have binding affinities to serum albumin 1/10,000th of that in the CNS; in addition, benzodiazepines are displaceable from albumin by L-tryptophan (*11*), in contrast to the binding site in cortex (Table 1). Benzodiazepine binding in rat kidney, liver, and lung also differs fundamentally from that in cortex (*4*). No diazepam binding to erythrocytes (*11*) or skeletal muscle has been observed (*4*). Pig CNS and calf CNS also contain [^{3}H]diazepam specific binding sites (*4*).

Note added in proof: The benzodiazepine receptor in human brain corresponds to that of rat brain in affinity stereospecificity and regional distribution (*4*).

The identification of the site of action of the benzodiazepines may provide new insight into their mechanism of action.

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Cyclic Nucleotides Injected Intracellularly into Rat Superior Cervical Ganglion Cells

Abstract. *Intracellular iontophoresis of either adenosine 3′,5′-monophosphate or guanosine 3′,5′-monophosphate produces a membrane depolarization and an increased membrane conductance in sympathetic ganglion cells of the rat superior cervical ganglion. Since adenosine 3′,5′-monophosphate did not cause a membrane hyperpolarization, it is difficult to assign it a second messenger role in the mediation of the slow inhibitory postsynaptic potential. However, these results do not rule out the possibility that the cyclic nucleotides, at the intracellular concentrations attained in these experiments, participate in cellular processes that contribute to conductance changes which result in depolarization of the ganglion cell membrane.*

Reports (*1*–*3*) indicate that the concentrations of cyclic nucleotides, adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP), determined by biochemical techniques, are increased in whole ganglia or slices thereof that have been treated with drugs such as dopamine, norepinephrine, or methylxanthines, or after orthodromic electrical stimulation. Thus a hypothesis has been proposed (*1*, *2*) that cyclic AMP mediates the slow hyperpolarizing response, the catecholamine-induced (*4*) inhibitory postsynaptic potential (IPSP), in sympathetic ganglia; and that cyclic GMP may mediate (*1*, *2*) the slow depolarizing response, the acetylcholine-induced (*5*) slow excitatory postsynaptic potential (slow EPSP).

According to the above hypothesis, a slow, hyperpolarizing response should be recorded from a ganglion cell when the intracellular concentration of cyclic AMP is increased. Electrophysiological investigations of this hypothesis have demonstrated that cyclic AMP or its dibutyryl derivative produce a hyperpolarization (*1*), no effect (*6*, *7*), or a depolarization (*6*, *8*). Because of the variability of results, interpretation has been difficult (*9*).

The hypothesis further suggests that if cyclic GMP is the mediator of the slow EPSP, then increasing the cyclic GMP within the cell should result in a slow depolarizing response. The results of studies with extracellular applications of cyclic GMP or its dibutyryl derivative have been consistent in that only a depolarization has been reported (*1*, *2*, *6*).

The variability in these results may be due to the fact that investigators have used only extracellular applications of cyclic AMP, cyclic GMP, their dibutyryl derivatives, or drugs that act by way of an adenyl cyclase mechanism. Thus, the biochemical and electrophysiological data are not in agreement as to the role of cyclic nucleotides in ganglionic transmission.

We have attempted to alter directly the intracellular concentrations of cyclic nucleotides in principal rat sympathetic ganglion cells by microiontophoretic injection of specific nucleotides into cells while recording the resultant effects on the effective membrane resistance (R_0), resting membrane potential (RMP), and orthodromic action potential. The rat superior cervical ganglion was used in this study because it produces an IPSP upon orthodromic stimulation (*10*), and because it possesses the highest density of small intensely fluorescent (SIF) cells in ganglia of commonly investigated mammals (*11*). The SIF interneurons are important because they are interposed between preganglionic axons and principal ganglionic neurons (*12*), and might be capable of modulating ganglionic transmission by releasing a postsynaptic inhibitory transmitter.

Rat superior cervical ganglia were maintained at 37°C in vitro, with Krebs solution aerated with 95 percent O_2 and 5 percent CO_2 (*13*). Double-barreled microelectrodes were used. The recording barrel (40 to 60 megohms) was filled with 3*M* KCl. The iontophoretic barrel (60 to 100 megohms) was filled with the acid of cyclic AMP (5 m*M*) or 5′-AMP (5 m*M*), or the sodium salt of cyclic GMP (0.1 m*M*) or 5′-GMP (0.1 m*M*) at *p*H 6.6 to 6.8. Only those cells in which an orthodromic action potential could be elicited were used in this study. Impaled cells were allowed to stabilize for a minimum of 10 minutes prior to nucleotide treatment. A bridge circuit was used to pass current and record the resultant electrotonic potentials simultaneously in order to obtain an estimate of membrane resistance. A cathodal holding current prevented leakage from the ejection barrel so that the nucleotide was only released during a 1-minute continuous anodal current of 2 to 50 na. The transfer number for cyclic AMP was determined by a technique similar to that described by Shoemaker *et al.* (*14*).

