Modulation of Cardiac Autonomic Neurotransmission by Epicardial Superfusion
Effects of Hexamethonium and Tetrodotoxin

Toshihisa Miyazaki, Harald P. Pride, and Douglas P. Zipes

The heart contains superficial cardiac nerves whose effects may be modulated by pericardial fluid bathing the epicardium. We tested this hypothesis in open-chest dogs anesthetized with secobarbital. Oxygenated normal Tyrode's solution (NT) or NT containing hexamethonium, a ganglionic blocker (500 μM), or tetrodotoxin, a blocker of axonal neurotransmission (5 μM, TTX), was instilled into the pericardial cavity to superfuse the epicardium of the whole heart. During each superfusion, effective refractory period (ERP) was determined in deep intramycocardium (≥4 mm in depth from the epicardium) of anterior and posterior left ventricle and in the subendocardium of the right ventricle in the baseline state and during bilateral cervical vagal stimulation (VS) or ansae subclaviae stimulation (SS). Lengthening of ERP induced by VS during superfusion with NT (6.9±0.3 msec, mean±SEM, n=36) was eliminated during subsequent superfusion with hexamethonium (0.9±0.5 msec, p<0.001). Hexamethonium also prevented sinus arrest induced by VS but did not affect shortening of ERP induced by SS (17.3±1.3 to 16.6±1.0 msec, n=26). TTX suppressed VS-induced changes in ERP (6.3±0.3 to 1.5±0.5 msec, n=32, p<0.001) and SS-induced changes in ERP (18.8±1.6 to 6.0±0.9 msec, n=23, p<0.001) but did not affect changes in ERP induced by intravenous administration of norepinephrine or methacholine. This indicates that the suppressive effect of TTX on neurally induced changes in ERP was due to an inhibition of neurotransmission and not on the response of the effector site to the neurotransmitter. We conclude that 1) vagal ganglia that innervate both ventricles as well as sinus node are distributed superficially and are blocked by epicardial superfusion with hexamethonium, 2) the epicardium lacks sympathetic ganglia, and 3) both vagal and sympathetic axons at some point travel superficially during their course in the heart and are blocked by TTX. These results suggest that substances in the pericardial fluid, whether normally secreted or present due to disease, have the potential to modulate autonomic neural transmission to the heart. (Circulation Research 1989;65:1212-1219)
be analogous to superfusing isolated nerves in a tissue bath.

In the present study, we tested this hypothesis by measuring the response of efferent vagal- and sympathetic-induced changes in ventricular refractoriness during epicardial superfusion with hexamethonium, a ganglionic blocker, and tetrodotoxin, a blocker of axonal neurotransmission.

**Materials and Methods**

**Surgical Preparation**

Forty-four mongrel dogs of either sex weighing 16–30 kg were anesthetized with secobarbital (30 mg/kg i.v.). Additional doses were injected as needed to maintain anesthesia. Dogs were intubated and ventilated with room air by use of constant volume-cycled respirator (model 607, Harvard Apparatus, South Natick, Massachusetts). A fluid-filled cannula placed in the right femoral artery was connected to a transducer (Statham p-23Db, Gould, Cleveland, Ohio) to monitor arterial pressure, and a femoral venous cannula was used to infuse normal saline at 100–200 ml/hr to replace spontaneous fluid losses. Lead II electrocardiogram was monitored throughout the study. In 12 dogs, right atrial pressure was measured through a venous cannula. The chest was opened through a median sternotomy, and a small incision was made on the anterior surface of the pericardium. The edges of the incision were tied with sutures at four points so that tension applied to the sutures produced a square opening approximately 2.5x2.5 cm. This provided a pericardial cavity sufficient to contain 45-120 ml (mean, 84 ml) of the solution to bathe the epicardium of the heart (Figure 1). The entire epicardial surface of the heart was superfused with the solution instilled into the pericardial cavity. This was confirmed by the staining of the entire epicardial surface with methylene blue instilled into the pericardial cavity.

