SUMMARY  The action potential of guinea pig papillary muscle exposed to the ceveratum alkaloid germitrine (2 μM) is followed by a long-lasting after-depolarization (maximal amplitude, 8 mV; half-time of decay, 32 seconds; total duration, ~75 seconds). This after-depolarization interrupts the terminal phase of repolarization. During repetitive stimulation (0.1-1.0 Hz; 80 nM germitrine) the after-depolarizations that follow consecutive action potentials are summed, causing persistent depolarization of up to 10 mV. The after-depolarization is reversibly abolished by tetrodotoxin (TTX). Test contractions evoked at various times during or after the germitrine-induced after-depolarization reveal a phase during which the ability of the muscle to develop force is transiently increased. This positive inotropic influence reaches its maximum 1 minute after the conditioning stimulus and thereafter decays with a half-time of 4.8 times longer than the half-time of decay of the after-depolarization. It is reversibly abolished by TTX and augmented by dihydro-ouabain (DHO). We conclude: Germitrine induces an after-depolarization by prolonging dramatically the Na permeability component which is mediated by the fast Na channels and normally restricted to the first few milliseconds of the action potential. The germitrine-induced selective and persistent increase of sarcolemmal sodium permeability (PNa) causes a positive inotropic effect, probably because intracellularly accumulating Na ions exchange for extracellular Ca ions.

TWO DIFFERENT mechanisms are currently thought to play a major role in determining the inotropic state of the cardiac muscle cell: inward Ca current during depolarization, and Na-Ca exchange across the sarcolemma. The latter concept predicts that a change in the Na concentration gradient across the cell membrane alters the transsarcolemmal distribution of Ca. For instance, an increase of [Na] at constant [Na], should increase [Ca]. Changes of [Na], by their effect on Na-Ca exchange, may be responsible for the positive inotropic response of cardiac muscle to increased stimulation rates or to cardiac glycosides. With regard to the functional significance of Na-Ca exchange it seemed desirable to test how an intervention which selectively increases the Na permeability (PNa) of the sarcolemma affects contractile force.

The ceveratum alkaloid veratridine increases PNa of excitable membranes by a specific interaction with the (fast) Na channels. The tetrodotoxin-sensitive prolongation of the cardiac action potential by veratrine or veratridine indicates that a drug-induced increase of PNa occurs during each excitation. If an increased sarcolemmal PNa causes a positive inotropic effect via accumulation of Na ions inside the cell, the inotropic response should lag behind the increase in PNa. The test of this prediction is complicated, however, by the relatively short duration of the Na permeability increase associated with veratrine or veratridine. The present publication shows that the germin triester germitrine, unlike monoesters such as veratridine or cevadine, or veratrine, which contains several monoesters, causes an after-depolarization in guinea pig papillary muscle that lasts longer than 1 minute and is due to an increased PNa. By choosing an appropriate concentration of the alkaloid, the amplitude of the after-depolarization can be adjusted to a level which is well below the known voltage threshold of slow inward Ca current and which leaves the muscle electrically excitable throughout the course of the after-depolarization. This allows one to ascertain the inotropic state of cardiac muscle during a long-lasting, selective increase of sarcolemmal Na permeability.

Methods

Guinea pigs (250-350 g) were killed by a sharp blow on the skull. Right ventricular papillary muscles were rapidly excised from the hearts and mounted in a two-chambered vessel with an internal circulation of bath solution (50 ml) as described in detail earlier. The muscles were mounted either vertically for the measurement of mechanical activity only or horizontally for the simultaneous measurement of mechanical and transmembrane electrical activity. To ensure adequate oxygenation of the preparation only muscles with a diameter of less than 1.0 mm were used. The bath solution was a modified Krebs-Henseleit solution of the following composition (mM): NaCl, 115; NaHCO3, 25; KCl, 4.7; KH2PO4, 1.2; CaCl2, 3.2; MgSO4, 1.2; glucose, 10; pH 7.5. In some experiments the CaCl2 concentration was increased to 5.0 mM, in others it was reduced to 1.6 mM. In some instances the KCl concentration was increased to 7.8 mM. The solutions were gassed continuously with 95% O2-5% CO2, and temperature was maintained at 35°C.

The muscles were stimulated electrically by rectangular pulses 1-2 msec in duration through two punctate platinum electrodes positioned close to the base of the muscle.
Unless otherwise mentioned the frequency of stimulation was 1 Hz and intensity was 1.1-1.2 times the excitation threshold. Force was recorded isometrically by means of an inductive force transducer (Q 11, 10 p; Hottinger Baldwin Messtechnik) connected to an oscilloscope and a pen recorder. The resting force was kept constant throughout the experiments at 0.4 g. An equilibration period of at least 1 hour at a contraction frequency of 1 Hz preceded each experiment.