The tracings in Fig. 1 depict the effect of intracellularly injected cyclic AMP on R_0 and RMP of one cell and the effect of intracellularly injected cyclic GMP on RMP and the orthodromic action potential of another cell. Both nucleotides decreased the membrane resistance (an increased conductance) and depolarized the membrane. This at times resulted in blockade of the orthodromic action potential. In each case, these effects were

Table 1. Effects of cyclic nucleotides and their derivatives, administered by intracellular iontophoresis (2 to 40 na) or extracellular perfusion, on effective membrane resistance (R_0) and resting membrane potential (RMP) in rat superior cervical ganglion cells. Abbreviation: NC, no change.

Nucleotide	Decrease in R_0 (%)	N	RMP depolarization (mv)	N
	Intracellular iontophoresis			
Cyclic AMP	20 to 50	14	0 to 20	14
Cyclic GMP	20 to 50	16	0 to 20	16
5′-AMP	NC	19	NC	19
5′-GMP	NC	11	NC	11
	Extracellular perfusion (2.5 mM)			
Dibutyryl cylic AMP	NC	6	NC	6
	Extracellular perfusion (25 μM)			
Dibutyryl cyclic GMP	NC	6	NC	6

consistently reproducible, concentration dependent, and reversible. Cyclic GMP was at least 50 times more potent than cyclic AMP in producing these effects. Cells injected with cyclic GMP (5 m*M*) in concentrations equimolar to cyclic AMP resulted in an irreversible shift of the membrane potential in the depolarizing direction at the lowest injection currents (2 na) employed. In no instance did either nucleotide produce a hyperpolarization or an increase in resistance.

The value obtained [0.0175 ± .002 (mean ± standard error), $N = 6$] for the transfer number is less than that (0.048) reported by Shoemaker *et al.* (*14*), but this can be explained by differences in the conditions used in our study—higher impedance electrodes and a more dilute solution of nucleotide. Based on this transfer number, current intensity, duration of injection, and the size of these cells, the estimated intracellular cyclic AMP concentrations were increased to 0.3 to 3 m*M* immediately after injection. This value is comparable to that reported by Tsien (*15*) after injection of cyclic AMP into dog Purkinje fibers. It is difficult to demonstrate that this concentration (0.3 to 3 m*M*) of injected cyclic nucleotides is normally present within a ganglion cell. If cyclic AMP were a mediator of the slow (IPSP) hyperpolarization and the amount injected experimentally was excessive, as the cyclic nucleotide concentration was reduced by the action of endogenous phosphodiesterase, a hyperpolarization should have become apparent (a biphasic effect). This effect was never observed. Nonetheless, two results support the fact that the cyclic nucleotides have an action at these concentrations: (i) lesser amounts were without effect, whereas injection of greater amounts produced an irreversible depolarization, and (ii) both the noncyclic derivatives, 5′-AMP and 5′-GMP, were ineffective under comparable conditions (Table 1).

Dibutyryl cyclic AMP produced no effect when applied extracellularly in concentrations as high as 2.5 m*M*, but others have reported a hyperpolarization (*1*, *2*) or a depolarization (*8*). Adenosine, $10^{-4}M$, also failed to produce a hyperpolarization, but rather produced a 2 to 20 mv depolarization in 6 out of 14 cells (*16*).

The membrane depolarization and increased conductance that occurred after intracellular injection of cyclic GMP was not readily demonstrable by the extracellular application of dibutyryl cyclic GMP, which was without effect at 25 μM. However, dibutyryl cyclic GMP at 250 μM did produce a 2 to 8 mv depolarization in each of three cells tested. Furthermore, guanosine (10^{-5} to $10^{-4}M$) produced no consistent effects, in contrast to the observation with adenosine.

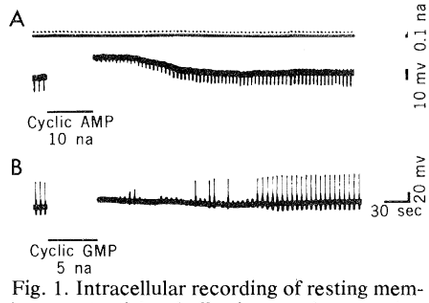

Fig. 1. Intracellular recording of resting membrane potential and effective membrane resistance (A) or orthodromic action potential (B), before and after injection of cyclic nucleotides. (A) Top: electrotonic current monitor, upward deflection represents pulsatile anodal currents. Bottom: thick horizontal tracing denotes resting membrane potential from which anelectrotonic potentials deflect downward. Note decreased amplitude of these tonic potentials at peak of depolarization after intracellular injection of cyclic AMP; upward deflection is depolarization. (B) Orthodromic action potentials (upward deflections) being blocked during depolarization after intracellular injection of cyclic GMP.

In biochemical analyses of the cyclic nucleotides (*6*) we have also confirmed the report by Greengard (*1*) that orthodromic stimulation increases the concentrations of cyclic AMP, and cyclic GMP in sympathetic ganglia. However, these biochemical changes may not necessarily imply a cause and effect relationship between ganglionic transmission and increased concentrations of cyclic nucleotides (*17*).

Thus, in our experiments, intracellular iontophoresis of cyclic AMP did not cause membrane hyperpolarization which would be required to support the hypothesis that cyclic AMP is a second messenger in the mediation of the hyperpolarizing IPSP in rat sympathetic ganglion cells. However, since both cyclic AMP and cyclic GMP produced a membrane depolarization, it is possible that both may be involved in the biochemical mediation of cellular processes which may lead to increases in sodium conductance or calcium conductance, or both (*18*, *19*), at the ganglion cell membrane.

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