Through the pericardial opening, four hook electrodes made from Teflon-coated wires, insulated except for their tips, were inserted in the anterior and posterior myocardium of the basal and apical left ventricle to a depth of 4–6 mm, and two additional electrodes were placed in the subendocardium of the right ventricular outflow tract and apex. These electrodes served as the cathode for unipolar stimulation to determine the ventricular effective refractory period (ERP). An anodal electrode was placed in the abdominal wall. Two bipolar plunge electrodes were placed in the atrial appendage and in the ventricle to record activation. A thermistor (model 430, series 400, Yellow Springs Instrument, Yellow Springs, Ohio) was used to monitor epicardial temperature, which was maintained between 36° and 38° C by adjusting the proximity of an operating table lamp.

**Measurement of Effective Refractory Period**

The ERP was determined at six test sites by the extrastimulus technique with a programmable stimulator (Kranert Medical Engineering, Indianapolis, Indiana) and a constant current isolator. Each ventricular test site was driven with a 2-msec rectangular stimulus twice the diastolic threshold, which was measured during each intervention. A train of eight stimuli (S1) was followed by a late premature stimulus (S2) that produced a propagated response. The S1-S2 interval was 240–250 msec in dogs tested for sympathetic response and 280–300 msec in dogs tested for vagal response and was kept constant throughout each experiment. The ventricular response to S2 was recorded in lead II electrocardiogram and from the bipolar ventricular electrode and displayed on a storage oscilloscope. The S1-S2 interval was shortened in 2-msec steps until S2 failed to produce a propagated response. The S1-S2 interval was then increased by 5 msec and was shortened by 1-msec decrements until S2 failed to produce a propagated response. The ERP was defined as the longest S1-S2 interval at which S2 failed to produce a propagated response. The ERP was determined twice at each test site. If the values were not within 1 msec of each other, the data were discarded and the determination was repeated.

**Neural Stimulation**

Decentralized bilateral ansae subclaviae were stimulated with shielded bipolar electrodes placed on the anterior and posterior ansae; separate constant current isolators driven by a programmable stimulator (Pulsar 4, Frederick Haer, Brunswick, Maine)
Experimental Protocols
during neural stimulation. It took about 15 minutes
ERP was determined at six test sites before and
minute period of each epicardial superfusion, the
subgroups in which either the efferent sympathetic
control and test groups. Each group contained two
three instillations of NT; test dogs received NT, test
pericardial cavity, allowed to remain 60 minutes,
tension reached about 400 mm Hg before they were
The effects of vagal stimulation were determined
during intravenous infusion of norepinephrine at a
stant background of sympathetic effect. The ERP
determined during norepinephrine infusion served as
control for the determination of the efferent vagal
response was tested. After a 45-
were used. Stimuli were rectangular 4-msec pulses
at a frequency of 2–4 Hz and 2–4 mA.
Both cervical vagi were stimulated through two
Teflon-coated wire electrodes embedded in the car-
diac end of each vagal nerve surgically interrupted
in the neck. Rectangular pulses of 4-msec duration
were delivered at a frequency of 20 Hz by use of
separate constant-current isolators. The current
strength was 0.05 mA greater than that required to
produce sinus arrest (>2 seconds) for the right vagus
and complete atrioventricular block for the left vagus.
The effects of vaginal stimulation were determined
during intravenous infusion of norepinephrine at a
constant rate of 0.125 µg/kg/min to achieve a con-
stant background of sympathetic effect. The ERP
was not changed 15 minutes after instillation of
NT into the pericardial cavity (Table 1). Mean
right atrial pressure was increased slightly. Base-
line ERP, measured at 20 ventricular test sites in
of six dogs, the effect of intravenous administration
of norepinephrine (0.25 µg/kg/min) or meth-
acholine (12.5 or 25 µg/kg/min) on the ERP was
examined to determine whether the ventricular myo-
cardium was normally responsive during epicardial
superfusion with tetrodotoxin. In these dogs,
changes in ERP induced by stimulation of ansae
subclaviae or cervical vagi were also measured
during epicardial superfusion with NT and during
subsequent superfusion with tetrodotoxin.
Also, the effect of epicardial superfusion with
tetrodotoxin on the fast component of local bipolar
electrograms recorded from subepicardial muscle
(<1 mm in depth from the epicardium) and intram-
ycardium (4–6 mm) of the left ventricle was deter-
ned in three dogs.
In three other dogs, NT containing fluorescein
(10⁻⁴ or 10⁻³ M) was instilled into the pericardial
cavity, and plasma concentration of fluorescein at
45 minutes after the onset of superfusion was mea-
sured by fluorescence spectrophotometer (model
MPF-66, Perkin-Elmer, Norwalk, Connecticut).

