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Stimulation of Neuronal Acetylcholine Receptors Induces Rapid Gene Transcription

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Cholinergic agonists rapidly and transiently induced transcription of the c-*fos* proto-oncogene and one or more actin genes in neuronally differentiated PC12 cells. Transcription was activated within minutes after stimulation of the nicotinic acetylcholine receptor and required an influx of extracellular Ca^{2+} ions through voltage-sensitive calcium channels. Nicotine activation proceeded by a different pathway from activation by nerve growth factor, whose stimulation of these genes is independent of extracellular Ca^{2+} ions. These findings suggest that neurotransmitters may rapidly activate specific gene transcription in nondividing neuronally differentiated cells. They also suggest a functional role for neurotransmitter induction of c-*fos* and actin expression in the nervous system.

ALTHOUGH THE BEST CHARACTERized function of neurotransmitters is the regulation of transsynaptic communication in the central and peripheral nervous systems, neurotransmitters also regulate longer term changes in the cellular properties of neurons and other postsynaptic cells. Well-studied examples of this include the transsynaptic regulation over a period of several days of levels of neuropeptide synthesis (*1*) and of synthetic enzymes such as tyrosine hydroxylase (*2*), the latter by a mechanism dependent on new RNA transcription (*3*). We report here that activation of cholinergic receptors also causes extremely rapid induction of specific gene expression. We show that two genes, the c-*fos* proto-oncogene and actin, which have previously been shown to be activated by growth factors (*4–8*), are induced within minutes after nicotinic agonists bind to their receptors on nondividing neuronally differentiated PC12 cells. Induction by nicotine and nerve growth factor (NGF) show distinctly different dependencies upon flux of extracellular Ca^{2+} into the cell, indicating different mechanisms for c-*fos* induction through the neurotransmitter and growth factor receptors. These results suggest that rapid gene activation in nondividing, neuronally differentiated cells may be closely coupled to neurotransmitter stimulation.

For these studies we used the pheochromocytoma cell line PC12 (*9–11*), which expresses both nicotinic and muscarinic acetylcholine receptors (*10, 12, 13*). PC12 cells differentiate to nondividing, sympathetic neuron–like cells when treated with NGF for several days (*9, 10*). In response to this treatment they become electrically excitable (*11*) and exhibit increased levels of both types of acetylcholine receptor (*12, 13*). Exposure of neuronally differentiated PC12 cells to 100 μ*M* nicotine, an agonist of nicotinic acetylcholine receptors, resulted in a tenfold increase in c-*fos* transcription within 5 minutes (Fig. 1, A and B) as measured by a nuclear run-off transcription assay (*4, 14*). Actin transcription was also rapidly induced but to a lesser extent. The activation of c-*fos* transcription by nicotine was transient; it was maximal within 5 to 15 minutes after drug addition and decreased to the level present in unstimulated cells within 30 to 60 minutes (Fig. 1, A and B). While the kinetics of induction and repression of c-*fos* and actin transcription in response to nicotine were very similar to those previously reported in response to growth factors such as epidermal growth factor and NGF in PC12 cells (*15–17*) or platelet-derived growth factor in 3T3 fibroblast cells (*4–8*), induction by nicotine is mediated through a distinct pathway. The stimulation of c-*fos* and actin by nicotine is specific inasmuch as this cholinergic agonist does not significantly affect the expression of several other genes including α-tubulin (Fig. 2) and c-*raf*, a proto-oncogene (*18*) that encodes a protein with sequence homology to tyrosine kinases (Fig. 1A). The level of transcription of another growth factor–inducible nuclear proto-oncogene, c-*myc* (*4, 16, 19, 20*), appears unaffected within the first hour after treatment with nicotine (Fig. 1A). However, a slight stimulation of c-*myc* transcription

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occurred 2 hours after nicotine addition. In some analyses c-*myc* transcription was also activated at earlier time points (Fig. 1C). Additional experimentation will be required to verify the reproducibility and significance of the small increases in c-*myc* expression.

