*., 1998), but the existence of PLD in bovine chromaffin cells had yet to be confirmed (Boarder, 1993; García *et al*., 1993; Glenn *et al*., 1998). Interestingly, the nicotine-evoked activation of PLD (Caumont *et al*., 1998) has not been invariably recorded, and nicotine has actually been reported to inhibit PLD activation induced by β amyloid in a neuroblastoma cell line (Singh *et al*., 1998).

The present study demonstrates the existence of a 105 kDa protein detected by anti-PLD antibody in bovine adrenal chromaffin cells. As sequences similar to the peptide CIIGSANINERS, against which the antibody was raised, are known only in the PLD proteins (Bairoch and Apweiler, 1999), the finding strongly suggests the existence of PLD in the chromaffin cells. Immunoreactivity was detected mainly in the particulate protein fraction. This is in agreement with previous findings that PLD is mainly associated with various membrane structures. However, the subcellular localisation of PLD may vary between cell types (Provost *et al*., 1996; Brown *et al*., 1998).

The present results clearly demonstrate that activation of PKC by PMA and receptor-mediated stimulation by Sar^1-angiotensin II and bradykinin increase transphosphatidylation activity in bovine adrenal chromaffin cells, in contrast to results previously obtained with bradykinin and PMA stimulations of [^{32}P]$_i$ prelabelled cells (Purkiss *et al*., 1991). Differences in labelling are unlikely, at least alone, to explain the different results. A possible explanation is the difference in the number of cells per sample, which was 15 times higher in the present study. An increased PLD activity was sustained for an hour upon continuous exposure to PMA. However, I was unable to demonstrate a prolonged activation of PLD by Sar^1-angiotensin II or bradykinin. In this sense, the role of PLD in chromaffin cells differs from that in adrenal cortical cells, which sustain PLD activity upon a continuous exposure to angiotensin II (Jung *et al*., 1998). When phosphatidylethanol formation was measured, nicotine and a depolarising concentration of potassium had no effect on PLD activity, while PMA again strongly stimulated the enzyme. This lies in stark contrast to a previous report despite largely similar methodology (Caumont *et al*., 1998). The signalling pathway leading to angiotensin- and nicotine-induced prolonged diacylglycerol production and protein kinase C activity in bovine chromaffin cells thus remains to be clarified. The putative PC-PLC may still have a crucial role as previously suggested (Liscovitch, 1992; Nishizuka, 1992).

PLD activation has been hypothesised to play an important role in exocytosis from various cell types, including PC12 phaeochromocytoma cells (Kanoh *et al*., 1992; Cockcroft, 1996). If PLD is crucial for chromaffin cell exocytosis, trapping phosphatidic acid by primary alcohols should inhibit secretion. In my study, this inhibition did occur. However, no selectivity of primary alkanols over *sec*- and *tert*-butanol was detected. This is in line with previous findings on alkanol effects (Harper and Littleton, 1990; Wood *et al*., 1991; Alifimoff *et al*., 1993), but largely conflicts with the results and interpretation of Caumont *et al*. (1998).

All the butyl alcohols tested efficiently inhibited induction of further Ca^{++} transients by secretagogues. Ethanol, at the same mass concentration, was less efficient. The

reason for reduced exocytosis by nicotine and K^+ from bovine chromaffin cells thus appears to lie on the cell membrane. The effects of the alkanols on exocytosis, at concentrations adopted from Caumont *et al*. (1998), seemingly are not PLD-dependent. The role of PLD in chromaffin cell exocytosis is further questioned in that highly active bacterial PLD does not reconstitute the Ca^{++}-dependent exocytosis from permeabilised bovine chromaffin cells (Glenn *et al*., 1998). The different results between the present and the previous studies (Purkiss *et al*., 1991; Caumont *et al*., 1998) cannot be fully explained, although differences in the cell preparation and culture conditions cannot be ruled out as a source of variation.

Interestingly, all the alcohols tested raised the $[Ca^{++}]_i$. The effect was reduced but not totally abolished in the absence of extracellular Ca^{++}, suggesting a release from the intracellular stores. Although *n*-butanol increased the $[Ca^{++}]_i$ roughly as much as nicotine, it only weakly stimulated the noradrenaline secretion. A similar phenomenon is known to occur upon exposure to thapsigargin (Xu *et al*., 1992), suggesting the possibility that the $[Ca^{++}]_i$ response is initiated at the level of endoplasmic reticular Ca^{++} stores, and the secretion remains small despite increased $[Ca^{++}]_i$. However, the possible store-emptying effect of alcohols does not explain the reduced response to nicotine (Mollard *et al*., 1995).

