Electrically Triggered All-or-None Ca^{2+}-liberation during Action Potential in the Giant Alga *Chara*

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ABSTRACT Electrically triggered action potentials in the giant alga *Chara corallina* are associated with a transient rise in the concentration of free Ca^{2+} in the cytoplasm (Ca^{2+}_{cyt}). The present measurements of Ca^{2+}_{cyt} during membrane excitation show that stimulating pulses of low magnitude (subthreshold pulse) had no perceivable effect on Ca^{2+}_{cyt}. When the strength of a pulse exceeded a narrow threshold (suprathreshold pulse) it evoked the full extent of the Ca^{2+}_{cyt} elevation. This suggests an all-or-none mechanism for Ca^{2+} mobilization. A transient calcium rise could also be induced by one subthreshold pulse if it was after another subthreshold pulse of the same kind after a suitable interval, i.e., not closer than a few 100 ms and not longer than a few seconds. This dependency of Ca^{2+} mobilization on single and double pulses can be simulated by a model in which a second messenger is produced in a voltage-dependent manner. This second messenger liberates Ca^{2+} from internal stores in an all-or-none manner once a critical concentration (threshold) of the second messenger is exceeded in the cytoplasm. The positive effect of a single suprathreshold pulse and two optimally spaced subthreshold pulses on Ca^{2+} mobilization can be explained on the basis of relative velocity for second messenger production and decomposition as well as the availability of the precursor for the second messenger production. Assuming that inositol-1,4,5,-trisphosphate (IP_3) is the second messenger in question, the present data provide the major rate constants for IP_3 metabolism.

KEY WORDS: calcium mobilization • Fura-2 • simulation • voltage-dependent second messenger production

INTRODUCTION

The membrane of the giant alga *Chara corallina* is electrically excitable. In the course of an action potential (AP),* the concentration of free Ca^{2+} in the cytoplasm (Ca^{2+}_{cyt}) increases transiently from ~100 nM to ~1 μM (Williamson and Ashley, 1982; Plieth et al., 1998). This transient rise in Ca^{2+}_{cyt} is considered central in the process of membrane excitation, because it is thought to activate Ca^{2+}-sensitive Cl^- channels and, hence, initiate membrane depolarization (for review see Tazawa et al., 1987; Thiel et al., 1997).

The causal relationship between stimulation of APs by positive membrane voltage and elevation of Ca^{2+}_{cyt} is still unknown. The current view of this process is that membrane depolarization causes, by some unknown mechanism, a liberation of Ca^{2+} from internal stores (Plieth et al., 1998; Thiel and Dityatev, 1998). The idea that Ca^{2+} is liberated from internal stores rather than entering through plasma membrane channels (Kikuyama and Tazawa, 1998) is supported by experiments using Mn^{2+} as a quencher of fura-2 fluorescence. It was found that transient calcium rises were not associated with a quenching of the fura-2 fluorescence in the presence of extracellular Mn^{2+} (Plieth et al., 1998). Such a quenching would have been expected if the bulk change in Ca^{2+} was due to influx of Ca^{2+} via plasma membrane channels (Merrit et al., 1989). The consequent notion that liberation from internal stores is responsible for the transient calcium rises was further supported by experiments in which the cytoplasm of *Chara* cells was preloaded with Mn^{2+}. With this preconditioning, APs were associated with a quenching of fura-2 even in the absence of any external Mn^{2+}, suggesting that in this case quenching was due to liberation of Mn^{2+} together with Ca^{2+} from internal stores (Plieth et al., 1998).

Experiments with *Chara* cells have shown that inhibitors of PLC caused a delay and a suppression of the electrically stimulated elevation of the Cl^- conductance, i.e., the conductance that causes the depolarization in an AP (Biskup et al., 1999). Furthermore, elevation of the concentration of the second messenger inositol-1,4,5,-trisphosphate (IP_3) in the cytoplasm of these cells was able to elicit APs (Thiel et al., 1990). Together these experiments lend support to the view that the mechanism linking electrical stimulation with mobilization of Ca^{2+} from internal stores includes IP_3 as a second messenger.

In the present investigation, we examined the relationship between the electrical stimulation and the kinetics of Ca^{2+} mobilization in the course of an AP. We

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*Abbreviations used in this paper: AP, action potential; IP_3, inositol-1,4,5,-trisphosphate.

found that transient calcium rises were triggered by current pulses in an all-or-none fashion. Furthermore, we found that APs could be stimulated by a pair of two subthreshold pulses if the second pulse was neither too closely nor too far separated from the leading pulse. Together these data provide the basis for a kinetic model describing the voltage-dependent production of a second messenger and its transient elevation as a link between electrical stimulation and Ca^{2+} mobilization.