An analysis of the concentration dependence of c-*fos* transcriptional activation by nicotine (Fig. 1, C and D) shows that the stimulation can be detected with as little as 10 μ*M* nicotine but that the response is maximal at 100 μ*M*. Other effects of nicotine on neuronally differentiated PC12 cells such as release of catecholamines (*21*) display a similar concentration dependence. In the experiments shown here actin transcription appears to be induced at a somewhat lower concentration of nicotine (Fig. 1C) however, because actin transcriptional activation is generally less robust it is more difficult to accurately quantify the concentration dependence of its stimulation.

The activation of c-*fos* transcription in response to nicotine, as measured by the nuclear run-off transcription assay, was followed by an increase in the level of 2.2-kb c-*fos* cytoplasmic messenger RNA (mRNA). Northern blot analysis shows that c-*fos* mRNA increased markedly when neuronally differentiated PC12 cells were treated with 100 μ*M* nicotine (Fig. 2, lanes 1 and 2). Under identical conditions, no change in α-tubulin mRNA levels was detected (Fig. 2). The ability to rapidly stimulate gene transcription is not unique to nicotine but also occurs in response to another nicotinic agonist, 1,1-dimethyl-4-phenyl-piperizinium iodide (DMPP) (Fig. 2, lane 7), and when PC12 cells are stimulated with the muscarinic cholinergic agonist oxotremorine (*22*).

We examined the specificity of the response of c-*fos* and actin transcription to cholinergic agonists by determining if the effects are exerted through the appropriate

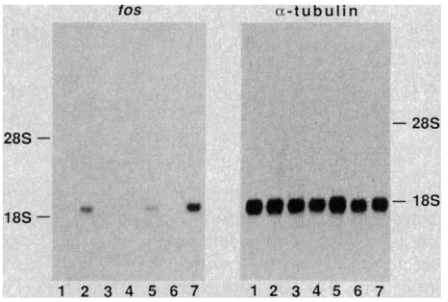

Fig. 2. Analysis of specificity of nicotine activation of c-*fos* cytoplasmic mRNA. Neuronally differentiated PC12 cells were left untreated (lane 1); or treated for 15 minutes with 100 μ*M* nicotine (lane 2); 100 μ*M* nicotine, 3 m*M* EGTA (lane 3); 100 μ*M* nicotine, 10 μ*M* mecamylamine (lane 4); 100 μ*M* nicotine, 10 μ*M* atropine, (lane 5); 100 μ*M* nicotine; 27.5 μ*M* verapamil (lane 6); or 100 μ*M* DMPP (lane 7). In the experiment shown in lane 3, EGTA was added immediately prior to nicotine. All other agents were added 20 to 30 minutes prior to nicotine. As previously described (*4, 16*) polyadenylated cytoplasmic RNA was isolated, fractionated on a formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled fragments of a 1-kb internal Pst I fragment from pv-*fos*-1 or α-tubulin.

