a second copulation. This competitive effectiveness is indicated by observations that unsuccessful males of such an aggregate cease courting and begin to disperse soon after their successful rival has achieved intromission (*9*). The plugs do not remain in place long enough to assure males of sole paternity.

In *Thamnophis*, mating generally precedes ovulation by several weeks (*10*), and a female could mate again after expelling a plug. However, the female may leave the area of high male density at the mating site before the plug is expelled. Also, while the plug is in place, the first male's sperm may reach and occupy the specialized sperm storage crypts in the walls of the anterior oviducts, where they would be in the most favorable position to survive until ova enter the oviducts (*10*). If the plugs impede further copulations, they should be prevalent in those species in which there might otherwise be a high probability of two or more matings in succession. While this is true of the three taxa considered here, some reptiles have behavioral attributes, such as territoriality or male combat, which may isolate a female from the advances of other males, thus eliminating the selective advantage for a plug. If the plugs function merely to retain sperm, they should be equally common in either group of reptiles.

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9 September 1974

Cyclic AMP and Cyclic GMP May Mediate Opposite Neuronal Responses in the Rat Cerebral Cortex

Abstract. *Electrophysiologically identified pyramidal tract neurons in the rat cerebral cortex were tested with norepinephrine, acetylcholine, adenosine 3',5'-monophosphate (cyclic AMP), and guanosine 3',5'-monophosphate (cyclic GMP) applied by microiontophoresis. The neurons were usually inhibited by norepinephrine and cyclic AMP, but excited by acetylcholine and cyclic GMP. These opposing responses of pyramidal tract neurons to cyclic AMP and cyclic GMP suggests that these two nucleotides could function as reciprocal intracellular second messengers for norepinephrine and acetylcholine, respectively.*

Extensive evidence now indicates that responses of cells in many tissues to hormones may be mediated intracellularly by the cyclic nucleotide adenosine 3',5'-monophosphate (cyclic AMP) or guanosine 3',5'-monophosphate (cyclic GMP) (*1*). In central nervous tissues, elevations of cyclic GMP content have been correlated with muscarinic cholinergic receptors (*2*), while cyclic AMP concentrations can be increased through beta-norepinephrine receptors (*3*) or responses to dopamine (*4*). Tests of the possible physiological actions of these cyclic nucleotides in neurons have relied heavily on the technique of iontophoretic application (*5*). These studies have led to conflicting interpretations of the actions of cyclic AMP because of different standards for the utilization and assessment of iontophoretic tests (*6–8*). In the rat cerebellar cortex, norepinephrine (NE) and cyclic AMP produce similar changes in discharge rate and membrane properties; furthermore, the proportion of Purkinje cells which give positive immunocytochemical reactions for cyclic AMP is increased only by topical application of NE or activation of the noradrenergic synaptic pathway arising in the locus coeruleus (*5, 9*). We now report that iontophoretic studies on cells electrophysiologically identified as pyramidal tract (PT) neurons in the rat cerebral cortex show distinctly opposite responses to cyclic AMP and to cyclic GMP: while NE and cyclic AMP predominantly depress all cells, acetylcholine (ACh) and cyclic GMP are mainly excitatory.

Adult male rats were anesthetized with urethane (1.25 g per kilogram of body weight, intraperitoneally) and prepared for recording in the parietal cortex (*10*). Standard microiontophoretic procedures (*11*) and five-barreled micropipettes with an overall tip diameter of 5 to 10 μm were employed. They were filled immediately before the experiment by centrifugation (*12*). Our iontophoresis circuitry minimizes polarizations of the electrode tip during drug ejection by use of automatic balancing (*11*); prevention of undesired diffusion of drugs between tests was effected by use of holding currents. Only spontaneously active cells were studied. Artificial activation of cells by excitatory amino acids was not employed in order to prevent complex drug interactions (*7*).

