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Modulation of Signal Transduction in the Nervous System
by Protein Phosphorylation

A thesis submitted to the Faculty of
The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

Jessica F. Hopfield

October 1990
The Rockefeller University
New York

To J.J. Hopfield

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TABLE OF CONTENTS

List of Figures	vi-vii
List of Tables	viii
List of Abbreviations	ix
Abstract	1
Chapter 1 Mechanisms of Signal Transduction	3
Chapter 2 Protein Phosphorylation in the Nervous System	7
Chapter 3 Tyrosine Phosphorylation of the Nicotinic Acetylcholine Receptor Regulates its Desensitization Rate	
Introduction	14
Methods	19
Results	23
Discussion	40
Chapter 4 D2 Dopamine Receptors in Striatal Neurons are Coupled to a Potassium Conductance that may be Regulated by Protein Phosphorylation	
Introduction	46
Methods	52
Results	56
Discussion	70
Chapter 5 Dopamine Inhibits Na ⁺ ,K ⁺ -ATPase Activity in Striatal Neurons Through D1 and D2 Receptor Synergism	
Introduction	74
Methods	77
Results	81
Discussion	98
Chapter 6 Conclusions	105
Bibliography	114

LIST OF FIGURES

Chapter 1

1. Receptor-ion channel interactions in excitable cells 5

Chapter 2

2. Signals in the nervous system 9
3. Schematic illustration of the four configurations for recording from a cell with a patch-clamp pipette 12

Chapter 3

4. Proposed secondary structure and sequenced primary structure of the nAChR 16
5. Phase photomicrographs of liposomes containing nAChR 25
6. Pharmacology of nAChR channel activity 26
7. Desensitization behavior of purified and reconstituted nAChR as seen in single patches 28
8. Purified nAChR preparations with various stoichiometries of tyrosine phosphorylation 32
9. Tyrosine phosphorylation modulates the rate of desensitization of the nAChR as measured by exponential decay with time in the frequency of channel openings 34
10. Increased levels of tyrosine phosphorylation increase the rate of the rapid phase of desensitization 35
11. Open time lifetimes of single channel events from records pooled across all experiments conducted at a given stoichiometry of tyrosine phosphorylation 38

Chapter 4.

12. Simplified schematic diagram of the synaptic organization of the major afferent and efferent connections of the caudate-putamen 47
13. Photomicrographs of dissociated neostriatum 58
14. Voltage-activated currents recorded from a cell in the whole-cell patch clamp configuration 61

15.	Current-voltage plots of peak current versus membrane potential for the cell shown in Figure 14	62
16.	Membrane currents elicited by dopamine application	64
17.	The D2-selective agonist quinpirole enhances an outward conductance	65
18.	Current-voltage plot of peak outward current versus membrane potential of the D2-specific conductance in the presence of various dopaminergic agents	66
19.	Forskolin inhibits quinpirole-activated conductances	68

Chapter 5

20.	Photomicrographs of dissociated medium spiny neurons	83
21.	Concentration dependence of dopamine inhibition of Na^+, K^+ -ATPase activity	85
22.	Time dependence of dopamine inhibition of Na^+, K^+ -ATPase activity	86
23.	D1 and D2 antagonists each prevent the inhibitory action of $1 \mu\text{M}$ dopamine	87
24.	Synergistic action of D1 and D2 agonists as inhibitors of Na^+, K^+ -ATPase activity	90
25.	Forskolin mimics D1 receptor activation	92
26.	Autoradiogram of western blots of guinea pig striatal homogenates probed for the basal ganglia enriched phosphoproteins ARPP-16, ARPP-19, ARPP-21 and DARPP-32	95
27.	Photomicrograph of immunoreactive DARPP-32 containing neurons in guinea pig striatum	97
28.	Hypothetical model for D1 and D2 synergistic inhibition of Na^+, K^+ -ATPase activity	102

Chapter 6

29.	Schematic diagram illustrating proposed regulation of the nAChR by three different first messenger systems	107
30.	Schematic illustration of a proposed model for D1 and D2 receptor antagonism in striatal neurons	110
31.	Schematic illustration of a proposed model for D1 and D2 receptor synergism in striatal neurons	112

LIST OF TABLES

Chapter 3

I. Acetylcholine concentration dependence of desensitization	29
II. Effect of tyrosine phosphorylation on the fast and slow time constants of desensitization	36
III. Effect of tyrosine phosphorylation on single channel properties of the nAChR	39
IV. Probability of phosphate occupation of the three potential phosphorylation sites at different stoichiometries of phosphorylation	41

Chapter 4

V. D1 and D2 receptors in the central nervous system	49
VI. Saturation binding parameters of [³ H]SCH 23390 and [³ H]raclopride to D1 and D2 receptors in control and trypsin-treated slices	59

Chapter 5

VII. Striatal dopamine levels in benserazide and vehicle treated animals as measured by HPLC	89
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LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
cAMP	Adenosine 3':5'-cyclic monophosphate
cGMP	Guanosine 3':5'-cyclic monophosphate
CGRP	Calcitonin gene-related peptide
CPM	Counts per minute
EDTA	Ethylenediamine tetra acetatic acid
EGTA	Ethylene glycol bis(β -aminoethyl ether)-N,N,N,N'-tetra acetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
kD	Kilodaltons
MES	2-[N-morpholino]ethane sulfonic acid
nAChR	Nicotinic acetylcholine receptor
PBS	Phosphate buffered saline
PCA	Perchloric acid
PIPES	Piperazine-N,N'-bis (ethanesulfonic acid)
PMA	Phorbol myristate acetate
SDS/PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEA	Tetraethylammonium
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl) aminomethane
TTX	Tetrodotoxin
SEM	Standard error of the mean

ABSTRACT

Signal transduction, the process by which an extracellular signal is translated into an intracellular message, underlies all brain function. This thesis examines the role of protein phosphorylation in mediating three different molecular mechanisms by which neurotransmitter receptors transduce their signals: chemically-gated ion channels, receptor-linked ion channels and receptor-linked ion pumps.

The nicotinic acetylcholine receptor, a neurotransmitter-gated ion channel, is phosphorylated by a protein tyrosine kinase in postsynaptic membranes *in vitro* and *in vivo*. Purified nicotinic receptor molecules from *Torpedo* electroplaques can be phosphorylated to known stoichiometries and reconstituted into lipid vesicles. Tyrosine phosphorylation increases the rate of the rapid phase of desensitization of the receptor as measured by single channel recording but does not alter other channel properties. These data provide direct evidence for the regulation of ion channel properties by tyrosine phosphorylation and suggest that tyrosine-specific protein phosphorylation, in addition to regulating cell transformation and proliferation, may regulate neuronal signal transduction.

Medium spiny neurons, the principal cell type of the neostriatum, contain D1 and D2 dopamine receptors. D2 receptor activation is coupled in dissociated neurons to an outward, voltage-dependent, transient K⁺ conductance, probably I_A, as measured by whole-cell patch clamping. This D2 coupled current can be inhibited by micromolar concentrations of forskolin suggesting that the receptor-channel linkage is regulated by protein phosphorylation. This inhibition of the D2 signal transduction

pathway by an adenylate cyclase-mediated process may underlie the electrophysiological antagonism of D1 and D2 receptor activation in striatum.

The activity of Na^+, K^+ -ATPase, an ion pump which couples the hydrolysis of ATP to the countertransport of Na^+ and K^+ ions across the plasma membrane, can be measured in permeabilized dissociated neostriatal neurons. The neurotransmitter dopamine, through a synergistic effect on D1 and D2 receptors, inhibits the Na^+, K^+ -ATPase activity of isolated striatal neurons. This mechanism appears to involve protein phosphorylation since cAMP-dependent protein kinase activation mimics D1 receptor stimulation and phorbol ester treatment mimics the effect of dopamine application. These data provide unequivocal evidence for regulation by a neurotransmitter of a neuronal ion pump. They demonstrate that synergism between D1 and D2 receptor activation, which underlies many of the electrophysiological and behavioral effects of dopamine in the mammalian brain, can occur on the same neuron. In addition, the data suggest that dopamine, in addition to its regulation of ion channels, regulates cell excitability through the novel mechanism of pump inhibition.

CHAPTER 1

Mechanisms of Signal Transduction

Signal transduction, the process by which a cell converts a stimulus into a response, is the very essence of neurobiology. In the mammalian brain, ten billion cells each communicate by releasing neurotransmitters or hormones that bind to specific receptors in postsynaptic target neurons. Cells must then transduce this extracellular chemical signal into an intracellular message that directly or indirectly alters their physiological properties. One powerful way to affect neuronal excitability is to regulate transmembrane electrochemical gradients. Neurotransmitter receptors accomplish this task through three distinct receptor mechanisms: chemically-gated ion channels, receptor-linked ion channels and receptor-linked ion pumps.

Chemically-gated ion channels include the nicotinic acetylcholine receptor, GABA_A receptor, glycine receptor and the NMDA and kainate/quisqualate subtypes of glutamate receptors. These receptors are multimeric transmembrane complexes that comprise both a neurotransmitter binding site and an ion channel. Recent cloning and sequencing of the cDNAs for three of these receptors has suggested that they are members of a receptor superfamily and share many structural features (Barnard et al, 1987). For each of these receptors, the selective permeation of specific ions serves as a signal by depolarizing or hyperpolarizing the subsynaptic area of the cell. Chapter 2 describes experiments on the modulation of the archetypal member of this class, the nicotinic acetylcholine receptor.

Some receptors are indirectly coupled to ion channels via guanine nucleotide binding proteins (G-proteins). Members of the G-protein linked receptor family include α and β adrenergic receptors, dopamine receptors, muscarinic acetylcholine receptors and many neuropeptide receptors. Gene cloning suggests that many or all G-protein coupled receptors are related and share such features as seven transmembrane α -helical domains and a cytoplasmic domain that activates neighboring G-proteins (Ross, 1989). G-proteins exist in their basal state as an $\alpha\beta\gamma$ trimer oligomer with GDP bound to the α subunit. During activation following ligand binding to a receptor, GDP is released and replaced with GTP and the trimer dissociates to form two potential regulatory molecules, α -GTP and $\beta\gamma$ (Gilman, 1987). These activated G-proteins can either regulate ion channels directly or regulate channels indirectly by inducing changes in the intracellular levels of cyclic nucleotides, inositol polyphosphates and arachidonic acid metabolites. The regulation of channel activity by the D2 dopamine receptor, a G_i -linked receptor, is the subject of Chapter 3.

The existence of both chemically-gated ion channels and receptor-linked ion channels provides many different possibilities for extracellular agents to regulate the intracellular biochemical environment (Figure 1). Most of these channels are in some way dependent on membrane voltage and their activation, in turn, modulates membrane potential. Electrical activity of neurons, however, is also dependent on the activity of ion translocating enzymes and a third class of neurotransmitter receptors is those that are linked to such ion pumps. Although pumps can be considered to be indirectly coupled to any neurotransmitter receptor

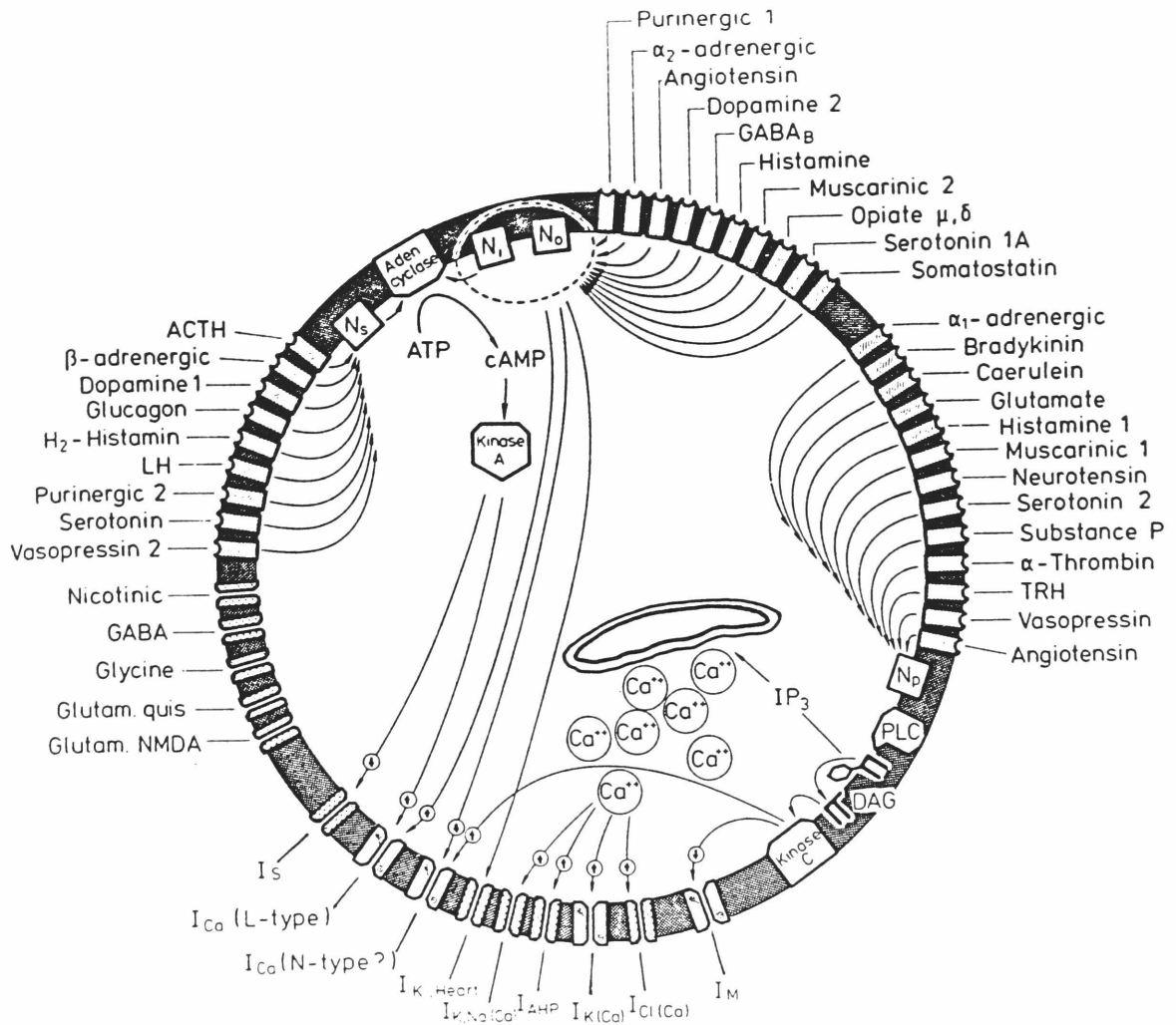


Figure 1 Receptor-ion channel interactions in excitable cells. Receptors are grouped according to whether they directly gate ions through channels that are part of the receptor complex or are linked via G-proteins to separate channels. N_s is a stimulatory G-protein, N_o and N_i are members of the inhibitory G-protein family and N_p represents receptor-mediated phosphoinositide breakdown. (From Neher, 1988)

that changes the concentration of the ion that the pump transports, direct regulation of ion pump kinetics by neurotransmitters has previously been demonstrated only in the kidney where dopamine regulates epithelial Na^+ transport by inhibiting the Na^+, K^+ -ATPase (Aperia et al., 1987). Chapter 5 demonstrates that the dopamine receptor in brain uses this third class of transduction mechanism which may also be used by receptors for opiates and epinephrine (Hajek et al., 1990; Beach et al., 1987).

CHAPTER 2

Protein Phosphorylation in the Nervous System

Protein phosphorylation, the reversible covalent addition of phosphate molecules to hydroxylated amino acids, is a major mechanism of the control of such varied cellular processes as glycogen metabolism, protein synthesis and oncogenic transformation (for review, see Cohen, 1982; Hunter et al., 1985). All phosphorylation reactions have three common components: a substrate that can exist in dephosphorylated or phosphorylated form, a protein kinase that catalyzes the phosphorylation of the substrate, and a protein phosphatase that catalyzes the reverse, dephosphorylation, reaction. The addition of the negatively charged phosphate molecule to the substrate induces local charge differences and structural changes which may result in functional changes in the protein.

Phosphoproteins, protein kinases and protein phosphatases are all highly enriched in the synaptic regions of neurons suggesting that protein phosphorylation modulates or mediates synaptic transmission (Nestler et al., 1984; Nairn et al., 1985). Across many species and brain regions, protein phosphorylation has been shown to regulate the efficacy of synaptic transmission both by modulating neurotransmitter release and by altering either the sensitivity of receptors or their underlying effector-coupling mechanisms (for review, see Hemmings et al., 1989). For example, phosphorylation of the vesicle-associated protein, synapsin I, facilitates neurotransmitter release in the squid giant synapse (Llinas et al., 1985) and postsynaptic long term potentiation in hippocampal CA1 neurons requires activity of both protein kinase C and calcium/calmodulin-

dependent protein kinase (Nicolle et al, 1988).

Phosphorylation mediates signal transduction through a multi-step amplification process as shown Figure 2 (Greengard, 1987). Extracellular signals, or first messengers, such as neurotransmitters interact with membrane bound receptors. This leads to increases in intracellular levels of specific second messengers such as cAMP, cGMP, calcium and diacylglycerol which, in turn, activate specific cAMP-dependent, cGMP-dependent, calcium/calmodulin-dependent and calcium/diacylglycerol-dependent protein kinases. These kinases phosphorylate a broad range of neuronal proteins including neurotransmitter receptors, ion channels, cytoskeletal proteins and synaptic-vesicle associated proteins. These phosphorylated substrates, or third messengers, may be the immediate effector for the biological response or they may in turn activate fourth, fifth, sixth, etc. messengers.

Protein biochemistry has been a powerful tool to identify such messenger molecules by studying the possible phosphorylation of known proteins and identifying novel proteins that can be demonstrated to be phosphorylated in response to physiological stimuli. To elucidate the functional consequences of such phosphorylation reactions, however, is often difficult since measuring the incorporation of ^{32}P in proteins extracted from cell homogenates is far removed from observing the effects of phosphorylation in intact, living cells. Electrophysiology has traditionally provided information about the functional state of neurons in terms of current and voltage but little information about the intracellular signalling and phosphorylation status of the cell.

Direct evidence for a role of protein phosphorylation in mediating

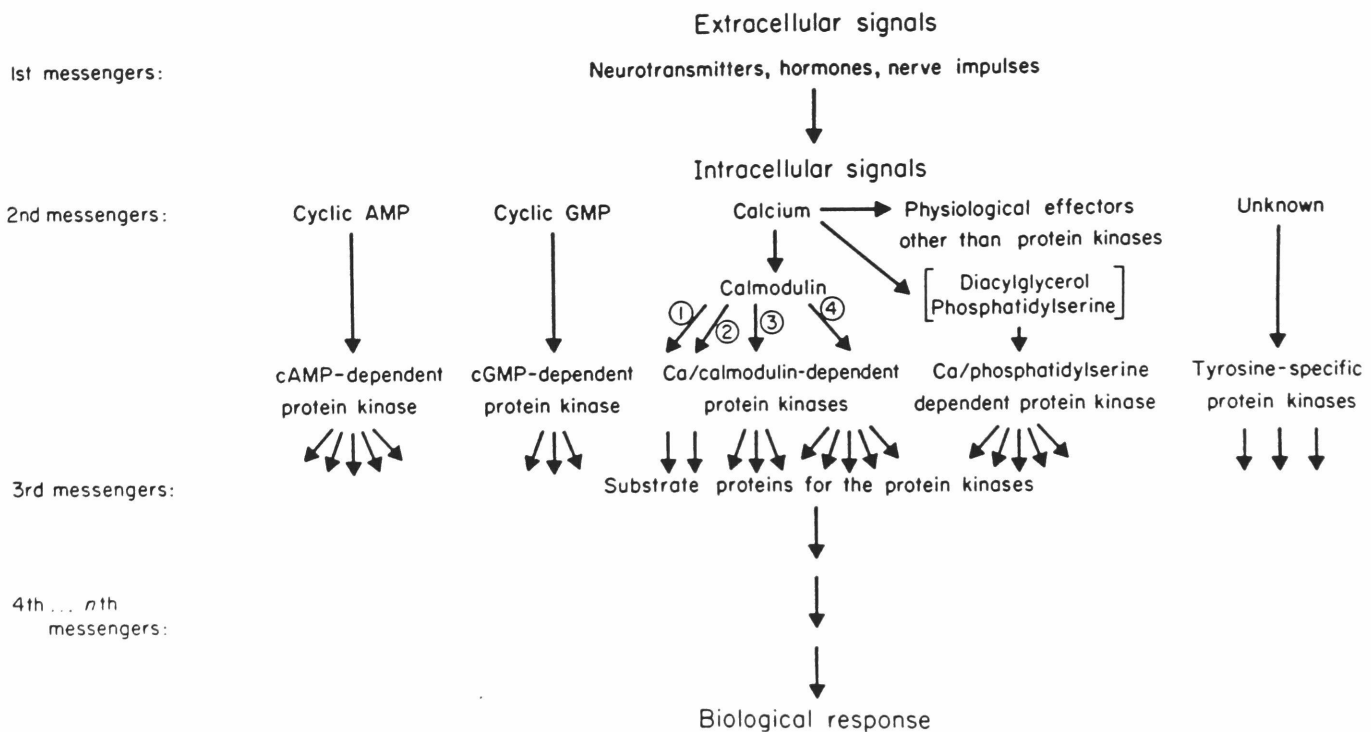


Figure 2 Signals in the nervous system. Extracellular signals produce specific biological responses in target neurons via a series of intracellular signals. First messengers such as neurotransmitters, photons of light and membrane depolarization regulate intracellular levels of second messengers that include cAMP, cGMP, calcium and diacylglycerol. These compounds activate a small family of kinases which phosphorylate, on kinase-specific residues, a large number of proteins. These third messengers, of which there are over seventy that are neuron-specific, either directly modulate cell physiology or produce biological responses by activating additional messengers. (From Greengard, 1987).

neurotransmitter action has been obtained by intracellular injection of protein kinases into nerve cells. Each of the four major classes of protein kinases that phosphorylate substrate proteins on serine or threonine residues has been demonstrated, upon intracellular injection, to mimic the physiological activation of nerve cells. cAMP-dependent protein kinase decreases the conductance of serotonin regulated potassium channels in *Aplysia* (Castelluci et al., 1980). cGMP-dependent protein kinase mediates serotonin regulation of voltage sensitive calcium channels in *Helix* (Paupardin-Tritsch et al., 1986). Calcium/calmodulin dependent protein kinase facilitates neurotransmitter release in the terminal digits of the squid giant synapse (Llinas et al., 1985). Calcium/diacylglycerol dependent protein kinase (protein kinase C) enhances the voltage sensitive calcium currents in *Aplysia* (DeRiemer et al., 1985). Dozens of examples exist of experiments using microinjection of either kinases to mimic or potentiate the actions of extracellular signals, or kinase inhibitors to prevent such physiological responses. With the notable exception of hippocampal neurons in which protein kinase C injection elicits features of long term potentiation (Hu et al., 1987), injection experiments have been limited almost entirely to large, invertebrate neurons. Vertebrate neurons are smaller and easily damaged by cell penetration with a conventional micropipette.

The recent development of the patch clamp technique has dramatically altered the approaches to studying protein phosphorylation-mediated cellular events. Patch clamping was initially designed to resolve single ion channels without shunt currents and excess background noise (Hamill et al., 1981a). Very fast unit currents can be measured by this method with

high signal to noise ratios thereby permitting many kinetic parameters of ion channel function to be quantified. The gigohm seal between the electrode and membrane surface is not only electrically tight but mechanically stable which permits the topological arrangement of small patches of membrane. Receptor sites can be deliberately oriented either inward or outward by manipulating the membrane/glass seal (Figure 3). Biochemical agents can be applied to either side of the membrane during single channel recording to reveal changes in channel conductance, open probability or availability (Sakmann et al., 1984). The cell-attached patch configuration can be used to investigate channel regulation by neurotransmitters acting via second messengers. By applying neurotransmitter to the cell membrane area outside the pipette, the modulation of channels in the membrane patch in the pipette tip opening can be examined. Such an approach has been used to demonstrate that serotonin and cAMP close single potassium channels in *Aplysia* sensory neurons (Siegelbaum et al., 1982).