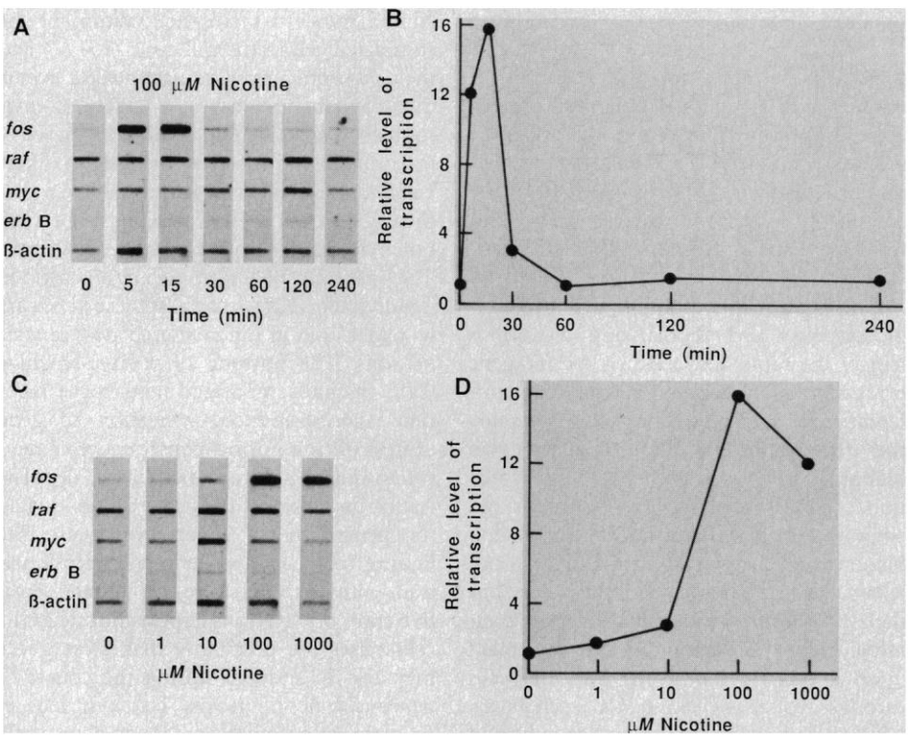

Fig. 1. The effect of nicotine on transcription in neuronally differentiated PC12 cells. In (A) and (B) nuclei were isolated from neuronally differentiated PC12 cells at various times after treatment with 100 μ*M* nicotine. In (C) and (D) nuclei were isolated from neuronally differentiated PC12 cells that had been incubated for 15 minutes with various concentrations of nicotine. Nicotine was added directly to the complete growth medium from a 1000× stock solution. In this and all subsequent experiments incubation was at 37°C in an atmosphere containing 7.5% CO_2. Cells were harvested and used for preparation of nuclei as described (*4, 16*). The ^{32}P-labeled RNA run-off transcripts were prepared (*4, 16*) and approximately 1 × 10^6 count/min in 1.8 ml of hybridization buffer were incubated for 36 hours at 65°C with 10 μg each of probes pv-*fos*-1, pv-*raf*, pM-c-*myc* 54, pv-*erb* B, and pH-β-actin DNA immobilized on nitrocellulose (*4, 16*). The overall level of incorporation of α^{32}P-UTP into RNA was found to be unchanged by addition of nicotine to PC12 cell cultures. In (B) and (D), relative signal intensities for c-*fos* were calculated by densitometric scanning of the autoradiographs shown in (A) and (C). PC12 cells were grown as previously described (*9, 10*) on collagen-coated 100-mm dishes in culture medium containing 85% RPMI 1640 medium, 10% horse serum, and 5% fetal bovine serum. To obtain neuronally differentiated cells, cultures were treated for 2 to 4 weeks with complete medium containing NGF (50 ng/ml) (*30*). In all experiments, approximately 20 × 10^6 to 40 × 10^6 cells were harvested for each determination.

nicotinic receptors. The stimulation of both c-*fos* and actin transcription and c-*fos* mRNA production by 100 μ*M* nicotine was completely abolished by the presence of 1 to 10 μ*M* mecamylamine (*23*), a specific nicotinic antagonist (Fig. 2, lane 4, and Fig. 3, lanes 4 and 5). The muscarinic antagonist atropine (10 μ*M*) was not nearly as effective as mecamylamine in blocking the induction of transcription by nicotine (Fig. 2, lanes 4 and 5, and Fig. 3, lanes 4 and 6). In contrast, 10 μ*M* atropine appears to inhibit c-*fos* and actin stimulation by the muscarinic agonist oxotremorine, while 10 μ*M* mecamylamine is not an effective blocker of this response (*22*). These experiments with specific antagonists of the cholinergic receptors strongly suggest that the induction of rapid gene transcription by nicotine shown here results from specific activation of the nicotinic receptor.