Spontaneously active cortical neurons were identified as PT cells on the basis of the antidromic action potential

Table 1. Responses of rat cerebral cortical neurons to cyclic nucleotides. Values are the numbers of cells (*N*) exhibiting excitation, inhibition, and no response. Unidentified cells are those which could not be identified as PT cells. For the effects of cyclic AMP and NE the correlation coefficient r is .97523 for PT cells and .96429 for unidentified cells; for cyclic GMP and ACh, $r = .99094$.

Response to NE or ACh	Response to cyclic AMP or cyclic GMP					
	PT cells (*N*)			Unidentified cells (*N*)		
	Excitation	Inhibition	No response	Excitation	Inhibition	No response
	NE and cyclic AMP					
Excitation	0	0	0	0	0	0
Inhibition	3	11	0	1	4	0
No response	1	1	2	0	0	2
	ACh and cyclic GMP					
Excitation	22	2	6			
Inhibition	2	6	2			
No response	0	0	1			

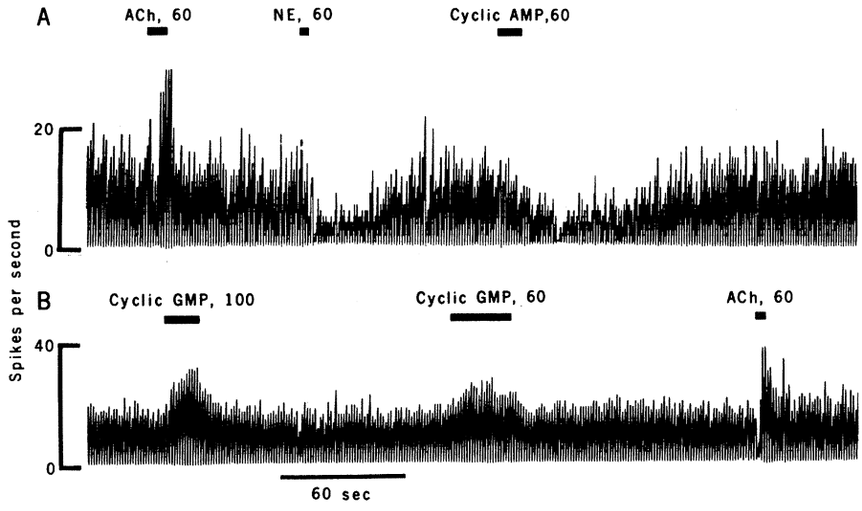

Fig. 1. Rate meter records of the firing rates of two different neurons in the cerebral cortex in response to the microiontophoretic application of (A) acetylcholine (ACh), 60 na; norepinephrine (NE), 60 na; and dibutyryl cyclic AMP, 60 na; and (B) cyclic GMP, 100 na and 60 na; and ACh, 60 na. The duration of drug application is indicated by the bars.

(spike) produced by stimulating the medullary pyramid, as previously described (*13*); almost all such PT cells respond to ACh with excitation and to NE with inhibition (*14, 15*).

When data from identified and unidentified cells were pooled, NE was seen to inhibit all responsive cells (19 of 25 tested). With four exceptions, cells that were inhibited by NE were also inhibited by cyclic AMP, while cells that were unresponsive to NE did not respond to cyclic AMP (Fig. 1A; Table 1). Of the units identified as PT cells, 72 percent (13 of 18 cells tested) responded in the same manner to both NE and cyclic AMP (Table 1). Seven of the 25 cells tested were not identified as PT cells. Within this group, six of the units responded in the same manner to both agents (Table 1). The responses to cyclic AMP in both groups of neurons were, in general, slower in onset and offset than responses of the same neurons to NE. The responses to cyclic AMP were also similar, whether the compound was monobutyryl or dibutyryl cyclic AMP. When comparable ejecting currents of 60 to 100 na were used, cyclic AMP had to be applied at least twice as long as NE; thus, if prolonged ejection periods had not been used with cyclic AMP, many potentially responsive units could have been judged unresponsive (*8*). Nevertheless, units that responded rapidly to NE (that is, within 5 seconds) also usually responded relatively quickly to cyclic AMP. An example of such a rapid response to both agonists is shown in Fig. 1A. Such responses could indicate that certain cells or areas of the cell membrane which are more sensitive to NE may also be more sensitive to cyclic AMP, or that spatial factors of pipette tip to neuron affected both responses similarly.

