Neurotransmitters Increase Cyclic Nucleotides in Postganglionic Neurons: Immunocytochemical Demonstration

Abstract. *Dopamine increases adenosine 3',5'-monophosphate (cyclic AMP) but not guanosine 3',5'-monophosphate (cyclic GMP) in slices of bovine sympathetic ganglion; this increase is localized to the postganglionic neurons. Conversely, acetylcholine increases cyclic GMP but not cyclic AMP in the ganglion; this increase also occurs within postganglionic neurons. Thus, different neurotransmitters can selectively alter cyclic nucleotide levels within the same neuronal population.*

Evidence (*1–4*) recently summarized (*5*) indicates that the cyclic nucleotides adenosine 3',5'-monophosphate (cyclic AMP) and guanosine 3',5'-monophosphate (cyclic GMP) may be involved in the mediation of dopaminergic and muscarinic cholinergic synaptic transmissions in vertebrate sympathetic ganglia. In the bovine superior cervical ganglion, exogenous dopamine causes a substantial increase in ganglionic cyclic AMP but not in ganglionic cyclic GMP (*2, 4*). Conversely, exogenous acetylcholine increases ganglionic cyclic GMP but not cyclic AMP; this response is sensitive to atropine (*4*). The superior cervical ganglion contains small interneurons, glia, fibroblasts, and vascular elements in addition to the preganglionic fibers and the postganglionic neurons. Therefore, our studies were undertaken to determine which of these cell types increases its intracellular content of cyclic nucleotide in response to the neurotransmitters. Our results demonstrate that both the dopamine-induced increase in cyclic AMP and the acetylcholine-induced increase in cyclic GMP can be visualized predominantly within the postganglionic neurons.

Slices of bovine superior cervical ganglia were prepared and incubated (*6*). Dopamine, 100 μM, as reported previously (*2, 4*), caused a severalfold increase in the content of ganglionic cyclic AMP. Likewise, dopamine, 100 μM, induced an accumulation of histochemically demonstrable cyclic AMP in this tissue (compare Fig. 1A with Fig. 1B). The most striking increase in the histochemically demonstrable cyclic AMP occurred in cells whose size, shape, and spatial distribution indicated them to be postganglionic neurons (*4*). As indicated by fluorescence intensity, the cyclic AMP content of the cytoplasm of the soma was uniformly increased, whereas the cyclic AMP content of the nucleus was increased to a much lesser extent than was that of the cytoplasm. In appropriate sections, the nucleus could be seen outlined as a negative image by the surrounding intensely stained cytoplasm. No axons or peripheral dendrites of the postganglionic neurons were observed with this cyclic AMP staining procedure. Few, if any, of the satellite cells which surround the postganglionic neurons could be demonstrated with this cyclic AMP method; however, intense staining could be demonstrated occasionally in small cells which did not appear to be postganglionic neurons and which may be fibroblasts. As reported previously (*4*), dopamine caused little or no increase in the cyclic GMP content of blocks of bovine superior cervical ganglion, in contrast with its ability to elevate the level of cyclic AMP. Likewise, little or no increase in histochemically demonstrable cyclic GMP was observed in tissue incubated with dopamine and stained for cyclic GMP (compare Fig. 1D with Fig. 1E).

Acetylcholine can increase the cyclic GMP content of blocks of bovine superior cervical ganglion by interaction with a muscarinic receptor; however, this neurotransmitter has little or no effect on the content of ganglionic cyclic AMP (*4*). Comparison of Fig. 1D with Fig. 1F demonstrates the marked accumulation of histochemically demonstrable cyclic GMP which occurred in the postganglionic neurons of tissue treated with 100 μM acetylcholine. In addition to the soma of the postganglionic neurons, the axon and dendrites leaving the soma could also be clearly identified (arrows in Fig. 1F). The other cell types, such as satellite cells or fibroblasts within the ganglion, were not stained for cyclic GMP. Thus, an acetylcholine-induced increase in cyclic GMP can also be visualized predominantly in the postganglionic neurons of the bovine superior cervical ganglion. The content of histochemically demonstrable cyclic AMP in the acetylcholine-treated tissue was comparable with that of the control tissue (compare Fig. 1A with Fig. 1C).

