When progesterone levels are also elevated, owing either to exogenous administration or to natural events during the menstrual cycle, the reverse should occur. Our data conform to this model.

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Preganglionic Stimulation Increases Calcium Uptake by Sympathetic Ganglia

Abstract. *Isolated superior cervical ganglia of rats accumulate more calcium during preganglionic stimulation than do unstimulated controls; uptake of calcium-45 is approximately doubled by stimulation of 12 impulses per second. The extra uptake is markedly reduced by a high concentration of magnesium, but not by mecamylamine hydrochloride or tetraethylammonium chloride, although all three agents eliminate the postsynaptic action potential. Uptake of calcium-45 is also greatly increased by a high external concentration of potassium.*

In 1940, Harvey and MacIntosh (*1*) observed that calcium was required for the release of acetylcholine from cat superior cervical ganglia as a consequence of presynaptic stimulation. This finding focused attention on the role of Ca^{2+} in transmitter release. These efforts have culminated in the enunciation of a "calcium hypothesis" by Katz and Miledi (*2*). According to this hypothesis, depolarization of a presynaptic terminal causes an increase in its permeability to Ca^{2+}; Ca^{2+} diffuses into the terminal, down its electrochemical gradient (*3*), and is then involved in transmitter release. The hypothesis is supported by the observation that presynaptic depolarization of the squid giant synapse (*4, 5*) and the frog neuromuscular junction (*2, 6*), which is dependent on Ca^{2+} and independent of Na^+, is a sufficient stimulus to trigger transmitter release. Under appropriate conditions, depolarization of squid presynaptic terminals induces a regenerative response and transmitter release which is dependent on Ca^{2+}, even in the absence of Na^+ (*5*).

One way to test the calcium hypothesis is to measure directly entry of Ca^{2+} into presynaptic terminals during activity. Although isolated presynaptic nerve terminals from rat brain have an increased rate of Ca^{2+} accumulation when depolarized in media with high concentrations of K^+ (*7*), these experiments are open to the criticisms that isolated terminals may not

Table 1. Uptake of ^{45}Ca by isolated superior cervical ganglia of rats. Ganglia were incubated in ^{45}Ca solutions for 25 to 30 minutes. The member of each pair denoted by a prime (′) was held on the electrodes and stimulated at the frequency shown, for 20 minutes, except where indicated. Uptake of ^{45}Ca in ganglia 9, 9′, 11, and 11′ was measured in Ringer with a low Ca^{2+} concentration after exposure to Ringer with a low concentration of Ca^{2+} and a high concentration of Mg^{2+}. The concentration of mecamylamine hydrochloride was 50 μmole/liter for ganglia 15 and 15′, and 38 μmole/liter for 16, 16′, 17, and 17′. Uptake of ^{45}Ca in ganglia 20 and 20′ was measured after exposure to Ringer with a low Ca^{2+} concentration and to Ringer with a low concentration of Ca^{2+} and a high concentration of Mg^{2+}.

Ganglion pair	Temperature (°C)	Stimulation frequency (sec^{-1})	Ca^{2+} uptake (pmole/mg)	Stimulated Ca^{2+} uptake (pmole mg^{-1} $impulse^{-1}$)
Normal Ringer				
1	34		339	
1′	34	10–12*	484	0.0103
2	32		357	
2′	32	12	773	.0289
3	32		426	
3′	32	12	777	.0244
4	25		258	
4′	25	12	762	.0350
5	26		398	
5′	26	12†	675	.0167
Normal Ringer, unstimulated pairs				
6	22		201	
6′	22		136	
7	23		374	
7′	23		236	
8	23		269	
8′	23		237	
Ringer with low Ca^{2+} concentration				
9	25		90	
9′	25	12	257	0.0116
10	25		143	
10′	25	12	320	.0124
11	32		133	
11′	32	6–12‡	363	.0168
Ringer with low Ca^{2+}, high Mg^{2+} concentration				
12	25		101	
12′	25	12	81	−0.0014
13	25		101	
13′	25	12	108	.0005
14	32		134	
14′	32	12	138	.0003
Treated with mecamylamine				
15	33		261	
15′	33	6	345	0.0117
16	33		212	
16′	33	12	444	.0161
17	31		252	
17′	31	12	404	.0105
Treated with 3 mM TEA				
18	32		264	
18′	32	6§	688	0.0470
19	32		264	
19′	32	12	638	.0260
20	33		211	
20′	33	12	677	.0324