To examine the role of protein phosphorylation in channel modulation, protein kinases can be applied to the cytoplasmic membrane surface of a membrane patch in the inside-out configuration. Activation of calcium-dependent potassium channels by cAMP-dependent protein kinase in *Helix* neurons has been shown with this technique (Ewald et al., 1985). To prove, however, that it is the channel itself rather than a regulatory molecule that is being phosphorylated, it is necessary to measure directly the phosphorylation state of the ion channel. This can be accomplished in a marriage of biochemistry and electrophysiology; channels are purified or synthesized, phosphorylated to known stoichiometries and reconstituted

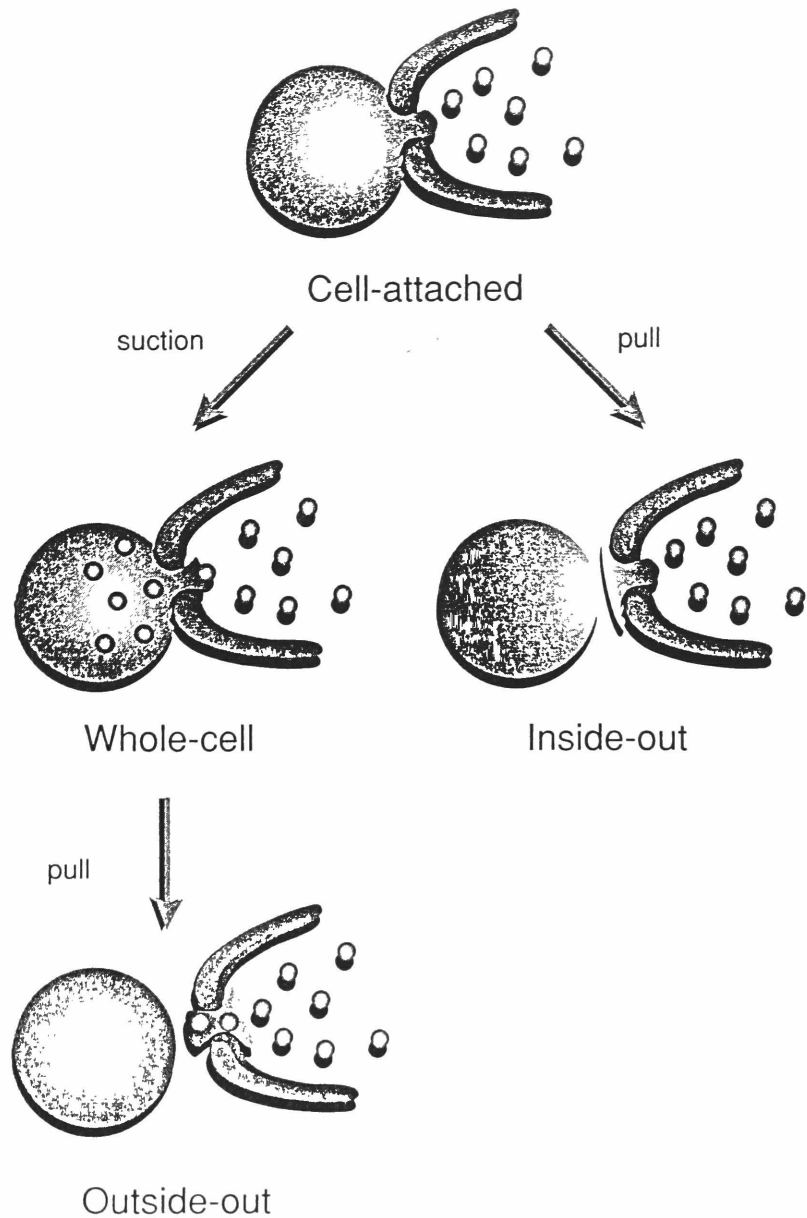


Figure 3 Schematic illustration of the four configurations for recording from a cell with a patch-clamp pipette. The **cell attached** configuration is generated by pressing a fire polished glass pipette (opening diameter approx $0.5\ \mu\text{M}$) against a clean membrane surface to form a high resistance, gigohm, seal. Pulling the pipette away from the cell generates an **inside-out** patch with the cytoplasmic side of the membrane facing outwards. Alternatively, a brief pulse of suction or voltage can be applied to rupture the membrane patch and form the **whole cell** configuration in which the electrode and cell interior are physically contiguous. If the cell is adhered to a surface, pulling the electrode away from the cell causes the membrane to reform in an **outside-out** orientation. (Modified from Hille, 1984 and Hammill et al, 1981a)

into lipid bilayers or vesicles for patch clamp recording. Chapter 3 presents data demonstrating functional modulation of the nicotinic acetylcholine receptor by tyrosine phosphorylation using such a reconstituted system.

An unanticipated use of the patch clamp technique has been the ability to record from small and/or fragile cells in the "whole-cell" configuration. Not only does this permit single electrode voltage and current clamp measurements with an excellent signal-to-noise ratio, it provides a method for infusing cells with compounds of interest since the electrode and cell interior are physically contiguous. Ions, second messengers and protein kinases and phosphatases can be loaded into cells without the trauma of microelectrode impalement and pressure injection. This technique has been used to provide direct evidence for protein phosphorylation on neuronal function. For example, perfusion of spinal cord neurons with the catalytic subunit of cAMP-dependent protein kinase reduces the amplitude of GABA_A evoked outward currents (Porter et al., 1990). The experiments described in Chapter 4 extend this technique to examine dopamine regulation of the basal ganglia by recording from dissociated guinea pig striatal neurons with concomitant extra and intracellular applications of protein phosphorylation-regulating agents.

CHAPTER 3

Tyrosine Phosphorylation of the Nicotinic Acetylcholine Receptor Regulates its Desensitization Rate

The nicotinic acetylcholine receptor (nAChR) is a receptor ion channel complex that mediates the depolarization of the postsynaptic membranes of nicotinic cholinergic synapses. The nAChRs from skeletal muscle and electric organs are pentameric complexes of four types of subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. Each subunit is a transmembrane protein that forms one stave of a pseudosymmetric barrel around a central ion channel that is permeable to Na^+ , K^+ , and Ca^{++} ions. The nAChR is present at accessible neuromuscular junction synapses for electrophysiological study and can be purified in hundred microgram quantities from single electroplaques of electric fish for biochemical assays, and therefore has become the model for the study of the structure, function and regulation of chemically-gated ion channels (for review, see Changeux et al., 1984).

Isolated postsynaptic membranes enriched in nAChR contain at least four classes of protein kinases: cAMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase, protein kinase C and a family of tyrosine-specific protein kinases (Miles et al., 1988). Three of these endogenous kinases stoichiometrically phosphorylate the nAChR. cAMP-dependent protein kinase rapidly phosphorylates serine residues on the γ and δ subunits (Huganir et al., 1983a). Protein kinase C rapidly phosphorylates a serine residue on the δ subunit and more slowly phosphorylates a serine residue on the α subunit (Huganir et al., 1983b). An endogenous tyrosine kinase rapidly phosphorylates tyrosine residues on

the β , γ and δ subunits (Huganir et al., 1984). Each of these kinases phosphorylates unique residues and thus the three kinases phosphorylate the nAChR on a total of seven distinct sites.

All four subunits of the nAChR have been cloned from many different species and are highly homologous to each other in their amino acid sequence (eg, Noda et al., 1983). Several models of transmembrane topology for the subunits have been proposed based on the hydropathy plots of their amino acid sequences (Guy et al., 1987). Although such models differ in the location of the carboxy terminus relative to the cell interior and in the number of transmembrane domains, they share the feature of a large intracellular loop between transmembrane α helix M3 and its neighbor (Figure 4a). Consensus sequences for the protein kinases have suggested, and peptide sequencing has proven, that the seven residues phosphorylated by the three kinases are located on this loop (Yee et al., 1987; Huganir et al., unpublished observations). The close proximity of these sites (the three sites on the δ subunit are within 20 amino acids of each other) suggests that the phosphorylation of the nAChR regulates a common property of the receptor (Figure 4b). These phosphorylation sites are conserved in the amino acid sequences of nAChR from all species except for those on the γ subunit which are not conserved in mammals (Miles et al., 1988).

The physiological significance of phosphorylation of the nAChR was first established by stop flow and quench flow kinetic experiments on purified and reconstituted receptors. Such reconstituted receptors maintain all the channel properties observed in vertebrate muscle acetylcholine receptors *in situ* (Tank et al., 1983). Phosphorylation of

the γ and δ subunits of the purified receptor by cAMP-dependent protein kinase increases the rapid phase of receptor desensitization as measured by $^{86}\text{Rb}^+$ flux but does not change the initial rate of ion transport or the dissociation constant of acetylcholine from its binding sites (Huganir et al., 1986). Quench flow analysis provides information about rapid kinetic events by measuring net flux but cannot determine channel open times or conductances. The rate of desensitization can be determined from the total flux rate only if mean channel current and open time are unchanged across preparations. To examine in more detail what receptor parameters change with phosphorylation, whole cell and single channel electrophysiological techniques have been applied to the study of nAChR in skeletal muscle. cAMP and forskolin (both activators of cAMP-dependent protein kinase) and phorbol esters (activators of protein kinase C) have been shown to increase the desensitization rate of acetylcholine-induced whole-cell currents *in situ* (Middleton et al., 1986; Albuquerque et al., 1986; Eusebi et al., 1985). Forskolin enhances receptor desensitization through a second messenger pathway without changing the properties of individual single-channel currents (Middleton et al., 1988).

In contrast, the functional role of tyrosine phosphorylation of the nAChR has not been examined. Phosphorylation of proteins on tyrosine residues regulates cell growth and transformation: cytoplasmic tyrosine-specific protein kinases were first discovered as the protein products of transforming retroviruses and many membrane-bound growth factor receptors are tyrosine kinases (for review see Hunter et al., 1985 and Ullrich et al., 1990). Although a number of substrate proteins are phosphorylated on tyrosine residues, the functional alteration of proteins by tyrosine

phosphorylation has previously been convincingly demonstrated only for protein tyrosine kinases (Rosen et al., 1983; Courtneidge, 1985; Cooper et al., 1986). A role for tyrosine phosphorylation in neuronal function might seem unlikely because mammalian brain is a nonproliferative tissue and phosphotyrosine constitutes as little as 0.01% of the total phosphoaminoacids of proteins of normal cells (Bishop, 1983). However, the normal cellular homologues of the *src* and *yes* viral oncogenes are very abundant in adult brain and tyrosine phosphorylation of synaptic-vesicle associated proteins can be seen in intact nerve terminals, suggesting that tyrosine phosphorylation may be involved in signal transduction (Hirano et al., 1988; Pang et al., 1988).

Highly purified postsynaptic membranes from *Torpedo* electroplaques contain a very active protein tyrosine kinase activity which phosphorylates the nAChR on the β , γ and δ subunits *in vitro* (Huganir et al., 1984). Isolated nAChR contain high levels of endogenous phosphotyrosine (1.5 mole phosphotyrosine/mole receptor). Since the tyrosine phosphorylated sites of the nAChR are close to the same sites phosphorylated by cAMP-dependent protein kinase and protein kinase C, it seemed possible that tyrosine phosphorylation of the receptor would also regulate its desensitization rate. To analyze quantitatively the functional effects of tyrosine phosphorylation of the receptor, small unilamellar vesicles containing purified and reconstituted receptor from *Torpedo californica* were fused by a simple freeze thaw procedure to form large liposomes. Giga-seal patch-recording techniques were used to form isolated inside-out patches of reconstituted liposome membrane and to measure the single channel properties of the receptor.

METHODS

Receptor Phosphorylation

Postsynaptic membranes highly enriched in the nAChR were prepared by subjecting freshly dissected and homogenized electric organs of *Torpedo californica* to a discontinuous sucrose gradient (Huganir, 1984). The postsynaptic membranes were diluted to 0.5 mg/ml with a phosphorylation reaction mixture containing, in mM, 20 Tris-HCl, 10 MgCl₂, 2 MnCl₂, 1 ouabain, 1 sodium vanadate, 0.5 EGTA, 0.5 EDTA, 10 2-mercaptoethanol plus 100 nM cAMP-dependent protein kinase inhibitor (Walsh peptide) and 10 µg/ml each of leupeptin and antipain. To obtain an autoradiograph of ³²P-labelled tyrosine phosphorylated receptor, the phosphorylation reaction was initiated with 0.5 mM [γ -³²P]-ATP and carried out for 30' at 30°. Samples were subjected to electrophoresis on 10% NaDodSO₄ polyacrylamide gels and the incorporation of ³²P quantified by liquid scintillation spectrophotometry. Receptor preparations were treated with agents to increase and decrease the stoichiometry of tyrosine phosphorylation from the endogenous level. To increase phosphorylation levels, the membranes were incubated with or without 0.5 mM ATP for 10' or 30' at 30°. The reactions were stopped by rapid cooling to 4°C and centrifugation. The pellets were resuspended at 2.5 mg/ml in 20 mM Tris-HCl pH 8.0, 20 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 20 µg/ml leupeptin, 20 µg/ml antipain, 20 units/ml Trasylol and 0.3% Na-cholate. To dephosphorylate the membranes, the suspended samples were incubated for 15' at 30°C with or without 0.25 or 0.5 mg/ml alkaline phosphatase.

To determine the stoichiometry of phosphotyrosine content of the nAChR subunits, parallel assays were run comparing ¹²⁵I-protein A labelling

of phosphotyrosine residues with ^{32}P -incorporation during the *in vitro* phosphorylation (Hopfield et al., 1988). The nAChR was phosphorylated *in vitro* using unlabelled ATP, purified and Western blotted using affinity purified antiphosphotyrosine antibodies and ^{125}I -protein A. The ^{125}I -protein A labelling of the β, γ and δ subunits under these conditions measured both the endogenous phosphotyrosine residues in the subunits prior to the *in vitro* phosphorylation and the phosphotyrosine residues phosphorylated *in vitro*. The stoichiometry of ^{32}P incorporation in each of these samples was measured by conducting the *in vitro* phosphorylation with $\gamma\text{-}^{32}\text{P}$ -ATP of known specific activity. The specific activity of ^{125}I -protein A labelling (CPM/mole phosphotyrosine) could then be calculated by comparing the stoichiometry of ^{32}P -incorporated with the increase in ^{125}I -protein A labelling of the nAChR upon *in vitro* phosphorylation using the formula: $[(\text{CPM of } ^{125}\text{I}\text{-protein A labelling of } \textit{in vitro} \text{ phosphorylated subunit}) - (\text{CPM of } ^{125}\text{I}\text{-protein A labelling of nonphosphorylated subunit})]/\text{mole } ^{32}\text{P}\text{-phosphate incorporated } \textit{in vitro}$. From this calculation, the stoichiometry of tyrosine phosphorylation of the subunits of all the receptor preparations was determined to within 0.1 moles phosphotyrosine/mole receptor.

Receptor Purification and Reconstitution

The phosphorylated receptor was purified to over 95% homogeneity from the Na-cholate extract using affinity chromatography on a choline carboxymethyl affinity gel with 20 mM carbamylcholine as eluate. The solubilized receptors were then incorporated via detergent dialysis into small unilamellar asolectin vesicles (Huganir et al., 1982) for final lipid-protein ratios in the range of 100:1 to 50:1 by weight. 50 μl

aliquots of vesicles were suspended at a concentration of 20 to 25 mg lipid per ml of a storage buffer containing, in mM, 100 NaCl, 50 KCl, 0.1 EDTA, 0.1 EGTA, 10 NaP_i, pH 8.0, then rapidly frozen and stored in liquid nitrogen.

Patch Clamp Recording

Aliquots of receptor were slowly thawed at 4°C overnight to permit the formation of large, multilamellar liposomes. 0.5 μ l portions were spotted onto the center of the bottom of 35 mm plastic petri dishes and covered with 2 ml of a salt solution containing (in mM) 150 NaCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.2. The largest of the multilamellar liposomes (>5.0 μ m in diameter) were selected under phase contrast microscopy (400X) for recording. Patch pipettes were pulled in a two step pull using a Kopf 720 vertical pipette puller from 100 μ l Boralex glass (Rochester Scientific) and fire polished with a Narishige microforge to a final resistance of 10–20 Mohm. Pipettes were backfilled with the salt solution plus varying concentrations of cholinergic compounds.

A patch clamp system, similar to that described by Hamill et al. (1981a), consisting of an Axopatch 1A amplifier, Zeiss motorized micromanipulator and a PDP 11-73 computer with a Cheshire data interface and digital display was used to apply a holding voltage to the pipette interior and record single channel currents. For each experimental trial, a fresh non-agonist-exposed sample of liposomes was used. Seals were formed by lowering the electrode to within 1 μ m of the selected liposome while maintaining positive pressure on the pipette interior and then abruptly releasing the pressure which caused the liposome to "jump" onto the electrode tip. Two seconds after seal formation (seal resistance > 25

Gohm), the pipette holding potential was clamped at +100 mV and the pipette was withdrawn several microns from the liposome, creating a unilamellar inside-out patch. This two second delay was required for seal stabilization. Currents were continuously sampled for 2 minutes at 4000 Hz (250 μ sec/pt) and filtered at 1500 Hz (-3 dB, 8 pole Bessel filter). Experiments were conducted at room temperature (18-22°C).

Data Analysis

Data were sampled, displayed, analyzed and plotted using a series of eighteen homewritten Basic-23 programs. Following acquisition, data were displayed in 128 msec blocks and the opening and closing of each channel event measured as a function of time after seal formation. Desensitization histograms of the number of channel events per time interval ($N(t)$) were created and fitted to exponential distributions using a reiterative interactive χ^2 minimization routine. Histograms containing at least 100 channel events over the 2 min. recording period were fitted to the following equation:

$$N(t) = \frac{D \left[(A/B) e^{-t/B} + (1-A/C) e^{-t/C} \right]}{Ae^{-2/B} + (1-A)e^{-2/C}}$$

In this three variable fit, A = % fast exponential's contribution to the observed exponential; B = τ_{fast} ; C = τ_{slow} ; D = total number of channel events X histogram bin width. The denominator of the equation used is a correction for the distribution of channel events missed in the two second delay prior to data collection.

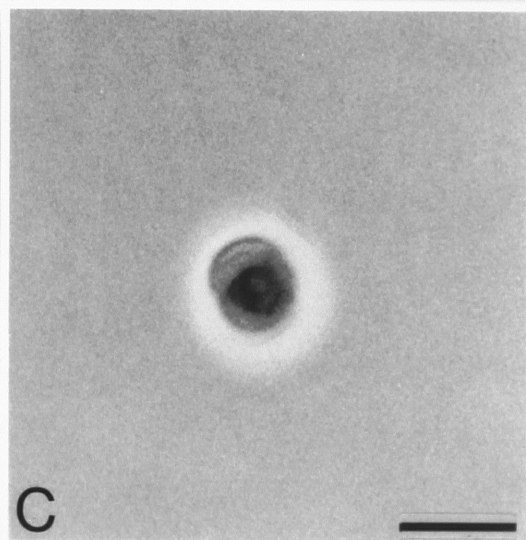
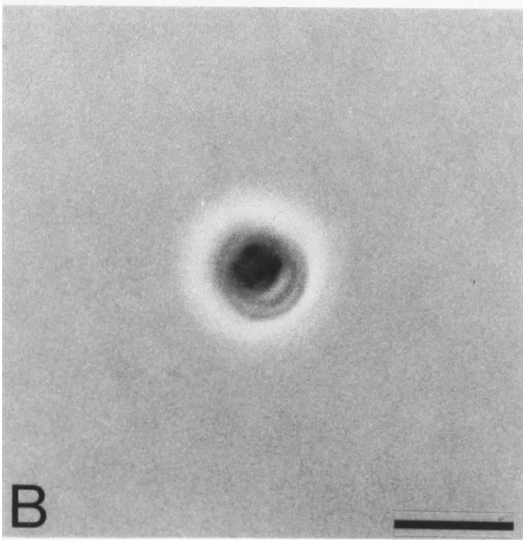
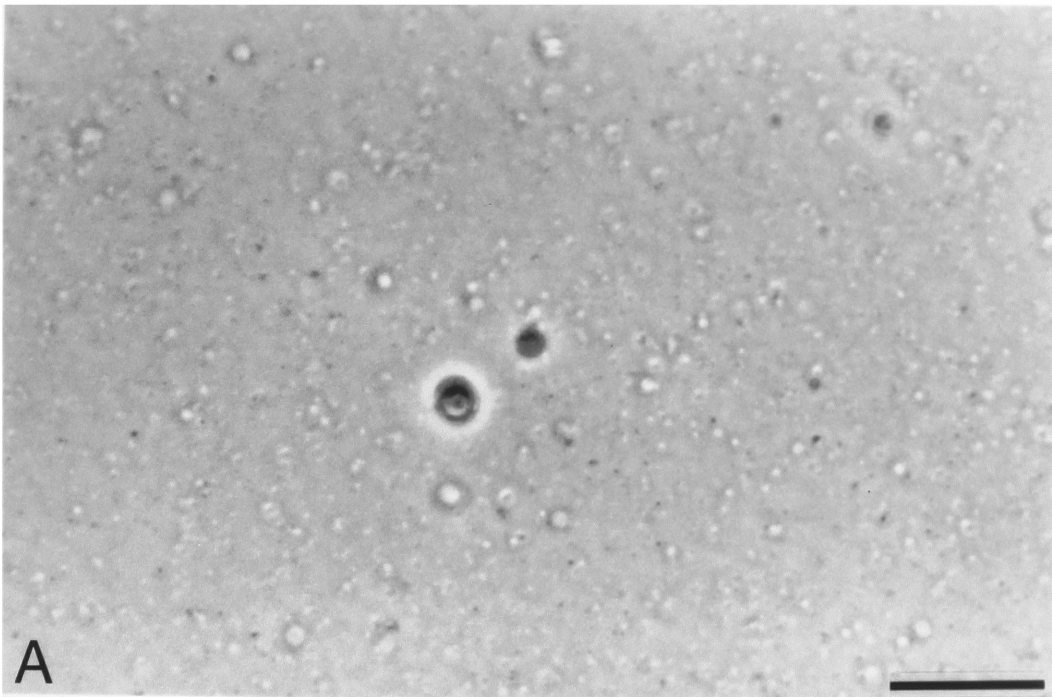
Histograms of open channel lifetimes were generated in a similar fashion using a bin width of 0.5 msec and fitted to double exponential distributions using a reiterative χ^2 fitting routine.

RESULTS

The nAChR containing liposomes, when diluted into saline for recording, formed a suspension of irregularly shaped bilayer structures ranging in size from below the resolution of light microscopy to several microns in diameter. The largest of these liposomes (5 μm in diameter) that were free from surrounding lipid material were selected for patch clamp recording. Such liposomes, as shown in Figure 5, exhibited complex membrane topology with bilayer protrusions on their surfaces. Ion flux assays have shown that in such bilayer structures, reconstituted receptors have a uniform orientation with the acetylcholine binding sites facing outward (Huganir et al., 1982).

The reconstituted nAChR displayed channel properties similar to those previously reported (Tank et al., 1983): patches of liposome membrane containing the nAChR displayed multiple channel openings corresponding to a unitary inward 4.2 pA current when the pipette contained 1 μM acetylcholine and the pipette interior was clamped at +100 mV (Figure 6a). No nAChR channel activity was ever observed in the absence of either agonist (Figure 6b) or voltage or in pure liposomes without reconstituted receptor. The reconstituted nAChR displayed the established pharmacological responses to a variety of cholinergic antagonists: bath application of 10 μM α -bungarotoxin prior to seal formation, or the presence of 100 μM tubocurarine in the patch pipette, blocked all channel activity. The inclusion of 500 μM procaine with acetylcholine in the patch pipette greatly reduced the frequency of channel openings (Figure 6c).

Figure 5 Phase photomicrographs of liposomes containing nAChR
A: Suspension of liposomes of varying appearance and size. Scale bar = 20 μm
B,C: Large liposomes selected for patch clamp recording. Scale bar = 5 μm



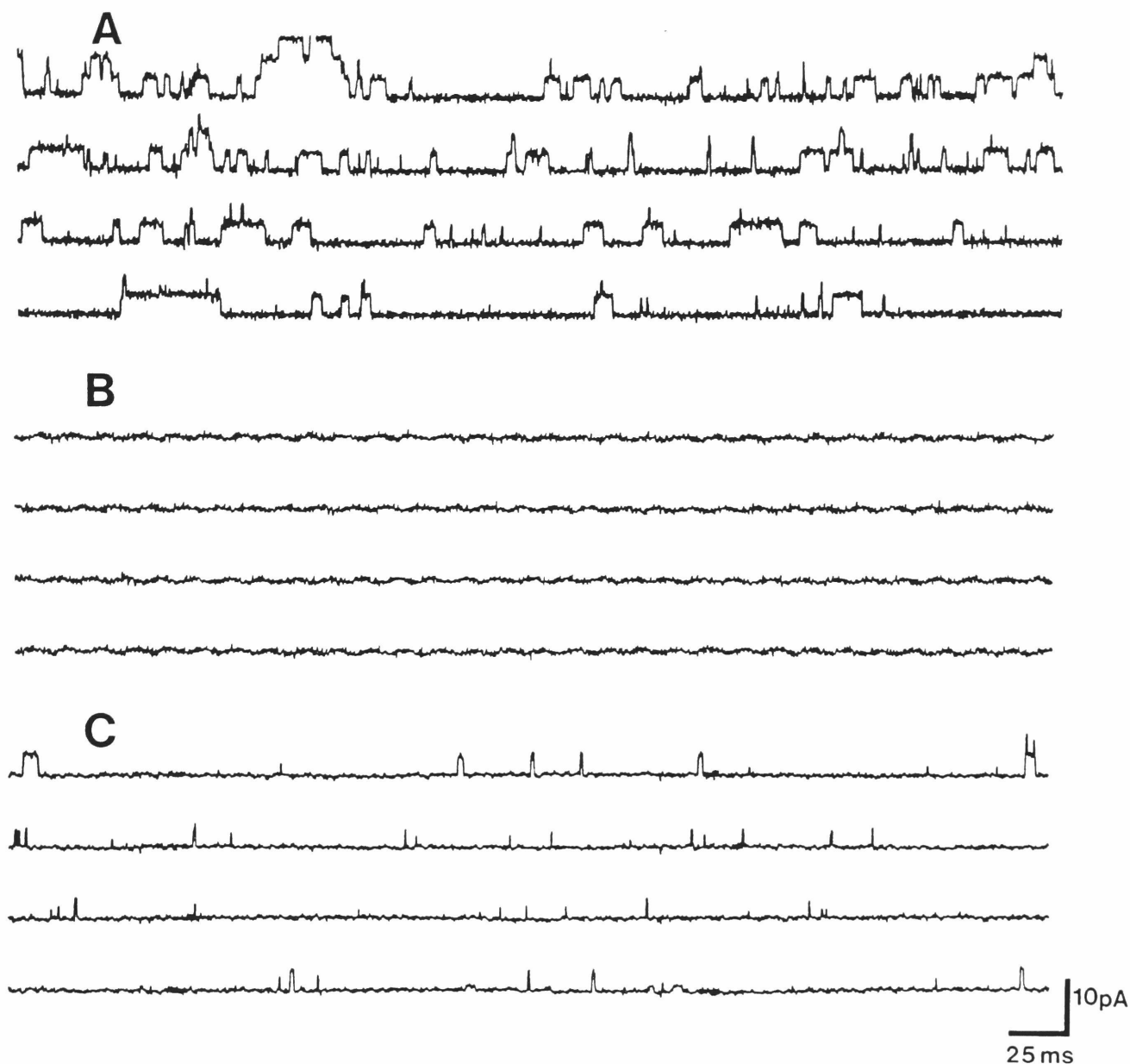


Figure 6 Pharmacology of nAChR channel activity. Traces are four contiguous 0.512 sec portions of individual records sampled immediately after seal formation and application of a +100 mV holding potential. Traces are plotted atypically with inward current as an upward deflection. The patch pipette contained, A: 1 μ M acetylcholine B: 0 μ M acetylcholine C: 500 μ M procaine and 1 μ M acetylcholine.

Plasma membrane vesicles from *Torpedo*, although most highly enriched in nAChR, also contain voltage-gated chloride channels (Tank et al., 1982). Such channels, readily distinguishable from nAChR by their 9 and 18 pS conductance substates, long open times, voltage dependence, agonist independence and failure to desensitize, were observed in only five of several hundred liposomes sampled (data not shown).

