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# Effect of increased potassium concentrations on particle motion within a neurosecretory structure

(laser light scattering/pericardial organ/swelling of cytoplasm)

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ABSTRACT **Intensity fluctuation spectroscopy was used to detect particle motion within the pericardial organ of the crab *Carcinus* while it was perfused with media with increased potassium concentrations. Replacement of some or all of the sodium ions of the normal medium with potassium ions caused a graded increase in motion. Evidence is presented which indicates that this effect is not a consequence of membrane depolarization or calcium ion influx, but is due to the flux of potassium chloride and water into the cytoplasm of the cells.**

Temporal analysis of intensity fluctuations of laser light scattered from moving light scatterers can yield information about the dynamics of the scatterers (1). Intensity fluctuation spectroscopy offers the possibility of studying *in vivo* the movements of intracellular macromolecules or organelles having dimensions below the resolution of the light microscope (2). Isotropic motion (either active movements or passive Brownian motion) causes intensity fluctuations that have a monotonically decreasing power spectrum, the width and amplitude of which are related to the velocity and extent, respectively, of particle motions.

Our interest in this technique derived from the report of Shaw and Newby (3) that the intensity fluctuations of laser light scattered from a locust ganglion showed a reversible increase when the sodium ions of the insect Ringer solution were exchanged for potassium ions. They suggested that nerve terminal depolarization caused a calcium ion influx, which liquified the axoplasm, thereby freeing the presynaptic vesicles to undergo Brownian motion. These results are of interest and are relevant to the problem of the mechanism of neurotransmitter release, because nerve terminal depolarization in the presence of calcium ions causes the release of neurotransmitters (4).

The observation of Shaw and Newby has been confirmed and extended by Piddington and Sattelle (5). They found, however, that omission of calcium ions from the medium had no effect on the potassium response (6). Despite this, they nevertheless attributed the scattering change to calcium entry.

We have investigated this phenomenon to determine whether it has any relevance to the mechanism of neurotransmitter release. The pericardial organ of the crab *Carcinus* was used. This is a specialized neurosecretory structure, the cortex of which is packed tightly with neurosecretory endings containing a variety of granules and vesicles (7). It usually has a bluish color when viewed under a dissecting lamp, probably due to light scattering by the granular inclusions of the nerve endings (8).

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## METHODS

Specimens of *Carcinus* were obtained from the Marine Biological Laboratory at Woods Hole and kept in a tank of artificial sea water at about 15°C. The pericardial organ was dissected from an animal in artificial sea water and transferred to a microscope slide to which it was fastened with Vaseline. The microscope slide formed the bottom of a perfusion chamber which could be covered and sealed with a cover slip. This chamber was then mounted on the stage of a specially modified Zeiss WL microscope (Fig. 1).

This microscope permits observation of preparations with the incandescent illuminator of the microscope as well as with laser light. The beam of a 4-mW He/Ne laser (Coherent Radiation model 80) passes through a lens and is reflected from a half-silvered mirror and focused onto the back focal plane of the microscope condenser (numerical aperture = 1.4). The beam then passes through the preparation at an angle determined by the lateral displacement of the beam off the optical axis of the microscope (9). This angle can be adjusted between 0° and almost 90° by means of the micrometer drive on the lens–mirror assembly.

An iris diaphragm in the image plane of the microscope can limit the field to an area 27 $\mu$m in diameter (using a 40× objective). This diaphragm and the image of the preparation can be observed through an ocular or, by removing a mirror from the system, the light scattered from the preparation can be passed through a pair of lenses that bring the back focal plane of the microscope objective (40×, numerical aperture = 0.65) to focus on an aperture in front of the cathode of a photomultiplier tube. The position of this aperture in the back focal plane determines the angle at which light is collected from the preparation by the photodetector. The size of this aperture was selected so that a few coherence areas (1) fall on the detector. By adjusting the position of this aperture and the position of the lens-mirror assembly, scattering angles ($\Psi$) from 0° to at least 90° can be obtained in practice (see Fig. 1). Angles were measured with a protractor placed on the microscope stage and corrected for refraction at interfaces.