The following parameters of the isometric contraction records were measured: force of contraction ($F_c$) = peak of developed force; time to peak force ($t_1$); relaxation time ($t_2$); total contraction time ($t_1 + t_2$); isotropic effect ($\Delta F_c$) = change of force of contraction; rate of development of force ($S_1$); kinetics effect ($\Delta S_1$) = change in rate of force development. We measured $t_1$ and $t_2$ at the 10% level of force of contraction. $S_1$ was determined as 50% of $F_c$ divided by the time elapsing between the 25% and 75% levels of force of contraction during the ascending phase. Most of the isotropic interventions reported will be characterized by the change in force of contraction, $\Delta F_c$; since positive isotropic effects were never accompanied by an increase in $t_1$ but usually by a decrease in this parameter, they reflect exclusively positive kinotrophic effects, i.e., increases in the degree, and not duration, of activation of the contractile elements.

Transmembrane electrical activity was recorded with conventional glass microelectrodes which were filled with 3 M KCl and had tip resistances of 10-30 MΩ. The tip potentials were less than 10 mV. Transmembrane potentials were measured by means of an electrometer amplifier providing capacity compensation (M 701, W-P Instruments) and were displayed on one or two oscilloscopes. Photographs were obtained and analyzed after magnification. Maximum rate of rise of the action potential upstroke was determined as the slope of a straight line drawn through the steepest part of the upstroke (sweep speed = 2 cm/msec). Only cells near the base of the muscle were impaled; the opposite end of the preparation was stimulated through a bipolar platinum electrode.

**DRUGS**

The following drugs were used: germitrine (Ayerst), tetrodotoxin (TTX) (Sankyo), dihydro-ouabain (DHO) (Hommel), reserpine (Merck). Germitrine is germine 3-ylbutyrate. Stock solutions of germitrine were made by dissolving the base in a slightly larger than equimolar value of 3 M HCl and had tip resistances of 10-30 MΩ. The tip potentials were less than 10 mV. Transmembrane potentials were measured by means of an electrometer amplifier providing capacity compensation (M 701, W-P Instruments) and were displayed on one or two oscilloscopes. Photographs were obtained and analyzed after magnification. Maximum rate of rise of the action potential upstroke was determined as the slope of a straight line drawn through the steepest part of the upstroke (sweep speed = 2 cm/msec). Only cells near the base of the muscle were impaled; the opposite end of the preparation was stimulated through a bipolar platinum electrode.

**EXPERIMENTS WITH TETRODOTOXIN**

TTX was used to block the fast Na channels. To achieve this result in guinea pig papillary muscle micromolar concentrations are necessary. As one would expect, the excitation threshold increases in the presence of TTX. Therefore, TTX-treated preparations were stimulated with pulses of 5-msec duration and 2-3 times the rheobase strength determined in the absence of TTX. For the experiments carried out at a high (1 Hz) contraction frequency the muscles were taken from guinea pigs pretreated with reserpine (5 mg/kg, ip) 24 hours prior to the experiment. This was done to avoid release of endogenous catecholamines by the electrical stimuli. This precaution seemed to be necessary because of the possibility that high intensity stimulation might directly depolarize nerve terminals within the muscle in addition to exciting the adrenergic nerve fibers. The former effect, but not the latter, would persist in the presence of TTX and thus necessitate depletion of the catecholamine stores. Experiments carried out at a later stage of this work revealed, however, that TTX (30 nm) completely (and reversibly) eliminated the positive isotropic effect resulting from suprathreshold stimulation ($n = 6$; intensity, 3 times threshold; frequency, 1 Hz; duration of pulses, 5 msec). The same result was obtained by Feinstein and Paimre for cat atrium. Thus, while reserpine pretreatment prevents any indirect sympathomimetic effect, it does not seem to be necessary to prevent stimulation-induced release of endogenous catecholamines in preparations treated with micromolar TTX concentrations. The positive isotropic effect of ceveratrum alkaloids is not affected by reserpine treatment of the animal14 (see also Fig. 7).

**CUMULATIVE CONCENTRATION-EFFECT CURVES**

The concentration-dependence of the positive isotropic effect of germitrine was assessed by using cumulatively increasing concentrations. The concentration was raised if the effect of the preceding concentration had fully developed, i.e., if no further increase in the force of contraction occurred during an observation period of 10 minutes. The final concentration of germitrine which caused no further increase of the force of contraction usually decreased $F_c$ gradually. In the absence of a steady state, the effect of a 30-minute incubation in this concentration was arbitrarily taken as a measure of the effect at this concentration and is shown in the concentration-effect curves.

**STATISTICAL EVALUATION**

Whenever appropriate the results are presented as mean values ± the standard error (SE) of the mean. Student's t-test was used to determine the significance of differences between means; n denotes the number of papillary muscles.

**Results**

**AFTER-DEPOLARIZATION INDUCED BY GERMITRINE**

In the isolated papillary muscle of the guinea pig contracting at a frequency of 1 Hz, nanomolar concentrations of germitrine produced a marked positive isotropic effect. The effect of germitrine on the cardiac action potential, on the other hand, was most obvious if the muscle was exposed to a relatively high (micromolar) concentration and stimulated at intervals of several minutes. This effect will be described first.