Additional experiments were performed to gain insight into the mechanism by which nicotinic agonists induce c-*fos* and actin gene expression and to compare it to the mechanism of induction by NGF. The binding of nicotine to the acetylcholine receptor causes the receptor channel to open, permitting the passive flow of ions across the plasma membrane (*13, 24*). This results in membrane depolarization that is followed by an activation of voltage-sensitive Ca^{2+} channels (*24, 25*) as well as the generation of action potentials (*11, 26*). When nicotine was added to neuronally differentiated PC12 cells in the presence of 3 m*M* EGTA, the activation

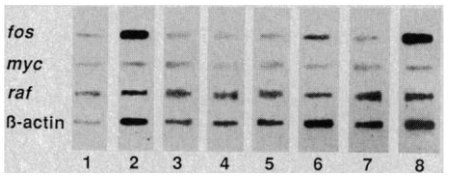

Fig. 3. Analysis of the specificity of nicotine activation of transcription. Neuronally differentiated PC12 cells were left untreated (lane 1); or treated for 15 minutes with either 100 μ*M* nicotine (lane 2); 100 μ*M* nicotine, 3 m*M* EGTA (lane 3); 100 μ*M* nicotine, 10 μ*M* mecamylamine (lane 4); 100 μ*M* nicotine, 1 μ*M* mecamylamine (lane 5); 100 μ*M* nicotine, 10 μ*M* atropine, (lane 6); 100 μ*M* nicotine, 27.5 μ*M* verapamil (lane 7); 100 μ*M* nicotine, 1 μ*M* tetrodotoxin (lane 8). After treatment, nuclei were isolated and run-off assays performed as previously described (*4, 16*). Isolated ^{32}P-labeled RNA's were hybridized to pv-*fos*-1, pM-c-*myc* 54, pv-*raf*, and pH-β-actin DNA's bound to nitrocellulose as described in the legend to Fig. 1. The radioactivity was visualized by autoradiography.

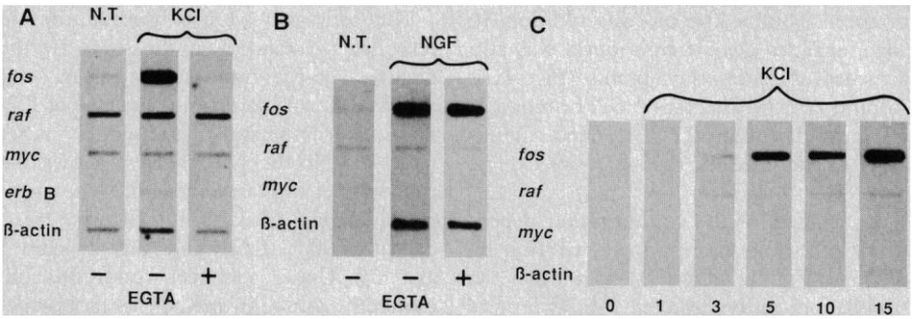

Fig. 4. Effect of elevated KCl on specific RNA transcription. (A) Neuronally differentiated PC12 cells. Cells were left untreated or incubated in the presence of 45 m*M* KCl for 15 minutes in the presence (+) or absence (−) of 3 m*M* EGTA. KCl was added from a 160 m*M* stock (1:4 final dilution) to maintain the osmolarity of the culture medium. (B) PC12 cells were left untreated or incubated for 15 minutes with NGF (50 ng/ml) in the presence (+) or absence (−) of 3 m*M* EGTA. (C) PC12 cultures were left untreated (0) or incubated with 45 m*M* KCl. EGTA (3 m*M*) was added to the KCl-treated cells at 1, 3, 5, 10, or 15 minutes after KCl addition. Nuclei were isolated from all samples 15 minutes after the initial treatment with KCl. The ^{32}P-labeled RNA run-off transcripts were prepared as previously described (*4, 16*) after isolation of nuclei and were hybridized with pv-*fos*-1, pv-*raf*, pM-c-*myc* 54, pv-*erb* B (A), and pH-β-actin DNA immobilized on nitrocellulose. Radioactivity is visualized by autoradiography.

of c-*fos* and actin transcription was blocked (Fig. 2, lane 3, and Fig. 3, lane 3). This inhibition by EGTA suggests that activation of gene expression by nicotine requires an influx of extracellular Ca^{2+}. This is substantiated by the observation that verapamil, a blocker of voltage-sensitive Ca^{2+} channels (*27*), abolishes gene activation by nicotine (Fig. 2, lane 6, and Fig. 3, lane 7). However, NGF induction of c-*fos* and actin transcription does not require extracellular Ca^{2+}. Transcriptional induction by NGF in neuronally undifferentiated PC12 cells was not blocked in the presence of 3 m*M* EGTA (Fig. 4B), consistent with previous findings that other NGF-triggered responses are also independent of the influx of extracellular Ca^{2+} (*28, 29*).