In the experiments reported here the ac component of the photodetector output was amplified (Princeton Applied Research model 113) and then analyzed with a homemade spectrum analyzer similar in principle to that described by Clark *et al.* (10). The square of the analyzer output is proportional to the power in a narrow band (adjustable) around a selected frequency. The analyzer output could be monitored at a single frequency to follow the the time course of changes in the intensity fluctuations. The photodetector output was averaged by a low pass filter (time constant = 10 sec) and monitored to check for changes in the average scattered intensity. The laser

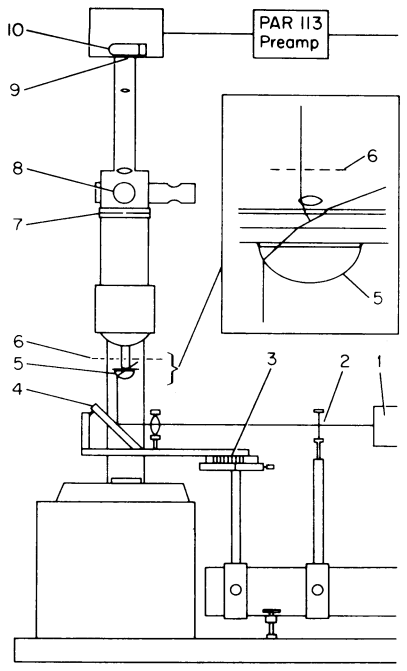


FIG. 1. Microscope modified for intensity fluctuation spectroscopy: 1, He/Ne laser with plane of polarization perpendicular to the paper; 2, attenuating filter; 3, lens–mirror assembly with micrometer drive; 4, half-silvered mirror; 5, microscope condenser lens; 6, back focal plane of objective lens; 7, iris diaphragm in the image plane; 8, ocular; 9, aperture in the back focal plane; 10, photomultiplier tube. *Inset* shows details of the scattering geometry. PAR, Princeton Applied Research.

intensity was adjusted by rotating a polarizing filter (Fig. 1) to maintain the average scattered intensity constant during an experiment. The amplified ac component of the photodetector output could be tape recorded (always using the same average scattered intensity) and played back through the spectrum analyzer repeatedly to construct a complete power spectrum. A digital voltmeter and a digital averaging circuit were used to average the analyzer output.

Noise in the laser output or the detection system was checked by directing the attenuated laser beam directly onto the aperture in front of the photomultiplier tube and measuring the power spectrum for an average light intensity equal to that used in experiments. This noise, though small, was subtracted from power spectra. The entire system was mounted on several heavy slabs of slate alternated with porous packing material or inner tubes to minimize vibration artifacts.

As an overall check of the system, intensity fluctuations of laser light scattered from a dilute suspension of carboxylated styrene butadiene spheres (approximately 2200-Å diameter, a gift of Polysar Corp., Sarnia, ON) were analyzed for different scattering angles ($\Psi$). Lorentzian-shaped curves with the minimum least squares error were fitted to the power spectra obtained. The halfwidths ($f_{1/2}$) of these curves are plotted in Fig. 2 against the square of the scattering vector, $|\vec{q}|$, given by

$$|\vec{q}| = \frac{4\pi n_0}{\lambda_0} \sin\frac{\Psi}{2} \qquad [1]$$

in which $n_0$ is the refractive index of the medium (1.33 for water) and $\lambda_0$ is the wavelength of light (6328 Å). The relationship expected for homodyne conditions (11),

$$f_{1/2} = \frac{D}{\pi} |\vec{q}|^2, \qquad [2]$$

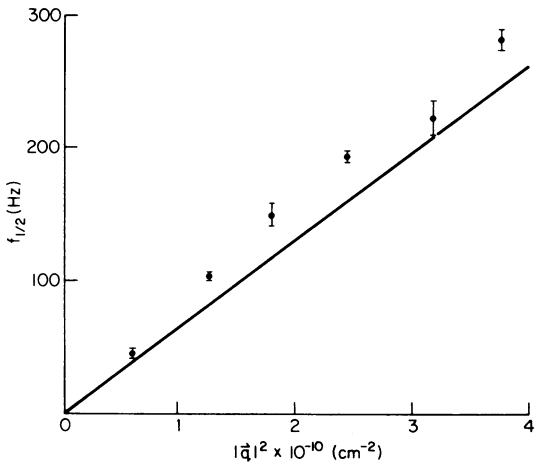


FIG. 2. The relationship between the halfwidth of the Lorentzian-shaped power spectrum and the square of the scattering vector, $|\vec{q}|$, for intensity fluctuations of laser light scattered from a dilute aqueous suspension of carboxylated styrene butadiene spheres, ~2200-Å diameter. Each data point shows the mean and SD for three measurements. The line gives the relationship expected from theory for the diffusion coefficient, $D = 2.1 \times 10^{-8}$ cm$^2$/sec, calculated from the Stokes–Einstein equation.