While NE and cyclic AMP mainly depress spontaneous discharge, PT cortical units are also known to be excited by ACh via an atropine-sensitive receptor (*13*); we therefore investigated the responsiveness of these cells to cyclic GMP as a possible cholinergic mediator and as a control for the effects of cyclic AMP. In contrast to the effects of NE and cyclic AMP, most cortical units, especially PT cells, responded to both ACh and cyclic GMP with excitation (Fig. 1B; Table 1, 22 of 30 cells excited by ACh). A smaller group of cells were inhibited by both ACh and cyclic GMP (6 of 10 cells which were not PT cells). Of the total cell population examined (41 cells), 29 responded similarly to ACh and cyclic GMP (72 percent). As in the case of NE and cyclic AMP, the responses to cyclic GMP appeared to be slower in onset and longer in duration than responses of the same neurons to ACh. Similarly, units that were extremely sensitive to ACh were also quite sensitive to cyclic GMP.

These results indicate that, in the cerebral cortex of the rat, cells characterized as PT neurons exhibit qualitatively opposing responses to cyclic AMP and cyclic GMP. Cyclic AMP, like NE (Table 1), tends to reduce spontaneous discharge, as has been reported for several other regions of the rat brain (*16*). Although interpretations vary, previous reports have indicated that some unidentified rat cortical neurons exhibit a potentiation of NE-induced inhibition by phosphodiesterase inhibitors and blockade of NE responses by prostaglandins of the E series (*7*). Similar effects of these compounds were also observed on identified neurons in the rat cerebellum (*5*) and hippocampus (*17*). In addition, there is evidence (*18*) that responses of identified PT neurons to NE are potentiated by papaverine and blocked by prostaglandin E_1. Cytochemical investigation of the rat motor cortex has not yet progressed sufficiently to define the existence of cholinergic or adrenergic synapses on PT or non-PT cells. However, light microscopic and neurochemical data suggest that such synapses are almost certainly present within these regions, as are the enzymes adenylate cyclase (*19*) and guanylate cyclase (*20*).

In view of the reproducible, but opposing effects of NE and ACh on rat PT cells and the similar antipodal responses to cyclic AMP and cyclic GMP, the data reported here suggest that separate intracellular mediation mechanisms are involved. Goldberg *et al.* (*21*) have proposed that cyclic AMP and cyclic GMP act in a dualistic opposing fashion as intracellular mediators of specific hormonal responses, and data obtained electrophysiologically in the sympathetic ganglion (*22*) support this dualistic or "yin-yang" hypothesis. Our results extend the dualism hypothesis of intracellular regulations mediated by cyclic nucleotides (*21*) into the function of cortical neurons. This is the first functional or physiological evidence favoring the yin-yang hypothesis in the central nervous system.

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Beta Cell Culture on Synthetic Capillaries: An Artificial Endocrine Pancreas

Abstract. *Beta cells from neonatal rats were cultured on bundles of artificial capillaries perfused with tissue culture medium. Cells continued to release insulin and remained responsive to changes in glucose concentration. The quantity of insulin released was similar to that of conventional flask cultures.*

The application of tissue culture techniques to the study of pancreatic beta cells offers a number of distinct advantages. Monolayers that have been prepared from pancreases of newborn rats can be maintained for periods varying from days to months (*1*). They have been successfully employed to study beta cell growth and replication, insulin biosynthesis and release, and beta cell morphology by both light and electron microscopy (*1*, *2*). In addition, small numbers of alloxan diabetic animals have been treated by implanation of these cultured cells (*3*).