Catecholamines other than dopamine can increase the cyclic AMP content of blocks of bovine superior cervical ganglion. Thus, treatment of blocks of ganglion with either *l*-norepinephrine (*2*) or *l*-isoproterenol (*7*) causes an accumulation of cyclic AMP; the norepinephrine effect is partially blocked and the isoproterenol effect is completely blocked by beta-adrenergic antagonists, compounds which fail to affect the dopamine-induced increase in cyclic AMP. Figure 1A (inset) demonstrates that *l*-norepinephrine, 100 μM, increased the cyclic AMP content of postganglionic neurons as well as of other cell types. These other cell types include a structure whose size and shape correspond to those of a blood vessel, as well as cells of a size and shape resembling fibroblasts. These results indicate that, in addition to its effect on cyclic AMP of the postganglionic neurons, norepinephrine is capable of activating receptors other than a dopamine receptor in nonneuronal elements of the bovine superior cervical ganglion.

The results of our study can be interpreted quite readily within the framework of current ideas concerning the synaptic organization of the mammalian sympathetic ganglion. Various anatomical, physiological, and biochemical evidence (*5*) indicate a role for cyclic nucleotides in the process of synaptic transmission in sympathetic ganglia. Preganglionic cholinergic fibers synapse directly on the postganglionic neurons; the activation of postsynaptic muscarinic receptors by acetylcholine increases the cyclic GMP content of the postganglionic neurons; this increase in cyclic GMP results in a slow depolarization of these neurons. Preganglionic cholinergic fibers also synapse on dopamine-containing interneurons which, in turn, synapse on the postganglionic neurons; physiological activity in the preganglionic fibers causes the release of dopamine from the interneurons, which then increases the cyclic AMP content of the postganglionic neurons; the electrophysiological sign of this increase in cyclic AMP is a slow hyperpolarization of these neurons.

Our results provide direct support for some elements of this scheme concerning the physiology and biochemistry of the sympathetic ganglia. Thus, dopamine increases the cyclic AMP content of the postganglionic neurons; and acetylcholine increases the cyclic GMP content of the postganglionic neurons. These and other (*1*) observations suggest that the electrophysiological events that accompany either dopaminergic or muscarinic cholinergic synaptic transmission in the ganglion may be the result of cyclic nucleotide–mediated activity within the postganglionic neurons. One attractive possibility concerning the nature of these biochemical actions is that the elevation in the levels of cyclic AMP and cyclic GMP initiates specific cyclic nucleotide–dependent phosphorylation of synaptic membrane proteins, which causes the alteration of the selective permeability properties of the membrane (*8*).

Several recent studies have demonstrated that the magnitude of the dopamine-stimulated increase in the cyclic AMP content of the superior cervical ganglion varies among different species. Thus, dopamine induces a five- to sevenfold increase in the cyclic AMP content

of the bovine superior cervical ganglion (*2, 4, 9*); however, this catecholamine causes only a 15 percent increase in the cyclic AMP content of the rabbit superior cervical ganglion (*3*) and a 74 percent increase in the cyclic AMP content of the cat superior cervical ganglion (*9*). Dopamine does not affect the cyclic AMP content of the rat superior cervical ganglion, although other catecholamines can elevate the cyclic AMP content of this ganglion (*10*). The reasons for the species variation in the sensitivity of the superior cervical ganglion to dopamine and other catecholamines is not immediately evident. Possibly the sensitivity of the cyclic GMP content of the sympathetic ganglia to regulation by acetylcholine might also vary among different species.

Our results also show the utility of the immunohistochemical procedure for the demonstration of cyclic nucleotides. This procedure is able to qualitatively demonstrate selective increases in either cyclic AMP or cyclic GMP within the same cell type. The application of this procedure to preparations of cerebellum has helped to elucidate the involvement of cyclic AMP in noradrenergic synaptic transmission in that structure (*11*).