in which $D$ is the translational diffusion coefficient calculated from the diameter of the spheres and the Stokes–Einstein equation, is shown by the line in Fig. 2. The deviations of the data from this line are probably due to nonlinearity and uneven frequency response of the spectrum analyzer, but this inaccuracy will not affect the essentially qualitative conclusions made here.

The normal perfusion medium used was the same as that used by Cooke (8): 448 mM NaCl/20.5 mM $Na_2SO_4$/ 11 mM KCl/13 mM $CaCl_2$/24 mM $MgCl_2$/9 mM boric acid and was brought to pH = 7.6 with concentrated NaOH. Sodium ions were replaced with $K^+$ to increase the $K^+$ concentration. In some experiments most of the $Cl^-$ was replaced with methanesulfonate ions to maintain the $[K^+][Cl^-]$ product approximately the same as that of the normal perfusion medium. In other experiments the $Ca^{2+}$ was omitted and the $Mg^{2+}$ concentration was increased to 32 mM and 5 mM disodium ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) was added. All preparations were perfused with the normal perfusion medium for about 30 min before perfusion with test solutions.

Some experiments were done at room temperature (about 23°C), while other experiments were done at a reduced temperature by precooling the medium before it entered the perfusion chamber with a thermoelectric cooling device (Cambion thermoelectrics) to maintain the temperature of the chamber as measured with a thermistor at about 16°C. The experimental results were essentially the same at the two temperatures.

In all experiments reported here scattered laser light was collected from an area of about 30 $\mu$m diameter near the edge of the ventral trunk of the pericardial organ, just posterior to the anterior bar (12).

## RESULTS

Perfusion of the laser-illuminated pericardial organ with a medium with an increased $K^+$ concentration caused the preparation to twinkle much more extensively than normally when observed through the microscope ocular. This was observed by Shaw and Newby (3) in the locust ganglion. Fig. 3 shows that there was much greater intensity fluctuation with