COTININE PHARMACOLOGY

In the present study, cotinine inhibition of nicotinic responses was detected in several parameters: phorbol-12,13-dibutyrate binding, PKC activity, noradrenaline release and $[Ca^{++}]_i$. As no inhibition of responses evoked by cell depolarisation by veratrine or high K^+ were detected, involvement of nicotinic receptors in the responses seems probable. In addition, cotinine as such evoked responses mimicking those of nicotine: it evoked noradrenaline release, stimulated phorbol-12,13-dibutyrate binding, increased membrane-associated PKC activity and raised $[Ca^{++}]_i$, many of these being reversed by nicotinic antagonists. Furthermore, cotinine inhibited its own effects on $[Ca^{++}]_i$, and its effects were also inhibited by nicotine. The maximum effect of cotinine on noradrenaline release was followed by a reduction in the response, as happens also upon exposure to nicotine (Kilpatrick *et al*., 1980; Livett *et al*., 1981). In prolonged incubations, the peak cotinine effect on noradrenaline release was smaller than that observed with 10 μM nicotine. These results altogether suggest that cotinine is a low-affinity partial nicotinic agonist at chromaffin cell acetylcholine receptors, also capable of desensitising the receptors.

My results on the effects of ion channel blockers on cotinine-evoked Ca^{++} transients are in accord with findings that micromolar tetrodotoxin has only a minor, if any, effect on exocytosis from chromaffin cells stimulated with 10 – 100 μM acetylcholine (Kilpatrick *et al*., 1981), and that at submicromolar concentrations, nimodipine reduces $^{45}Ca^{++}$ uptake evoked by nicotinic stimulation into chromaffin cells only in part (Gandia *et al*., 1991). At micromolar concentrations, nimodipine and various other L-type calcium channel antagonists inhibit nicotinic receptor ion fluxes (Donnelly-Roberts *et al*., 1995), hence raising nimodipine concentrations from the 300 nM used would have been inadequate. Therefore, I reason that the cotinine-evoked Ca^{++} influx largely occurs through nicotinic cholinergic receptors. The submicromolar increases in $[Ca^{++}]_i$ detected might well be mediated by chromaffin cell nicotinic receptor channels, as previously calculated (Zhou and Neher, 1993).

The binding of cotinine to chromaffin cell nicotinic acetylcholine receptors at concentrations roughly comparable with those needed to evoke responses was also established in the present study. Thus, the questions raised originally and along this work have largely been answered. Nevertheless, the affinity of cotinine to nicotinic acetylcholine receptors in the bovine chromaffin cells was weak, and the low micromolar concentrations reached upon human nicotine administration (Benowitz *et al*., 1983; Benowitz and Jacob, 1994) are unlikely to stimulate the receptors. The same appears true for frontal cortical and hippocampal nicotinic receptors, although they more avidly bind cotinine. Indeed, the affinity data are in agreement with the observed functional potency difference of cotinine between chromaffin and central neuronal preparations (Dwoskin *et al*., 1999).

In the present model, cotinine effects are seemingly evoked by activation of the nicotinic acetylcholine receptors. This leads to Ca^{++} influx through the receptors and voltage-operated calcium channels, activation of PKC and noradrenaline release. The PKC isoenzymes activated include cPKCα and nPKCε in chromaffin cells. The PKC activation is probably mediated by Ca^{++}-stimulated PI-PLC, since the nPKCε *per se* is Ca^{++}-independent. Yet, cotinine increased $[Ca^{++}]_i$ similarly in both thapsigargin-treated and control cells. This suggests that in cotinine-induced chromaffin cell activation, the release of Ca^{++} from endoplasmic reticulum plays only a minor, if any, role. Despite being considered important in the exocytotic process of various cell types (Kanoh *et al*., 1992; Cockcroft, 1996), PLD does not appear to be involved in the nicotinic or depolarisation-evoked signalling in bovine chromaffin cells, although the converse has also been reported (Caumont *et al*., 1998).

Bovine chromaffin cells express genes encoding the α3, α5, α7 and β4, but not the β2, nicotinic acetylcholine receptor subunits (Campos-Caro *et al*., 1997; Wenger *et al*., 1997). Thus, α3β4* heteromers, containing or lacking the α5 subunit, and α7 homomers are the possible pentameric subunit combinations. The human α7 homomers are only weakly activated but readily inhibited by cotinine (Briggs and McKenna, 1998), and their activation by nicotine requires high micromolar concentrations (Briggs *et al*., 1999). When my findings on cotinine effects on noradrenaline release and Ca^{++} transients are reflected against the existing data on mammalian α7 receptors, these do not appear the probable target of cotinine in bovine adrenal chromaffin cells.