Analysis of Data
Data from the test site with less than 9 msec
shortening of ERP induced by bilateral ansae subcla-
vae stimulation or less than 3 msec lengthening of
ERP induced by bilateral vagal stimulation during
the first control superfusion with NT were discarded
because of possibly insufficient neural effect at that
particular site for the accurate detection of alter-
ations in neurotransmission by the following epicar-
dial superfusion. This resulted in exclusion of data
from a total of 15 sites. Data are expressed as
mean±SEM. Repeated-measures ANOVA was used.
When multiple comparisons were made, a modified t
test by Bonferroni was used. A statistical signifi-
cance was set at a value of p<0.05.

Results
Effects of Epicardial Superfusion on
Hemodynamic Parameters and Ventricular Refractoriness

Heart rate and mean arterial blood pressure
were not changed 15 minutes after instillation of
NT into the pericardial cavity (Table 1). Mean
right atrial pressure was increased slightly. Bas-
line ERP, measured at 20 ventricular test sites in

<table>
<thead>
<tr>
<th>TABLE 1. Effects of Epicardial Superfusion With Tyrode’s Solution on Hemodynamic Parameters and Ventricular Refractoriness</th>
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<tbody>
<tr>
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<tr>
<td>Heart rate (/min) (N=12)</td>
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<tr>
<td>Mean arterial pressure (mm Hg) (N=12)</td>
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<tr>
<td>Mean right atrial pressure (mm Hg) (N=12)</td>
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<tr>
<td>Baseline ERP (msec) (n=20)</td>
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</table>

Values are mean±SEM. ERP, effective refractory period of the ventricular test sites; N, number of test dogs; n, number of test sites; NS, not significant.

Solutions
Normal Tyrode’s solution (NT) was used for con-
trol epicardial superfusion. The composition of the
solution was (mM) MgCl₂ 0.5, NaH₂PO₄ 0.9, CaCl₂
2.0, NaCl 137.0, NaHCO₃ 12.0, KCl 4.0, and glucose
5.0. This resulted in a pH of 7.35. Test solution was
prepared by adding hexamethonium (5x10⁻⁴ M; Sigma
Chemical, St. Louis, Missouri) or tetrodo-
toxin (5x10⁻⁶ M; Sigma Chemical) to the NT. The
solutions were prewarmed on a hot plate stirrer to
36–38°C and gassed with 95% O₂-5% CO₂; oxygen
tension reached about 400 mm Hg before they were
instilled into the pericardial cavity.

Experimental Protocols
In each dog, the solution was instilled into the
pericardial cavity, allowed to remain 60 minutes,
and then removed by suction. Control dogs received
three instillations of NT; test dogs received NT, test
solution, and then NT again, separated by 10-
minute intervals. The dogs were randomized to
control and test groups. Each group contained two
subgroups in which either the efferent sympathetic
or efferent vagal response was tested. After a 45-
minute period of each epicardial superfusion, the
ERP was determined at six test sites before and
during neural stimulation. It took about 15 minutes
to determine each set of ERPs. In a separate group

of six dogs, the effect of intravenous administration
of norepinephrine (0.25 µg/kg/min) or meth-
acholine (12.5 or 25 µg/kg/min) on the ERP was
examined to determine whether the ventricular myo-
cardium was normally responsive during epicardial
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ERP induced by bilateral vagal stimulation during
the first control superfusion with NT were discarded
because of possibly insufficient neural effect at that
particular site for the accurate detection of alter-
ations in neurotransmission by the following epicar-
dial superfusion. This resulted in exclusion of data
from a total of 15 sites. Data are expressed as
mean±SEM. Repeated-measures ANOVA was used.
When multiple comparisons were made, a modified t
test by Bonferroni was used. A statistical signifi-
cance was set at a value of p<0.05.