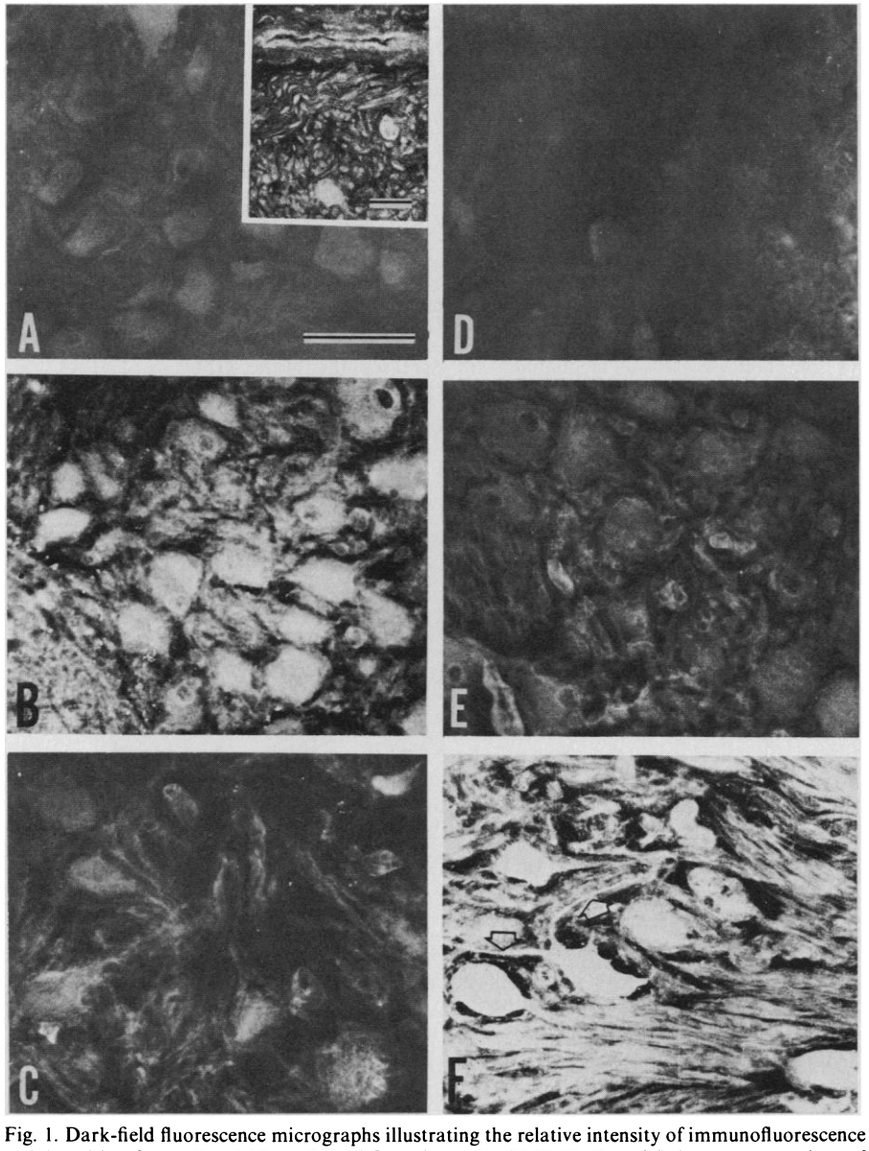

Fig. 1. Dark-field fluorescence micrographs illustrating the relative intensity of immunofluorescence staining either for cyclic AMP (A, B, and C) or for cyclic GMP (D, E, and F) in cryostat sections of bovine superior cervical ganglion incubated in Krebs-Ringer bicarbonate buffer as follows. (A and D) Tissue was incubated for 5 minutes in 1 m*M* SQ 20,006; (B and E) tissue was incubated for 5 minutes in 1 m*M* SQ 20,006 plus 100 μM dopamine; (C and F) tissue was incubated for 2 minutes in 1 m*M* SQ 20,006 plus 100 μM acetylcholine. All samples of stained tissue [except (A) inset] were photographed at the same exposure with Tri-X film, using transmitted dark-field fluorescence (× 16). The light source was a 150-watt xenon lamp; the exciting light passed through a double interference FITC filter, and the fluorescent light through a 50-nm barrier filter. All conditions used in the preparation of the photomicrographs [except (A) inset] were identical, so that the relative brightness of the staining in (A) through (F) is directly comparable. The bar in (A) represents 100 μm and applies to all photomicrographs [except (A) inset]. The inset in (A) illustrates the results of a 5-minute incubation in 100 μM norepinephrine plus 10 m*M* theophylline; positive staining is observed in two large neurons, the intima of a large vessel (above) and many small fusiform cells resembling fibroblasts; the calibration bar represents 100 μm. The incubated tissue contained: (A and D) 8.3 pmole of cyclic AMP per milligram of protein, 0.3 pmole of cyclic GMP per milligram of protein; (B and E) 39.6 pmole of cyclic AMP per milligram of protein, 0.44 pmole of cyclic GMP per milligram of protein; and (C and F) 8.0 pmole of cyclic AMP per milligram of protein, 0.95 pmole of cyclic GMP per milligram of protein (values are means of duplicate determinations on two to four samples of tissue).