My results demonstrating two agonist affinity classes of receptors on bovine adrenal chromaffin cells are different from a report based on [^{3}H]nicotine as the label (Lee *et al*., 1992). The different affinities may reflect different receptor compositions or they may indicate the presence of receptors with different affinity states. The 15-fold difference between the high- and low-affinity sites observed in the present work agrees with the 15- to 50-fold difference observed in α3* receptors of human neuroblastoma SH-SY5Y cells (Wang *et al*., 1996; Peng *et al*., 1997). However, the cell line also expresses β2 subunits, foreign to bovine chromaffin cells. Furthermore, the affinity I obtained for dihydro-β-erythroidine is similar to that reported for rat α3β4 receptors (Xiao *et al*., 1998). Another possibility is that the low-affinity receptor class represents the α7 homomeric receptors, which are less sensitive to epibatidine than the heteropentameric neuronal nicotinic receptors (Gerzanich *et al*., 1995; Delbono *et al*., 1997). However, α-bungarotoxin displaced only one sixth of the label when 1 nM [^{3}H]epibatidine was used, and it was totally ineffective in displacing 100 pM epibatidine. The relatively low affinity of methyllycaconitine to the receptors and the micromolar concentrations required also argue against a major involvement of α7 homomers in

the two-site binding, but fits better to the data previously obtained with α3β4 receptors (López *et al*., 1998).

In light of the pre-existing data and my results, the apparent two-site binding probably reflects different affinity states of the α3β4* receptors. However, the apparent two-site binding is a mathematical model of the results obtained, and the actual binding of the label may be, and probably is, more complicated. Therefore, caution is needed in deducing the real nature of the apparent binding sites. In the brain, the apparent one-site binding most likely occurs at the α4β2 receptors. The cotinine affinity to the brain receptors was over an order of magnitude higher than to the chromaffin cell receptors. The binding of cotinine to brain receptors also explains cotinine effects detected on brain preparations *in vitro* (Dwoskin *et al*., 1999).

Most likely, activation is not the primary means of cotinine affecting cholinergic receptors *in vivo*. This is supported by the present results that relatively low cotinine concentrations inhibit nicotinic responses, while high concentrations are required for receptor activation. In fact, most nicotinic agonists desensitise their receptors at lower concentrations than are required for activation (Marks *et al*., 1996). As the desensitisation is well established in both repeated and continuous exposure of cells to nicotinic agonists (Boksa and Livett, 1984; Marley, 1988), it may apply to cotinine as well. Data suggesting such a phenomenon in oocyte-expressed human α7 receptors has been presented (Briggs and McKenna, 1998). By silencing nicotinic receptors, cotinine may counteract some of the effects of nicotinic agonists at the concentrations reached with tobacco or alternative nicotine use, even without detectable stimulation. The phenomenon may have a role in the clinical effects of cotinine, such as a worsening of tobacco withdrawal symptoms (Keenan *et al*., 1994; Hatsukami *et al*., 1998). However, the effects of smoking and nicotine replacement therapy may well be secondary to receptor desensitisation. This is even likely since the concentrations of nicotine reached upon voluntary administration (Benowitz *et al*., 1988; Gourlay and Benowitz, 1997) are insufficient to activate nicotinic acetylcholine receptors, but rather desensitise them (Marks *et al*., 1996).

CONCLUSIONS

1 Bovine adrenal chromaffin cells possess PLD protein and PLD1-like activity that can be activated by G-protein coupled receptor activators angiotensin II and bradykinin, and by phorbol ester stimulation of PKC.

2 PLD activation is not necessary for chromaffin cell exocytosis. Neither does PLD participate in nicotine signalling nor in sustained chromaffin cell responses to angiotensin II or bradykinin.

3 Cotinine inhibits nicotinic responses, such as elevation of $[Ca^{++}]_i$, increase in PKC activity and noradrenaline exocytosis, in bovine adrenal chromaffin cells.

4 Cotinine efficiently stimulates bovine adrenal chromaffin cells, but high concentrations are required. The cotinine-evoked responses include elevation of $[Ca^{++}]_i$, increase in PKC activity, and noradrenaline exocytosis.

5 Cotinine binds to nicotinic binding sites at acetylcholine receptors. The nicotinic acetylcholine receptor agonism initiates the chromaffin cell responses to cotinine.

6 Cotinine affinity for nicotinic acetylcholine receptors is low, and the cotinine concentrations achieved in nicotine consumption are likely to affect the receptor activity states.

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