While these experiments show that Ca^{2+} influx through voltage-sensitive Ca^{2+} channels, a consequence of membrane depolarization, is required for nicotine stimulation of gene expression, the generation of the Na^+ action potential itself appears not to be necessary. Pretreatment with 1 μ*M* tetrodotoxin, a Na^+-channel blocker that is effective on PC12 cells (*11, 26*), had no effect on the ability of nicotine to induce c-*fos* or actin transcription (Fig. 3, lane 8). Moreover, the finding (*22*) that nicotine can stimulate c-*fos* and actin in neuronally undifferentiated PC12 cells that express nicotinic receptors but are not capable of generating action potentials (*11, 26*), further indicates that influx through the Na^+ channel is not important for rapid activation of these genes.

We have found that depolarization of PC12 cells (± NGF pretreatment) by incubation with elevated KCl induces c-*fos* but not actin transcription (Fig. 4A) (*16*). The induction of c-*fos* by KCl is also Ca^{2+}-dependent as it was inhibited when 3 m*M* EGTA was added to the medium. When PC12 cells were depolarized for as short a time as 3 minutes, followed by the addition of 3 m*M* EGTA to prevent Ca^{2+} influx, c-*fos* transcriptional activation still occurred 15 minutes after the initial depolarization event (Fig. 4C). The observation that nicotine induces actin while KCl does not implies that gene regulatory signals provided by the acetylcholine receptor may, in part, differ from those arising from membrane depolarization.

Our experiments establish that cholinergic agonists and depolarizing conditions trigger the rapid activation of specific gene expression in nondividing, neuronally differentiated PC12 cells. The experiments further demonstrate that there are at least two different pathways for c-*fos* and actin activation in PC12 cells. These pathways are employed by the neurotransmitter analog nicotine and the growth factor NGF. Both agents trigger c-*fos* and actin expression, but their activating mechanisms differ in their initial steps. Induction by nicotine relies upon a flux of Ca^{2+} ions into the cells through voltage-sensitive Ca^{2+} channels, while induction by NGF is independent of extracellular Ca^{2+}. Our observation that c-*fos* induction after KCl depolarization of the membrane is also blocked by EGTA provides further support for a c-*fos* activation mechanism that depends upon voltage-sensitive Ca^{2+} channels.

Activation of the c-*fos* proto-oncogene was first observed during the transition of quiescent fibroblasts from the G_0 to G_1 growth state (*4–7*), and the nuclear protein encoded by c-*fos* was initially suggested to have a pivotal regulatory function during cell cycle progression (*4*). More recent evidence suggests that the rapid induction of both c-*fos* and actin transcription is closely coupled to the provision of transmembrane stimuli rather than to the position of the stimulated cell in the cell cycle (*15–17*) and the c-*fos* protein may regulate nuclear events that occur in response to a variety of environmental stimuli. The demonstration of c-*fos* and actin induction by nicotine in differentiated PC12 cells provides evidence that these genes can also be rapidly induced in nondividing cells by nonmitogenic signals. The possibility of activating c-*fos* and actin in nondividing cells is crucial if these genes are to play a role in differentiated post-mitotic neurons. The opening of voltage-sensitive Ca^{2+} channels implicated here in the nicotinic agonist–induced activation of gene expression is a normal consequence of neurotransmitter-induced membrane depolarization in neuronal cells and occurs during the generation of action potentials. The finding that c-*fos* and actin transcription is transiently activated through voltage-sensitive entry of Ca^{2+} ions in post-mitotic PC12 cells raises the possibility that these genes may also be induced during the course of neuronal function in vivo. In the nucleus, c-*fos*, actin, or a similarly regulated gene could mediate long-term responses to enhanced neurotransmission, including expression of tissue-specific genes or information storage.