MATERIALS AND METHODS

Plant Material and Ca^{2+}_{cyt} Measurements

Chara corallina Klein ex Wild was grown as reported previously (Thiel et al., 1993). The concentration of free Ca^{2+} in the cytoplasm (Ca^{2+}_{cyt}) was measured with a fluorescence ratio imaging method using the dual excitation dye fura-dextran as Ca^{2+} indicator (Grynkiewicz et al., 1985). Individual internodal cells of ~40 mm in length were loaded with fluorescent dye via pressure injection using a custom-built injection device (Plieth and Hansen, 1996). Cells loaded with dye were stored overnight in experimental solution (artificial pond water 0.5 mM KCl, 0.5 mM $CaCl_2$, 1 mM NaCl, and 2 mM HEPES/NaOH, pH 7.5).

For Ca^{2+}_{cyt} measurements, the dye was excited with monochromatic light from a xenon lamp altering rapidly between 340 and 380 nm (T.I.L.L. Photonics). Emitted light from a square area of ~10 μm^2 to 25 μm^2 was collected using a Zeiss objective (Fluar 40*/1.3 oil immersion) and transmitted through a 390-nm dichroic mirror before being detected by a photomultiplier (Seefelder Meßtechnik). A band pass filter (510 ± 30 nm; Schott) in the light path served to reduce auto fluorescence of chloroplasts. The EPC-9 unit with PULSE and X-chart software (Heka Elektronik) was used to control switching between excitation wavelength and recording of the photomultiplier output. Data were collected with a frequency of 5 or 10 Hz.

Ratiometric measurements were calibrated in vitro as described in Plieth and Hansen (1996) using standard Ca^{2+} solutions (calibration kit, C-3722; Molecular Probes). APs were triggered by current pulses of variable amplitude and length via extracellular electrodes. These were placed close to the area for Ca^{2+}_{cyt} recording to assure that Ca^{2+}_{cyt} changes were picked up from the site of excitation.

The Model

The model to describe a transient calcium rise in response to short single and double pulses is based on the variation of the concentration of a second messenger (here termed Q_2) in the cytoplasm. We assume that Q_2 is generated from a pool Q_1 and degraded to Q_3. We further assume that a threshold concentration of Q_2 is required for mobilization of Ca^{2+} from internal stores. Beyond this threshold, Q_2 causes a transient calcium rise that is largely independent of pulse duration and strength. For the model no other parameters (e.g., diffusion, cell geometry) than production of Q_2 and decay were considered. All calculated values are relative changes with reference to the resting concentrations of Q_2 and Q_1 that were set to 0 and 1, respectively.

The model is governed by the two following differential equations:

$$\frac{\partial [Q_1]_t}{\partial t} = k_{Q1}([Q_1]_0 - [Q_1]_t) - k_{Q2}[Q_1]_t, \quad (1)$$

$$\frac{\partial [Q_2]_t}{\partial t} = k_{Q2}[Q_1]_t - k_{Q3}[Q_2]_t \quad \text{(Wang et al., 1995)}. \quad (2)$$

where k_{Q1} and k_{Q3} are time- and voltage-independent rate constants for pool Q_1 refilling and decay of Q_2. k_{Q2} is the voltage-dependent rate constant for production of Q_2 and associated depletion of Q_1, respectively. In pulse intervals and after the second pulse, k_{Q2} is zero and the differential equations are then governed by k_{Q3} and k_{Q1}, respectively.

The term $k_{Q1}([Q_1]_0 - [Q_1])$ in Eq. 1 is equivalent to the assumption that a controller for Q_1 homeostasis is used. Any deviation from the set point $[Q_1]_0$ (as caused by its conversion to Q_2; Eq. 2) leads to restoring of the set point value. The detailed mechanism used for this task may be complicated. A minimum scheme for such a homeostatic controller was suggested by Hansen (1990). The reaction there was furnished for homeostasis in a compartment by mean of transmembrane transport, however, the same equations hold for homeostasis by chemical reactions. The important feature is the requirement of ATP.