* Ten pulses per second for 2.5 minutes, then 12 per second for 17.5 minutes. † Twelve pulses per second for 23 minutes. ‡ Six pulses per second for 2 minutes, then 12 per second for 18 minutes. § Six pulses per second for 25 minutes.

be physiologically intact and that the electrical activity and the magnitude of depolarization cannot be measured directly. We therefore designed experiments to measure Ca^{2+} uptake in an intact preparation. The isolated superior cervical ganglion of the rat was chosen because of its small size, ready availability, and known ability to survive in vitro for many hours (*8*).

White female rats (200 to 250 g) were anesthetized with pentobarbital (10 mg/kg injected intraperitoneally). Both superior cervical ganglia were excised, including at least 10 mm of the cervical sympathetic trunk and at least 3 to 4 mm of the internal carotid nerve. The ganglia were immediately placed in bicarbonate-buffered Ringer solution containing in final concentration: 136 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 1.2 m*M* NaH_2PO_4, 16.2 m*M* $NaHCO_3$, 5.6 m*M* glucose, and 0.03 m*M* choline chloride. Other solutions used for these experiments were: Ringer solution with a low concentration of Ca^{2+}, in which the $CaCl_2$ content was 0.4 mmole/liter; Ringer solution with a low concentration of Ca^{2+} and a high concentration of Mg^{2+}, containing 0.4 m*M* $CaCl_2$, 21.2 m*M* $MgCl_2$, and only 106 m*M* NaCl; and Ringer solution with 62 m*M* K^+, in which 56 m*M* NaCl was replaced by 56 m*M* KCl. The addition of drugs will be mentioned below. All solutions were equilibrated with 95 percent O_2 and 5 percent CO_2.

Excised ganglia were desheathed, the cervical sympathetic trunk was drawn into a miniature suction electrode for stimulation, and the internal carotid nerve was drawn into a similar recording electrode (*9*); the assembly was placed in a 5-ml chamber modified from that of Larrabee *et al.* (*10*). After both members of a pair were checked for excitability, one ganglion was retained on the electrodes as the "test" (stimulated) ganglion, while the other was permitted to float freely in the medium, as the control.

Table 2. Effect of external K^+ concentration on uptake of Ca^{2+} by isolated superior cervical ganglia of rats. Isolated ganglia were incubated in Ringer solutions containing either 5.6 m*M* or 62 m*M* K^+ for 15 minutes.

Ganglion pair	Temperature (°C)	K^+ (mmole/liter)	Ca^{2+} uptake (pmole/mg)	Increase in 62 m*M* K^+ (%)
21	23	5.6	124	
21′	23	62.	506	308
22	23	5.6	174	
22′	23	62.	715	311
23	33	5.6	216	
23′	33	62.	679	214
24	33	5.6	177	
24′	33	62.	801	352

The experimental chamber was then emptied and rinsed, and 5.0 ml of the experimental solution was added. Excitability of the test ganglion was monitored periodically (*11*). About 5 minutes was allowed for the solution to diffuse into the ganglia, and for the effect of the high concentration of Mg^{2+} or drug to reach a steady level. Ten microcuries of ^{45}Ca (10 mc/mg) was added to the solution in the chamber, and another 5 minutes was allowed to elapse to permit the isotope solution to equilibrate with the interstitial fluid of the ganglia. The presynaptic nerve was then stimulated with supramaximal (1.5 to 2 times maximum) stimuli, usually at a rate of 6 to 12 per second for 20 minutes (Table 1). Immediately thereafter, both ganglia were placed in 20 ml of ice-cold normal Ringer solution; the solution was changed every 2 minutes for a total of six changes. The ganglia were then frozen, weighed, and prepared for liquid scintillation counting to determine ^{45}Ca uptake (*12*).