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Cliff Swallow Colonies as Information Centers

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Colonies of cliff swallows (Aves: *Hirundo pyrrhonota*) appear to be information centers in which colony residents acquire information on the location of food sources. Individuals that have been unsuccessful on a foraging trip return to the colony, locate a successful forager, and follow that individual to a food source. Individuals often follow, and are followed by, their neighbors within the colony, possibly because neighbors can observe foraging success through food brought back to nestlings. All individuals are equally likely to follow others or be followed, and thus all individuals benefit from opportunities to receive information.

ONE MAJOR ADVANTAGE OF LIVING in a group is the opportunity provided to observe other group members find food (*1*). Transfer of information about the location and quality of food often occurs at a fixed location such as a breeding colony, and a colony is considered an "information center" in such cases (*2, 3*). The best examples of information centers occur in social insects, especially honey bees (*Apis* spp.), where individuals (that are often related) inform each other about food location and quality (*4*). However, few if any unequivocal examples of information centers have been found among nonhuman vertebrates (*3, 5*). Breeding colonies and communal roosts of birds are prime candidates in which to expect the evolution of information centers (*2, 6*). I report a case of an information center in a colonial vertebrate, the cliff swallow (*Hirundo pyrrhonota*).

Cliff swallows are small migratory passerines that nest in colonies throughout much of western North America. The birds arrive in the southern and coastal parts of their breeding range in March and in most other areas by early May. Most cliff swallows leave North America in August and September for their wintering range, which extends from southern Brazil to Argentina and Chile (*7*). The birds build gourd-shaped nests out of mud, and the nests are fastened underneath overhanging rock ledges on the sides of cliffs and canyons or, more recently, on artificial structures such as bridges and highway culverts. These birds feed exclusively on insects caught in flight. Cliff swallows feed, preen, gather mud for their nests, and migrate in synchronized groups (*8, 9*). There is no evidence that cliff swallow colonies are composed of close genetic relatives (*10*), and thus kin selection is probably unimportant in the evolution of their social behavior. Nesting within each colony is highly synchronous, and these usually monogamous birds typically raise a single brood (*8–11*).

This study was done in Keith and Garden counties, Nebraska, from May to August, 1982 through 1985. In this area, cliff swallows nest on natural cliff sites, bridges, culverts, and occasionally buildings. My assistants and I studied 167 cliff swallow colonies totaling 53,308 nests (*9*). Colony size ranged from 1 to 3000 nests (mean, 319.2; SD, 522.0).

For an animal colony to be an information center, individuals living there that have recently been unsuccessful at finding food either (i) must be informed of food sources by successful individuals through active signals (such as a language or a form of chemical communication) or (ii) must recognize successful individuals on the basis of appearance or behavior and follow them to food sources. I focus on the second alternative because there is no evidence that cliff swallows (or any other birds) communicate about food sources with language or pheromones (*12*).

Cliff swallows feed on localized concentrations of aerial insects that occur unpredictably in both space and time (*9*). These concentrations are caused principally by localized convection currents that create high densities of insects within each patch. The birds also feed on insects that temporarily congregate in mating swarms and mass emergences. A patch of insects can often support more than 500 foraging swallows, but patches seldom last longer than 20 to 30 minutes, after which time the birds must locate another one (*9*). Thus, to continuously receive information on the location of a current foraging location is important to an individual in maintaining a high level of foraging efficiency. We discovered that when an individual cliff swallow is naïve about the present location of a food resource, that individual follows a knowledgeable neighbor from the colony to the food resource.

When feeding nestlings, cliff swallows in all colonies larger than ten nests clustered their departures from the nests (*9*). Clustered departures usually occurred as one individual followed another one away from the colony. We examined whether the birds that left together then fed together. Our study colonies were surrounded by treeless, open terrain, making it possible to observe with binoculars all foraging by colony residents. At two colonies, we visually tracked departing birds and kept them in sight until they reached a foraging location and began catching insects (*13*), or until members of the group drifted apart before ever starting

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