After exposure for 74 minutes to germitrine ($2 \mu M$) the terminal phase of repolarization of the action potential was altered. While repolarization was complete in the...
absence of germitrine (Fig. 1A, upper panel), the repolarization was interrupted in the presence of germitrine at a level 5 mV positive to the resting potential before the action potential upstroke (Fig. 1B, upper panel). A simultaneous record obtained with a second oscilloscope at a much slower sweep speed and higher gain revealed that complete repolarization eventually occurred after a long-lasting after-depolarization (Fig. 1B, lower panel). It is impossible to clearly display both the action potential and after-depolarization on a single oscilloscope sweep because the after-depolarization lasted about 400 times longer than the normal action potential. Thus, the upper panel of Figure 1B shows the action potential and the initial part of the after-depolarization, while the trace in the lower panel shows the complete time course of the after-depolarization and a small interruption (arrow) caused by the action potential. Apart from the delayed final repolarization, the resting and action potentials showed little change (Fig. 1 and Table 1). The amplitude of repeatedly elicited after-depolarizations (i.e., the maximal reduction of the membrane potential during the course of an after-depolarization) increased slowly during the 1st hour of exposure to the alkaloid and later stabilized (four muscles). Evaluation of 29 records obtained from six muscles after equilibrium for at least 1 hour in 2 μM germitrine disclosed that the after-depolarization regularly exhibited an initial phase of progressive depolarization (duration, 9.5 ± 0.4 seconds), followed by a phase of gradual repolarization to the original level of the resting potential. Semilogarithmic plots of the decaying phase (from 100% to 10% of the amplitude of the after-depolarization) were not linear but showed a progressively steepening decline. The half-time of decay of the after-depolarization was 32.3 ± 0.7 seconds. The time elapsed between the rapid upstroke of the action potential and repolarization to within 1 mV of the resting potential was approximately 75 seconds. The average amplitude of the after-depolarization amounted to 8.1 ± 0.4 mV. While the amplitude increased during the 1st hour of exposure to germitrine, its half-time of decay remained practically constant.

TTX (10 μM) reversibly abolished the after-depolarization (Fig. 2); the same result was obtained during a continuous microelectrode impalement from another preparation. Concomitantly, TTX reversibly reduced the maximum rate of rise of the action potential by 55% in the experiment illustrated in Figure 2, while not affecting the duration of the plateau (0-mV level). The sensitivity to TTX strongly suggests that the after-depolarization is mediated by those Na channels which normally operate only during the first few milliseconds of the action potential and which are known to be specifically blocked by TTX.15 We conclude that the after-depolarization reflects an increase in Ps. It seems unlikely that the slow inward current whose voltage threshold is normally at −35 mV is activated by the germitrine-induced depolarization which at most reduced the membrane potential to −70 mV. Moreover, the threshold stimulus voltage for eliciting an action potential in a muscle treated with 65 μM TTX ("slow response") was not altered during exposure to 2 μM germitrine for 1 hour.

**RELATION BETWEEN AFTER-DEPOLARIZATION AND POSITIVE INOTROPIC EFFECT**

Eight papillary muscles were preincubated in 2 μM germitrine for 1.5 hours and were not stimulated during this time. Subsequently, during maintained exposure to germitrine, a contraction was elicited and thereafter, following a certain ("test") interval, a "test" contraction was elicited. The first contraction had the same small ampli-

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**Figure 1** Effect of a high concentration of germitrine (2 μM) on the action potential and contraction of a guinea pig papillary muscle stimulated once every 10 minutes. The upper panels show the action potential and isometric contraction at a slow sweep speed and the upstroke of the action potential at a fast sweep speed. The records shown in the lower panels were obtained simultaneously with the corresponding upper records on a second oscilloscope at higher gain and very slow sweep speed. They show the membrane potential before and during a period of 1.5 minutes after the action potential. The action potential which is displayed in the corresponding upper panel causes the interruption (at arrow) of these traces. Records under panel A were obtained before, and records under panel B 74 minutes after, addition of germitrine. The dashed lines indicate the level of the resting potential before the action potential was elicited. Note incomplete repolarization (B, upper panel) which is due to an after-depolarization (lower panel). Continuous microelectrode impalement. Calibrations of A apply also to B.

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**Table 1** Effect of 2 μM Germitrine on Transmembrane Electrical Activity of Guinea Pig Papillary Muscles Stimulated Once Every 10 Minutes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Germitrine</th>
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<tbody>
<tr>
<td>Resting potential (mV)</td>
<td>−81.4 ± 1.0</td>
<td>−83.6 ± 1.0</td>
</tr>
<tr>
<td>Maximum rate of rise (V/</td>
<td>338 ± 20</td>
<td>338 ± 20</td>
</tr>
<tr>
<td>sec)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overshoot (mV)</td>
<td>47.8 ± 1.0</td>
<td>45.0 ± 0.8</td>
</tr>
<tr>
<td>Action potential duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mV level (msec)</td>
<td>134.5 ± 4.1</td>
<td>125.5 ± 3.0</td>
</tr>
<tr>
<td>−70 mV level (msec)</td>
<td>165.0 ± 2.7</td>
<td>170.0 ± 3.0</td>
</tr>
<tr>
<td>Total (sec)*</td>
<td>0.182 ± 0.003</td>
<td>74.2 ± 2.5</td>
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</tbody>
</table>