The frequency of acetylcholine-induced channel events in untreated receptors with an endogenous phosphotyrosine content of 1.5 mole/mole receptor diminished with time after seal formation and voltage application (Figure 7, top). This desensitization behavior could be quantified from histograms of the number of channel openings per unit time after seal formation (Figure 7, bottom) and could be fitted to a sum of two exponentials. Two desensitization processes, a rapid and a slow phase, have previously been observed for the nicotinic acetylcholine receptor both *in situ* and after reconstitution (Sakman et al., 1980; Hamill et al., 1981a; Walker et al., 1982). Desensitization of the nAChR was very rapid and channel events could be observed only if seal formation occurred within the first few seconds of the pipette entering the bath solution and if recording began immediately after seal formation. Such traces typically contained several hundred channel events during the two minute period of continuous recording. Recordings obtained after longer delays to seal formation (> 10 sec) contained only a very low steady-state level of channel activity.

The rates of the rapid and slow phases of desensitization were dependent on the concentration of acetylcholine applied (Table I). Increasing agonist concentrations increased the rates of both the rapid

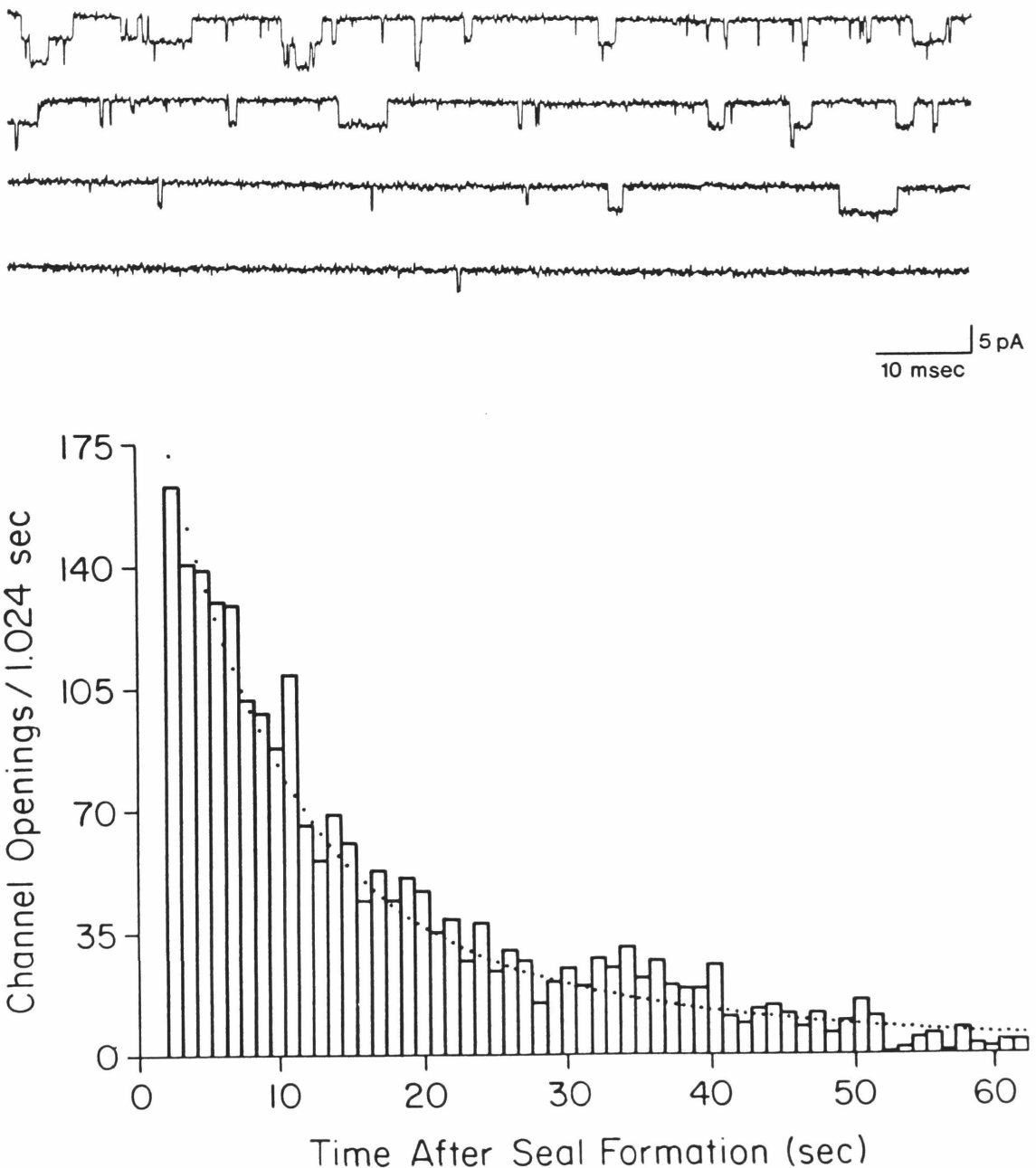


Figure 7 Desensitization behavior of purified and reconstituted nAChR as seen in single patches. Top: 0.512 sec portions of a single record sampled 0, 15, 30 and 60 sec following seal formation and voltage application. Pipette holding potential, +100 mV; 1 μ M acetylcholine. Bottom: exponential decay with time of the frequency of channel opening events. The number of channel openings in successive 1.024 second intervals was plotted versus the midpoint of these time intervals. The dotted curve shows a double exponential distribution with $\tau_{\text{fast}} = 7.8$ sec, $\tau_{\text{slow}} = 30.2$ sec, % fast = 50. (From Hopfield et al., 1988)

ACh, μ M	τ_{fast}	τ_{slow}	% fast	n
0.5	11.5(0.8)	28.8(3.9)	61(25)	4
1.0	6.5(0.9)	25.5(8.0)	71(15)	6
2.5	3.9(0.8)	20.9(1.5)	68(19)	4
5.0	1.6(0.5)	12.0(2.2)	79(22)	6

Table I Acetylcholine concentration dependence of desensitization. Fast and slow time constants (secs) were determined by double exponential fits to histograms of channel openings over time. All experiments were conducted with a single preparation of reconstituted receptor with a stoichiometry of 0.5 mole phosphotyrosine per mole each of β , γ and δ subunits. Data are mean values (\pm standard deviations).

and slow phases of desensitization. The observed rates and dose dependence of both phases of desensitization were consistent with the rates and dose dependence of desensitization of the nAChR from *Torpedo* determined using rapid mixing ion flux techniques (Walker et al., 1982). Experiments were confined to a narrow agonist concentration range of 0.5 to 5.0 μM . Below 0.5 μM , the slow desensitization process dominated and the total number of channel events was too low for exponential analysis and above 5.0 μM , desensitization was so fast that the initial decay of channel activity occurred during the several second seal formation procedure followed by only occasional channel bursting behavior. The optimal concentration of acetylcholine that yielded a large total number of channel events and enabled determination of both phases of desensitization was 1 μM which was therefore used for subsequent experiments.

The nAChR from *Torpedo californica* is phosphorylated on tyrosine residues to a high stoichiometry *in vivo* (Smith et al., 1987). To determine the endogenous phosphotyrosine content of the purified nAChR, quantitative immunoblotting techniques employing an anti-phosphotyrosine affinity purified antibody were used (Hirano et al., 1988; Ohtsuka et al., 1984). The purified nAChR contained 0.9–1.5 moles of endogenous phosphotyrosine per mole of receptor depending on the preparation. The phosphotyrosine was distributed among the β , γ , and δ subunits (Figure 8, lane 5). This endogenous phosphotyrosine content could be decreased *in vitro* by treatment of the nAChR with alkaline phosphatase (Figure 8, lanes 3 and 4). Under the conditions used, alkaline phosphatase did not dephosphorylate nAChR phosphorylated *in vitro* on serine residues by

Figure 8 Purified nAChR preparations with various stoichiometries of tyrosine phosphorylation (from Hopfield et al., 1988)

Lane 1: Protein stain of purified receptor preparation

Lane 2: Autoradiograph of ^{32}P -labeled tyrosine phosphorylated receptor

Lanes 3-7: Western blot analysis of nAChR preparations with increasing phosphotyrosine content using affinity-purified anti-phosphotyrosine antibody

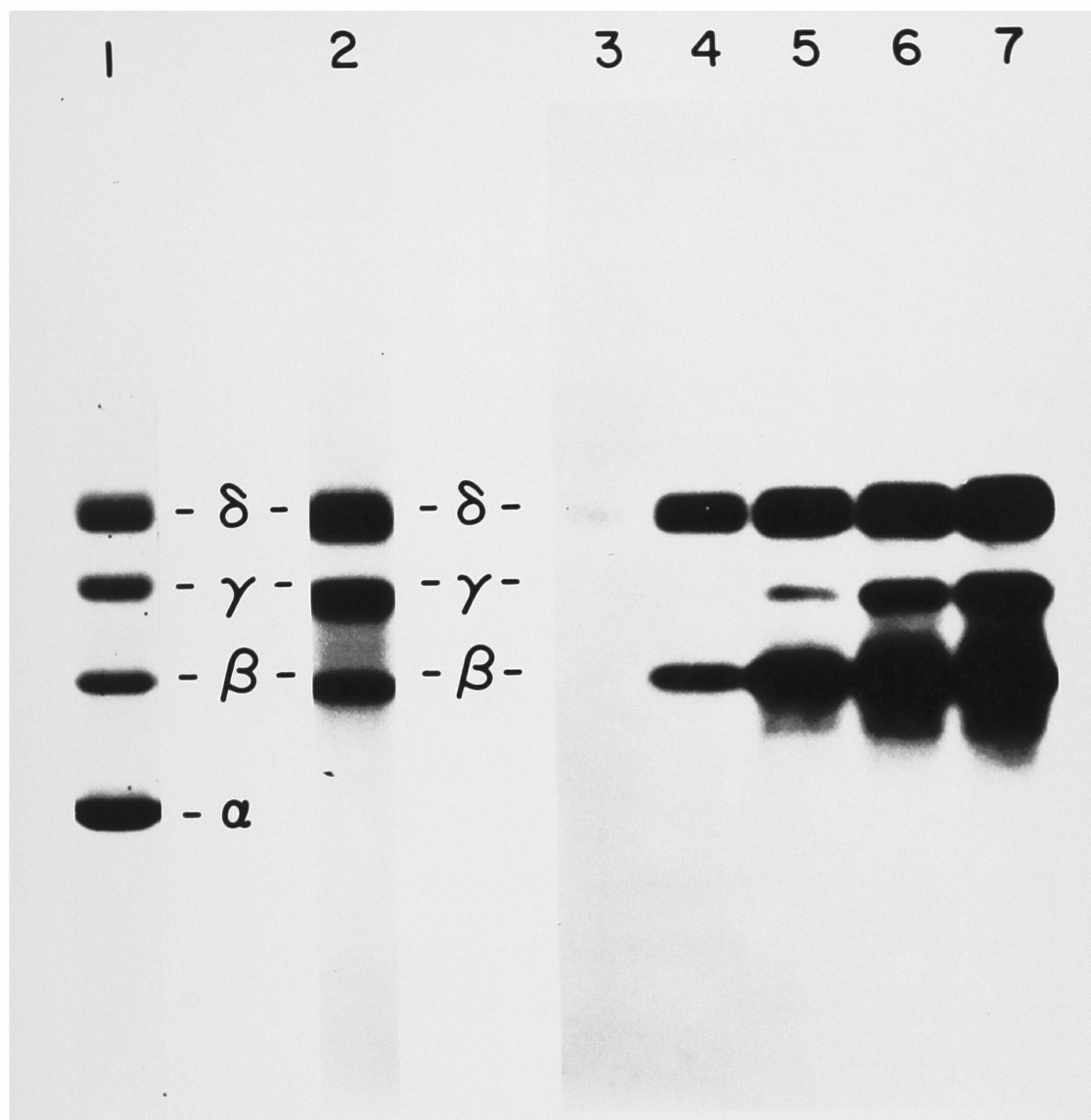
Lane 3: *in vitro* phosphatase-treated nAChR (0.50 mg/ml)

Lane 4: *in vitro* phosphatase-treated nAChR (0.25 mg/ml)

Lane 5: untreated receptor

Lane 6: phosphorylation reaction mixture-treated nAChR (10')

Lane 7: phosphorylation reaction mixture-treated nAChR (30')



protein kinase C or by the catalytic subunit of cAMP-dependent protein kinase using γ -³²ATP. Conversely, the stoichiometry of tyrosine phosphorylation of the receptor could be increased *in vitro* by phosphorylation of the receptor using the endogenous protein tyrosine kinase activity in the postsynaptic membrane (Figure 8, lanes 2, 6 and 7). Using a combination of these dephosphorylation and phosphorylation procedures, purified receptor preparations with a broad range of stoichiometries of tyrosine phosphorylation were prepared and reconstituted into phospholipid vesicles. Samples with eight different stoichiometries ranging from 0.6 to 2.7 moles phosphotyrosine/mole receptor were used for recording and analyzed for changes in channel kinetics.

The rate of desensitization of the nAChR showed a striking dependence on the stoichiometry of tyrosine phosphorylation. Examples of data recorded from two individual patches of reconstituted receptor with stoichiometries of tyrosine phosphorylation of 0.6 and 2.3 moles phosphate/mole receptor are shown in Figure 9. The overall rate of desensitization was dramatically increased with increased stoichiometries of phosphorylation. The major portion of this effect was on the rapid phase of desensitization which showed a seven-fold change in rate over the range of stoichiometries of tyrosine phosphorylation examined (Figure 10). In addition, a two-fold change in the slow rate of desensitization was observed (Table II). The subunits appear to contribute equally to the overall desensitization rate of the receptor: two receptor preparations which had identical total stoichiometries of 1.5 moles phosphotyrosine/mole receptor but with the phosphates differently distributed across the

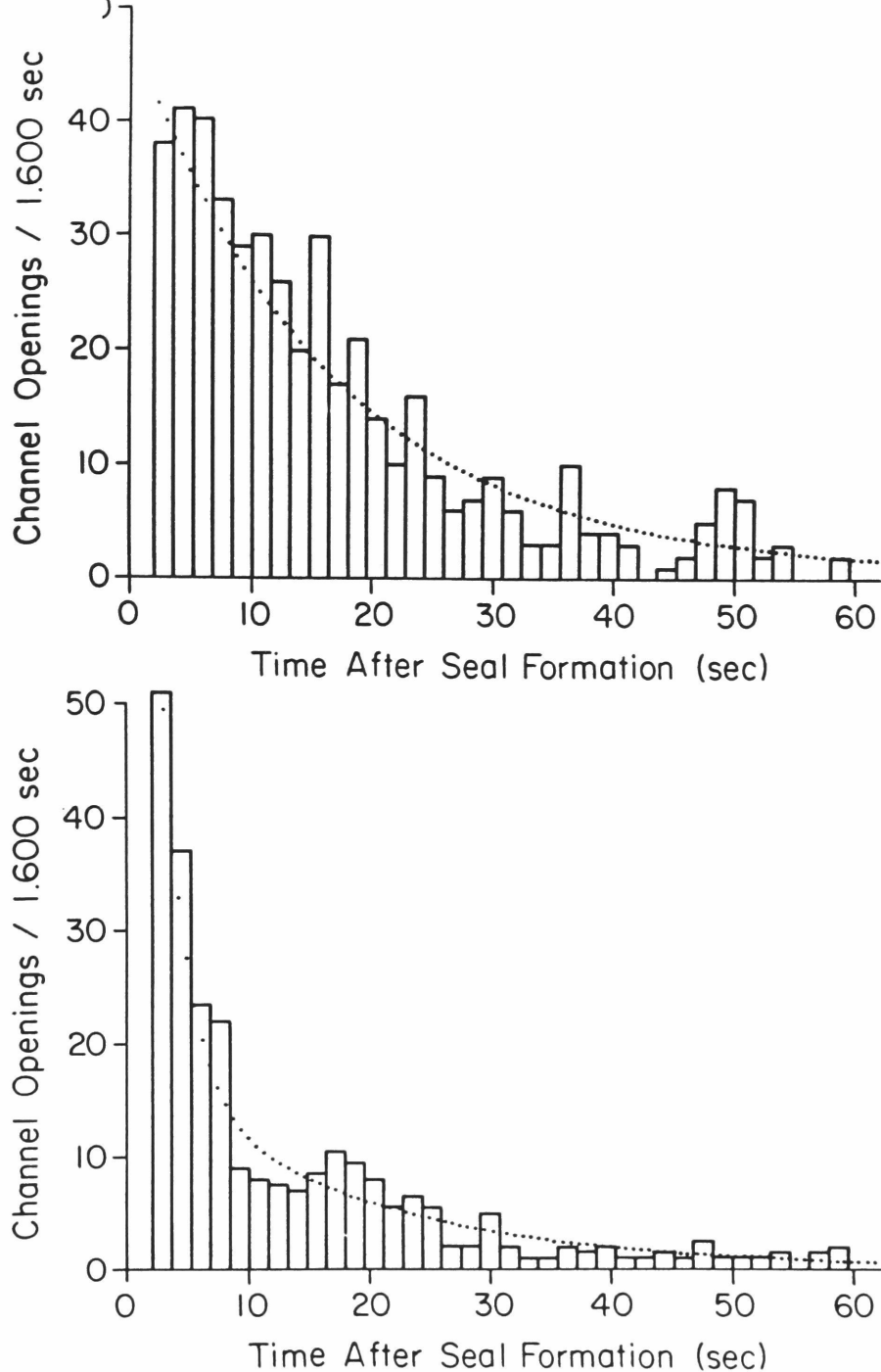


Figure 9 Tyrosine phosphorylation modulates the rate of desensitization of the nAChR as measured by exponential decay with time in the frequency of channel opening events. Pipette holding potential +100 mV; 1 μ M acetylcholine. Top: 0.6 moles phosphotyrosine/mole receptor. Fitted double exponential: $\tau_{\text{fast}} = 15.1$ sec, $\tau_{\text{slow}} = 32.4$ sec, % fast = 76. Bottom: 2.3 moles phosphotyrosine/mole receptor. Fitted double exponential: $\tau_{\text{fast}} = 2.1$ sec, $\tau_{\text{slow}} = 17.6$ sec, % fast = 47. (From Hopfield et al., 1988)

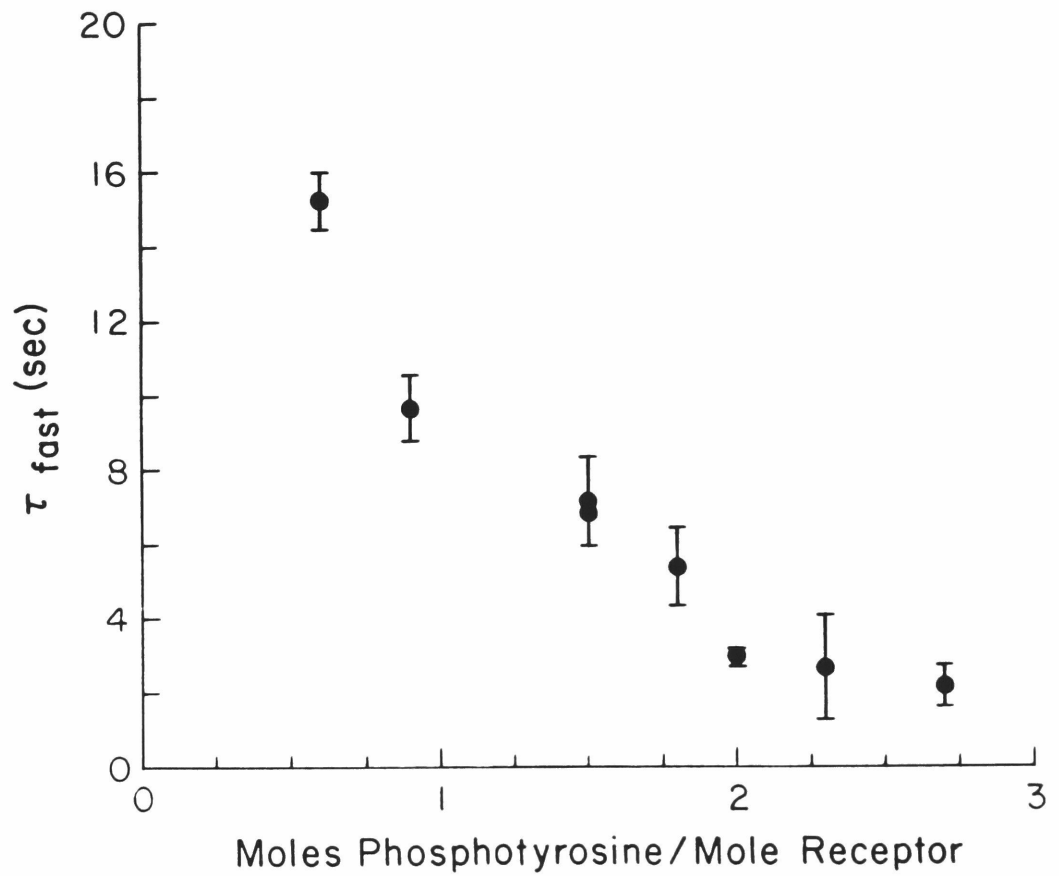


Figure 10 Increased levels of tyrosine phosphorylation increase the rate of the rapid phase of desensitization. Time constants of the rapid phase of desensitization are plotted versus the stoichiometry of tyrosine phosphorylation. Data are plotted as mean values \pm standard deviations. (From Hopfield et al., 1988)

Total Stoich.	Subunit Stoich.			τ_{fast}	τ_{slow}	% fast	n
	β	γ	δ				
0.6	.2	.1	.3	15.3(0.7)	36.3(8.7)	87(11)	4
0.9	.3	.3	.3	9.7(0.9)	23.1(4.3)	86(13)	3
1.5	.6	.3	.6	7.2(1.2)	23.6(3.1)	45(22)	6
1.5	.5	.5	.5	6.9(0.9)	25.5(8.0)	71(15)	6
1.8	.6	.5	.6	5.4(1.1)	20.6(8.1)	87(8)	7
2.0	.7	.5	.8	2.9(0.2)	19.9(6.7)	44(25)	6
2.3	.7	.7	.9	2.7(1.4)	19.1(2.7)	40(9)	4
2.7	.9	.9	.9	2.2(0.6)	14.8(2.7)	55(21)	3

Table II Effect of tyrosine phosphorylation on the fast and slow time constants (secs) of desensitization. Pipette holding potential +100 mV; 1 μ M acetylcholine. All experiments at a given stoichiometry (moles phosphotyrosine/mole receptor) were conducted with a single preparation of reconstituted receptor. All data are mean values (\pm standard deviations). (From Hopfield et al., 1988)

subunits, had nearly identical desensitization rates (Table II).

Tyrosine phosphorylation of the nAChR had no detectable effect on other parameters of ion channel function. Open time analysis of nAChR in a single patch from records without simultaneous channel openings indicated that most open channel lifetimes were in a range from 1-15 msec. Histograms of the frequency of open channel lifetimes, generated by pooling all single channel openings observed across experiments with a given stoichiometry of phosphorylation, indicate that the open channel distributions could be fitted to the sum of two exponentials with characteristic fast and slow time constants of 1 and 7 msec respectively. Figure 11 illustrates two such histograms for receptors with 0.6 and 2.3 moles phosphotyrosine/mole receptor. The apparent mean channel open-time distributions and single channel conductances were essentially identical in all of the receptor preparations examined (Table III) and similar to those previously reported for untreated receptor (Tank et al., 1983).

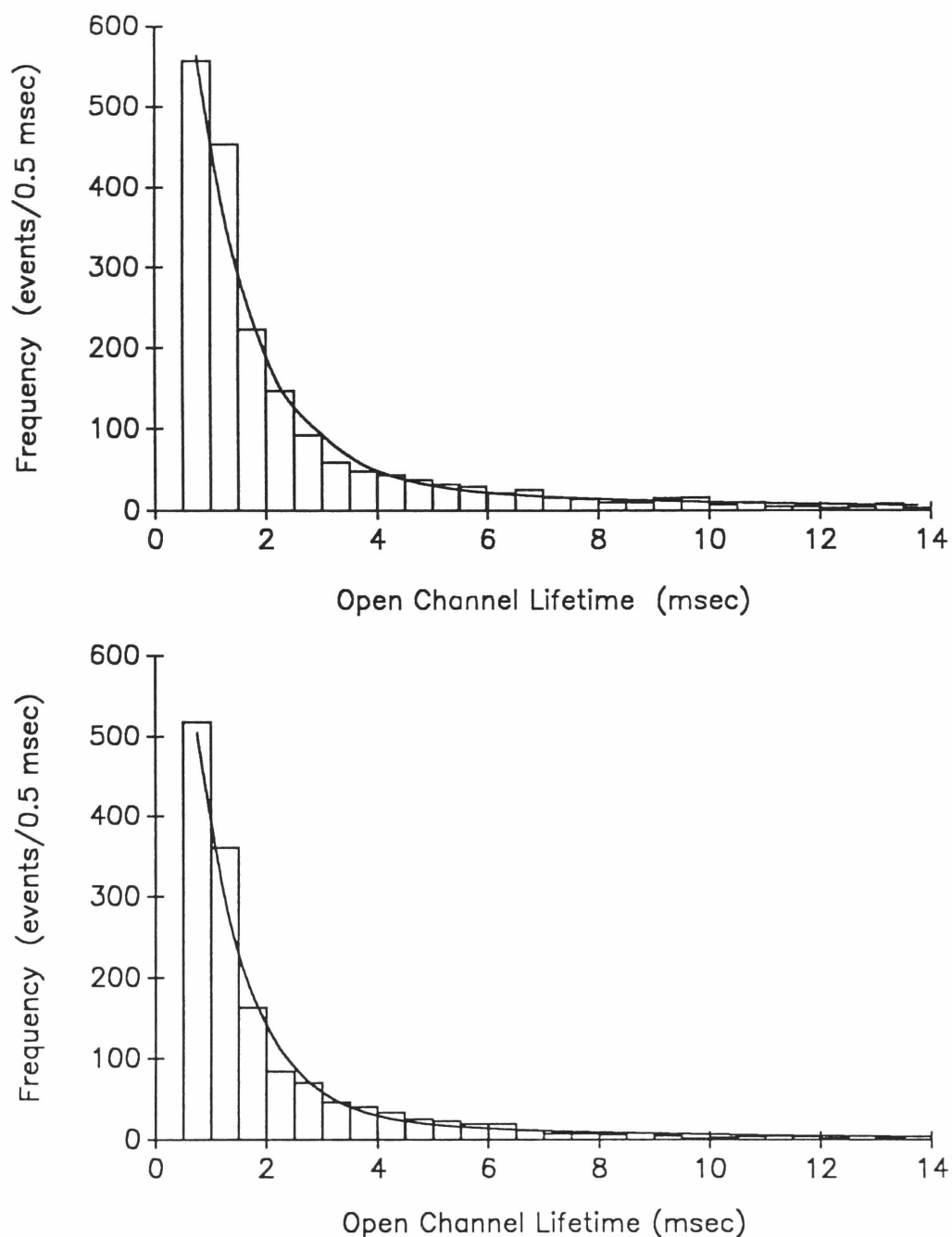


Figure 11 Open channel lifetimes of single channel events from records pooled across all experiments conducted at a given stoichiometry of tyrosine phosphorylation. Pipette holding potential +100 mV; 1 μ M acetylcholine. Top: 0.6 moles phosphotyrosine/mole receptor. Fitted double exponential: $\tau_{fast}=1.1$ msec, $\tau_{slow}=7.3$ msec, % fast=80. Bottom: 2.3 moles phosphotyrosine/mole receptor. Fitted double exponential: $\tau_{fast}=0.8$ msec, $\tau_{slow}=7.0$ msec, % fast=83.