Until recently these monolayers were cultivated on the surfaces of plastic or glass petri dishes or flasks. In 1972 Knazek *et al.* (*4*) reported culturing cells from established lines on bundles of artificial capillaries formed from plastic. In the present studies these methods were adapted to culturing normal diploid pancreatic beta cells.

The overall rationale was: (i) to examine possible means for improving the growth of pancreatic beta cells compared to conventionally utilized petri dish or flask systems; (ii) to develop a convenient method for studying short-term insulin secretory dynamics by using preparations maintained for days, weeks, or months under varying controlled conditions; and (iii) to develop a prototype for an artificial endocrine pancreas, since cells cultivated in this way are protected by a membrane barrier, and thus are not vulnerable to immune rejection (*5*).

One hundred fibers (Amicon XM-50) were sealed into the ends of a small glass jacket. The lumen of each fiber was 190 μm in diameter and the wall thickness 75 μm. It was permeable to substances with molecular weight below 50,000. The total capillary surface available for cell attachment was 75 cm^2. Plastic flasks with surface area 75 cm^2 were used for control cultures. Cells were prepared from the pancreases of newborn rats as previously described (*1*, *2*) and seeded into either capillary units or flasks (*6*). Capillary units were perfused with 100 ml of tissue culture medium recirculated at a rate of 2 to 3 ml per minute. Silastic tubing connecting the capillary units

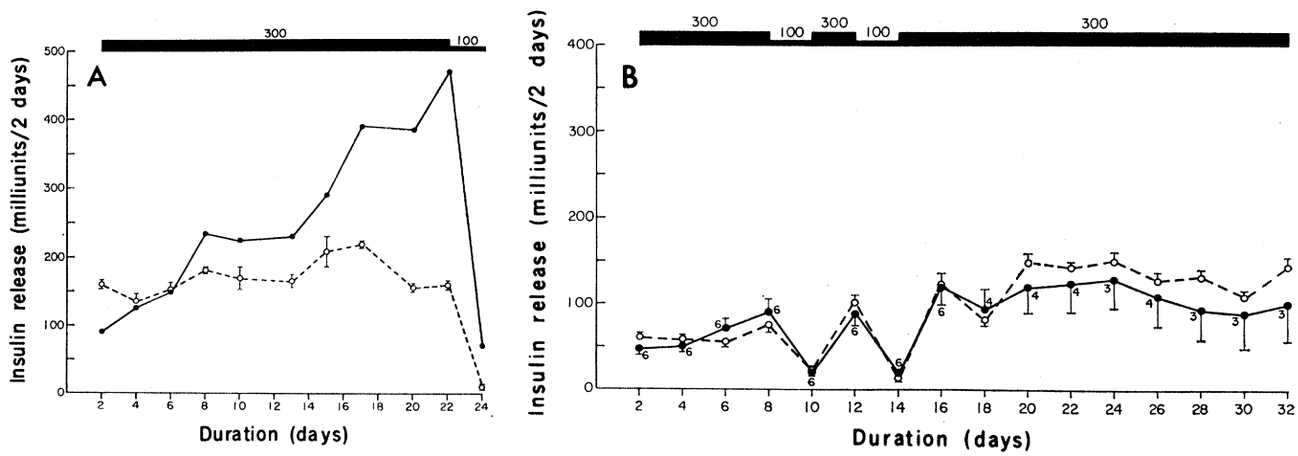

Fig. 1. Insulin release from representative capillary units versus insulin release from conventional flask cultures. The glucose concentration (300 or 100 mg per 100 ml) was varied as indicated by the labels on the heavy bars. (A) Solid line: one capillary unit; dotted line: mean of three flasks, plus or minus the standard error of the mean. (B) Solid line: mean of capillary units (*N* as indicated) ± S.E.M.; dotted line: mean of eight flasks ± S.E.M.