In each of five muscles a single cell was impaled and two action potential records were evaluated, one obtained before (control) and one during exposure to germitrine. Mean values ± SE of the measurements on these five cells are given. Time of incubation with germitrine was 30 minutes in one muscle and 58-74 minutes in the remaining four muscles.
Figure 2: Reversible block by tetrodotoxin (TTX) of germitrine-induced after-depolarization in guinea pig papillary muscle. Each horizontal row shows three different parts of the same action potential. The left panel shows the upstroke, the middle panel shows the action potential with the exception of the terminal phase of repolarization (greatly prolonged by germitrine), and the right panel shows the membrane potential before and during a period of 1.5 minutes after the action potential at higher gain. In the panels on the right the upstroke and fast phase of repolarization of the action potential are underexposed because of their short duration; they cause the interruption of the traces. The records were obtained from a different cell of the preparation shown in Figure 1 during maintained exposure to germitrine (2 μM) before (top row), during exposure to 10 μM TTX (middle row) and after washout of TTX (bottom row). Continuous microelectrode impalement. Contraction frequency = 0.1 min⁻¹.

In six control muscles (diameter = 0.70 ± 0.08 mm) test contractions at all intervals had essentially the same amplitude as the normal “rested state contraction” of ventricular muscle, because germitrine lacked a positive inotropic effect on contractions which followed long rest intervals (cf. Figs. 1 and 8). After a rest interval of 12 minutes, during which the electrical and inotropic aftereffects (see below) of the test contraction disappeared, another rested state contraction was evoked, followed by a test contraction after a different test interval. Each rested state contraction was preceded by a rest period of 12 minutes or more. The rest intervals between rested state contractions and test contractions (= test intervals) ranged from 10 seconds to 10 minutes. Test intervals were of progressively increasing or decreasing order. The size of the rested state contractions showed no systematic variations during the time necessary to establish the interval-dependence of the test contractions (usually 3 hours).

In six control muscles (diameter = 0.70 ± 0.08 mm) test contractions at all intervals had essentially the same amplitude as the preceding rested state contractions (52 ± 9 mg). In the presence of germitrine, test contractions exceeded the rested state contractions at all intervals less than 10 minutes in duration (Fig. 3). Hence, the ability of the muscle to develop force after a (conditioning) rested state contraction was increased by germitrine. The time courses of the membrane potential during the after-depolarization and of the positive inotropic aftereffect following a rested state contraction in the presence of 2 μM germitrine are compared in Figure 3. Clearly, the development of the after-depolarization preceded the positive inotropic effect. During the 1st minute following a rested state contraction, ΔFt, rose to its maximum value, while the membrane potential passed through maximum depolarization and repolarized to about 30% of its maximum value. During the following minute the after-depolarization subsided completely, while the ability of the muscle to develop force decayed only slightly. Thereafter, ΔFt declined with a half-time 4.8 times longer than the half-time of decay of the after-depolarization.

To further define the relationship between the electrical and the inotropic effect we examined the question of whether TTX suppresses the germitrine-induced phase of increased contractility. Since the absolute positive inotropic effect shown by the most strongly affected test contraction was relatively small (less than 80 mg) (Fig. 3), it seemed desirable to increase this effect if its abolition by TTX was to be shown. For this purpose [Ca], was increased to 5.0 mm throughout these experiments. At the 1-minute test interval, ΔFt (four muscles) was 126 ± 48 mg in the presence of 2 μM germitrine, 0.3 ± 0.6 mg in the presence of 10 μM TTX and germitrine, and 115 ± 51 mg after washout of TTX. Fc of the conditioning rested state contractions was 80 ± 15, 69 ± 13, and 82 ± 7 mg, respectively. Thus, TTX completely and reversibly eliminated the positive inotropic influence induced by germitrine.

The blocking effect of TTX and the temporal dissociation between after-depolarization and inotropic effect suggest that an increase of PNa causes a positive inotropic effect after some delay. Such a delay would, indeed, be expected if the effects were causally linked by intracellular