Total Stoich.	τ_{fast}	τ_{slow}	% fast	Total events examined	n	conductance pS
0.6	1.1	7.3	80	1971	4	42
1.5	0.9	5.8	91	4720	6	44
1.5	1.2	7.3	79	2035	3	42
2.3	0.8	7.0	83	1516	4	43
2.7	0.9	6.9	86	725	3	40

Table III Effect of tyrosine phosphorylation on single channel properties of the nAChR. All single channel openings across n separate experiments from a receptor preparation at a given stoichiometry were pooled. Fast and slow time constants (msec) of open channel lifetimes were determined by double exponential fits to histograms of the frequency of open channel lifetimes.

DISCUSSION

The data in Figure 10 and Table II suggest that the effect of phosphorylation of each subunit is additive and that the more highly phosphorylated species of the nAChR desensitize at more rapid rates. Kinetic interpretations of the data are complicated by the fact that the calculated stoichiometries of tyrosine phosphorylation are non-integer numbers that represent composites of receptors with integer values of phosphorylation. Given the assumption that the phosphorylation/dephosphorylation of individual subunits occurs independently of each other, each receptor preparation would consist of eight different receptor populations, present in varying proportions and ranging in the number (0-3) of phosphates per receptor and in the individual subunits phosphorylated. As shown in Table IV, the probability of phosphate occupancy at the three potential phosphorylation sites can be calculated for the different stoichiometries of phosphorylation. With increasing stoichiometry, the percentage of triply phosphorylated receptor increases while the percentage of non-phosphorylated receptor decreases. The percentage of singly and doubly phosphorylated receptor first increases and then decreases with increasing stoichiometry of phosphorylation. These calculations assume phosphorylation only on tyrosine residues since phosphorylation on serine residues was extremely low (0 to 0.1 mole phosphoserine/mole receptor) and did not change with treatment to regulate the level of tyrosine phosphorylation.

The measured rates of desensitization presumably represent composites of the rates of desensitization of these variously

Total Stoich.	Subunit Stoich.			Probability of sites phosphorylated			
	β	γ	δ	0	1	2	3
0.6	.2	.1	.3	.50	.40	.10	.00
0.9	.3	.3	.3	.34	.44	.19	.03
1.5	.6	.3	.6	.11	.38	.40	.11
1.5	.5	.5	.5	.12	.38	.38	.12
1.8	.6	.6	.6	.06	.29	.43	.22
2.0	.7	.5	.8	.03	.22	.47	.28
2.3	.7	.7	.9	.00	.12	.43	.44
2.7	.9	.9	.9	.00	.03	.24	.73

Table IV Probability of phosphate occupation of the three potential phosphorylation sites at different stoichiometries of phosphorylation. Assuming that the sites are independent, the probabilities can be calculated from the following formulae:

$$P_3 \text{ sites} = P_\beta P_\gamma P_\delta$$

$$P_2 \text{ sites} = P_\beta P_\gamma (1-P_\delta) + P_\beta P_\delta (1-P_\gamma) + P_\gamma P_\delta (1-P_\beta)$$

$$P_1 \text{ site} = P_\beta (1-P_\gamma) (1-P_\delta) + P_\gamma (1-P_\beta) (1-P_\delta) + P_\delta (1-P_\beta) (1-P_\gamma)$$

$$P_0 \text{ sites} = (1-P_\beta) (1-P_\gamma) (1-P_\delta)$$

phosphorylated species. The rapid and slow phases of desensitization would themselves be expected to be of multi-exponential character reflecting the multiple phosphorylation states of the receptor. However, the relatively low number of channel events per patch does not permit such complex exponential analysis.

The contributions of these subpopulations are particularly important when considering the accuracy of the rapid rates of desensitization measured for the highly phosphorylated preparations. Most of the records from patches isolated from such preparations showed few channel events due to the extremely rapid rate of desensitization. In such cases, most of the channel openings appear to have occurred during the several second delay prior to data collection inherent in the recording paradigm because when patches were isolated with an unusually short delay, a significant portion of the rapid phase of desensitization could be observed. For the most highly phosphorylated preparation used (2.7 moles phosphotyrosine/mole receptor), 73% of the receptor molecules would be expected to be phosphorylated on the β , γ and δ subunits. It is possible that the two second time constant observed may be largely determined by the 27% of the nAChR molecules not phosphorylated on all three subunits and therefore, the rate constant of the rapid phase of desensitization for the triply phosphorylated receptor may be considerably faster than the two seconds determined for this preparation. Flux analysis of fast desensitization kinetics from these liposomes will be required to resolve this issue.

Tyrosine phosphorylation of the nAChR had no effect on the open time distributions of single channel events (Table III). Increased probability of channel opening with phosphorylation by cAMP-dependent protein kinase

in nAChR reconstituted into lipid bilayers has been reported (Montal et al., 1987). Such experiments, however, measure reactivation of the receptor following desensitization rather than the initial phase of desensitization examined here. It was technically impossible to examine the effect of tyrosine phosphorylation on receptor recovery from the desensitized state because inside-out patches were used, requiring agonist to be applied in the patch pipette. At the time these experiments were designed, removal and reapplication of solutions within the patch pipette on a subsecond time scale was not feasible. Repeated attempts to form outside-out patches were unsuccessful because of the complex onion-like structure of the liposomes which precludes the "whole-cell" recording configuration.

It should be noted that because such recovery experiments could not be done, the existence of desensitization in the preparation could not be proven. However it is extremely likely that desensitization, rather than a non-physiological process that shuts off the channel, is taking place for three reasons. First, true desensitization of the nAChR is seen in reconstituted liposomes during flux measurements in which acetylcholine can be repeatedly washed off and reapplied (Walker et al., 1982). Second, the concentration dependence of this desensitization is almost identical to the concentration dependence of the presumed desensitization measured in these patch clamp experiments. Finally, desensitization of the nAChR following agonist application has been seen in many electrophysiological studies of receptors *in situ* (Boyd, 1987; Feltz et al., 1982; Magleby et al., 1981).

The increased desensitization rate with tyrosine phosphorylation

cannot be explained by an indirect effect of tyrosine phosphorylation on channel density since the desensitization measurement used here is independent of channel number. Tyrosine phosphorylation of the nAChR or associated proteins may have such effects on channel clustering or cytoskeletal interactions: pp60^{src}, the tyrosine-specific protein kinase product of Rous sarcoma virus prevents nAChR clustering on cultured chicken muscle fibers (Anthony et al., 1984). Tyrosine phosphorylation may also play a role in the developmental regulation of the receptor since innervation during development and denervation of adult muscle regulates the level of tyrosine phosphorylation (Qu et al., 1990).

The results presented here demonstrate that tyrosine phosphorylation of the nAChR dramatically increases the rate of the rapid phase of desensitization. Moreover, dephosphorylation of the endogenous phosphotyrosine residues decreases the rate of the rapid phase of desensitization to the point where it approaches the rate of the slow phase of desensitization (Table III). These results suggest that the rapid phase of desensitization may have an absolute dependence on phosphorylation. It was not possible to obtain a completely dephosphorylated preparation of the nAChR by alkaline phosphatase treatment to test this hypothesis. A completely dephosphorylated preparation could also address the possibility that phosphorylation is necessary for receptor activation as has been suggested for the neuronal nAChR and the GABA_A receptors (Margiotta et al., 1987; Stelzer et al., 1988). If this were the case, one would expect to see a change in channel number with phosphorylation since a given receptor would be either dephosphorylated (off) or phosphorylated to a stoichiometry of one (on).

Site-directed mutagenesis of nAChR prior to expression in oocytes may be the best way to test the absolute requirement for receptor phosphorylation for activation or desensitization as well as to determine if all seven phosphorylation sites are in fact equivalent and additive in their effects.

This demonstration that tyrosine phosphorylation increases desensitization is the only existing electrophysiological proof that phosphorylation of a receptor-gated ion channel alters its channel properties. The data are consistent with studies on skeletal muscle nAChR that suggest that agents which raise levels of second messengers and therefore probably phosphorylate the receptor, modulate receptor desensitization (Albuquerque et al., 1986; Middleton et al., 1986). This confirmation that phosphorylation of the receptor increases its desensitization rate has become important in light of several recent reports that modulation of nAChR desensitization by forskolin is independent of cAMP (Wagoner et al., 1988; White, 1988). Concentration range experiments with forskolin have indicated that it may, at high concentrations, exert direct anesthetic-like effects on the nAChR independent of its activation of a second messenger pathway (Middleton et al., 1988). Phosphorylation of purified receptor-linked ion channels by identified kinases sacrifices information about intact cell function but permits analysis of the direct functional consequences of protein phosphorylation free from such ambiguities of second messenger activation.

CHAPTER FOUR

D2 Dopamine Receptors in Striatal Neurons are Coupled to a Potassium Conductance that may be Regulated by Protein Phosphorylation

Striatal neurons are the principal cell type of the caudate-putamen, a kidney shaped nucleus that is a member of the group of forebrain structures known collectively as the basal ganglia. Over 95% of striatal neurons are of one type, medium spiny type II. These GABAergic neurons receive a large excitatory projection from the cerebral cortex, consisting primarily of glutamate fibers, and a second afferent projection of dopamine fibers from the substantia nigra, pars compacta (Figure 12). Branching efferent collaterals project to the globus pallidus and the substantia nigra as well as to the cell bodies of neighboring medium spiny neurons (for review, see Gerfen, 1988)

The basal ganglia primarily coordinate sensory and motor signals to generate programmed and planned movement (Evarts et al., 1984). The nigro-striatal dopamine pathway appears to be critical in this circuit; degeneration of dopamine projections from the substantia nigra to the caudate-putamen results in the rigidity and tremor of Parkinson's disease (German et al., 1989) and the medium spiny neurons of the caudate atrophy and become aspiny in Huntington's disease (Martin et al., 1986). In addition, cocaine addiction and major mental pathologies such as schizophrenia and obsessive-compulsive disorders have been linked to abnormalities in the basal ganglia and, specifically, to changes in the dopamine pathways (Seeman, 1987).

Dopamine receptors in mammalian brain can be separated into two subtypes, D1 and D2, which differ in their anatomical distributions and

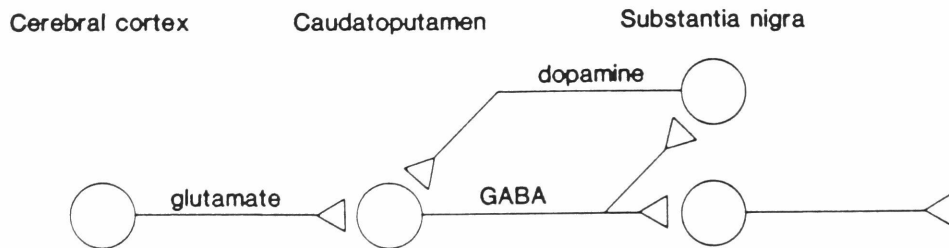


Figure 12 Simplified schematic diagram of the synaptic organization of the major afferent and efferent connections of the caudate-putamen. The GABAergic medium spiny neurons, the major cell type of the striatum, receive afferent projections from the cerebral cortex and substantia nigra and have efferent projections to the substantia nigra. (From Hemmings et al., 1987)

pharmacological profiles and mediate distinct behavioral effects (Table V). The D1 receptor has recently been cloned and has the deduced amino acid sequence of a G-protein coupled receptor (Deary et al., 1990). D2 receptors exist in at least two molecular forms in the caudate which are generated by alternative splicing of a gene that encodes for a seven transmembrane domain G-protein coupled receptor (Giros et al., 1989).

The electrophysiology of dopamine systems is not well understood. The dopamine containing neurons that project from the substantia nigra to striatum have been extensively characterized both in terms of their firing patterns and their modulation by dendritic dopamine release (for review, see Grace, 1987). However, the actions of dopamine on target neurons in the striatum remain ambiguous. Ionophoretic application of dopamine onto striatal neurons recorded intracellularly has been reported to result in: depolarization and increased firing (Akaike et al., 1987), depolarization and decreased firing (Bernardi et al., 1978) and hyperpolarization and decreased firing (Herrling et al., 1980). This failure of dopamine to elicit responses that can be characterized as solely excitatory or inhibitory has led to the frequent classification of dopamine as a neuromodulator. Dopamine antagonizes the excitatory effect of glutamate on striatal neurons (Brown et al., 1983). However, dopamine has also been reported to enhance both glutamate-induced excitation and GABA-induced inhibition (Chiodo et al., 1986).

The lack of a consensus on the actions of dopamine in the basal ganglia has several probable causes. Dopamine binds with similar affinities to D1 and D2 receptor subtypes in striatum and therefore activates at least two signal transduction pathways that may vary in their

	D1	D2
Anatomical Distribution	Neostriatum Frontal cortex N. Accumbens Amygdala	Neostriatum Pituitary Retina
Location	Postsynaptic	Presynaptic Postsynaptic
Adenylate Cyclase Coupling	Stimulation	Inhibition
Prototypical Selective Agonist	SKF 38393	Quinpirole
Prototypical Selective Antagonist	SCH 23390	Sulpiride
Behavioral Effect of Stimulation	Grooming Oral movements	Locomotion

Table V D1 and D2 dopamine receptors in the central nervous system. For a review of the D1/D2 classification scheme, see reviews by Anderson et al., 1990 and Stoof et al., 1984.

relative inputs among cells and preparations. Dopamine receptors may be differently coupled to ion channels in dendrites and cell bodies. This could account for the variation in cell responsiveness seen with the method of dopamine application; endogenous (dendritic) dopamine application by stimulation of the substantia nigra generates EPSPs in striatal neurons whereas exogenous (somatic) dopamine application decreases the spontaneous activity of cells in the caudate (see for example, Williams et al., 1990). To further complicate analysis, medium spiny neurons have recurrent collaterals that synapse onto and rapidly inhibit neighboring cells making direct versus indirect responses to dopamine difficult to distinguish (Lighthall et al., 1983).

To examine the signal transduction pathways utilized by D1 and D2 receptors requires an experimental preparation that maximizes the spatial and temporal resolution of dopamine responses. Striatal slices, the standard preparation for both physiology and pharmacology, are synaptically complex and require tedious post-recording dye injection to identify the recorded cells. Cultured cells, although simple to record from and easily identifiable, are prone to *in vitro* developmental artifacts in their signal transduction mechanisms (Hoch et al., 1989). A third preparation is acutely dissociated adult neurons which lack synaptic contacts yet are fully differentiated, morphologically distinct cells. Dissociated CA1 hippocampal neurons have been used to study GABA_A and glutamate receptor interactions (Stelzer et al., 1989).

This chapter presents preliminary data using dissociated neostriatal neurons to examine the electrophysiological correlates of D2 receptor activation. This work is part of a long term project to determine the

signal transduction pathways of the striatal D1 and D2 receptors and the role of protein phosphorylation in mediating and modulating these responses.

METHODS

Cell Dissociation

Acutely dissociated neostriatal neurons were prepared from guinea pig brain in a modification of an isolation procedure developed for hippocampal neurons (Kay et al., 1986a). For each recording session, one 120–200g female guinea pig was decapitated, the brain was removed as rapidly as possible and placed in cold (5°C) PIPES saline containing, in mM, 120 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 25 D-glucose, 20 PIPES, pH 7.0. Right and left striata were rapidly dissected out using glass probes and hand sliced with a platinum razor blade into approx. 500 μ m thick coronal slices. Individual slices were transferred with a fine paintbrush to cold PIPES saline that had been bubbled with 100% O₂.

Once both striata had been cut, the twenty to twenty-five resulting slices were transferred to a Wheaton Cell-Stir flask containing 15 ml PIPES saline with 15 mg trypsin added (Sigma type XI, from bovine pancreas). The time from animal sacrifice to tissue incubation could not exceed four minutes to retain cell viability. Slices were incubated for 60 to 90 min, at 30°C with continuous 100% O₂ bubbling the solution. Slices were stirred in the flask at a rate sufficient to buoy up the slices and prevent settling on the bottom but not so rapidly as to disintegrate the slices or cloud the solution. After incubation, the trypsin solution was removed and replaced with 15 ml of fresh PIPES saline. The flask was allowed to come to room temperature while continuously introducing 100% O₂ and stirring the slices. Slices could be kept alive in this manner for up to six hours post-digestion.

To harvest neurons, two slices were removed from the flask and

placed in 1 ml of a 2 mM MgCl_2 , calcium-free PIPES saline held in a small glass vial. The slices were triturated with a series of increasingly fine fire-polished Pasteur pipettes with inner diameters of 0.5–0.2 mm. The resulting cell suspension was transferred to a glass bottom recording chamber and permitted to settle for 5 min. The PIPES saline was replaced with unoxygenated HEPES saline containing, in mM, 140 NaCl, 5 KCl, 1 CaCl_2 , 1 MgCl_2 , 25 D-glucose, 10 HEPES, pH 7.4. Isolated cells, as assessed by physiology, morphology and trypan blue exclusion, remained viable for at least one hour post-trituration.

Receptor Binding

To assay the effects of trypsin incubation on dopamine receptors, parallel incubations of striatal slices were run with and without trypsin and permitted to recover for 30 min at 20° in fresh PIPES saline. The slices from both groups were then separately homogenized with a Tisumizer in 30 ml of a 50 mM Tris-HCl solution, pH 7.4 and centrifuged at 20,000 x g for 10 min at 4°C. The pellets, after one wash by suspension and recentrifugation, were resuspended in an incubation buffer containing, in mM, 120 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 50 Tris-HCl, pH 7.4. Protein concentrations of the membrane preparation were determined (Bradford, 1976) and the samples were diluted in incubation buffer to final protein concentrations of 0.05 mg/ml (D1) and 0.10 mg/ml (D2).

Radioligand binding assays were conducted using either [^3H]SCH 23390 to label D1 receptors or [^3H]raclopride to label D2 receptors. Following a 45 min. (D1) or 60 min. (D2) incubation at 30°C, the samples were rapidly filtered through Whatman GF/C glass fiber filters, followed by two

washes in 5 ml of cold incubation buffer. Radioactivity was determined by liquid scintillation spectrophotometry. Specific binding was defined to be the total binding minus the nonspecific counts in control samples incubated with both the radioligand and a competitive receptor subtype-specific non-radiolabelled ligand (30 μ M SKF 38393 for D1 and 300 μ M sulpiride for D2). Saturation binding parameters of [3 H]SCH 23390 and [3 H]raclopride were determined by Scatchard analysis.

Patch Clamp Recording

Patch-clamp electrodes were pulled in two stages on a Kopf 720 vertical puller and fire-polished to a final resistance of 2 to 4 Mohm with a Narishige microforge. Electrodes were back-filled with one of the following solutions (in mM):

Potassium Fluoride: 120 KF, 1 CaCl₂, 1 MgCl₂, 11 EGTA, 10 HEPES, pH 7.4

Potassium Methanesulfonate: 100 KMeSO₃, 30 KCl, 4 MgCl₂, 2 ATP, 10 EGTA, 10 HEPES, 0.1 leupeptin (a thiol-protease inhibitor), pH 7.4

All dopaminergic drugs were dissolved in HEPES saline and either bath applied or picospritzed onto isolated cells. Solutions containing dopamine included 250 μ M ascorbate to slow oxidation and were kept on ice. For the experiments with kinase injection, catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart (Kaczmarek et al., 1980) and added to potassium methanesulfonate electrode solutions (final concentration 12 μ g/ml (300 nM)). HEPES saline was used as the bath solution for all recordings and in some experiments, as noted in the text, was supplemented with blocking agents.

Dissociated cells were observed with a Zeiss IM-35 inverted

microscope equipped with phase contrast and differential interference contrast (Nomarski) optics. Voltage clamping in the whole cell configuration was conducted using a patch clamp system, similar to that described by Hamill et al., 1981a, consisting of an Axopatch 1A amplifier, Narishige hydraulic micromanipulator and TL-1 DMA interface (Axon Instruments). Pipette capacitance and series resistance were electronically compensated. Current records were sampled at 200 to 10,000 Hz and filtered appropriately (-3dB, 8 pole Bessel filter). Data were sampled, stored and analyzed using Pclamp versions 4.0-5.1 (Axon Instruments) and a Zenith 386 computer. All experiments were conducted at room temperature (18-22°C).

RESULTS

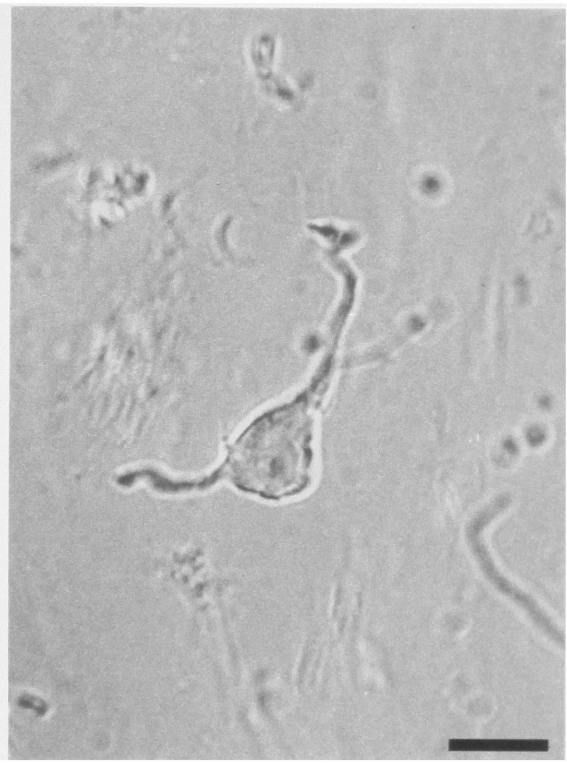
Healthy isolated cells, viewed under phase contrast microscopy, had uniformly bright cell bodies and retained proximal dendrites. Medium spiny neurons preserved their characteristic morphology displayed in Golgi-stained preparations consisting of a 10–15 μm pyramidally-shaped cell body and branched apical dendrites (Figure 13a). Medium spiny neurons were readily distinguishable from glia which tended to remain in large sheets, and from minor subpopulations of other cells such as the large aspiny cholinergic neurons which differed dramatically in size and dendritic morphology (Figure 13b). The existence of smooth somatic surfaces when viewed under Nomarski optics was a good indicator of neuronal vitality. As cells were perfused and began to "run-down" during whole cell recording, cells developed grainy plasma membrane textures and nuclei could be observed (Figure 13c).

Trypsin digestion has been reported to alter membrane-bound proteins (Lee et al., 1977). To investigate if D1 or D2 receptors were damaged by the dissociation procedure, dopamine receptors were labelled with the D1 antagonist [^3H]SCH 23390 or the D2 antagonist [^3H]raclopride from slices that had been incubated with and without trypsin. Saturation curves of specific radioligand binding to the receptors and Scatchard analyses were used to obtain binding parameters of dopaminergic compounds to the treated and non-treated receptors (Table VI). The only statistically significant change in binding with trypsin treatment was a 50% decrease in the number of D1 binding sites (B_{max}). The affinity (K_D) of SCH 23390 for the D1 receptor and both the affinity and number of D2 receptors as assayed by

Figure 13 Photomicrographs of dissociated neostriatum. **A:** Medium spiny neurons, marked with arrows, under phase contrast. The cells retain their characteristic somatic shape and branching proximal dendrites. **B:** A large cholinergic aspiny neuron viewed with Nomarski optics. Such non-dopamine receptor containing neurons comprise less than 5% of the identifiable dissociated cells. **C:** A medium spiny neuron after 10 min. of whole-cell patch clamp recording. As the cell "runs down", cellular organelles become visible. Inclusion of the protease inhibitor leupeptin in the patch pipette prevents the processes from retracting during recording. Scale bar= 20 μm



Before Cold Shock



After Cold Shock

		B_{\max} (fmol/mg protein)	K_D (nM)
D1	control	1931 \pm 326 (6)	0.45 \pm 0.10 (6)
	trypsin	1094 \pm 368 (5)	0.73 \pm 0.31 (6)
D2	control	477 \pm 102 (5)	7.82 \pm 1.08 (5)
	trypsin	391 \pm 89 (3)	6.13 \pm 3.08 (3)

Table VI Saturation binding parameters of [H^3]SCH 23390 and [H^3]raclopride to D1 and D2 receptors in control- and trypsin-treated guinea pig striatal slices. The lower B_{\max} of D1 receptors in trypsin-treated cells compared to non-trypsin-treated controls was the only binding parameter that significantly changed with enzymatic digestion ($p < 0.05$). Data are expressed as means \pm standard deviations and were compared using Student t-tests. These results indicate that guinea pig dopamine receptor binding parameters differ from those of other mammalian species previously examined: the D1 receptors are five fold lower in affinity than those in rat striatum (Faedda et al., 1989) and the D2 receptors are ten fold lower in affinity than those in rat and rabbit (Dewar et al., 1989). Whether these differences reflect true species variations or merely differences in the method of tissue preparation remains to be determined.

raclopride binding were unchanged.

Isolated medium spiny neurons ($n = 76$) had mean resting potentials of -59 mV (range, -75 to -47 mV) and mean input resistances of 600 Mohm (range, 300 to 1100 Mohm). They could be held for up to twenty minutes in the whole-cell voltage clamp configuration with longevity being determined both by individual cell health and the choice of intracellular dialysis solution. Inclusion of ATP and Mg^{++} in the patch pipette delayed "run down" as did the use of potassium fluoride as the dominant ionic species. During recording, spontaneous firing of action potentials was rare, as has been reported in striatal slices and the caudates of awake behaving animals (Calabresi et al., 1990).