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Heart rate and mean arterial blood pressure
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TABLE 2. Baseline Effective Refractory Period of the Ventricular Test Sites

<table>
<thead>
<tr>
<th></th>
<th>First superfusion</th>
<th>Second superfusion</th>
<th>Third superfusion</th>
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<tbody>
<tr>
<td>Vagal Study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group (n=35)</td>
<td>145±1</td>
<td>146±1</td>
<td>146±2</td>
</tr>
<tr>
<td>Hexamethonium group (n=36)</td>
<td>142±2</td>
<td>145±2</td>
<td>142±2</td>
</tr>
<tr>
<td>Tetrodotoxin group (n=32)</td>
<td>154±1</td>
<td>159±2*</td>
<td>153±2</td>
</tr>
<tr>
<td>Sympathetic study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group (n=25)</td>
<td>160±1</td>
<td>160±1</td>
<td>163±3</td>
</tr>
<tr>
<td>Hexamethonium group (n=26)</td>
<td>162±2</td>
<td>165±2</td>
<td>167±2*</td>
</tr>
<tr>
<td>Tetrodotoxin group (n=23)</td>
<td>159±3</td>
<td>159±3</td>
<td>158±3</td>
</tr>
</tbody>
</table>

Values are mean±SEM in msec. In test groups, the second epicardial superfusion was done with test solution containing hexamethonium or tetrodotoxin. n, number of test sites.

*p<0.05 vs. the values during the first superfusion with normal Tyrode’s solution.

Effects of Epicardial Superfusion With Hexamethonium and Tetrodotoxin on Changes in Ventricular Refractoriness Induced by Vagal Stimulation

Lengthening of ERP induced by bilateral vagal stimulation during the first control superfusion with NT was suppressed after a 45-minute period of epicardial superfusion with hexamethonium and tetrodotoxin (Figure 2). Although the baseline ERP during superfusion with tetrodotoxin was prolonged as a whole compared with that during the first control superfusion with NT (Table 2), it was constant (≤2-msec change) at 10 of 32 sites (151±3 to 151±2 msec). At these 10 sites, lengthening of ERP induced by vagal stimulation was suppressed by superfusion with tetrodotoxin (5.6±0.4 to 1.3±0.7 msec, p<0.001) as well as at the other 22 sites (6.7±0.3 to 1.6±0.7 msec, p<0.001). Significant recovery of vagal-induced lengthening of ERP was noted after removal of either test solution from the pericardial cavity and a 45-minute period of subsequent superfusion with NT. However, the changes in ERP did not quite reach the control values obtained during the first superfusion with NT. In six control group dogs, vagal-induced lengthening of ERP was not changed through three 45-minute periods of epicardial superfusion with NT.

Effects of Epicardial Superfusion With Hexamethonium on Changes in Ventricular Refractoriness at Each Test Site Induced by Vagal Stimulation

Vagal-induced lengthening of ERP during epicardial superfusion with NT at test sites located in the anterior and posterior left ventricle and right ventricle was eliminated or attenuated during subsequent superfusion with hexamethonium (Figure 3).

Effects of Epicardial Superfusion With Hexamethonium on Sinus Arrest Induced by Vagal Stimulation

Sinus arrest (>2 seconds) induced by bilateral vagal stimulation during the first epicardial superfusion with NT was prevented by subsequent superfusion with hexamethonium in all six test dogs. After removal of the hexamethonium solution and a 45-minute period of subsequent superfusion with NT, sinus arrest was induced again by vagal stimulation in five of six test dogs. A representative example is shown in Figure 4.
Effects of Epicardial Superfusion With Hexamethonium and Tetrodotoxin on Changes in Ventricular Refractoriness Induced by Ansae Subclaviae Stimulation

Shortening of ERP induced by bilateral ansae subclaviae stimulation during the first epicardial superfusion with NT was suppressed after a 45-minute period of subsequent superfusion with tetrodotoxin (Figure 5). Significant recovery was noted after removal of the tetrodotoxin solution from the pericardial cavity and a 45-minute period of subsequent superfusion with NT. Three 45-minute periods of epicardial superfusion with NT in five dogs and with NT-hexamethonium-NT in five test dogs did not alter shortening of ERP induced by ansae subclaviae stimulation.