Figure 3: Time course of membrane potential during the after-depolarization and of the positive inotropic effect following a rested state contraction in germitrine-treated guinea pig papillary muscles. Ordinate: • = membrane potential during after-depolarization which followed an action potential elicited at 0 time (100% = 8.1 ± 0.4 mV); O = peak force of test contraction minus peak force of prior rested state contraction (100% = 79 ± 16 mg). Abscissa: as indicated for the different symbols. The time course of the after-depolarization is shown by means (± SE) of 27-29 records obtained from six muscles incubated with 2 μM germitrine for at least 1 hour. The positive inotropic effect (mean values ± SE of six to eight muscles) was determined in different muscles; diameter: 0.69 ± 0.05 mm; force of contraction (Fc) of rested state contractions = 40 ± 7 mg. Inset: superimposed pen recordings of a rested state contraction (trace 1) and a test contraction elicited 1 minute later (trace 2) in a germitrine-treated muscle. See text for further explanations.
accumulation of Na ions. In agreement with this hypothesis, the cardiac glycoside dihydro-ouabain (DHO) augmented the positive inotropic aftereffect of germitrine. In six muscles treated with germitrine (2 μM), ΔF, after a 1-minute test interval was 73 ± 21 mg before, and 245 ± 93 mg after, a 30-minute incubation with 20 μM DHO. The preceding rested state contractions had amplitudes of 76 ± 18 and 95 ± 19 mg, respectively. In the absence of germitrine, this concentration of DHO had a small positive inotropic effect on test contractions (six different muscles): at the 1-minute test interval, 26% of the maximal positive inotropic effect under control conditions. The magnitude of the direct positive inotropic effect during the 1-Hz "staircase" rose in a staircase-like manner to a much higher value after the start of stimulation at 1 Hz resulted in a steady depolarization by 7 mV after approximately 24 seconds (Fig. 4D). This phase of slow repolarization strongly resembled the after-depolarization which followed a single action potential in the presence of a preceding rested state contraction. The membrane potential was reduced by 5 mV after 15 stimuli at 1 Hz, and the subsequent repolarization of the unstimulated preparation had a half-time of about 4 seconds during the following period of rest. In the presence of germitrine (80 nm), the membrane potential was reduced by 5 mV after 15 stimuli at 1 Hz, and the subsequent repolarization was approximately 24 seconds (Fig. 4D). This phase of slow repolarization strongly resembled the after-depolarization which followed a single action potential in the presence of a higher germitrine concentration. Prolonged stimulation at 1 Hz resulted in a steady depolarization by 7 mV after about 50 excitations (Fig. 4E), while the force of contraction rose in a staircase-like manner to a much higher value than under control conditions (Fig. 4F vs. 4C). Like the after-depolarization induced by 2 μM germitrine, the depolarization of repetitively stimulated muscles treated with 80 nm germitrine preceded a positive inotropic effect. The time to half-maximal depolarization after the start of stimulation at 1 Hz was 11 ± 2 seconds (nine measurements from six muscles), and the time to reach half of the maximal positive inotropic effect under these conditions was 90 ± 8 seconds (six different muscles; inotropic effect obtained as the difference between corresponding forces of contraction during the 1-Hz "staircase" before and after addition of germitrine). The magnitude of the germitrine-induced steady depolarization increased with increasing frequencies. During one continuous microelectrode impalement, it was found to be 2 mV at 0.1 Hz, 5 mV at 0.3 Hz, and 10 mV at 1.0 Hz.

These results suggest that low concentrations of germitrine depolarize cardiac muscle only if it is excited at intervals shorter than a certain critical interval which is necessary for the complete decline of the basic electrical effect, a small after-depolarization due to increased PNa. Accordingly, TTX abolished or prevented (see below, Fig. 6, and Table 2) the depolarization induced by 80 nm germitrine in muscles stimulated at a frequency of 1 Hz.

**SUMMATION OF AFTER-DEPOLARIZATIONS DURING REPETITIVE STIMULATION**

If germitrine-treated muscles were stimulated during the after-depolarization, the second action potential gave rise to another after-depolarization which led to a further reduction of the membrane potential. This phenomenon is very similar to a well known feature of veratrine-treated nerve: the summation of negative after-potentials. If muscles were exposed to a low concentration of germitrine (80 nm), action potentials elicited at intervals of several minutes lacked a conspicuous after-depolarization. During repetitive stimulation at 1 Hz, however, a cumulative loss of resting potential indicated the presence of small after-depolarizations. Results of such an experiment are shown in Figure 4. In the absence of germitrine, stimulation (1 Hz) of a papillary muscle, previously quiescent for several minutes, resulted in a small but transient depolarization of the resting membrane potential (Fig. 4B). After 15 excitations (Fig. 4A) the membrane was depolarized by 2.5 mV and repolarized with a half-time of about 4 seconds during the following period of rest. In the presence of germitrine (80 nm), the membrane potential was reduced by 5 mV after 15 stimuli at 1 Hz, and the subsequent repolarization of the unstimulated preparation had a half-time of approximately 24 seconds (Fig. 4D). This phase of slow repolarization strongly resembled the after-depolarization which followed a single action potential in the presence of a higher germitrine concentration. Prolonged stimulation at 1 Hz resulted in a steady depolarization by 7 mV after about 50 excitations (Fig. 4E), while the force of contraction rose in a staircase-like manner to a much higher value than under control conditions (Fig. 4F vs. 4C).

Like the after-depolarization induced by 2 μM germitrine, the depolarization of repetitively stimulated muscles treated with 80 nm germitrine preceded a positive inotropic effect. The time to half-maximal depolarization after the start of stimulation at 1 Hz was 11 ± 2 seconds (nine measurements from six muscles), and the time to reach half of the maximal positive inotropic effect under these conditions was 90 ± 8 seconds (six different muscles; inotropic effect obtained as the difference between corresponding forces of contraction during the 1-Hz "staircase" before and after addition of germitrine). The magnitude of the germitrine-induced steady depolarization increased with increasing frequencies. During one continuous microelectrode impalement, it was found to be 2 mV at 0.1 Hz, 5 mV at 0.3 Hz, and 10 mV at 1.0 Hz.

These results suggest that low concentrations of germitrine depolarize cardiac muscle only if it is excited at intervals shorter than a certain critical interval which is necessary for the complete decline of the basic electrical effect, a small after-depolarization due to increased PNa. Accordingly, TTX abolished or prevented (see below, Fig. 6, and Table 2) the depolarization induced by 80 nm germitrine in muscles stimulated at a frequency of 1 Hz.