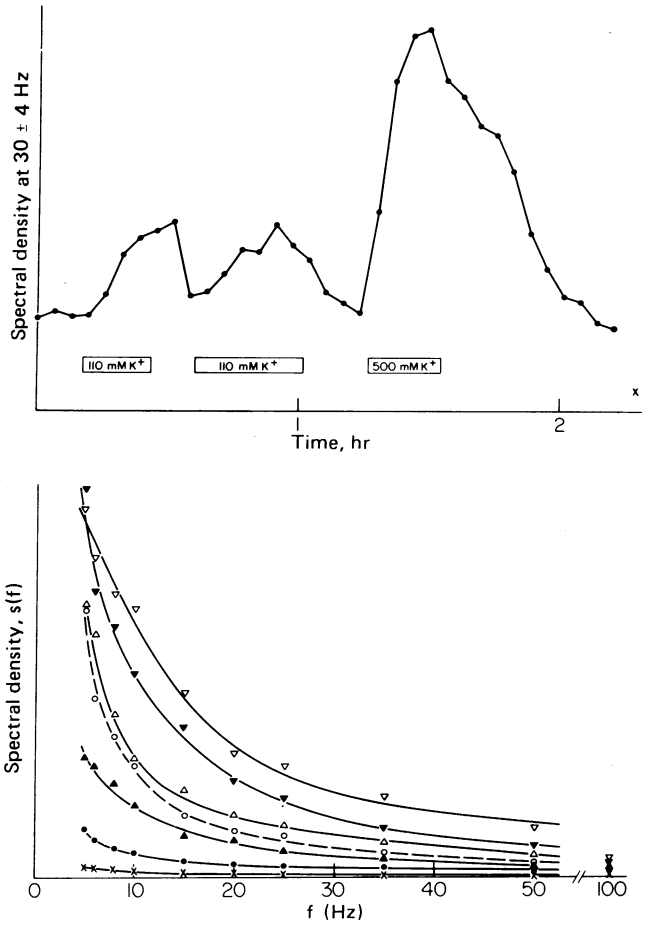


FIG. 3. The effect of elevated $K^+$ concentrations on the intensity fluctuations of laser light scattered from the pericardial organ. (*Upper*) The square of the analyzer output was monitored to record changes in the power spectral density at 30 ± 4 Hz. The perfusion medium was the normal medium except for periods indicated, when the $K^+$ concentration was increased to 110 or 500 mM. At the end of the experiment the preparation was perfused with 95% ethanol (×). $\Psi = 90°$, room temperature. (*Lower*) The intensity fluctuations from a different preparation were tape recorded and complete power spectra were measured for the pericardial organ initially in normal medium (●) and in media with elevated $K^+$ concentrations: 110 mM (▲), 220 mM (△), 330 mM (▼), and 500 mM (▽). Twenty-five minutes elapsed after each solution change before the fluctuations were recorded. The preparation recovered partially after 90 min in normal medium (○, broken line) and then was perfused with 95% ethanol (×). The average scattered light intensity was the same in each case. $\Psi = 87°$; temperature = 16.5°C.

increased $K^+$ concentration, indicating more extensive motion. Increased $K^+$ concentrations sometimes caused changes in the average scattered intensity, but these changes were not observed consistently and the laser intensity was always adjusted to maintain the average scattered intensity constant. The amount of intensity fluctuation was graded over a wide range of $K^+$ concentrations (Fig. 3). While the effect was largely reversible after one or two challenges with 500 mM $K^+$ (Figs. 3 *upper* and 5), it was usually only partially, if at all, reversible after repeated or prolonged exposure to 500 mM $K^+$ (Fig. 3 *lower*). Fig. 3 also shows that fixation with ethanol decreased the intensity fluctuations to a level below that measured in normal medium.

The intensity fluctuation spectra for a pericardial organ in normal medium and in 500 mM $K^+$ at different scattering angles ($\Psi$) are given in Fig. 4. Lorentzian-shaped curves were fitted to these spectra. The interpretation of the data is com-

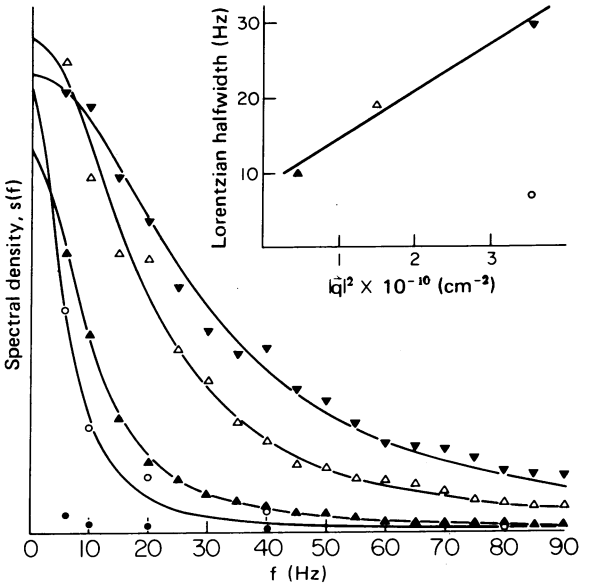


FIG. 4. The dependence of intensity fluctuation spectra on the scattering angle ($\Psi$) for a pericardial organ in normal medium [$\Psi$ = 29° (●) and $\Psi$ = 91° (○)] and in medium with 500 mM $K^+$ [$\Psi$ = 29° (▲), $\Psi$ = 55° (△), and $\Psi$ = 91° (▼)]. The lines drawn through the points are Lorentzian-shaped curves fitted to the data by a least-squares procedure. The *Inset* gives the halfwidths of these curves as a function of the square of the scattering vector, $|\tilde{q}|$.

plicated in a system such as living tissue where light scatterers may be anisotropic, polydisperse, concentrated and bound. Nevertheless, for intensity fluctuations arising from translational or rotational motion, the halfwidths of the power spectra should be smaller for smaller scattering angles and should approach zero for very small scattering angles (11). The power spectra for the pericardial organ in normal medium and in 500 mM $K^+$ are more narrow at smaller scattering angles, but plots of $f_{1/2}$ versus $|\tilde{q}|^2$ do not extrapolate through the origin. This may be a consequence of poor definition of the scattering angle due to refraction of the laser beam by structures within the preparation. Intensity fluctuations with power spectra independent of $|\tilde{q}|$ may arise from fluctuations in optical polarizability of nonmoving scatterers. It is possible that such fluctuations contribute to the observed spectra, but the intensity fluctuations from the pericardial organ must be due at least partly to motion of light scatterers.