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6. Superior cervical ganglia were removed from freshly slaughtered adult steers; the ganglia were rapidly desheathed and cut freehand with a razor blade into thin slices. The slices were quickly brought back to the laboratory in ice-cold Krebs-Ringer bicarbonate buffer which contained (mmole/liter): NaCl, 122; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.3; KH_2PO_4, 0.4; $NaHCO_3$, 25; and D-glucose, 10. This buffer had been equilibrated with a gas mixture of 95 percent O_2 and 5 percent CO_2 and had a *p*H of 7.4 at 25°C. The slices of ganglia were cut into small pieces with a Smith-Farquhar tissue chopper (setting, 0.52 mm). After sectioning, the pieces of tissue were placed in approximately 50 ml of ice-cold Krebs-Ringer bicarbonate buffer at 0°C. When all the tissue (approximately 2 g, wet weight) had been sectioned, it was incubated in oxygenated Krebs-Ringer bicarbonate buffer for approximately 30 minutes at 37°C. Portions of tissue were then incubated for 5 minutes with test substances in the presence of 1 m*M* SQ 20,006 [l-ethyl-4-hydrazino-1*H*-pyrazolo-(3,4-b)-pyridine-5 carboxylic acid ethyl ester HCl] as described (*2, 4*). At the end of the incubation period, the tissue samples were homogenized in acidified alcohol, and cyclic nucleotides were determined (*4*). Samples of tissue for histochemical observations were incubated for 5 minutes under conditions identical to those used for the biochemical studies. The tissue was then removed from the incubation medium, blotted dry, and mounted on cryostat chucks and rapidly chilled. Frozen sections were cut, and the tissues were stained for cyclic nucleotides by the procedure of H. J. Wedner, B. J. Hoffer, E. Battenberg, A. L. Steiner, C. W. Parker, F. E. Bloom [*J. Histochem. Cytochem.* **20**, 293 (1972)].

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Genetic Control of Chloroform Toxicity in Mice

Abstract. *Mouse strain differences suggest intermediate or multifactorial genetic control of chloroform-induced renal toxicity and death. The chloroform dose lethal to 50 percent of animals was four times higher in C57BL/6J males than in DBA/2J males. Twice as much chloroform accumulated in the kidneys of the sensitive as the resistant strain. First generation offspring were midway between parental strains for both parameters.*

Chloroform, which is used widely for medicinal and industrial purposes, is damaging to the liver and kidney of man (*1*) and experimental animals (*2*). Long-term chloroform administration produces liver tumors in mice (*3*), and nonanesthetizing doses are toxic to the fetal rat (*4*). Our experiments indicate that genetic factors control chloroform toxicity in mice, that the degree of toxicological responsiveness correlates with the extent of renal accumulation of chloroform, and that the mode of inheritance differs from that previously described.

Accidental exposure of different mouse strains to chloroform vapor has demonstrated that response varies from one strain to another (*5–7*). We chose to investigate the mode of genetic control of this differential susceptibility in two inbred strains: C57BL/6J, a resistant strain, and DBA/2J, an exceedingly sensitive strain (*6, 7*).

Susceptibility to the lethal effects of chloroform was studied at 9 weeks ± 4 days of age in these two strains and in their F_1 hybrid (B6D2F_1/J). All mice were obtained from Jackson Laboratory in Bar Harbor, Maine. Each group of eight mice received the same dose of chloroform (certified ACS, Spectranalyzed; Fisher Scientific) on the basis of body weight. The compound was dissolved in peanut oil; each mouse received a total volume of solution of 0.1 ml per 10 g of body weight. The drug was given by gavage, and the frequency of death was recorded over a 10-day period.