**ELECTRICAL AND INOTROPIC EFFECTS AT A CONTRACTION FREQUENCY OF 1 Hz**

Records of isometric force developed by a papillary muscle contracting at a frequency of 1 Hz and exposed to cumulatively increasing concentrations of germitrine are shown in Figure 5. The increase of peak force of contrac-
tion is due to an increased rate of force development. Time to peak force is shortened, relaxation time is moderately prolonged, and total contraction time is slightly reduced by germitrine. These features also were observed in studies on seven additional preparations. The average half maximally effective concentration (EC50) for the positive inotropic effect was 24 ± 3 nM (n = 8); thus, on a molar basis, germitrine is about 16 times more potent than veratridine. The positive inotropic effect of the maximally effective concentration of germitrine amounted to 81 ± 8% and the simultaneous klinotropic effect, ΔS1, to 92 ± 6%, of those of DHO tested on the same muscle (mean values ± se of 12 muscles; sequence of drug application reversed in six preparations). Germitrine concentrations exceeding the inotropically effective range regularly induced spontaneous contractions and contracture if stimulation at 1 Hz was continued. Mean exposure times necessary for half-maximal and maximal positive inotropic effects of 40 nM germitrine (EC50) were 6 and 28 minutes, respectively (n = 6). Washing with alkaloid-free solution completely reversed the positive inotropic effect of germitrine.

The first two panels of Figure 6 illustrate the effects of 80 nM germitrine on the action potential and contraction of a papillary muscle contracting at a frequency of 1 Hz. Mean values of the results obtained from six muscles are depicted in Table 2. The marked positive inotropic effect was accompanied by the following significant effects on resting and action potential: a reduction of the resting potential by 11%; reduction of the overshoot by 23%; reduction of the maximum rate of rise of the action potential, by increasing [K]0, reduces the overshoot and shortens the action potential. In guinea pig papillary muscle, however, potassium depolarization has little effect on these parameters. Thus, the depolarization itself probably was not responsible for the decreased overshoot and action potential duration. The reduction in the upstroke velocity, on the other hand, is likely to be caused by the depolarization. In control experiments on three muscles (1 Hz; [Ca]0 = 1.6 mM), reduction of the membrane potential from −75.8 ± 0.4 mV to −65.9 ± 0.5 mV after a contraction frequency to 0.2 min⁻¹ (Table 2). These procedures also prevented the decrease in the resting potential, the decrease of the maximum rate of rise of the action potential, and the positive inotropic effect (Table 2). In Purkinje fibers, reduction in the resting potential, by increasing [K]0, reduces the overshoot and shortens the action potential. In guinea pig papillary muscle, however, potassium depolarization has little effect on these parameters. Thus, the depolarization itself probably was not responsible for the decreased overshoot and action potential duration. The reduction in the upstroke velocity, on the other hand, is likely to be caused by the depolarization. In control experiments on three muscles (1 Hz; [Ca]0 = 1.6 mM), reduction of the membrane potential from −75.8 ± 0.4 mV to −65.9 ± 0.5 mV after an increase of [K]0 from 5.9 to 9.0 mM resulted in a

![Figure 6](http://circres.ahajournals.org/)

**Figure 6** Effect of tetrodotoxin (TTX) on the action potential and isometric contraction of a germitrine-treated guinea pig papillary muscle. The oscilloscope records were obtained (from left to right) before (control), 80 minutes after addition of 80 nM germitrine, 10 minutes after subsequent additional application of 10 μM TTX, and several minutes after washout of TTX. Each record is from a different impalement; muscle is from a reserpine-pretreated guinea pig. Contraction frequency = 1 Hz; [Ca]0 = 1.6 mM. Calibrations apply to all records. Upstrokes of action potentials were retouched.