Effects of Epicardial Superfusion With Tetrodotoxin on Changes in Ventricular Refractoriness Induced by Intravenous Administration of Norepinephrine and Methacholine and on Ventricular Electrograms

In three dogs, shortening of ERP induced by intravenous infusion of norepinephrine (0.25 μg/kg/min) during epicardial superfusion with NT was not changed by subsequent superfusion with tetrodotoxin despite a significant attenuation of the response to bilateral ansae subclaviae stimulation (Figure 6). Also, lengthening of ERP induced by methacholine (12.5 or 25 μg/kg/min) remained unchanged by superfusion with tetrodotoxin despite a significant attenuation of the response to vagal stimulation in three other dogs (Figure 6).

Supерfusion with tetrodotoxin for 45 minutes did not affect the rate of change (dV/dt) of the fast component of bipolar electrograms recorded from subepicardial muscle (903±259 to 916±262 mV/sec, n=3) and deep intramyocardium of the left ventricle (1,597±247 to 1,606±247 mV/sec, n=3).

Systemic Absorption of Fluorescein From the Pericardial Cavity

Plasma level of fluorescein at 45 minutes after instillation of NT containing fluorescein (10⁻⁴ or 10⁻³ M) is shown in Table 3.

Discussion

Major Findings

The present data indicate that epicardial superfusion with hexamethonium prevented lengthening of ERP in deep ventricular myocardium and sinus arrest induced by cervical vagal stimulation. Hexamethonium did not suppress shortening of ERP elicited by ansae subclaviae stimulation. Epicardial superfusion with tetrodotoxin suppressed both vagal- and sympathetic-induced changes in ventricular ERP without affecting changes in ERP induced by intravenous administration of norepinephrine and methacholine. These effects of hexamethonium and tetrodotoxin were reversible after washout of the solution from the pericardial cavity.

Consideration of the Experimental Model

Using the present model, we attributed attenuation of the neurally induced responses to the effects of epicardial superfusion on neurotransmission through superficial cardiac nerves. Although it is possible that the hexamethonium and tetrodotoxin might have been absorbed into systemic circulation from the pericardial cavity via the pericardium and/or subepicardial capillaries and affected the deep myocardial test sites directly, this does not seem likely. Studies of pericardial transport function have shown very slow rates of absorption, about 1.3 ml/hr from the rabbit pericardial cavity. It took more than 24 hours for 2.0 ml phenolsulfonphthalein dye, whose molecular weight (354 MW) was comparable with that of hexamethonium (273 MW) and tetrodotoxin (319 MW), to be absorbed completely from the pericardial cavity in a

Downloaded from http://circres.ahajournals.org/ by guest on July 9, 2017
A normal Tyrode's solution

RA

RV

ECG II

V1

1 SEC

B hexamethonium

RA

RV

ECG II

V1

C normal Tyrode's solution

RA

RV

ECG II

V1

Figure 4. A representative example of recordings showing the effect of epicardial superfusion with hexamethonium on vagal-induced sinus arrest. Bipolar electrograms recorded from the right atrial appendage (RA) and right ventricle (RV) and electrocardiogram lead II (ECG II) are shown. During the first control superfusion with normal Tyrode's solution (panel A), bilateral cervical vagal stimulation (VS) with stimulation parameters set to produce sinus arrest (>2 seconds) for the right vagus (20 Hz, 4-msec duration, 0.25 mA) and complete atrioventricular block for the left vagus (20 Hz, 4-msec duration, 0.72 mA) caused sinus arrest. After 45 minutes of superfusion with hexamethonium (panel B), neither sinus arrest nor second degree or complete atrioventricular block was induced by VS at the same stimulation parameters. After a 45-minute period of the third superfusion with normal Tyrode's solution (panel C), sinus arrest was again induced by VS.