The C57BL/6J strain was about four times more resistant to the lethal effects of chloroform than the DBA/2J strain: dose lethal to 50 percent of animals (LD_{50}) values for chloroform were 0.33 ml/kg and 0.08 ml/kg, respectively. The value for the B6D2F_1/J animals (0.20 ml/kg) was midway between those of the two parental strains (Fig. 1) (*8*). These results suggest either single gene intermediate inheritance or multifactorial genetic control over chloroform toxicity. The LD_{50} values differ significantly from each other. The three curves in Fig. 1 do not vary significantly from parallelism.

Resistance to chloroform has been reported to behave as a dominant trait: all males of the C3H/He strain died from an injection of chloroform at 0.18 ml/kg, whereas those of the C57BL/6JN strain and the F_1 hybrid between these strains survived (*9*). Similar findings were reported for the resistant BN strain and F_1 animals from the mating of BN and C3H/He strains (*9*). We performed a chloroform lethal dose response study on 9-week-old C3H/HeJ mice in which the LD_{50} value, 0.27 ml/kg (95 percent confidence limits are 0.22 to 0.33 ml/kg), was not significantly different from that of the C57BL/6J strain. Comparison of the log dose–probability plots for the two strains indicated that, in contrast to the above report (*9*), no dose of chloroform which was lethal to all C3H/HeJ males spared all C57BL/6J males.

Microscopic examination of tissues from mice dying of chloroform revealed dose-dependent changes in pathology (*3, 10*). Mice receiving chloroform doses of 0.17 ml/kg or less died with necrosis of the proximal convoluted renal tubules. However, mice of all three genotypes that received more than 0.17 mg/kg exhibited both renal tubular and hepatic centrolobular necrosis. Thus, at high chloroform doses all mice developed hepatic toxicity, whereas strain differences in LD_{50} values involved primarily the dose at which renal damage caused death. The lowest concentration of chloroform given to C57BL/6J mice was 0.27 ml/kg; only one of eight mice died of combined renal and hepatic lesions. By contrast, DBA/2J animals appear to be susceptible to death from renal lesions at doses of chloroform greater than about 0.05 ml/kg (Fig. 1). Males and females of the same strain exhibit similar thresholds to hepatic damage (*3*), but differ in regard to renal toxicity (*7, 11*); females die of chloroform-induced hepatic damage without developing renal lesions.

Sex hormones have been implicated in this sex difference in renal toxicity: immature male mice and castrated adult male mice are resistant to chloroform-induced damage, but are sensitized by testosterone; estrogen-treated males are resistant; and testosterone-treated females become sensi-

Table 1. Accumulation of [^{14}C]chloroform in mouse tissues. Each of four 9-week-old male mice (19 to 21 g) of the three genotypes received intraperitoneally [^{14}C]chloroform (0.07 ml/kg; 10 μc) in 0.2 ml of peanut oil at 7 p.m., before the lights in the room went off; food was withheld thereafter. Animals were killed 12 hours later. Genotype comparisons are presented as the ratio of specific activity in one group of animals to that in C57BL animals. Specific activities for all C57BL fractions are given as the mean ± standard error of the mean.

Tissue	Specific activity relative to that of C57BL			C57BL specific activity	
	DBA	F_1	C57BL	Wet weight (10^4 dpm/g)	Protein (10^2 dpm/mg)
		Tissue homogenates			
Liver	0.82	0.96	1.00	28.8 ± 2.5	
Kidney	2.41*	1.64	1.00	19.0 ± 1.7	
		Subcellular fractions			
Liver					
Nuclei	0.67	0.76	1.00		0.9 ± 0.1
Mitochondria	1.14	1.14	1.00		12.5 ± 2.0
Microsomes	0.64	0.73	1.00		28.0 ± 4.0
Cell sap	0.98	1.09	1.00		13.5 ± 1.3
Kidney					
Nuclei	2.20*	1.67	1.00		0.7 ± 0.1
Mitochondria	3.67*	1.97	1.00		13.9 ± 1.6
Microsomes	1.74*	1.44	1.00		13.7 ± 0.5
Cell sap	1.65*	1.23	1.00		7.8 ± 0.9

*Difference between DBA and C57BL means significant at $P < .05$, Student's t-test.