Whole-cell voltage clamp current recordings indicated that the cells remained electrically excitable after dissociation. Cells in HEPES saline that were recorded from with potassium fluoride-containing electrodes could be held at -90 mV. Step depolarizations for 75 msec to voltages up to $+30$ mV resulted in fast inward and delayed outward currents (Figure 14). The voltage-activated outward current (Figure 15a) had a mean peak amplitude at $+40$ mV of 2.2 nA (range, 400 pA to 5.3 nA) with a threshold of -50 to -70 mV. No inactivation was observed during voltage steps as long as 250 msec. This outward current could be abolished by bath application of 20 mM TEA (data not shown). The voltage-activated inward current (Figure 15b) had a mean peak amplitude of 4.1 nA (range, 750 pA to 14 nA) with a threshold of -40 to -50 mV. This current rapidly inactivated within 10 msec of voltage application and was TTX sensitive ($5\mu M$).

For dopamine application experiments, potassium methanesulfonate

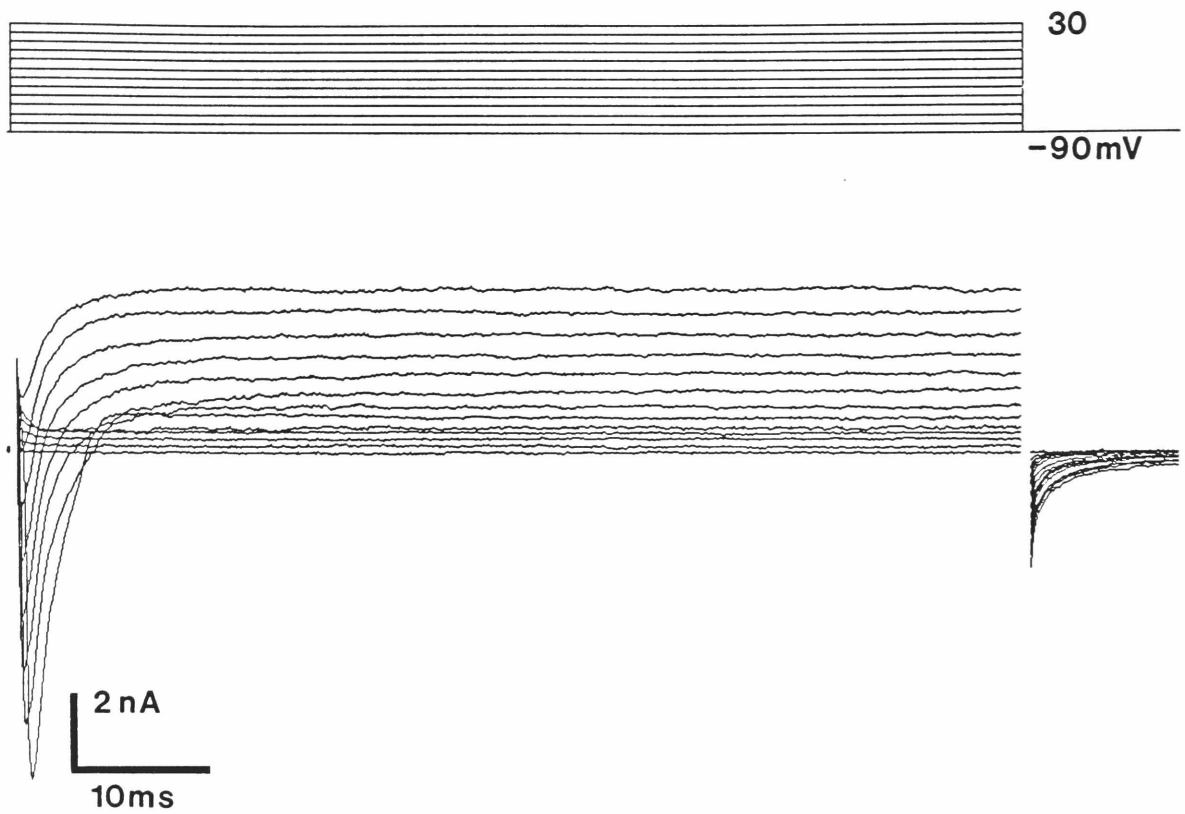


Figure 14 Voltage-activated currents recorded from a cell in the whole-cell patch clamp configuration. Ionic currents were elicited by voltage steps from -90 mV to +30 mV in 10 mV increments from a prepulse potential of -90 mV. Capacitative artifacts have been removed.

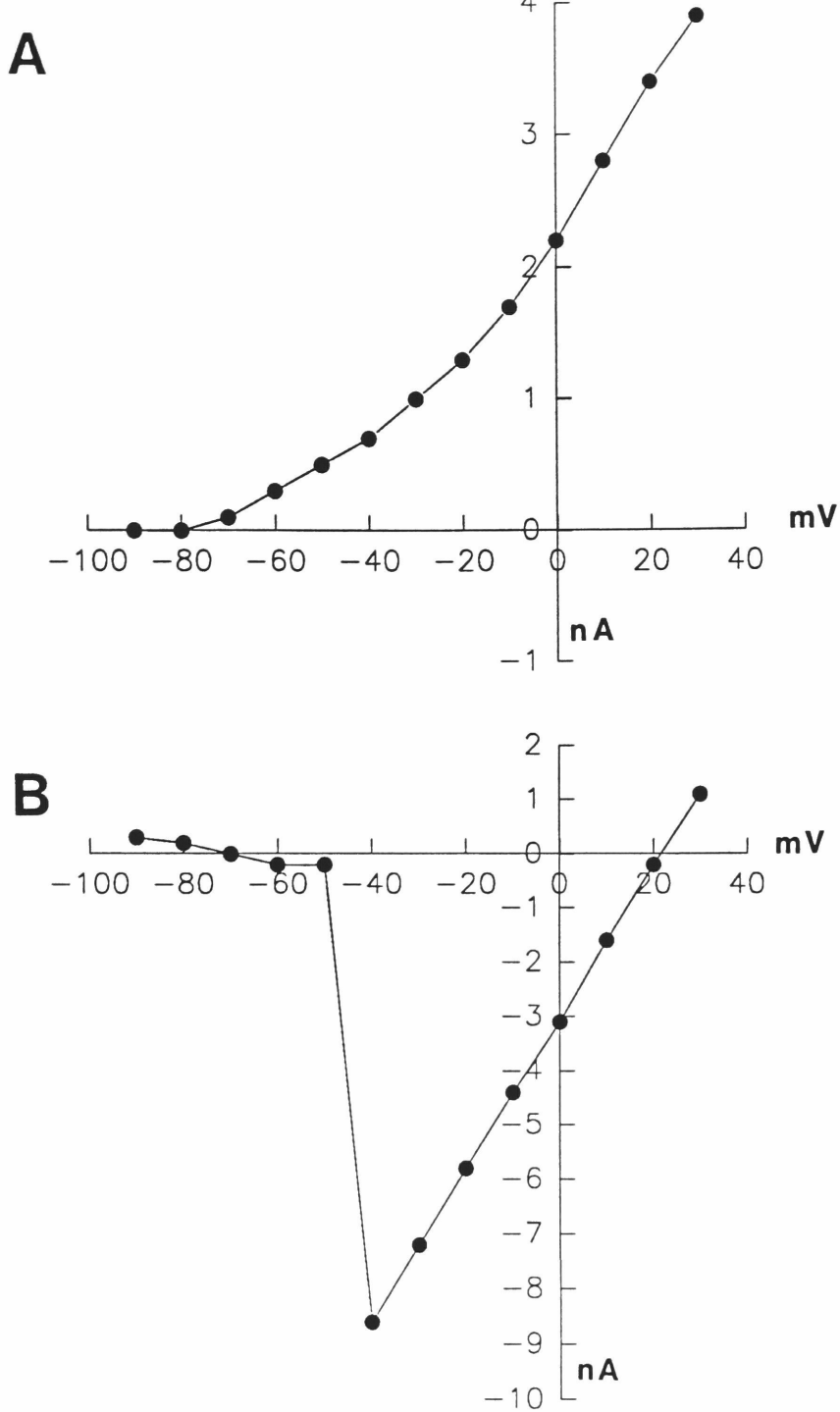


Figure 15 Current-voltage plots of peak current versus membrane potential for the cell shown in Figure 14. **A**: Peak outward current during the 75 msec voltage step application. **B**: Peak inward current during the 75 msec voltage step application.

internal solutions were used. Although potassium fluoride-containing electrodes promoted long-lived whole cell clamping, fluoride has been reported to alter the kinetic parameters of calcium currents (Kay et al., 1986b). Dopamine application to isolated striatal neurons elicited two types of responses. In cells that were poorly space clamped, 10 μ M picrospritzed dopamine evoked repetitive action potentials when neurons were sampled in HEPES saline without channel blockers (Figure 16a). More frequently, bath application of dopamine elicited a complex, non-spiking, response in 12 out of 19 cells sampled. This consisted of an increase in basal conductance that could be reversed by washing the recorded cell with HEPES saline (Figure 16b).

To investigate the role of dopamine receptor subtypes in mediating cell responsiveness, it was necessary to prevent the activation of non-agonist specific conductances. Highly selective D2 receptor agonists were therefore applied to cells in a 20 mM TEA, 5 μ M TTX-containing HEPES saline. The D2-selective agonist, quinpirole, enhanced a time-dependent outward conductance in 7 of 13 cells sampled (Figure 17a). The size of this conductance increase at +30 mV ranged from 100 to 400 pA and was dependent on the concentration of agonist; higher doses of quinpirole elicited larger outward currents (Figure 17b). This outward conductance could also be evoked by bathing cells in dopamine coapplied with the D1 antagonist SCH 23390, further suggesting that the conductance is D2-specific (Figure 18). The size of the outward current increased with time after drug application, appearing within seconds of drug application and reaching a peak within 60 seconds. The conductance could not be evoked by replacing the bath solution with fresh control HEPES saline or by applying

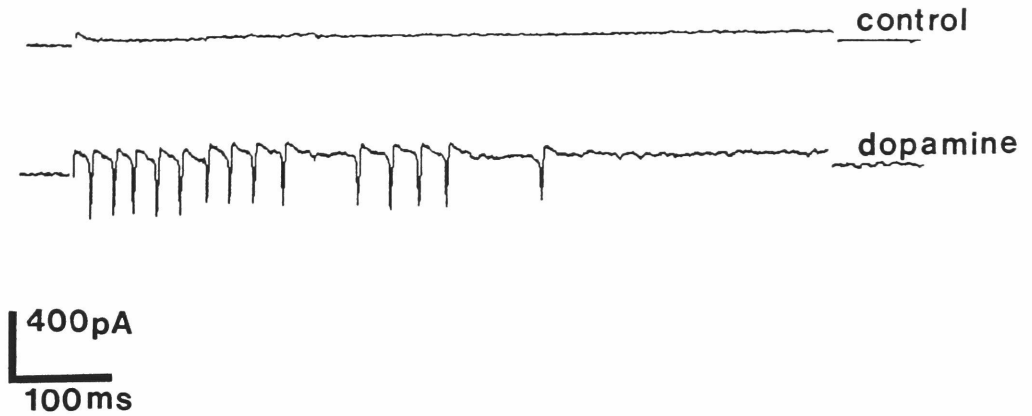
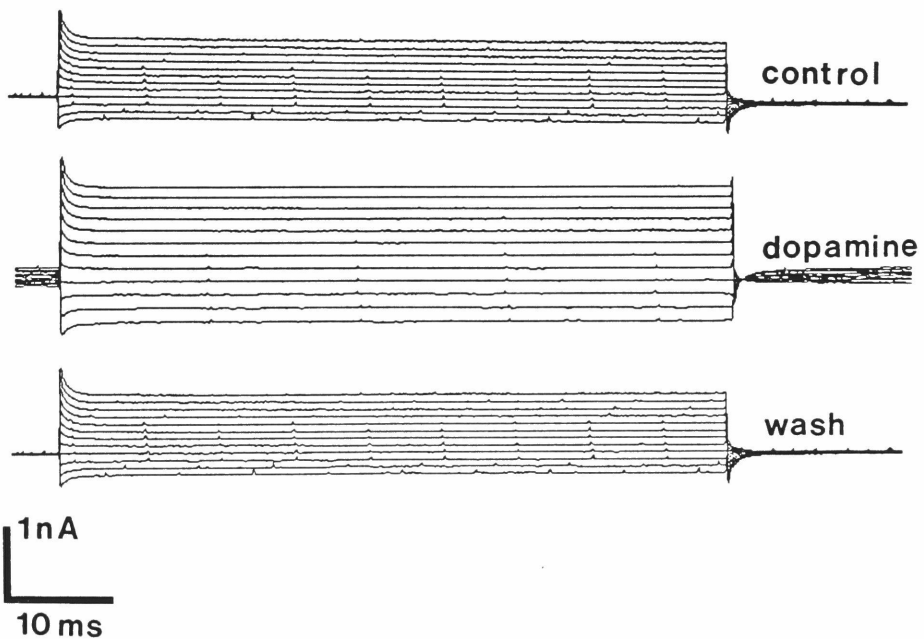
A**B**

Figure 16 Membrane currents elicited by dopamine application. A: The neuron was held at -60 mV and step depolarized for 800 msec to -30 mV simultaneous with picospritzer-application of either HEPES saline (control) or $10 \mu\text{M}$ dopamine. B: A different neuron bathed in $5 \mu\text{M}$ TTX to block action potentials and step depolarized in 10 mV increments from a holding potential of -60 mV up to $+30$ mV. Bath application of $10 \mu\text{M}$ dopamine elicited a change in voltage-dependent currents that could be abolished by washing the recorded cell with HEPES saline.

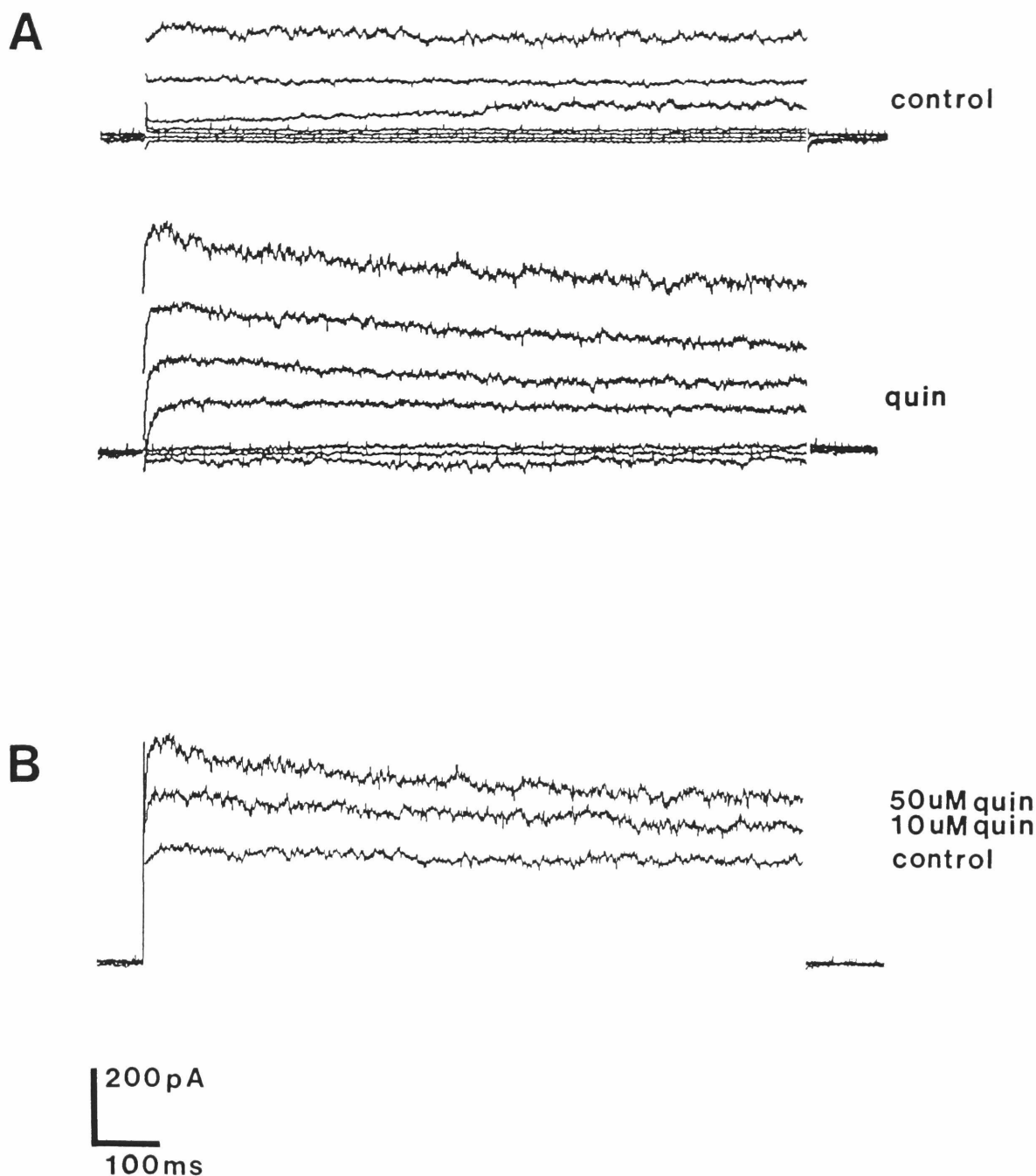


Figure 17 The D2-selective agonist quinpirole enhances an outward conductance. **A:** Step-depolarizing pulses of -90 to +30 mV in 20 mV increments from a holding potential of -60 mV. Control=HEPES saline bath solution containing TEA and TTX, Quin=HEPES plus 10 μ M quinpirole bath solution. **B:** Current responses of a different cell picospritzed in succession with HEPES, 10 μ M quinpirole and 50 μ M quinpirole when depolarized to +30 mV. Capacitative artifacts have been removed.

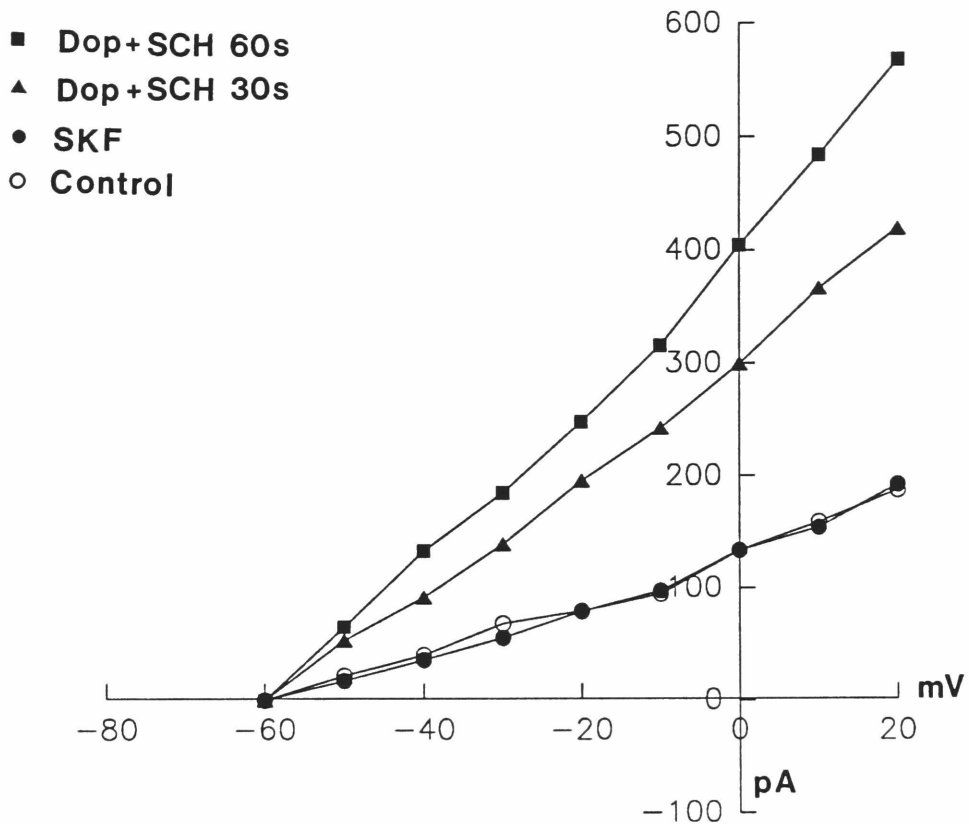


Figure 18 Current-voltage plot of peak outward current versus membrane potential of the D2-specific conductance in the presence of various dopaminergic agents. Data are from one cell which was bathed sequentially in HEPES (control), HEPES plus 50 μ M SKF 38393 (D1 agonist), HEPES plus 10 μ M dopamine and 50 μ M SCH 23390 (D1 antagonist) sampled 30 and 60 seconds post-application. The potassium equilibrium potential in these experiments is approximately -80 mV.

the D1 agonist SKF 38393.

To investigate the role of protein phosphorylation in mediating this D2 response, cells were bathed in the potent adenylate cyclase activator forskolin, following whole-cell patch clamp and D2 agonist application. In 3 out of 4 cells examined, forskolin elicited a time-dependent reduction in the outward current evoked by bath application of 10 μ M quinpirole (Figure 19a). This reduction in current was maximal within two minutes and did not recover with washing of the recorded cell and reapplication of agonist.

Although the majority of the experiments were conducted using whole-cell patch clamping, high resistance cell attached seals were occasionally formed. In three such cases, bath application of quinpirole elicited voltage-dependent channel activity within the isolated membrane patch which was not itself exposed to agonist (Figure 19b). This suggests that a second messenger system couples the D2 receptors to their associated channels. Forskolin, applied to the bath during one such cell-attached patch recording, resulted in closure of the quinpirole-activated channels (Figure 19b). Since the forskolin was applied to membrane surfaces isolated from the membrane sampled in the patch pipette, the decrease in channel activity is likely to stem from a second messenger-mediated effect rather than an anesthetic-like block of the channels. It is possible that forskolin may diffuse in the plane of the membrane into the patch. Experiments with non-adenylate cyclase activating forskolin analogues can address this question.

Forskolin stimulation of adenylate cyclase leads to the formation of cAMP and the activation of cAMP-dependent protein kinase. To test if this

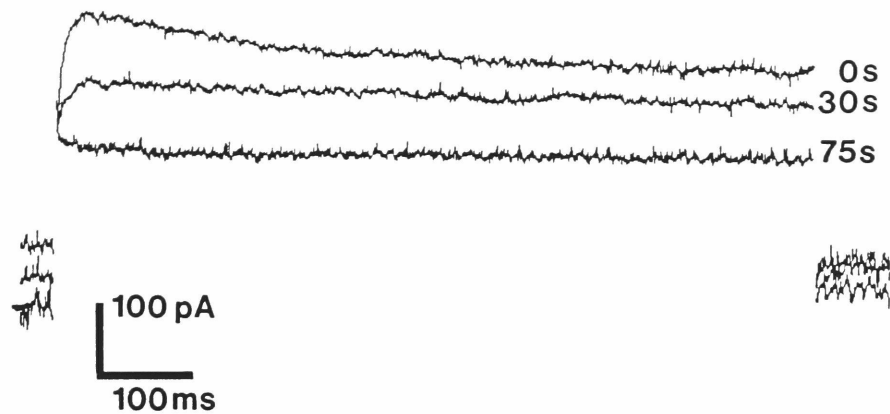
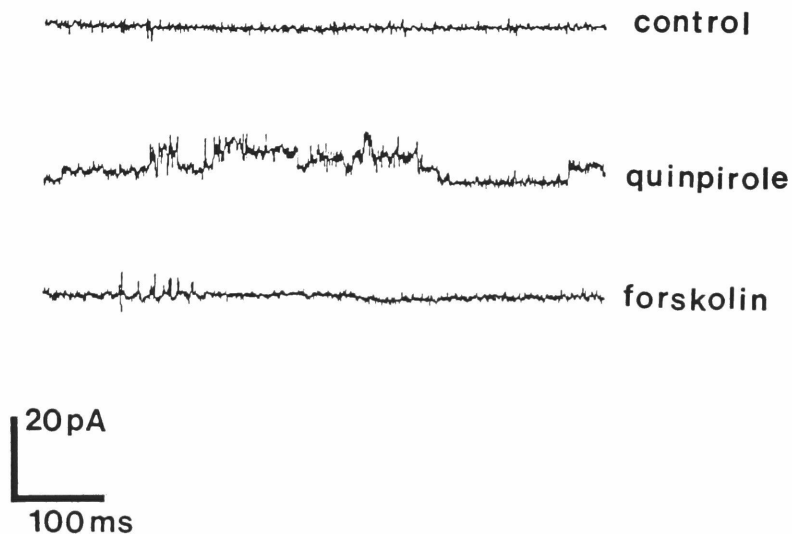
A**B**

Figure 19 Forskolin inhibits quinpirole-activated conductances. **A:** Step depolarizations from a membrane holding potential of -60 mV up to $+30$ mV elicit currents when the cell is picospritzed with 10 μM quinpirole. Following bath application of 10 μM forskolin (0 s), the size of this evoked current diminishes over time (30 and 75 s). **B:** Single channel recording of outward current conducting channels from a cell-attached membrane patch held at $+30$ mV to generate a 90 mV potential across the membrane. The 4 pA channel events are not seen in the absence of agonist (control) and are elicited by bath application of 50 μM quinpirole. Agonist-induced channel events are irreversibly blocked by the addition of 10 μM forskolin to the bath.

kinase was involved in the second messenger mediated inhibition of dopamine-evoked currents, cells were perfused with the catalytic subunit of cAMP-dependent protein kinase. In two out of two cells recorded from with 300 nM of catalytic subunit in the patch pipette, no outward currents were elicited by D2 receptor stimulation (data not shown). Although such preliminary data lends support to the idea that phosphorylation of an unidentified molecule inhibits the D2 receptor to channel pathway, it must be noted that cells dialyzed with catalytic subunit had suppressed basal, time-independent, conductances as well. The kinase may therefore be having non-selective effects on cell excitability. Control experiments with heat-inactivated catalytic subunit are planned.