In Fig. 4 the halfwidth of the Lorentzian-shaped curve fitted to the power spectrum for 500 mM $K^+$ medium is greater than that for normal medium for the same scattering angle. While the analysis is limited by the absence of spectral information below 5 Hz, this was also the case in each of three other experiments analyzed this way, including the experiments of Figs. 3 *lower* and 6 *lower*. The apparently broader spectra for preparations in 500 mM $K^+$ medium could be interpreted as indicating that intracellular particles were diffusing in a less viscous environment or were otherwise less constrained than when the preparation was in normal medium.

The release of neurosecretory material from the pericardial organ in response to electrical stimulation requires the presence of $Ca^{2+}$ in the bathing medium (13). However, the increase in motion caused by increased $K^+$ was found in solutions in which the $Ca^{2+}$ concentration was extremely low (<10 nM) (Fig. 5). The effect cannot be attributed to the influx of $Ca^{2+}$ into the cells.

An increase in the $K^+$ concentration increases the $[K^+]\,[Cl^-]$ product of the medium and this usually leads to an entry of KCl

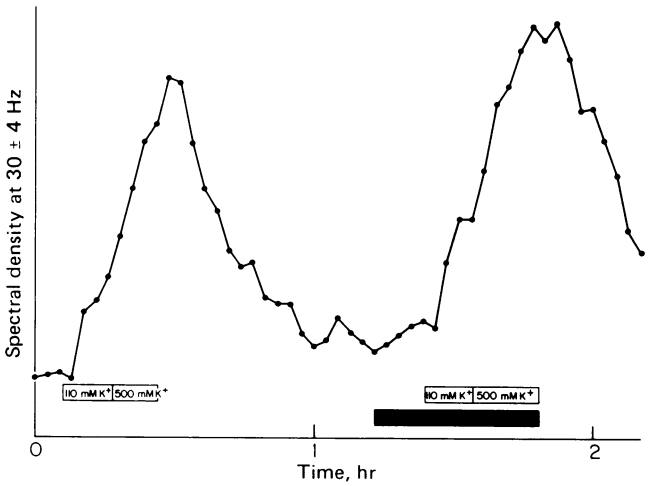


FIG. 5. The effect of calcium omission on the $K^+$-induced increase in motion in the pericardial organ. The plot is similar to that of Fig. 3 *upper* except that during the period indicated by the solid bar $Ca^{2+}$ was omitted from the medium and 5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid was present. $\Psi = 88°$; temperature = 17°C.

into excitable cells (14). This entry of ions would be accompanied by water entry and the cytoplasm would swell. When the $Cl^-$ concentration of the high $K^+$ medium was reduced by replacement with the relatively impermeant methanesulfonate ion to maintain $[K^+][Cl^-]$ approximately constant, only a very small change in the intensity fluctuations was seen (Fig. 6). This $KMeSO_3$ medium should depolarize cell membranes without causing appreciable swelling (15). When the normal $Cl^-$ concentration was reintroduced, however, a typical increase in intensity fluctuations was observed, apparently due to the entry of KCl (and water) into the cells. This effect was reversed by again reducing the $Cl^-$ concentration. The intensity fluctuation increase could also be produced by perfusing the pericardial organ with hyposmotic solutions (Fig. 6).