Figure 5. Bar graphs showing effects of epicardial superfusion with hexamethonium and tetrodotoxin (TTX) (abscissa) on changes in ventricular refractoriness induced by bilateral ansae subclaviae stimulation (ordinate). ERP, effective refractory period; NT, normal Tyrode's solution; n, number of test sites.

Effects of Epicardial Superfusion With Hexamethonium

Hexamethonium impairs ganglionic transmission by competing with acetylcholine for ganglionic cholinergic sites and prevents the development of postganglionic depolarization. It has no effect on nerve conduction or on the release of transmitter substance from the nerve terminals. Therefore, suppression, by epicardial superfusion with hexamethonium, of lengthening of ventricular refractoriness and sinus arrest elicited by vagal stimulation is likely due to a blockade of cardiac vagal ganglia. This suggests that vagal ganglia that innervate the ventricles are distributed superficially in a manner similar to the ganglia that selectively innervate the sinus and AV nodes. Recently, Blomquist et al presented data indicating that postganglionic vagal axons, arising from supraventricular ganglia, cross the AV groove to innervate the canine ventricles. Our data suggest that these ganglia must be located in the epicardial tissues. More precise localization requires further studies.

The fact that epicardial superfusion with hexamethonium did not block the efferent sympathetic response elicited by ansae subclaviae stimulation suggests that the epicardium lacks sympathetic gan-
TABLE 3. Plasma Fluorescein Level Determined by Fluorescence Spectrophotometry

<table>
<thead>
<tr>
<th>Fluorescein in the pericardial solution</th>
<th>Plasma fluorescein level</th>
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<tbody>
<tr>
<td>Dog 1</td>
<td>$10^{-4}$ M, $9.9\times10^{-8}$ M</td>
</tr>
<tr>
<td>Dog 2</td>
<td>$10^{-4}$ M, $9.7\times10^{-8}$ M</td>
</tr>
<tr>
<td>Dog 3</td>
<td>$10^{-3}$ M, $1.1\times10^{-6}$ M</td>
</tr>
</tbody>
</table>

glia. This result is consistent with the widely accepted concept that the peripheral ganglia of the sympathetic nerves are located near the spine and that the ganglia associated with nerves at the level of or within the heart usually are vagal.4

Effects of Epicardial Superfusion With Tetrodotoxin

In the present study, tetrodotoxin was used to block the neurotransmission in the axons, which it does by blocking the inward sodium current at doses that do not alter muscle depolarization or contractility.10,16,17 The suppressive effects of epicardial superfusion with tetrodotoxin on efferent autonomic responses in the ventricles suggest that both sympathetic and vagal efferent fibers are distributed superficially at some point in their course to the heart where they must come in contact with the pericardial fluid. These conclusions are concordant with previous studies using epicardial phenol applications, which established that vagal efferent fibers cross superficially at the atrioventricular groove and are then located subendocardially,18 whereas sympathetic efferent fibers are located in the superficial subepicardium of the ventricles.2

Implications of the Study

In the present study, we demonstrated that epicardial superfusion with solutions containing hexamethonium and tetrodotoxin could affect neural regulation of cardiac electrophysiological properties. This observation raises the distinct possibility that substances in the pericardial fluid, which are produced by the pericardium and/or epicardium normally or during disease, might also modulate cardiac autonomic neurotransmission in a similar way. For example, prostacyclin and prostaglandin E2, which are known to act as modulators of sympathetic neurotransmission,19-20,21 are produced by the pericardium in response to the changes in the loading conditions of the heart22-23 and may constitute a physiological feedback mechanism. It is quite possible that the pericardium plays an important, hitherto unrecognized, role in the physiology of cardiac autonomic neurotransmission.

Acknowledgment

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References

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**KEY WORDS** • autonomic ganglia • postganglionic axons • efferent sympathetic stimulation • efferent vagal stimulation • refractory period
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