DISCUSSION

The results suggest that D2 receptor stimulation in guinea pig neostriatal neurons induces a transient outward potassium conductance at membrane potentials positive to -60 mV. Dopamine stimulation of a voltage-dependent transient outward current has been observed in clonal pituitary cells that express D2 receptors (Login et al., 1990). In rat striatum, dopamine application has been reported to open an 85 pS potassium channel that may be D2-specific (Freedman, 1988). This large channel, which has the unusual pharmacological property of blockade with nanomolar concentrations of quinine (Freedman, 1989), is probably distinct from the current described here whose single channel conductance is in the 35 to 45 pS range.

The increase in outward conductance has at least two temporally distinct components that may reflect different signal transduction mechanisms. The initial increase in outward conductance occurs within seconds of agonist application (Figure 17) which may reflect direct G-protein gating of a channel. This outward conductance increases over the next minute of recording (Figure 18). This slower coupling mechanism could be due to the down regulation of cAMP-dependent protein kinase activity and the subsequent dephosphorylation of a substrate by active protein phosphatases.

One intriguing possibility is that the voltage-dependent outward current observed here is I_A , a transient potassium conductance present in most excitable cells. This current differs from the delayed outward current of Hodgkin and Huxley (I_K) in its activation at relatively

hyperpolarized values (threshold, -45 to -60 mV), rapid inactivation and selective block with 4-aminopyridine (Rogawski, 1985). Although experiments varying potassium concentration have not yet been conducted on these striatal neurons, the current's insensitivity to TEA and its voltage sensitivity suggest that it may be I_A . The current is unlikely to be a calcium or calcium-dependent potassium conductance because the patch pipette contained high levels of EGTA to chelate intracellular calcium. I_A has been demonstrated to be modified by protein phosphorylation in several systems. The reduction of I_A in *Hermissenda* photoreceptors with associative learning can be mimicked with intracellular injection of calcium/calmodulin-dependent protein kinase (Farley et al., 1985). I_A in *Aplysia* bag cells is similarly reduced in amplitude by the application of forskolin (Strong, 1984).

Muscarinic acetylcholine receptors in neostriatal neurons have been shown to increase the peak conductance of I_A and shift its voltage dependence towards more negative membrane potentials (Akins et al., 1990). The D2 dopamine receptor and the muscarinic receptor may therefore be coupled to the same conductance in neostriatal neurons. Such a convergence of multiple receptors onto the same channel has a precedence in the literature: GABA_A and serotonin receptors are coupled to a common potassium channel in hippocampal pyramidal cells (Andrade et al., 1986). Indirect evidence suggests that D2 and GABA_B receptors may share a potassium conductance in substantia nigra neurons (Lacey et al., 1988).

This postulated convergence of D2 and muscarinic receptors of striatal neurons onto a common potassium conductance is an attractive hypothesis because protein phosphorylation has been implicated in the

desensitization of muscarinic responses. Under basal conditions, M2 muscarinic acetylcholine receptors increase potassium conductances in heart and central neurons through an intervening G-protein. This appears to be a direct coupling mechanism independent of a second messenger cytoplasmic pathway although the precise G_K protein subunit that mediates the response (α versus $\beta\gamma$) is unresolved (Logothetis et al., 1987; Yatani et al., 1988). Protein phosphorylation-inducing agents mimic the desensitization of the muscarinic receptor responses, suggesting that phosphorylation of the receptor or its associated G-protein uncouples their interaction (Kwatra et al., 1987). In the experiments described here, protein phosphorylation may also be uncoupling the D2 receptor to channel connection by phosphorylating the receptor, the channel or a regulatory molecule (e.g. a G-protein).

Dopamine's D2 receptor mediated action on striatal neurons described here is similar to dopamine's activation of potassium conductances in other mammalian central neurons. Dopaminergic inhibitory postsynaptic potentials are mediated by an increased potassium conductance, although not I_A , in both substantia nigra and pituitary (Lacey et al., 1987; Williams et al., 1989). Invertebrate neurons, however, have dopamine receptors that are differently coupled. In *Helix*, dopamine decreases a voltage-dependent calcium current via a molluscan G-protein that is immunologically related to G_o (Harris-Warrick et al., 1988).

The data presented here are initial steps towards defining the D2 signal transduction pathway in neostriatal neurons. Definitive identification of the D2-coupled conductance, determination of whether the response is G-protein mediated and the molecular dissection of the role of

protein phosphorylation in mediating this response remain to be done. The preparation of acutely dissociated striatal neurons developed for this project is ideal for whole-cell patch clamp experiments because the cells have clean surface membranes, aiding seal formation, and lack distal dendritic processes, reducing space clamp problems. There is, however, the possibility that trypsin digestion may irreversibly alter membrane inserted channels or receptors. Receptor binding assays indicated that although the D2 receptors on the striatal neurons were unaffected by the trypsin treatment, D1 receptor binding sites were halved in number. For this reason, D1-specific conductances were not examined in these experiments. A better system for examining the D1 signal transduction pathway may be the NS20Y neuroblastoma cell line which contains high levels of D1 receptors that are positively coupled to adenylate cyclase (Barton et al., 1989).

CHAPTER 5

Dopamine Inhibits Na^+, K^+ -ATPase Activity in Striatal Neurons through D1 and D2 Receptor Synergism

Na^+, K^+ -ATPase, an integral membrane protein located in virtually all animal cells, couples the hydrolysis of ATP to the counter-transport of Na^+ and K^+ ions across the plasma membrane (Skou, 1965). The enzyme consists of an $\alpha\beta$ heterodimer. The α subunit, a 112 kDa protein with six to eight transmembrane domains, contains the binding sites for Na^+ , K^+ , and ATP and exists in three genetically distinct isozymes, α_1 , α_2 and α_3 (Shull et al., 1986). The β subunit, a 40–60 kDa glycoprotein that may regulate transport and insertion of the catalytic subunit, has at least two enzyme forms, β_1 and β_2 (Young et al., 1987). The Na^+, K^+ -ATPase is a member of the family of P-type ATPases that includes the Ca^{++} -ATPases of plasma membrane and sarcoplasmic reticulum, the gastric H^+, K^+ -ATPase and H^+ pumps in yeast and bacteria. Activity of these pumps is associated with phosphorylation and dephosphorylation of the active site of the catalytic subunit and E_1 - E_2 conformational transitions (Blostein, 1989).

Na^+, K^+ -ATPase activity controls either directly or indirectly many essential cell functions (for review, see Rossier et al., 1987). The transmembrane electrochemical potential gradient for Na^+ ions generated by the ATPase drives the transport of amino acids, sugars, and Ca^{++} , Cl^- and H^+ ions across the membrane. The ATPase maintains osmotic stability and therefore cell volume as well as the high intracellular K^+ levels needed for many enzymes. ATPase activity also generates heat, accounting for more than 20% of the basal metabolic rate of adult mammals.

In neurons and other excitable tissues, the enzyme maintains the transmembrane ionic gradients that underlie resting and action potentials and permit active uptake of nutrients and neurotransmitters. Na^+, K^+ -ATPase activity in such excitable tissues critically influences electrical activity. Each pump cycle of 3 Na^+ out/2 K^+ in generates a net extrusion of positive charge and hence an outward current that contributes to the cell membrane potential. The enzyme also indirectly regulates electrical excitability by maintaining the ionic gradients for Na^+ and K^+ that are continually dissipated by ionic fluxes across the cell membrane.

It is tempting, given the critical role the Na^+, K^+ -ATPase plays in electrical excitability, to hypothesize that the enzyme is regulated as a form of neuromodulation. Na^+, K^+ -ATPase activity can be directly regulated by monovalent cations (particularly intracellular Na^+ and extracellular K^+ ions), ATP and cardiac glycosides (Glynn, 1985). These endogenous regulators have been implicated in such physiological processes as renal sodium excretion, heart failure, insulin responsiveness and hypertension (Hamlyn et al., 1984). However, modulation of the enzyme by a neurotransmitter has only been convincingly demonstrated in the kidney, where dopamine and epinephrine inhibit pump activity (Aperia et al., 1987; Beach et al., 1987). This regulation requires an intact cell system; dopamine has no effect on the activity of purified enzyme from rat kidney. This suggests that dopamine stimulation activates intracellular second or third messengers that themselves regulate the ATPase activity.

Neostriatal neurons in the basal ganglia contain both D1 and D2 dopamine receptors (see Chapter 4). To examine if activation of dopamine receptors in brain modulates pump activity, dissociated neostriatal

neurons were incubated with dopaminergic drugs and Na^+, K^+ -ATPase activity measured as ouabain-sensitive ATP hydrolysis.

METHODS

Cell Dissociation

Acutely dissociated striatal neurons from guinea pig brain were prepared by trypsin digestion of striatal slices as described in Chapter 4. Following the incubation with trypsin, the slices were allowed to recover for 30 min at 22°C in fresh PIPES saline. The slices were then triturated in 1 ml of calcium-free, 2 mM magnesium, PIPES saline with a series of increasingly fine-bore pasteur pipettes to disperse the neurons. The resulting milky suspension was allowed to settle for 15 sec to permit sedimentation of undissociated neuropil and fiber tracts.

Na⁺,K⁺-ATPase activity

Na⁺,K⁺-ATPase activity was measured by a procedure modified from that developed for isolated kidney cells (Bertorello et al., 1989). Preincubation for a variable period at 22°C was started by transferring 45 μ l aliquots of the cell suspension to HEPES saline containing either dopaminergic drugs or vehicle (final volume 100 μ l). The preincubation period was stopped after 1 to 15 min. by rapid cooling to 4°C. 10 μ l aliquots of the cell suspension (2-7 μ g protein) were transferred to the Na⁺,K⁺-ATPase assay medium (final volume 100 μ l) containing, in mM, NaCl 50, KCl 5, MgCl₂ 10, EGTA 1, Tris-HCl 100 and ³²P-Na₂ATP 10 (sp.act. 3000 Ci/mmol). Cells were transiently permeabilized by placing the samples at -20°C for 10 min to permit the entry of ³²P-ATP and stabilize the intracellular Na⁺ concentration at 70 mM (50 mM NaCl and 10 mM ³²P-Na₂ATP). Cells did not freeze during this procedure and they maintained their morphology (see Figure 20). Samples were then incubated at 37°C for 15

min. The reaction was stopped by rapid cooling to 4°C and TCA/charcoal (5%/10%) was added immediately to bind free ATP. Na^+, K^+ -ATPase activity, measured by liquid scintillation spectrophotometric counting of the liberated ^{32}P , was calculated as the difference between the averages of duplicate test samples (total ATPase activity) and duplicate samples assayed in a Na^+ - and K^+ -free solution containing 1mM ouabain (ouabain-insensitive ATPase activity). Protein was measured by the method of Bradford (1976).

Dopamine Depletion

For the experiments using dopamine-depleted tissue to investigate D1 and D2 receptor synergism, guinea pigs were anesthetized with methoxyflurane and tube fed 300 mg benserazide chloride per kg bodyweight using 1 ml water as vehicle, one hour prior to sacrifice. The cell dissociation and Na^+, K^+ -ATPase assay were run as described above. The extent of dopamine depletion in the intact slices or triturated cell suspensions was measured in a separate series of experiments using trypsin digested striata from dopamine-depleted animals. Tissue samples were homogenized in 0.4M perchloric acid (PCA), and the dopamine was adsorbed onto alumina at pH 8.6 and eluted in 0.1 M PCA. Dopamine in the eluate was analyzed by reverse-phase HPLC with electrochemical detection using a BAS LC-4B detector and a BAS PM-48 pump (Sharp et al., 1986). Protein was analyzed by the procedure of Lowry et al (1951).

Western blotting

Three right and left guinea pig striata were dissected, frozen in

liquid nitrogen and kept at -70°C until analysis. Tissue pieces were homogenized by sonication in 1 ml 1% (wt/vol) boiling SDS and maintained in a boiling water bath for 10 min. Protein concentrations were determined by the procedure of Lowry et al., (1951). Aliquots of this homogenate (5–75 μg protein/lane) were subjected to SDS/PAGE (15% acrylamide) and electrophoretically transferred to nitrocellulose membranes (0.2 μM pore size) at pH 8.9 (Towbin et al., 1979). Protein aliquots for the detection of the phosphoprotein ARPP-21 were transferred using modified conditions of a 50 mM MES buffer at pH 5.5 with 20%(v/v) methanol and 0.025 μM nitrocellulose. Nitrocellulose membranes were blocked in a solution containing 5% milk and 0.1% TWEEN 20 in phosphate buffered saline (PBS) for 30 min. The membranes were then incubated for 2 hrs. in 15 ml of blocking solution containing one of three antibodies: a rabbit antiserum against both ARPP-16 and ARPP-19 (antisera number G153, 1:500 dilution), a mouse monoclonal antibody directed against DARPP-32 (antibody number C24-6A, 1:1000 dilution) or a mouse monoclonal antibody directed against ARPP-21 (antibody number 21-6A, 1:1000 dilution). After rinsing in PBS (3 x 5 min.), nitrocellulose membranes that had been probed with mouse monoclonal antibodies were then incubated serially in blocking solution containing rabbit anti-mouse antibody (1:500 dilution) for 1 hr., then in ^{125}I -protein A (1:1000 dilution) for two hrs., whereas those membranes probed with rabbit antisera (G153) were directly incubated in ^{125}I -protein A for 2 hrs. Nitrocellulose membranes were then dried and exposed for autoradiography at -70°C for 2 to 5 hrs. using Kodak XAR-5 film.

Immunocytochemistry

To investigate the presence of basal ganglia-specific phosphoproteins, a 260g female guinea pig was anesthetized with chloral hydrate (400 mg/kg) and perfused transcardially with 4% paraformaldehyde in a 0.1 M sodium phosphate buffer. 50 μ m thick coronal slices of striatum were incubated in a 1:30,000 dilution of a mixture of three murine monoclonal antibodies against DARPP-32 (Ouimet et al., 1984). The tissue was processed for immunocytochemistry using an avidin-biotin complex method with biotinylated goat anti-mouse IgG as the secondary antibody (Gustafson et al., 1990). Antibodies were visualized with 3,3'-diaminobenzidine and hydrogen peroxide treatment. The sections were mounted on gelatin coated slides, dehydrated, cover-slipped and viewed under Nomarski optics.

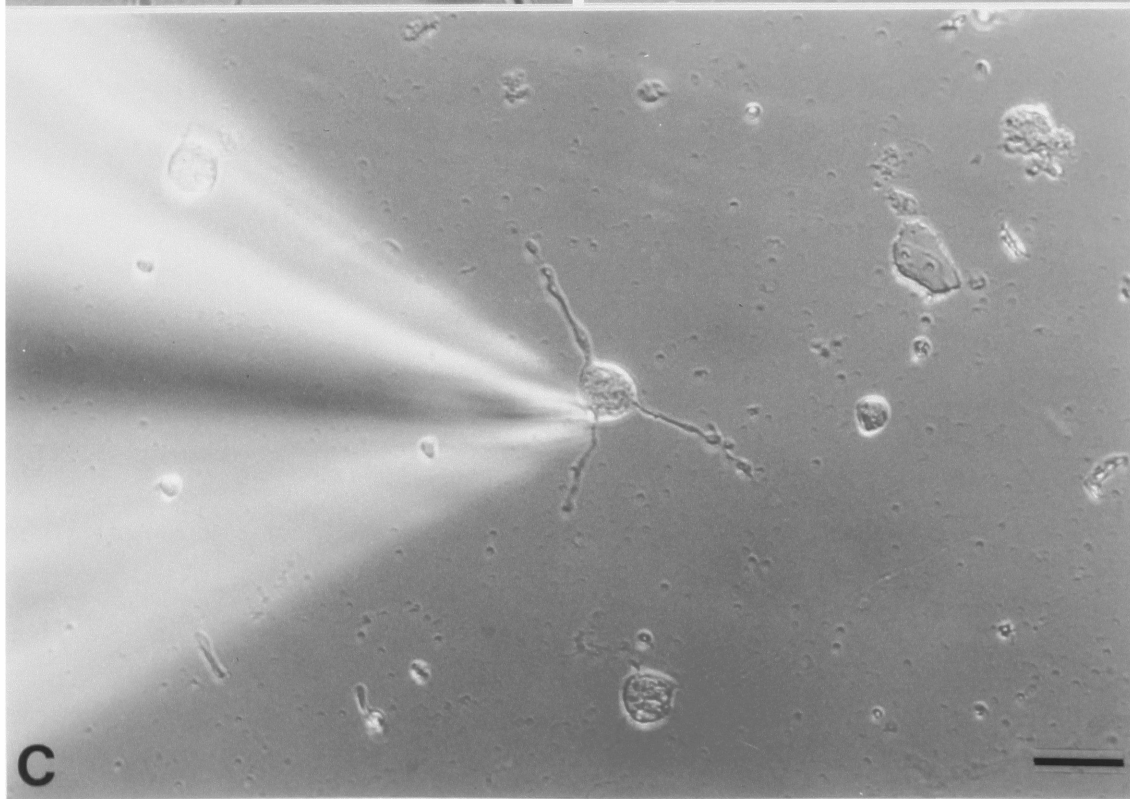
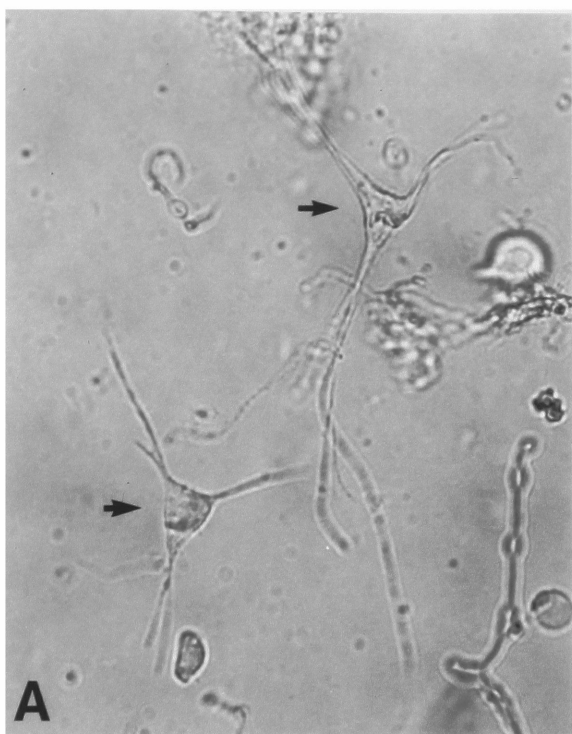
RESULTS

Na^+, K^+ -ATPase activity was assayed as ouabain-sensitive ATP hydrolysis in a cell suspension of acutely dissociated striatal neurons from guinea pig. Dissociated striatum yields a cell population which, morphologically, is nearly homogeneous: an estimated 95% of striatal neurons are of a single type, namely medium spiny type I (Gerfen, 1988), and almost all astrocytes and myelinated fibers settle out of the cell suspension. Isolated cells retained their characteristic medium spiny morphology and proximal dendritic structure and displayed voltage- and neurotransmitter-coupled ionic conductances (see Chapter 4).

To measure pump activity, intact cells were preincubated with dopaminergic drugs and then permeabilized by cold shock to load cells with ^{32}P -ATP and stabilize the intracellular Na^+ concentration at 70 mM. This cold shock procedure did not significantly disrupt the morphology of the isolated cells which retained their dendritic structures and somatic shape (Figure 20). Cell membranes maintained their integrity both before and after the permeabilization procedure; no detectable ^{32}P was liberated when cells were incubated with ^{32}P -ATP without prior cold shock and following permeabilization, cells did not leak ^{32}P into the surrounding media.

Na^+, K^+ -ATPase activity in control samples averaged 199 ± 13 nmol Pi/mg protein/min ($n=19$). Ouabain insensitive ATP hydrolysis (Mg^{++} -ATPase activity) averaged 209 ± 14 nmol Pi/mg protein/min ($n=19$) and accounted for approximately half of the total ATPase activity as has been described in other tissues and species (Katz et al., 1979). This ouabain-insensitive component of ATPase activity was unaffected by any of the experimental treatments.

Figure 20 Photomicrographs of dissociated medium spiny neurons viewed under Nomarski optics. Left: isolated cell after 10 min incubation with dopamine and before cold shock. Right: a different cell from the same experiment after incubation with dopamine and subsequent permeabilization by cold shock. Scale bar = 15 μ m.



Incubation of the cell suspension with dopamine inhibited Na^+, K^+ -ATPase activity in a concentration dependent manner (Figure 21). 1 nM dopamine was sufficient to induce significant inhibition of the enzyme activity to 76% of control level. The maximal effect, 50% inhibition, was obtained at dopamine concentrations of 10 μM .

This inhibition developed over time following an initial lag of 2 to 3 minutes (Figure 22). The inhibitory effect was maximal within 5 minutes of dopamine application and persisted for at least another 10 minutes. This decrease in enzyme activity over time did not represent a rundown of the striatal neurons; control samples without added dopamine at 1 and 15 minute incubation intervals had mean Na^+, K^+ -ATPase activities of 230 ± 17 (n=3) and 201 ± 23 (n=4) nmol/mg protein/min respectively.

To investigate the pharmacology of this inhibition, cells were incubated with dopamine in the absence or presence either of the selective D1 antagonist SCH 23390 or of one of the selective D2 antagonists, YM-09151 or S-sulpiride (Figure 23). Addition of either a D1 or D2 specific antagonist (10 μM) to the cell suspension was sufficient to prevent dopamine (1 μM)-induced inhibition of Na^+, K^+ -ATPase activity and maintain enzyme activity at control levels. The antagonists might have been expected to, but did not, raise the enzyme activity to above control levels by blocking the inhibitory effect of endogenous dopamine. Stimulation of the ATPase may not have been kinetically possible under the assay conditions of 70 mM Na^+ which is close to the V_{max} for the enzyme.

To examine the contributions of D1 and D2 dopamine receptor subtypes in mediating this effect of dopamine, it was necessary to prevent

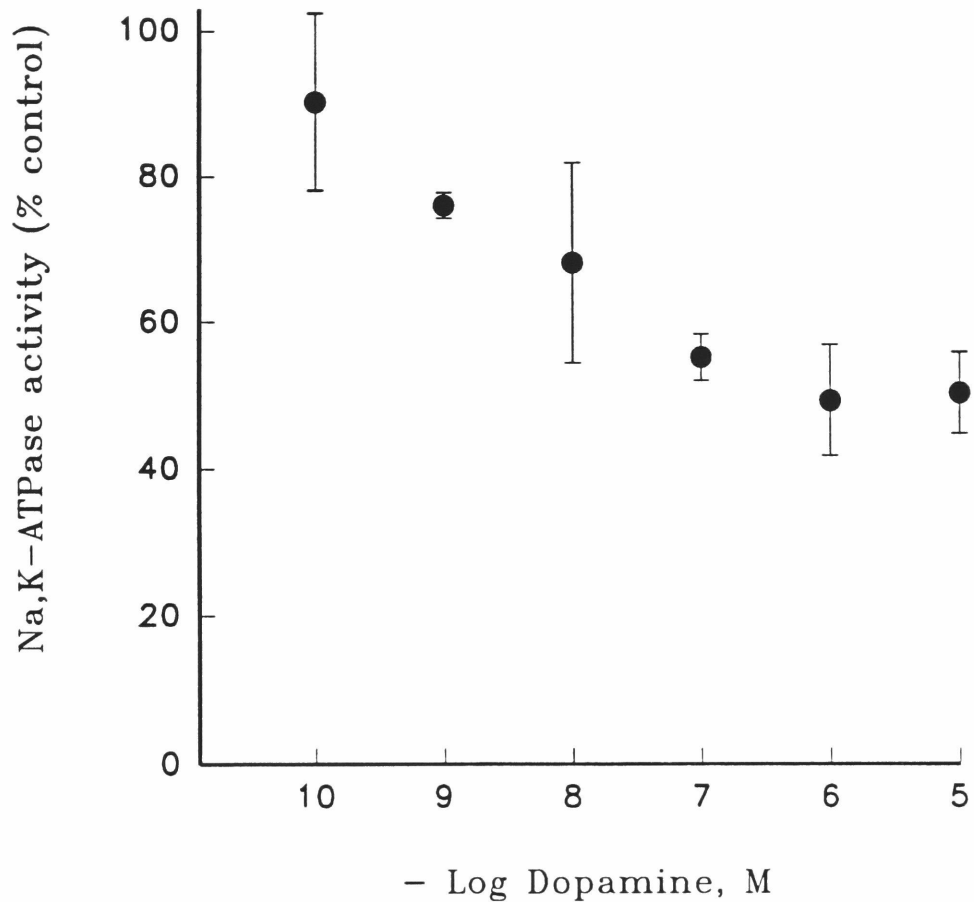


Figure 21 Concentration dependence of dopamine inhibition of Na^+, K^+ -ATPase activity. Cells were incubated for 10 min with the indicated concentrations of dopamine and compared with control samples incubated without dopamine for 10 min (mean activity = 206 ± 15 nmol Pi/mg protein/min, $n=28$). Data represent means \pm s.e.m. of 3-7 experiments. No change in enzyme activity was observed in samples of control cells incubated without dopamine for up to fifteen minutes. (From Bertorello et al., 1990)