<table>
<thead>
<tr>
<th>Action potential</th>
<th>Resting potential (mV)</th>
<th>Maximum rate of rise (V/sec)</th>
<th>Overtone (mV)</th>
<th>Duration at 0-mV level (msec)</th>
<th>Duration at 90% repolarization (msec)</th>
<th>Force of contraction (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (82)</td>
<td>−76.6 ± 0.3</td>
<td>271 ± 6</td>
<td>33.6 ± 0.4</td>
<td>136.0 ± 1.5</td>
<td>194.1 ± 1.1</td>
<td>240 ± 35</td>
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<td>Germitrine (58)</td>
<td>−67.8 ± 0.5</td>
<td>212 ± 5</td>
<td>25.8 ± 0.4</td>
<td>51.5 ± 2.4</td>
<td>132.1 ± 2.9</td>
<td>1017 ± 76</td>
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<tr>
<td>P</td>
<td>&lt;0.001</td>
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<tr>
<td>Contraction frequency = 1 Hz, 10 μM TTX (n = 4)</td>
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<tr>
<td>Control (33)*</td>
<td>−75.9 ± 0.5</td>
<td>111 ± 5</td>
<td>32.2 ± 0.6</td>
<td>128.3 ± 1.6</td>
<td>179.8 ± 1.1</td>
<td>135 ± 20</td>
</tr>
<tr>
<td>Germitrine (33)</td>
<td>−75.3 ± 0.5</td>
<td>110 ± 5</td>
<td>31.9 ± 0.4</td>
<td>125.5 ± 1.6</td>
<td>177.8 ± 1.1</td>
<td>163 ± 37</td>
</tr>
<tr>
<td>P</td>
<td>&gt;0.4</td>
<td>&gt;0.9</td>
<td>&gt;0.7</td>
<td>&gt;0.2</td>
<td>&gt;0.2</td>
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<tr>
<td>Contraction frequency = 0.2 min⁻¹ (n = 3)</td>
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<tr>
<td>Control (3)</td>
<td>−84.7 ± 0.3</td>
<td>355 ± 22</td>
<td>37.3 ± 0.3</td>
<td>126.7 ± 5.5</td>
<td>166.7 ± 4.2</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Germitrine (3)</td>
<td>−83.7 ± 1.9</td>
<td>354 ± 10</td>
<td>36.7 ± 0.3</td>
<td>130.0 ± 5.0</td>
<td>175.8 ± 5.8</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Quiescent preparations (n = 3)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control (28)</td>
<td>−79.2 ± 0.5</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Germitrine (32)</td>
<td>−79.0 ± 0.3</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>P</td>
<td>&gt;0.6</td>
<td></td>
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</table>

Values are means ± se; n indicates the number of muscles; numbers in parentheses indicate the number of impalements. P denotes the significance of the difference between values obtained before (control) and 1.0–1.5 hours after addition of germitrine as calculated by Student's t-test. [Ca]0 = 1.6 mM.

* Control records were taken 15 minutes after addition of tetrodotoxin.

TABLE 2 **Effect of 80 nm Germitrine on Transmembrane Electrical Activity and Force of Contraction of Guinea Pig Papillary Muscles**
The positive inotropic effect of germitrine (Fig. 8). Frequency was altered by stepwise ranges of contraction durations before and during exposure to 80 nM values of force of contraction were determined over a wide range of frequencies. In six muscles steady state values of force of contraction were determined over a wide range of frequencies before and during exposure to 80 nM germitrine (Fig. 8). Frequency was altered by stepwise reduction. It is evident that the positive inotropic effect of germitrine suggested that the positive inotropic effect associated with a sustained increase of P Na, also should increase inotropically effective range of germitrine concentrations. Cumulative concentration-effect curves revealed that the effect of 10 μM TTX was to increase the EC50 to 0.40 ± 0.04 μM (Fig. 7). The maximum positive inotropic effect of germitrine in the presence of TTX was slightly larger than in the absence of the toxin (two different groups of muscles); this may be related to the negative inotropic effect of TTX. Taking into account that the inotopically effective range of germitrine concentrations (at 1 Hz) reflects only an initial fraction of the range pertinent to the interaction with sarcolemmal P Na, and that 10 μM TTX leaves a considerable fraction of Na channels unblocked, the observed antagonism is probably not at variance with the noncompetitive antagonism between veratrum alkaloids and TTX (or saxitoxin) suggested by more direct experiments.

Since the positive inotropic effect resulting from a single germitrine-induced after-depolarization was increased by DHO, it was of interest to determine whether the positive inotropic effect associated with a sustained increase of P Na in continually active preparations treated with much lower germitrine concentrations would also be sensitive to the cardiac glycoside. A germitrine concentration of 40 nm and a frequency of 0.25 Hz were used in these experiments. In five muscles, DHO (10 μM) was added first in the absence and later in the presence of germitrine. Five other preparations were first incubated with germitrine and, after measurement of the superimposed positive inotropic effect of DHO and subsequent washing in drug-free solution, the effect of DHO was determined in the absence of germitrine. Fc was 167 ± 29 mg and 839 ± 103 mg in the absence and presence of germitrine, respectively (n = 10). DHO increased Fc by 68 ± 13 mg and 855 ± 84 mg in the absence and presence of germitrine, respectively. Thus, the positive inotropic response to the combination of the two drugs was 2 times larger than the sum of the responses to either alone.

**Discussion**

Our results suggest that a selective increase in the Na permeability of cardiac sarcolemma may enhance contrac-
tility to an extent which is only slightly less than that seen with the maximally effective concentration of a cardiac glycoside. The conclusion that germitrine increases the permeability to Na ions is based on the observation that TTX blocked in reversible fashion the depolarizing effect of the alkaloid. For a variety of excitable cell membranes including those of amphibian and mammalian heart, TTX is known to selectively block an ion channel which has a high selectivity for Na ions and normally is characterized by fast activation and inactivation following depolarization (the "fast" Na channel). The conclusion that the inotropic effect of germitrine is entirely due to the effect of the alkaloid on sarcolemmal Psa is based on the finding that the concentration of TTX (10 μM) which abolished or prevented the germitrine-induced depolarization likewise abolished or prevented its positive inotropic effect. It is to be expected that TTX, because of its inability to penetrate through the excitable membrane, interferes exclusively with sarcolemmal effects of germitrine.