Figure 1 shows the electrical activity recorded from the internal carotid nerve at its exit from the superior cervical ganglion, when the cervical sympathetic trunk is stimulated (*13*). The postganglionic response in Ringer solution with a low concentration of Ca^{2+} is shown in Fig. 1A. Its shape and amplitude were similar to that observed in the normal (2.2 m*M* Ca^{2+}) Ringer solution (Fig. 1E). Immediately after replacement of the solution with a low Ca^{2+} content by Ringer with a low concentration of Ca^{2+} and a high concentration of Mg^{2+}, the large biphasic spike began to decrease, and within 4 to 5 minutes (Fig. 1, B and C), only a small early response, the presynaptic action potential, and excitatory postsynaptic potential (EPSP) (*13*) remained; the EPSP, but not the presynaptic action potential, was abolished by a more prolonged exposure to this solution. Upon return to the medium with a low Ca^{2+} concentration and containing 1.2 m*M* Mg^{2+}, the ampli-

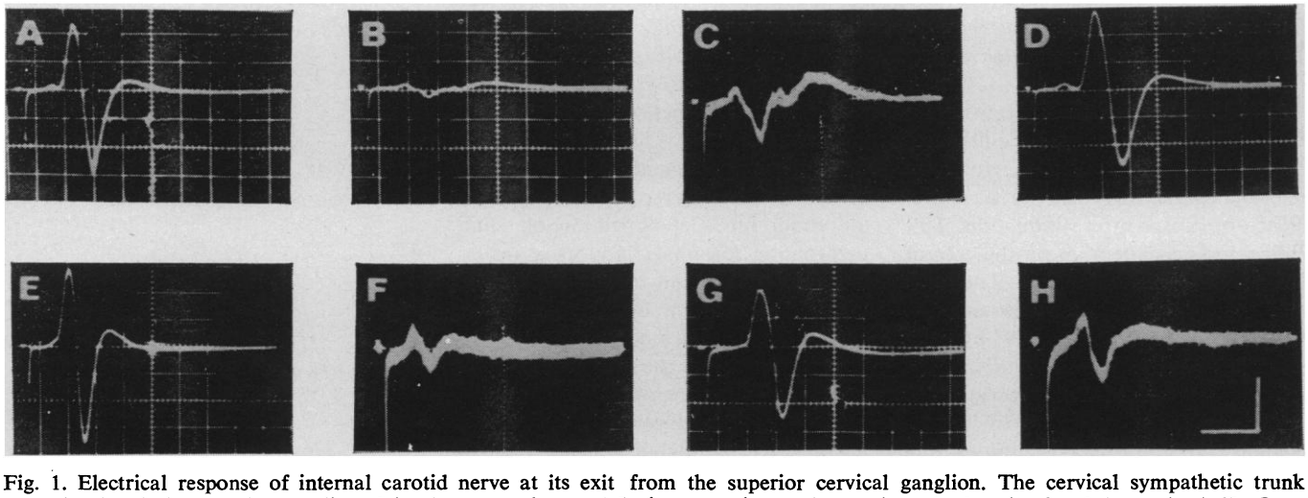

Fig. 1. Electrical response of internal carotid nerve at its exit from the superior cervical ganglion. The cervical sympathetic trunk was stimulated close to the ganglion. Stimulus strength was 1.5 times maximum (approximately 3 volts for 0.4 msec). A–F, Ganglion 20′ (Table 1); G and H, ganglion 17′ (Table 1). A and D, Ringer with low Ca^{2+} concentration; B and C, Ringer with low concentration of Ca^{2+}, high concentration of Mg^{2+}; E and G, normal Ringer; F, 3 m*M* TEA in normal Ringer; H, 38 μM mecamylamine hydrochloride in normal Ringer. In C, F, and H, the upward deflection 7 msec after the shock artifact is the presynaptic action potential; in C, the deflection at 20 msec represents the EPSP (*10*). Calibrations: Horizontal bar, 10 msec. Vertical bar, 1 mv in A, B, D, E, and G; 0.1 mv in C and F; 0.05 mv in H.

tude of the postganglionic response recovered completely (Fig. 1D). Although not shown here, increasing the Mg^{2+} concentration to 21.2 mmole/liter in the presence of 2.2 m*M* Ca^{2+} only partially reduced the postganglionic action potential.

The effects of two other ganglionic blocking agents were also examined. Mecamylamine hydrochloride (*14*), at concentrations as low as 38 μmole/liter, and tetraethylammonium chloride (TEA), at a concentration of 3 mmole/liter, both blocked the postsynaptic electrical activity when added to the normal Ringer solution (Fig. 1, E–H). With either drug, the recorded electrical activity was similar to that observed in the medium with low Ca^{2+} concentration and high Mg^{2+} concentration (Fig. 1C).