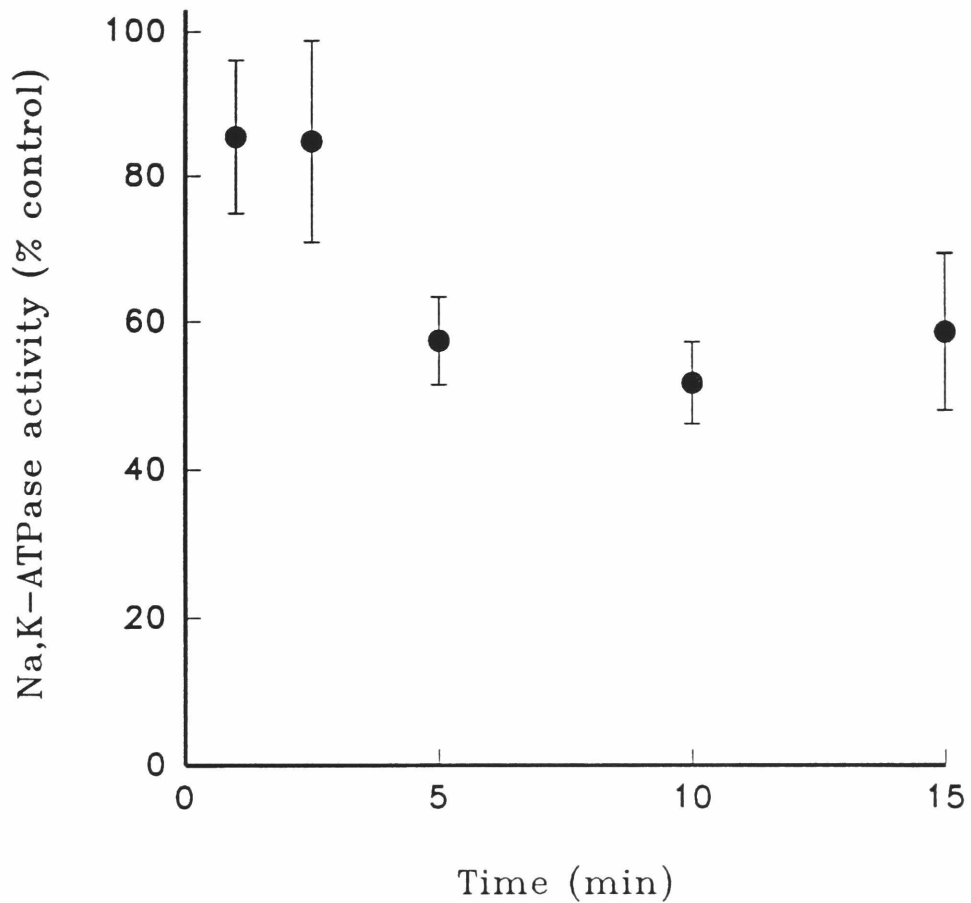


Figure 22 Time dependence of dopamine inhibition of Na^+, K^+ -ATPase activity. Cells were incubated with $1 \mu\text{M}$ dopamine for the indicated periods of time and compared with control samples incubated without dopamine (mean activity = 206 ± 15 nmol Pi/mg protein/min, $n=28$). Data represent means \pm s.e.m. of 3-5 experiments. (From Bertorello et al., 1990)

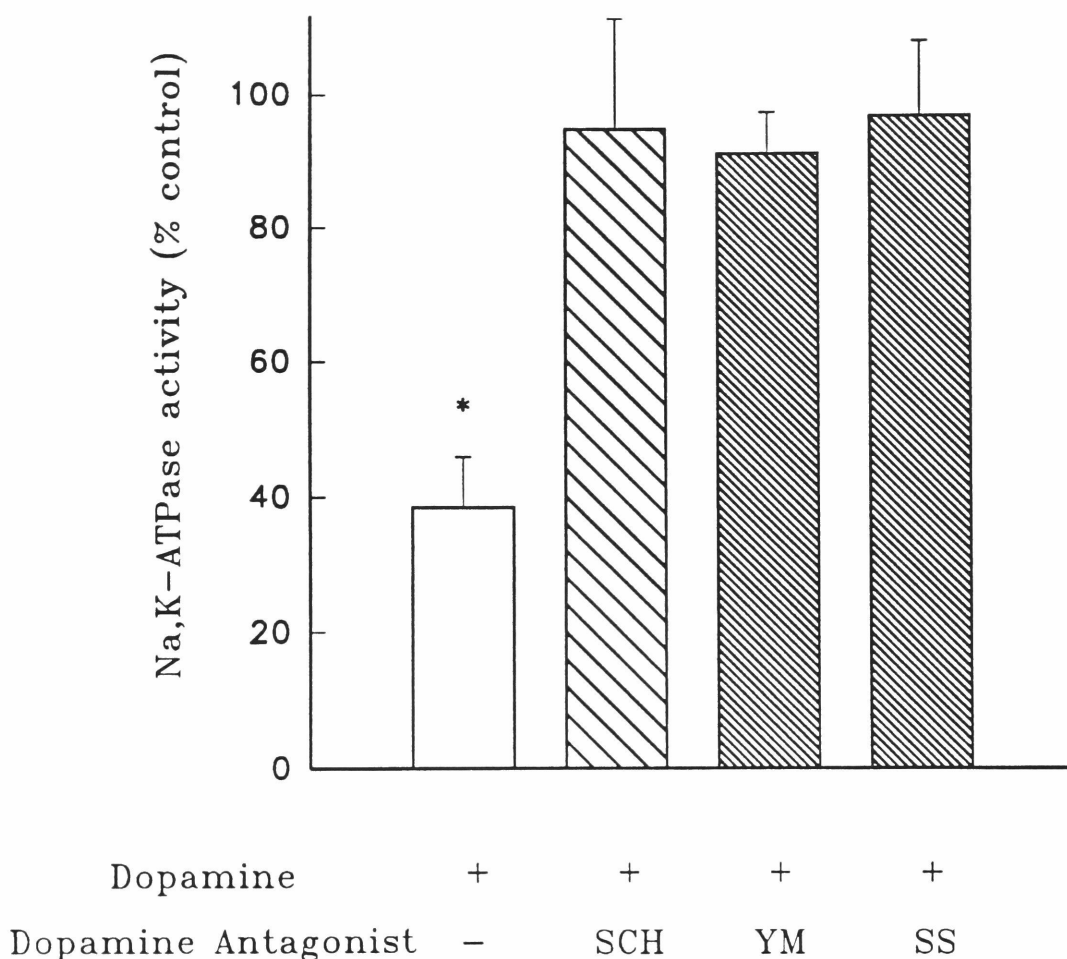


Figure 23 D1 and D2 antagonists each prevent the inhibitory action of 1 μ M dopamine. SCH = 10 μ M of the D1 antagonist SCH 23390, YM = 10 μ M of the D2 antagonist YM-09151, SS= 10 μ M of the D2 antagonist S-sulpiride. Results represent means \pm s.e.m. of 4 experiments. For each antagonist, data represent enzyme activity in the presence of dopamine expressed as percentage of that observed in the absence of dopamine (mean activity = 147 ± 11 nmol Pi/mg protein/min, $n=14$). * $P < 0.005$ compared to control (no dopamine) and compared to dopamine plus dopamine antagonists. (From Bertorello et al., 1990)

activation of receptors by endogenous dopamine. For this purpose, animals were pretreated with the L amino acid dopa decarboxylase inhibitor, benserazide, to deplete endogenous dopamine stores (Da Prada et al., 1984). This drug has been used to deplete dopamine stores in renal tissue prior to ATPase activity measurements and has no known effect on pump activity or number (Bertorello et al., 1988a). Benserazide treatment reduced the dopamine concentrations by two to three fold both in striatal slices and in triturated single cell suspensions from such slices compared to vehicle treated controls (Table VII).

In such benserazide-treated animals, neither 100 nM of the D1 selective agonist SKF 82526 nor 100 nM of the D2 selective agonist LY 171555 was itself sufficient to produce significant inhibition of Na^+, K^+ -ATPase activity (Figure 24). However, addition of 100 nM SKF 82526 with 100 nM LY 171555 resulted in an inhibition to about one-half of control level, indicating that both receptor subtypes must be activated to induce enzyme inhibition. This synergistic effect of D1 and D2 receptor co-activation was not a reflection of higher total agonist concentration in the agonist mixture: 1 μM doses of SKF 82525 and LY 171555 presented alone resulted in Na^+, K^+ -ATPase activity of $104 \pm 38\%$ ($n=4$) and $90 \pm 10\%$ ($n=2$) of control values respectively.

In vehicle-treated animals that did not receive benserazide ($n=3$), 100 nM SKF 82526 and 100 nM LY 171555 each given alone reduced Na^+, K^+ -ATPase activity to 78 and 70% of control levels, respectively (Figure 24). The two agonists, when present together, reduced enzyme activity to 65% of control. This ability of either selective agonist alone to inhibit Na^+, K^+ -ATPase activity suggests that endogenous dopamine in the cell suspension,

	<u>Benserazide</u>	<u>Vehicle</u>
Slices (n=4)	100.9(22.1)	234.7(64.0)
Dissociated Cells (n=2)	103.4(35.9)	349.7(123.3)

Table VII Striatal dopamine levels of benserazide and vehicle-treated animals as measured by HPLC. Striatal slices were trypsin digested and then either left intact or triturated into a dissociated cell suspension before homogenization and subsequent dopamine adsorption. Data represent mean values in pmol dopamine/mg protein (\pm standard deviations).

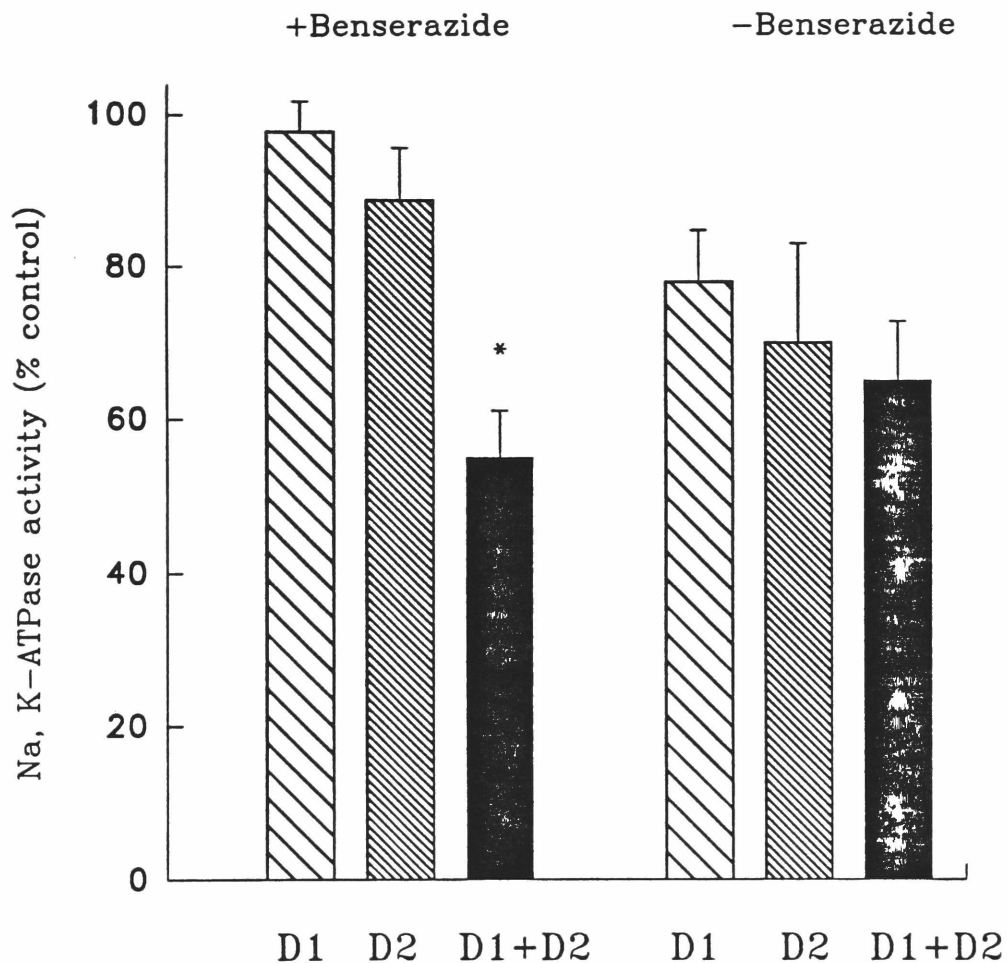


Figure 24 Synergistic action of D1 and D2 agonists as inhibitors of Na^+, K^+ -ATPase activity. Animals had been pretreated with benserazide to deplete endogenous dopamine or control treated with vehicle. D1 = 100 nM of the D1 agonist SKF 82526, D2 = 100 nM of the D2 agonist LY 171555. Results represent means \pm s.e.m. of 3 to 4 experiments. In each experiment, results were calculated as percentage of a non-agonist treated control. * = $P < 0.005$ compared to control and compared to D1 and D2 agonists alone. (Adapted from Bertorello et al., 1990)

released during mechanical trituration of the slices, may have caused some activation of both receptor subtypes. In support of this interpretation, basal enzyme activity in samples from vehicle-treated animals was slightly lower (154 ± 22 nmol Pi/mg protein/min, $n=3$) than in samples from benzerazide-treated animals (179 ± 10 nmol Pi/mg protein/min, $n=4$), possibly reflecting tonic inhibition of enzyme activity by endogenous dopamine in the cell suspensions.

D1 receptor activation in striatum stimulates adenylate cyclase leading to the formation of cAMP and the activation of cAMP-dependent protein kinase (Kebabian et al., 1972; Hemmings et al., 1987). Experiments with the potent adenylate cyclase activator forskolin suggest that this second messenger pathway and protein phosphorylation underlie the receptor interaction observed here. $10 \mu\text{M}$ forskolin, when co-applied with 100 nM of the D2 agonist LY 17155, produced a synergistic reduction of Na^+, K^+ -ATPase activity to 50% of control (Figure 25). Forskolin or LY 17155 applied alone reduced enzyme activity nonsignificantly to 81% and 91% of control levels.

D2 receptor activation has been linked to the polyphosphoinositol/diacylglycerol pathway and activation of protein kinase C. A ten minute incubation with $1 \mu\text{M}$ PMA mimicked the effect of dopamine by inducing inhibition to 42% of control levels (281 ± 35 nmol Pi/mg protein/min, $n=4$). This PMA effect on enzyme activity required an unusually high dose of the phobol ester and did not require D1 receptor activation, suggesting that it may be acting through a non-D2 selective pathway. No change in ATPase activity was seen with the nanomolar concentrations of PMA that are sufficient in neuronal cells to stimulate protein kinase C activity.

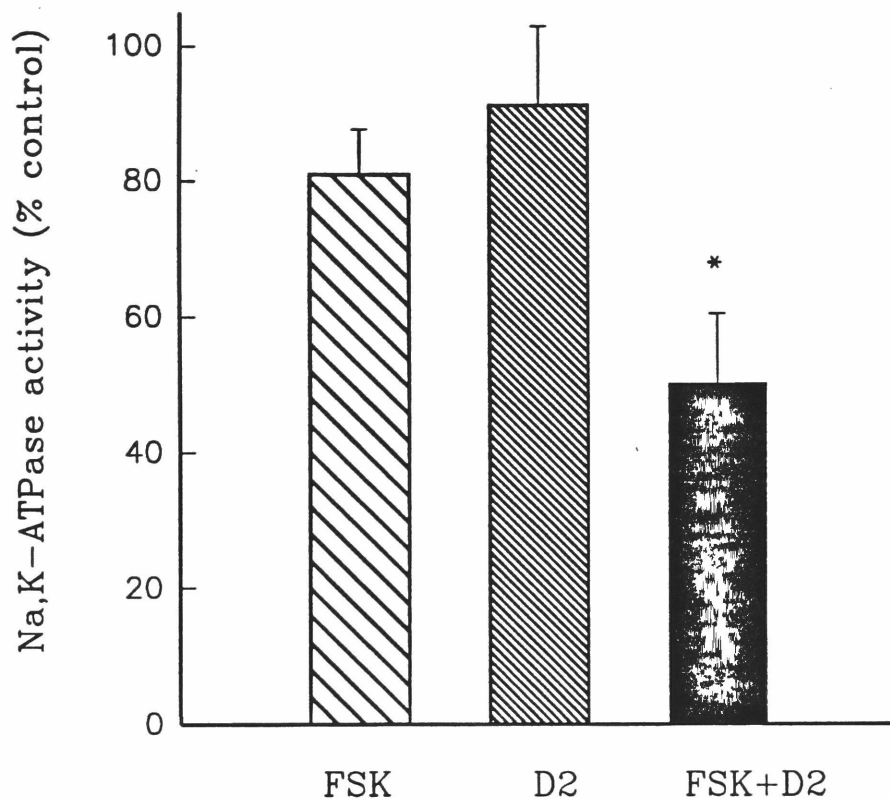


Figure 25 Forskolin mimics D1 receptor activation. FSK = 10 μ M forskolin, D2 = 100 nM of the D2 antagonist YM-09151. Results represent means \pm sem of 3 experiments. For each drug application, data represent enzyme activity expressed as a percentage of non-treated control with mean activity of 106 ± 30 nmol Pi/mg protein/min. *= $p < 0.005$ compared to control and compared to forskolin and D2 antagonist alone.

As an initial step towards defining the role of protein phosphorylation in the Na^+, K^+ -ATPase inhibition, guinea pig striata were screened for the existence of adenylate cyclase-regulated phosphoproteins. Antibodies raised in mouse or rabbit against four basal ganglia specific phosphoproteins, ARPP-16, ARPP-19, ARPP-21 and DARPP-32 were used to probe guinea pig striatal homogenates by standard immunoblotting procedures (Figure 26). All four of these phosphoproteins were found at high levels. One of these phosphoproteins, DARPP-32, is known in other mammalian species to be selectively localized to cells containing D1 receptors (Hemmings et al., 1987). Immunocytochemistry of guinea pig striatal slices with monoclonal antibodies to DARPP-32 revealed that the medium spiny neurons used in the Na^+, K^+ -ATPase assay have high levels of this protein (Figure 27).

Figure 26 Autoradiogram of western blots of guinea pig striatal homogenates probed for the basal ganglia enriched phosphoproteins, ARPP-16, ARPP-19, ARPP-21 and DARPP-32. 75 μ g of protein was loaded per lane. The additional bands in the DARPP-32 lane probably represent degradative breakdown products of this rapidly proteolyzed protein.

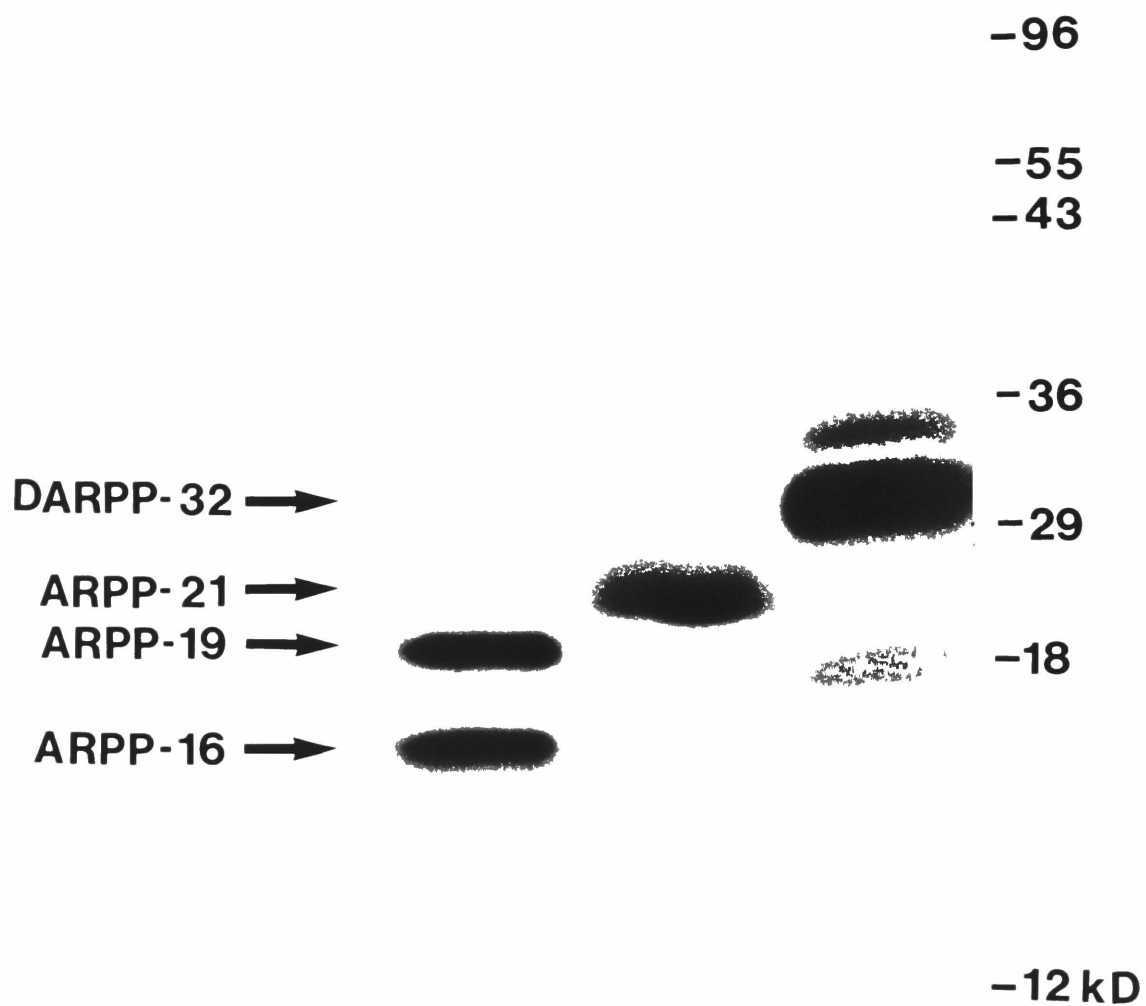
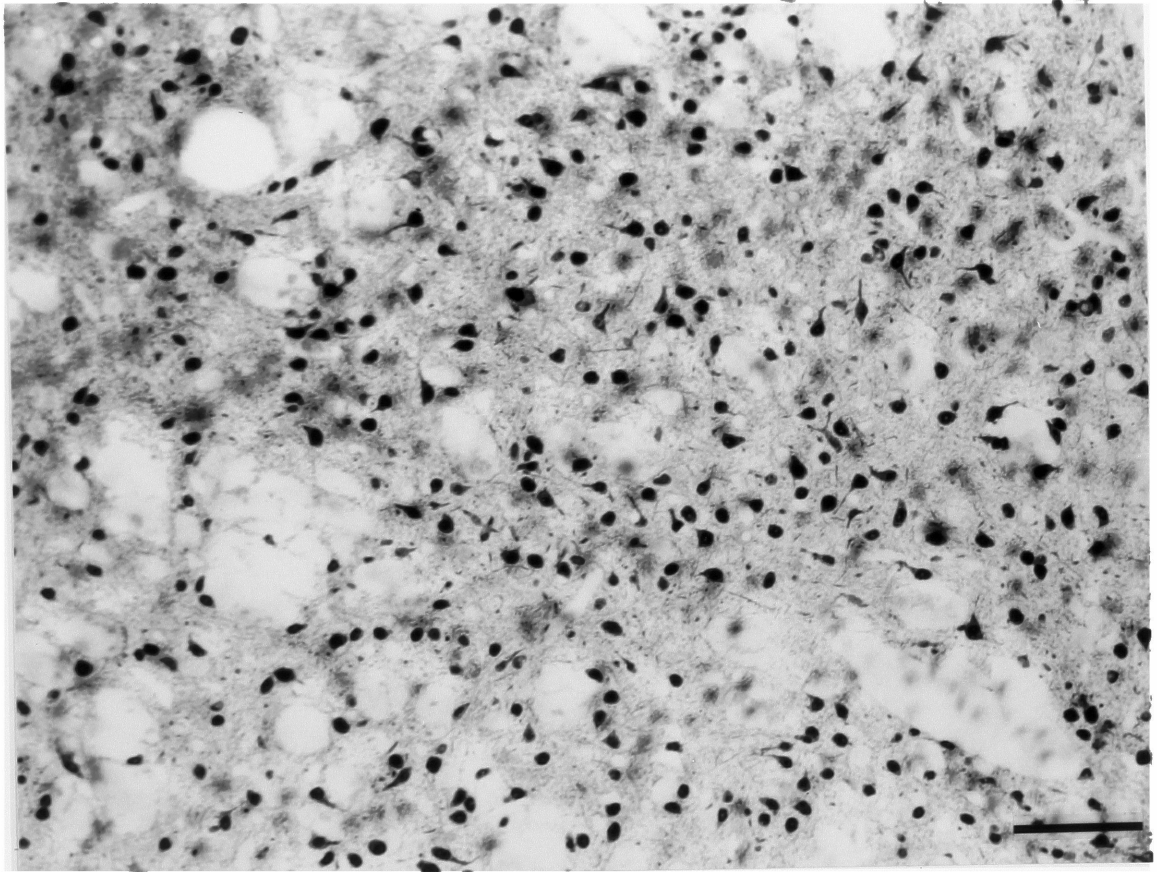


Figure 27 Photomicrograph of immunoreactive DARPP-32 containing neurons in guinea pig striatum. The cell bodies and processes of the medium spiny neurons are heavily stained. The surrounding white matter of corticofugal fibers is unstained. Scale bar = 100 μm



DISCUSSION

A large and generally unconvincing literature describes catecholamine-induced stimulation, inhibition, and non-regulation of Na^+, K^+ -ATPase activity (for review, see Phillis et al., 1981 and Gadsby, 1990). Direct regulation of the enzyme by neurotransmitters in brain has been difficult to demonstrate for two reasons. First, in broken cell preparations, catecholamines appear to nonspecifically enhance Na^+, K^+ -ATPase activity by chelating divalent metal ions that normally inhibit the enzyme (Van der Krogt et al., 1980). Second, activity of the enzyme is dependent upon and extremely sensitive to the Na^+ and K^+ concentrations near the cell membrane which may be altered as a result of neurotransmitter binding and ion channel activation (Gadsby, 1983). Indeed, the hyperpolarizing actions of catecholamines on sympathetic ganglia, once thought to be due to direct stimulation of Na^+, K^+ -ATPase, have been determined to arise from an increased potassium conductance which elevates the extracellular potassium concentration and thereby indirectly modulates the enzyme's kinetics (Smith, 1984).

In contrast, the inhibition of Na, K -ATPase by dopamine described here must reflect direct neurotransmitter induced changes in either the enzyme itself or a closely associated molecule. Intact cells were preincubated with dopaminergic compounds, and then permeabilized by cold shock to load them with ^{32}P -ATP and stabilize intracellular Na^+ concentration at 70 mM. This procedure should have dissipated any neurotransmitter-induced changes in ion concentrations before enzyme activity was measured. The inhibition cannot be explained by a down regulation in the number of membrane inserted Na^+, K^+ -ATPase molecules since

the ATP hydrolysis assay measures activity from both plasma membrane inserted and internalized pumps.

The present results provide strong evidence in support of the idea that activation of both D1 and D2 receptor subtypes is required for the inhibition of Na^+, K^+ -ATPase activity. An extensive literature describes an interaction between the D1 and D2 receptor subtypes in brain despite the fact that the receptors differ in their anatomical distributions and pharmacological profiles and mediate distinct behavioral and electrophysiological effects (for review, see Clark and White, 1987). Synergism has previously been demonstrated on both the behavioral and electrophysiological levels; D1 and D2 receptor co-activation is required for the expression of dopamine-induced stereotypic behaviors in the rat (Walters et al., 1987) and D1 receptor stimulation is required for the inhibition of nucleus accumbens neurons by D2 receptor agonists (White, 1987).