The slow time course of the germitrine-induced after-depolarization indicates that germitrine, like other everatum alkaloids, slows the kinetics of Na channels. Veratrine and a monoester of germine, germine-3-acetate, are known to have such an effect in nerve fibers. The rather long duration of the after-depolarization which the polyster germitrine induced in guinea pig papillary muscle was not unexpected, since Graham and Gasser noted 45 years ago that proteratrine, a mixture of two protevrine tetraesters, induces a longer lasting "negative after potential" in nerve than veratrine. We have adopted the term "after-depolarization" rather than "negative after potential" because "depolarization" precisely indicates the direction of the change in transmembrane potential.

As shown in Figure 3, the period of increased Psa is associated with a progressive increase of the ability to develop force. The subsequent loss of the inotropic influence, however, proceeds more slowly than the after-depolarization. This points to an indirect connection between both events. Considering that a sudden increase of Psa will cause a time-dependent increase of [Na] until Na efflux matches Na influx and that the Na pump needs apparently several minutes to adapt to a sudden change of passive Na influx, it is tempting to attribute the transiently increased ability to contract to a transient increase of the subsarcolemmal Na concentration. The fact that the inotropic response was highly sensitive to DHO, a known inhibitor of active Na extrusion, further strengthens this interpretation. The positive inotropic influence of an elevated [Na] can be explained if the sarcolemma contains an Na-Ca exchange system. According to this concept, an increase of [Na] will increase Ca influx. By this mechanism a greater amount of Ca could be made available for intracellular release during subsequent excitation.

The positive inotropic effect of germitrine was accompanied by a marked shortening of the duration and a significant reduction of the overshoot of the action potential. In line with evidence from other tissues, experiments on Purkinje fibers suggest that an increase of [Ca] hastens repolarization by increasing potassium permeability. This mechanism may account for the changes of the action potential in germitrine-treated papillary muscles.

It seems unlikely, but is not completely ruled out by the present experiments, that some so far unknown mechanism underlies the positive inotropic effect of germitrine. Such a mechanism should respond with a delay of a few minutes to a minor reduction in the resting potential and be intensified in the presence of DHO. Although voltage clamp experiments would possibly help to settle this question, available evidence indicates that the transient or maintained depolarization itself (i.e., in the absence of a pharmacologically increased Psa) would probably have either no, or the opposite, effect on contraction. In dog ventricular trabeculae or sheep Purkinje fibers, reducing the membrane potential during an interval of several seconds between two consecutive contractions decreases the force of the second contraction. The depolarization up to 10 mV which germitrine induced during maximal positive inotropic effect is insufficient to activate a slow inward Ca current whose voltage threshold is at —35 mV. The threshold stimulus intensity for eliciting an action potential, presumably mediated by slow inward current in the presence of 65 μM TTX, was not altered by germitrine.

The present findings suggest that a sudden, selective and persistent increase of sarcolemmal Psa causes, with some time lag, a positive inotropic effect. In germitrine-treated guinea pig papillary muscle, the change in internal Na concentration, which is expected to occur during altered passive Na influx, seems to be an important determinant of the efficacy of the excitation-contraction coupling mechanism.

Acknowledgments
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References
Alterations in Canine Myocardial Excitability during Ischemia

VICTOR ELHARRAR, PETER R. FOSTER, THOMAS L. JIRAK, WINSTON E. GAUM, AND DOUGLAS P. ZIPES

SUMMARY Changes in the ventricular diastolic excitability threshold following occlusion of the left anterior descending coronary artery (LAD) were studied in open-chest anesthetized dogs by using a new automatic threshold-following pacemaker (ATFP). The ATFP measures the diastolic excitability threshold by successively decreasing the duration of regularly occurring pacing stimuli until the ventricle fails to respond. Under control conditions, the threshold stimulation duration was 60 ± 4 (mean ± SEM) msec. In the first 1-3 minutes following occlusion of the LAD, the diastolic excitability threshold in the ischemic zone (IZ) decreased to 51 ± 5 msec and then rapidly increased to 600 msec at 5 minutes. The initial decrease in excitability threshold at IZ could be abolished by elevating the serum K+ concentration prior to the LAD occlusion. These changes in excitability threshold at IZ could be prevented by infusing nonoxygenated solutions into the LAD at a site distal to the occlusion. As the excitability threshold increased in IZ during ischemia, the earliest time at which IZ could be reactivated by a stimulus with a voltage equal to twice the predilation diastolic voltage threshold was increased. In nine of 16 dogs, after 5 minutes of LAD ligation, the IZ to normal zone (NZ) activation time (when stimulating at NZ) exceeded the NZ to IZ activation time (when stimulating at NZ) by an average of 10 msec. We also found that in four dogs the NZ to IZ activation time exceeded the IZ to NZ activation time by an average of 9 msec. We conclude from these findings that a gradient of increasing excitability threshold exists as one moves from normally perfused to more ischemic tissue, passing through a heterogeneous border zone that manifests some areas which have a decreased excitability threshold and other areas which have an increased excitability threshold, and that these changes in excitability importantly influence the determination of refractory period durations and conduction times.
Sarcolemmal sodium permeability and contractile force of guinea pig papillary muscle: effects of germitrine.
P Honerjäger and M Reiter

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