The data for uptake of ^{45}Ca are presented in Table 1. Uptake of Ca^{2+} by unstimulated ganglia is considerably less than that by paired stimulated ganglia. There is little difference in Ca^{2+} uptake when neither member of the pair is stimulated. These results indicate that preganglionic stimulation of the rat superior cervical ganglion enhances Ca^{2+} uptake; the stimulation-induced uptake from normal (2.2 m*M* Ca^{2+}) Ringer solution averages 0.023 pmole of Ca^{2+} per milligram, wet weight, per impulse. This value is probably lower than the actual uptake, because some of the accumulated Ca^{2+} may have been extruded during the periods of stimulation and washing, perhaps in exchange for external Na^{+} as may occur elsewhere in the rat nervous system (*3*).

If this extra uptake of Ca^{2+} is associated with the presynaptic terminals in the superior cervical ganglia, as implied by the calcium hypothesis, it should be altered in a predictable fashion by drugs known to affect synaptic transmission. The three agents examined for their effects on Ca^{2+} uptake were high concentrations of Mg^{2+}, mecamylamine, and TEA; all of these block ganglionic transmission (Fig. 1) although their mechanisms and sites of action are different. Elevated concentrations of Mg^{2+} block ganglionic transmission presynaptically by interfering with transmitter release as a consequence of competition with Ca^{2+} (*15*). Mecamylamine and TEA, on the other hand, appear to exert their blocking action primarily postsynaptically at acetylcholine receptors, since they prevent the stimulatory effect of cholinomimetic agents (*16*). Release of transmitter at invertebrate presynaptic terminals (*4, 5*) and at the neuromuscular junction (*2, 6*) is not prevented by TEA and may, in fact, be enhanced (*5, 6, 17*) by the prolonged depolarization associated with TEA (*18*). A possible presynaptic depressive action of mecamylamine (but not TEA) in addition to its postsynaptic effect, may also occur (*19*).

Despite these differences in the mechanism of producing ganglionic block, the effects of these three agents on the postganglionic electrical activity are indistinguishable; they all block the postsynaptic, but not the presynaptic potentials (Fig. 1). But their effects on Ca^{2+} uptake are not identical (Table 1); Ca^{2+} uptake induced by stimulation is markedly inhibited by high concentrations of Mg^{2+}, but not by mecamylamine or TEA. Since the latter agents block transmitter action at postsynaptic sites, whereas Mg^{2+} primarily acts presynaptically, these results may be interpreted as indicating that the presynaptic terminals are the likely sites of most of the extra uptake of Ca^{2+} associated with stimulation. The partial reduction of extra uptake of Ca^{2+} caused by mecamylamine (Table 1) (*20*) might then reflect a presynaptic action of this agent (*19*). The slight increase in uptake of Ca^{2+} (on the average) observed with TEA (Table 1) may be interpreted as a consequence of the ability of this agent to slow repolarization in the presynaptic elements (*18*), thus prolonging a concomitant increase in permeability to Ca^{2+} (*2, 4, 5*).

Finally, incubation in a high concentration of K^{+} also enhances uptake of Ca^{2+} by the superior cervical ganglion (Table 2), providing additional evidence that increased Ca^{2+} uptake is a consequence of depolarization. These results are consistent with previous observations that isolated presynaptic terminals from rat brain, when depolarized in media with a high concentration of K^{+}, take up Ca^{2+} (*7*) and release neural transmitter substances (*21*).

The main conclusion from these experiments, in support of the Katz and Miledi calcium hypothesis (*2*), is that stimulation and depolarization of presynaptic nerve elements increases their uptake of Ca^{2+}.

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12. After being washed, the ganglia were blotted on filter paper, and the pre- and postganglionic nerves were cut away. The ganglia were frozen on dry ice, and weighed on a torsion balance in a cryostat. They were then placed in counting vials and digested with 1.0 ml of 1*N* NaOH at 70°C for 30 minutes. The digested material was neutralized with 1.5 ml of 0.67*N* HCl (containing 2 m*M* $CaCl_2$ as carrier) and decolorized with three drops of H_2O_2. After addition of 15 ml of Bray's solution, the samples were assayed for ^{45}Ca in a liquid scintillation counter. Samples of the incubation solution were also treated with NaOH, HCl, and H_2O_2, and analyzed for radioactivity, in order to compute the specific activity of the ^{45}Ca in the incubation solutions.
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