It is not clear at what level of cellular organization such synergism is occurring since D1 and D2 receptors are widely but unevenly distributed across the cell types of the basal ganglia and their subcellular distribution is unknown (Richfield et al, 1989). The co-activation of both receptor subtypes required for many dopamine-mediated effects could reflect interaction of receptors on a single cell, on different cells from the same brain region or on cells from different brain regions. Dopamine receptor synergism has previously been postulated to reflect interactions between D1 receptors of the substantia nigra and D2 receptors in the neostriatum (Robertson et al., 1987). The synergism required for the Na^+, K^+ -ATPase inhibition demonstrated here, however, must

be occurring on the level of single cells. Assays were conducted on a dissociated cell population, nearly homogeneous morphologically, and lacking synaptic contacts. D1 and D2 receptor co-activation resulted in a synergistic inhibition of Na^+, K^+ -ATPase activity distinct from the additive effect expected from subpopulations of neurons each containing only one of the two receptor subtypes.

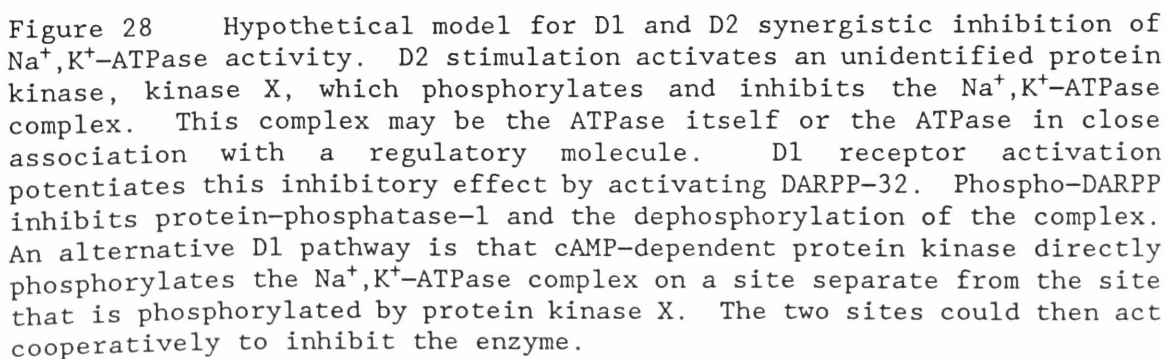
The biochemical mechanism of this synergism must reflect the signal transduction mechanisms of the D1 and D2 receptors in striatal neurons. D2 receptor activation in striatal neurons inhibits adenylate cyclase activity via the inhibitory G-protein G_i (Ohara et al., 1988). In pituitary lactotrophs, D2 receptor activation has a second coupling mechanism to polyphosphoinositol hydrolysis (Vallar et al., 1989). In striatum, D2 receptor activation is not thought to be coupled to this second pathway and may instead be linked to calmodulin translocation and calcium-regulated protein phosphorylation (Rubinstein et al., 1990; Lau, 1984).

D1 receptor activation in neostriatum leads to adenylate cyclase activation, cAMP formation and the activation of cAMP-dependent protein kinase (Hemmings et al., 1987). This kinase phosphorylates members of the ARPP family of basal ganglia-enriched phosphoproteins (Walaas et al., 1983a). One of these phosphoproteins, DARPP-32 (a dopamine and cyclic AMP regulated phosphoprotein, $M_r = 32,000$), is co-localized in cells which have D1 receptors and has been shown, in striatal neurons, to be phosphorylated in response to dopamine (Walaas et al., 1983b). Guinea pig medium spiny neurons contain high levels of this protein as shown in Figure 27. DARPP-32, when phosphorylated, becomes a potent inhibitor of a promiscuous

phosphatase, protein phosphatase-1. Phospho-DARPP-32 therefore can be considered to potentiate the phosphorylation of substrates by protein kinases by inhibiting the reverse dephosphorylation reaction (Hemmings et al., 1987). Intracellular loading of renal tubule cells with phospho-DARPP-32 inhibits Na^+, K^+ -ATPase activity (Aperia, personal communication).

Based on this information, a model for the receptor synergism underlying Na^+, K^+ -ATPase inhibition can be constructed (Figure 28). For simplicity, the functional unit of Na^+, K^+ -ATPase regulation can be considered to be a Na^+, K^+ -ATPase complex, consisting either of the enzyme alone or the enzyme in close association with a regulatory molecule. The complex can exist in two forms: dephosphorylated and ion translocating, and phosphorylated and inhibited. D2 receptor activation increases intracellular levels of diacylglycerols, Ca^{++} ions etc. which activate an unidentified kinase, protein kinase X. This kinase phosphorylates the Na^+, K^+ -ATPase thereby inhibiting its activity. D1 receptor activation stimulates cAMP-dependent protein kinase which phosphorylates DARPP-32 which in turn inhibits protein phosphatase 1. Phosphatase-1 inhibition prevents the dephosphorylation of the Na^+, K^+ -ATPase complex thereby potentiating the phosphorylation of the complex by protein kinase X. This model for D1 and D2 receptor synergism is currently being tested in renal tubule cells which contain DARPP-32 and in which D1 and D2 receptor activation also acts synergistically to inhibit Na^+, K^+ -ATPase activity (Meister et al., 1989; Bertorello et al., 1988b).

Of particular interest is the identification of the molecule that may be phosphorylated. Indirect evidence for inhibition of Na^+, K^+ -ATPase activity by protein phosphorylation has been obtained in rat synaptosomal



membranes (Lingham et al., 1983). The putative intracellular domains of the α catalytic subunit contain consensus amino acid sequences for phosphorylation by several protein kinase (Nairn, personal communication). Catalytic subunit of cAMP-dependent protein kinase has been reported to phosphorylate a protein in kidney membrane extracts which migrates on polyacrylamide gels in a similar position to the α subunit (Mardh, 1983).

The micromolar concentrations of D1 and D2 agonists used to inhibit Na^+, K^+ -ATPase activity suggest that the observed regulation of enzyme activity by dopamine may have physiological significance. Inhibition of the electrogenic Na^+, K^+ -ATPase leads to transient membrane depolarization and a rise in intracellular Na^+ in excitable cells (Phillis and Wu, 1984). In cardiac Purkinje fibers, pump inhibition by strophanthidin produces an 8 mV depolarization of the membrane potential (Gadsby et al., 1979). Attempts to measure dopamine modulation of striatal cell excitability using patch clamp techniques were unsuccessful due to the small size of the pump current relative to leakage through more rapidly conducting ion channels. Although the channels can be pharmacologically blocked, the small size of the pump current (< 3 pA) makes detection of its modulation a formidable task. The extent and time-frame of the presumed depolarization of the striatal neurons by inhibiting the electrogenic Na^+, K^+ -ATPase remains to be determined.

Regulation of Na^+, K^+ -ATPase activity has been implicated in both the development and pathology of the nervous system. Neuronal differentiation of the amphibian embryo can be inhibited if Na^+, K^+ -ATPase activity is reduced (Breckenridge et al., 1982) and neurotrophic agents such as nerve growth factor are necessary to maintain the enzyme's activity during

development (Skaper et al., 1986). Low Na^+, K^+ -ATPase activity is found in the brains of patients with Alzheimer's disease and lower activity in erythrocytes is correlated with bipolar affective disorders (Liguri et al., 1990; Hokin-Neaverson et al., 1989). Inhibition of Na^+, K^+ -ATPase activity in hippocampal slices leads to an increase in extracellular K^+ and onset of spreading depression, a model for seizure activity (Haglund et al., 1990).

The present results suggest that dopamine, in addition to its regulation of ion channels (see Chapter 4), regulates cell excitability through the novel mechanism of pump inhibition. If this is so, then this action of dopamine on Na^+, K^+ -ATPase activity may be an important component of the mechanism by which this neurotransmitter alters the responsiveness of dopaminoceptive neurons to other neurotransmitters such as glutamate and GABA (Chiodo et al., 1986).

CHAPTER SIX

Conclusions

This thesis describes three different signal transduction mechanisms, each of which may be mediated or modulated by protein phosphorylation. The rate of desensitization of the nicotinic acetylcholine receptor (nAChR), a chemically-gated ion channel, is dependent upon the stoichiometry of phosphorylation of the receptor. Dopamine receptors are coupled both to ion channels and to an ion pump, the Na^+, K^+ -ATPase, through a mechanism involving second messenger activation and protein phosphorylation. These different receptor-effector mechanisms illustrate two themes of signal transduction. A given cellular response may be subject to multiple first messenger controls as in the case of the nAChR. Alternatively, stimulation by a single first messenger, such as the activation of D1 and D2 receptors by dopamine, can result in divergent physiological responses.

Convergence of cellular signalling can be seen in the regulation of desensitization of the nAChR. Three different kinases, cAMP-dependent protein kinase, protein kinase C and a tyrosine-specific protein kinase, each phosphorylate a common region of the receptor increasing its rate of desensitization. The neurotransmitters and hormones responsible for the physiological regulation of the activity of the different protein kinases have been investigated recently. Calcitonin gene-related peptide (CGRP) is a co-transmitter with acetylcholine at the neuromuscular junction which raises intracellular cAMP levels (Laufer et al., 1987). CGRP binding to cultured myotubes increases the rate of nAChR desensitization and

stimulates phosphorylation of the α and δ subunits of the receptor in an identical pattern to that elicited by cAMP-dependent protein kinase (Mulle et al., 1988; Miles et al., 1989). Acetylcholine itself may be the first messenger that regulates phosphorylation of the nAChR by protein kinase C. The nicotinic agonist, carbamylcholine, stimulates phosphorylation of the receptor in a pattern similar to that elicited by protein kinase C (Miles et al., 1990). Protein kinase C may therefore be activated by calcium ions that permeate the receptor channel during agonist binding. Regulation of signal transduction by tyrosine phosphorylation is a new, uncharacterized pathway. The factors that regulate tyrosine phosphorylation of the receptor are unknown but are likely to be molecules co-released with acetylcholine at the neuromuscular junction. The nAChR is therefore regulated by three protein kinase systems that are activated by at least three independent first messenger systems leading to a common functional modulation of the receptor (Figure 29).

The functional role of receptor desensitization in the nervous system has been controversial. In classic neuromuscular junction preparations, desensitization develops only after seconds of agonist application or extremely high frequency stimulation (Katz et al., 1957). At nicotinic synapses, however, acetylcholine is present for millisecond long pulses, suggesting that desensitization does not have time to develop *in vitro*. Accordingly, receptor desensitization has been traditionally assigned the mundane function of a safety mechanism to make cells refractory to overdoses of agonist.

Two recent lines of evidence suggest that desensitization is a molecular mechanism for the regulation of synaptic efficacy at the post

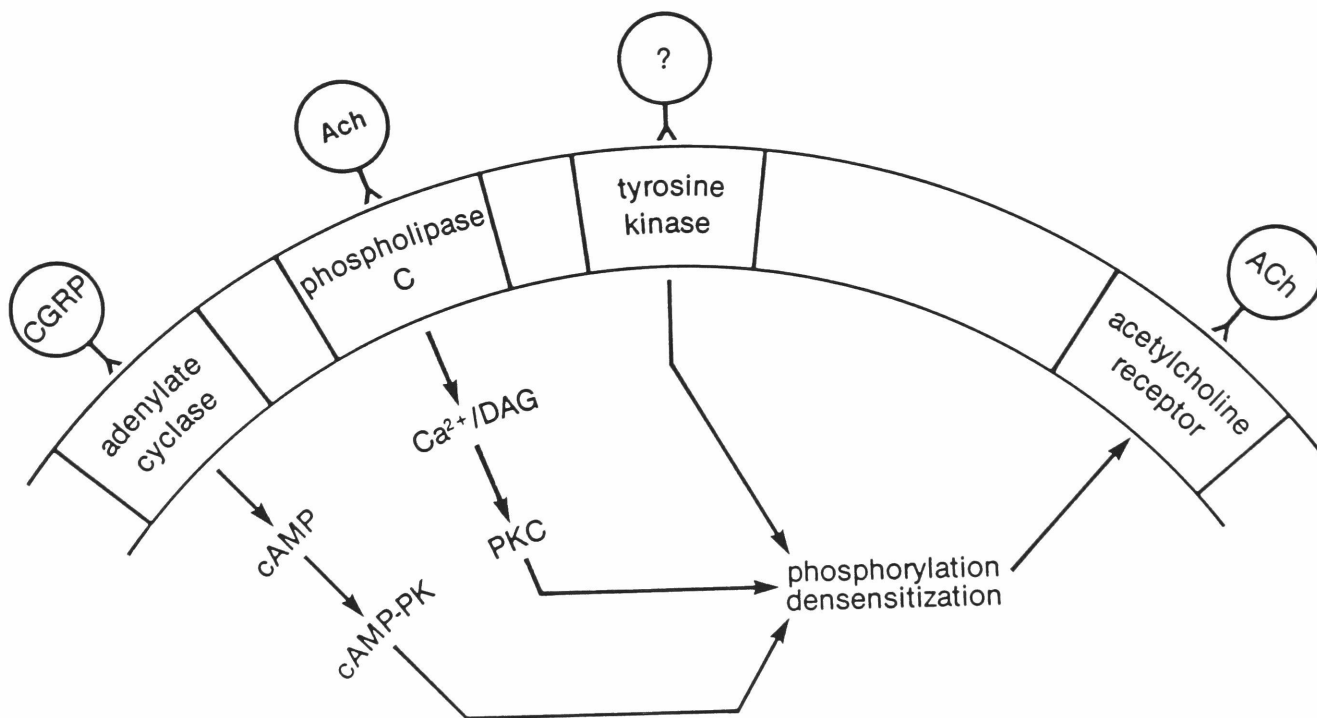


Figure 29 Schematic diagram illustrating proposed regulation of the nAChR by three different first messenger systems. CGRP, acetylcholine and an unidentified neurotransmitter, through the activation of their respective receptors and associated kinase systems, each brings about the phosphorylation and increased desensitization rate of the nAChR. (From Huganir et al., 1987)

synaptic level (Changeux, 1984). First rapid flux and electrophysiological measurements have revealed a rapidly developing desensitized state following millisecond long exposures to acetylcholine, providing a mechanism for desensitization to be manifested on a physiologically relevant time scale (Hess et al., 1979; Magleby et al., 1981). Second, several energy-expensive phosphorylation pathways have co-evolved to modulate the desensitization process, suggesting that it plays an important role in neuronal function. Modulation of the stoichiometry of phosphorylation, and thereby the potential inactivation of receptors, provides a mechanism for cells to regulate their sensitivity to incoming signals. The biphasic desensitization process at the nAChR creates several potential time frames of regulation. Desensitization over milliseconds to seconds may regulate the decay rate of synaptic currents and responses to closely-spaced repetitive stimuli. Slower desensitization rates may serve to tonically inhibit nicotinic synapses.

The molecular mechanism underlying desensitization is not well understood. At least three desensitized states exist which can be distinguished by the millisecond, second and minute time frames at which they develop. Desensitized nAChRs bind agonist with a higher affinity ($K_D=3$ to 5 nM) than receptors in the resting state ($K_D=50$ to 100 nM) (Boyd et al., 1980). Spectroscopic evidence suggests that the desensitized receptor has a different conformation than basal receptors (Barrantes, 1978). Phosphorylation is likely to regulate desensitization by enhancing this conformational change of the receptor to a non-conducting state by modifying the charge around amino acids that are critically involved in the transition. Phosphorylation does not change the initial binding of

acetylcholine to the α subunits (Huganir et al., 1986).

Receptor desensitization appears to be a common mechanism of regulation of chemically-gated ion channels. The kainate/quisqualate subtype of glutamate receptor desensitizes within 10 milliseconds of agonist application (Trusell et al., 1988). GABA_A receptors also desensitize and, interestingly, this desensitization is enhanced with forskolin application (Tehrani et al., 1989). Protein phosphorylation may therefore be a common mechanism for modulating desensitization across members of the receptor-gated ion channel family.

The action of dopamine on striatal neurons is an example of a divergent signal transduction pathway in which one neurotransmitter system is coupled to several intracellular cascades. Dopamine, acting on both D1 and D2 receptor subtypes, has both antagonistic and synergistic effects in the basal ganglia. These divergent actions can be explained, in part, by activation of either ion channels or ion pumps.

Antagonism of D1 and D2 receptor subtypes can be seen at the biochemical, electrophysiological and behavioral levels. D1 and D2 receptor activation is oppositely coupled to adenylate cyclase activity, GABA release, firing frequency and stimulation of locomotion (for review, see Clark et al., 1987). These antagonistic actions have led to the traditional classification of D1 receptors as excitatory and D2 receptors as inhibitory. The transient voltage-dependent outward conductance coupled to D2 receptors described in Chapter 4 provides a molecular basis for this antagonism (Figure 30). D2 receptor stimulation, by enhancing this outward current at membrane potentials positive to -60 mV, will tend to hyperpolarize and thereby inhibit striatal neurons. D1 receptor

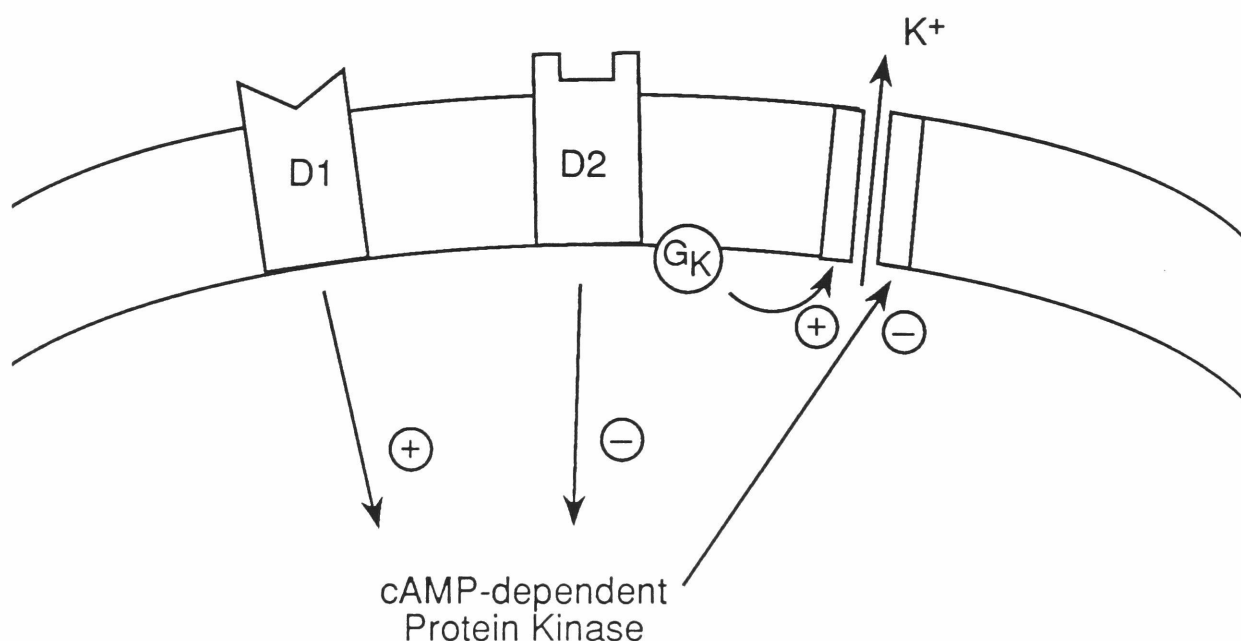


Figure 30 Schematic illustration of a proposed model for D1 and D2 receptor antagonism in striatal neurons. D2 receptor activation enhances an outward current through a G-protein linked potassium channel. D2 receptor stimulation also inhibits adenylate cyclase activity thereby downregulating cAMP-dependent protein kinase. D1 receptor stimulation activates adenylate cyclase leading to the production of cAMP and activation of cAMP-dependent protein kinase. This kinase phosphorylates an unidentified substrate (probably the D2 receptor, its associated G-protein or the ion channel) which uncouples the receptor-channel linkage.

activation, by activating cAMP-dependent protein kinase and functionally uncoupling the D2 receptor from the conductance, may abolish this inhibitory response.

Synergism of D1 and D2 receptors in the basal ganglia is frequently described in behavioral studies in which the behavioral sequelae of D2 receptor occupancy appear to require D1 stimulation for their expression (Gershanik et al., 1983). D1 and D2 receptor coactivation produces potentiated responses in nucleus accumbens and globus pallidus as assayed by extracellular recording techniques (Carlsson et al., 1987). Although some synergism undoubtedly reflects interactions across neurons, regulation of Na^+, K^+ -ATPase activity by the combined signal transduction pathways of the D1 and D2 receptors provides a molecular mechanism for synergism at the level of single cells (Figure 31). In this hypothetical model, as described in Chapter 5, D1 and D2 receptor stimulation activates two protein kinase systems one of which phosphorylates the ATPase complex and inhibits ion translocation while the other inhibits the reverse dephosphorylation reaction.

The physiological result of D1 and D2 receptor co-activation on a single striatal neuron may depend on which of these two dopamine signal transduction mechanisms (channel conductances or Na^+, K^+ -ATPase regulation) is operating. The subcellular distributions of D1 and D2 dopamine receptors and the Na^+, K^+ -ATPase are not known. The relative contributions of these two transduction systems to the neuron's electrophysiological state and decision to fire are therefore difficult to estimate. It is likely, however, that there is heterogeneity across striatal neurons in such dopamine-activated responses. Recent *in situ* hybridization with D1

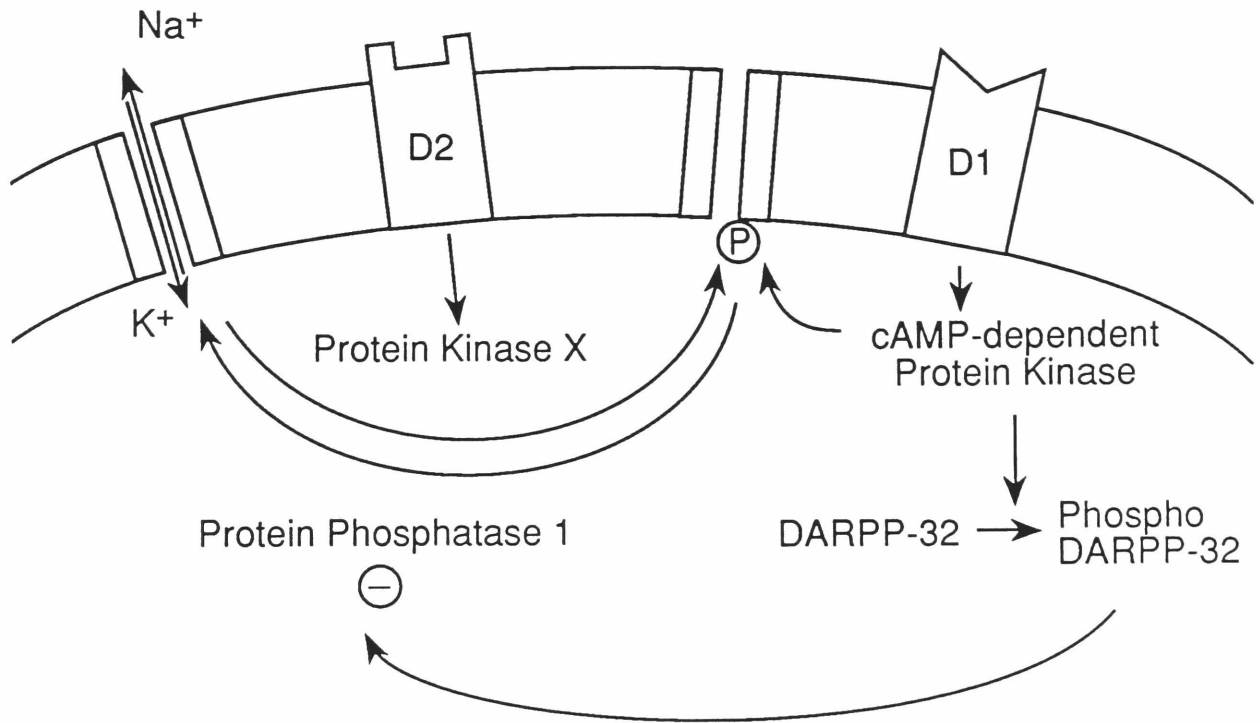


Figure 31 Schematic illustration of a proposed model for D1 and D2 receptor synergism in striatal neurons. D2 receptor occupancy activates protein kinase X which phosphorylates the Na^+/K^+ -ATPase, converting it from an ion translocating pump to an inactive molecule. D1 receptor occupancy stimulates cAMP-dependent protein kinase which phosphorylates DARPP-32. Phospho-DARPP-32 inhibits protein phosphatase 1 and the dephosphorylation of the ATPase back to an ion translocating pump. cAMP-dependent protein kinase may also directly regulate the Na^+/K^+ -ATPase by phosphorylating the ATPase. For simplicity, the site of phosphorylation is drawn as an intracellular portion of the Na^+/K^+ -ATPase molecule but the data do not preclude the existence of a closely associated regulatory molecule as the phosphorylated moiety.

and D2 specific probes has revealed that although most striatal neurons express mRNA for the D2 receptor, only 50% of striatal neurons express mRNA for the D1 receptor (Mansour et al., 1990; Sunahara et al., 1990). Both receptor subtypes are co-expressed, therefore, in only a subpopulation of striatal neurons. This suggests that some striatal neurons will exhibit pure D1 or D2-specific responses to dopamine application and other cells may have more complex responses reflecting the antagonistic and synergistic interactions of both receptor subtypes.

Mammalian neostriatum is not homogenous and is compartmentalized into striosome and matrix regions that are distinguishable by their neuropeptide content and the cortical layer of their afferent projections (Graybiel, 1990). Although the dendritic morphology and resting membrane properties of medium spiny neurons appear to be identical in the two compartments (Kawaguchi et al., 1989), an unexamined question is whether they differ in their distributions of dopamine receptors and therefore in their physiological responses to the neurotransmitter. Since striosome/matrix boundaries can be visualized only in fixed tissue stained for specific peptides, dopamine electrophysiology is blind to the origin of the sampled cells. The molecular basis of D1 and D2 signal transduction, how these mechanisms are modulated by protein phosphorylation and interact within a cell and how this interaction varies across the cells of the basal ganglia, await further investigation.

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