## DISCUSSION

The apparatus described here detects relative movements of light scatterers, regardless of their size, over distances greater than about $1/|\vec{q}|$ ($\simeq 0.05$ μm) in time periods of 200 msec or less. The data indicate that there is more extensive and possibly more rapid motion within the pericardial organ over distances greater than about $1/|\vec{q}|$ when the cytoplasm is swollen either by increasing the $K^+$ concentration in the presence of high $Cl^-$ concentration or by diluting the normal medium. The relatively small difference in intensity fluctuations between the pericardial organ in normal medium and fixed in ethanol suggests that diffusion of light scatterers is normally rather limited in the cytoplasm. Inclusions may be essentially bound in a cytoplasmic gel (16) and able to undergo only limited displacements over distances greater than about 0.05 μm. A microfilamentous network surrounding synaptic vesicles has been described in presynaptic terminals in rat brain (17). In the pericardial organ, swelling probably causes a dilution and disruption of the cytoplasmic gel, allowing granules, vesicles, and other cytoplastic materials to undergo larger diffusive displacements. Swelling may break crosslinks in the cytoplasm, resulting in a looser structure with decreased viscosity. This effect may be similar to the sol-to-gel transformation of actin/heavy meromyosin mixtures, where larger and more rapid intensity fluctuations are measured in the presence of ATP (18).

Piddington and Sattelle (5) reported that dilution of the

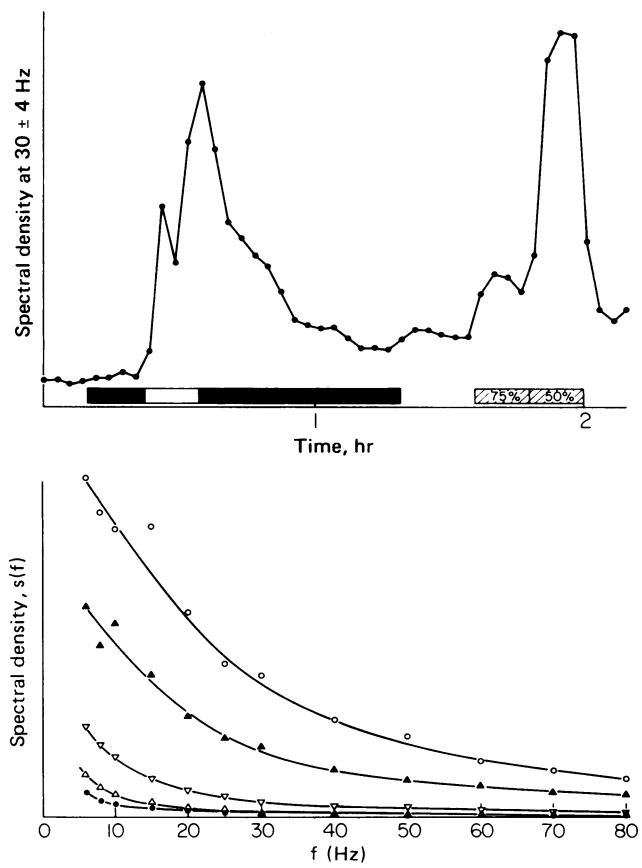


FIG. 6. The effects of $Cl^-$ substitution and of hyposmotic solutions on the intensity fluctuations of laser light scattered from the pericardial organ. (*Upper*) A plot similar to that in Fig. 3 *upper*. During periods of time indicated by the solid bars 500 mM $K^+$ and only 12 mM $Cl^-$ were present. The normal $Cl^-$ concentration, 533 mM, was present along with 500 mM $K^+$ during the period indicated by the open bar. The normal medium was diluted to 75% and subsequently to 50% with distilled water during the periods of time indicated by the hatched bars. $\Psi = 89°$, room temperature. (*Lower*) Power spectra for another experiment like that in *upper*. The preparation, initially in normal medium (●), was perfused with a high $K^+$, low $Cl^-$ medium (△) and then with a high $K^+$, high $Cl^-$ medium (▲). The preparation recovered partially in high $K^+$, low $Cl^-$ medium (▽) and then was perfused with the normal medium diluted to 50% (○). $\Psi = 79°$, room temperature.

normal insect Ringer to 75% of its normal osmolarity had no effect on motion recorded from the locust ganglion and discounted a swelling mechanism to explain the $K^+$ response. These workers, however, did not reduce the $Cl^-$ of the medium when elevating the $K^+$ concentration, so that a swelling mechanism cannot be excluded. The $K^+$ response is not dependent on $Ca^{2+}$ in the bathing medium in either the pericardial organ or the locust ganglion (6) and the response is not a result of membrane depolarization in the pericardial organ. It is therefore not likely that this phenomenon is related to the mechanism of neurotransmitter release (4).

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