To simulate the effect of small increments in pulse strength we assumed:

$$k_{Q2} = \frac{c_2(I - I_0)}{q} \quad (3)$$

for $I > I_0$ and $k_{Q2} = 0$ for $I \leq I_0$, where I is the current of the stimulating pulse. The minimum current I_0 and the charge q were determined by fitting the measured strength-duration relationship by Eq. 11. c_2 is a fitting parameter without a dimension. The introduction of a threshold such as I_0 is unusual for chemical reactions, as this does not fulfil the law of mass action. However, mechanisms can be assumed that can result in such a rate constant. For example, the presence of Ca^{2+}-sensitive PLC in plants (Kopka et al., 1998) as well as the strong dependency of membrane excitation on extracellular Ca^{2+} (Williamson and Ashley, 1982; Thiel et al., 1993) may suggest the following scenario: Ca^{2+} enters the cells in a voltage-dependent manner. This results in a local rise of Ca^{2+} in the vicinity of the plasma membrane, which remains undetected by our method. Significant PLC activity with a quasi-linear dependency on voltage would then only be stimulated for sufficient positive voltage excursions. The present linear approach is only an approximation but justified by the linear stimulus-quantity law that we found for triggering transient calcium rises in *Chara* (see Fig. 3). For larger I increments, we expect an exponential function for $k_{Q2}(I)$.

$[Q_1]$ and $[Q_2]$ was then calculated by:

$$[Q_1]_t = a_{i0} - a_{i2} \cdot e^{-(k_{Q1} + k_{Q2}) \cdot (t - t_{i0})} \quad (4)$$

and

$$[Q_2]_t = b_{i0} - b_{i1} \cdot e^{-k_{Q3} \cdot (t - t_{i0})} - b_{i0} \cdot e^{-(k_{Q1} + k_{Q2}) \cdot (t - t_{i0})}, \quad (5)$$

respectively for four different time intervals $\Delta t_i = t_{i,end} - t_{i0}$. t_{i0} and $t_{i,end}$ are the times for onset and end of each interval i (i = 1...4). During the first and the third interval, the cell is excited by a pulse, in the second and the forth interval the system is undisturbed. The constants a_{ij} and b_{ij} represents the boundary conditions for each interval. Their specific values are $a_{i0} = k_{Q1}/(k_{Q1} + k_{Q2})[Q_1]_0$, $a_{i1} = a_{i0} - [Q_1]_{i0}$, wherein $[Q_1]_{i0}$ is the concentration of Q_1 at the beginning of the i-th interval. Further are $b_{i0} = k_{Q1}k_{Q2}/(k_{Q3}(k_{Q1} + k_{Q2}))[Q_1]_0$, $b_{i1} = b_{i0} - b_{i2} - [Q_2]_{i0}$ and $b_{i2} = k_{Q2}a_{i2}/(k_{Q3} - k_{Q1} - k_{Q2})$. With start of the first pulse at t_{10}, $[Q_2]_{10}$ is zero and $[Q_1]_{10}$ is 1. In the pulse interval and after the second pulse, k_{Q2} and with it b_{0i} and b_{2i} are zero, and $[Q_2]$ is determined by the second right term of Eq. 5.

The time when $[Q_2]$ reaches its maximum value during the second pulse could be expressed in dependency of the time between both pulses $\Delta t_2 = t_{2,end} - t_{20}$ if pulse duration and pulse current were fixed. The time t_{max2} is given by the null of the derivative of Eq. 3, which is resolved to t:

$$t_{\max 2}(\Delta t_2) = t_{10} + \Delta t_1 + \Delta t_2 + \frac{1}{k_{Q3} - k_{Q1} - k_{Q2}} \cdot \ln\left[\frac{k_{Q3} \cdot (b_{30} - [Q_2]_{30} - b_{32}(\Delta t_2))}{-(k_{Q1} + k_{Q2}) \cdot b_{32}(\Delta t_2)}\right], \quad (6)$$

where $\Delta t_1 = t_{1,\text{end}} - t_{10}$ is the duration of the stimulus. $b_{30}(\Delta t_2)$ and $b_{32}(\Delta t_2)$ are dependent of $[Q_1]_{30}$ and $[Q_2]_{30}$ at the beginning of the second pulse, which are functions of Δt_2; their values are given by Eqs. 4 and 5, respectively. If the argument of the logarithm is equal to or smaller than zero, $[Q_2]$ does not reach a maximum even with infinite pulse duration. It rather approaches an asymptote. Then $[Q_2]$ reaches its maximum value at the end of the second pulse at $t_{\max 2} = t_{10} + \Delta t_1 + \Delta t_2 + \Delta t_3$.

$[Q_2]_{\max 2}$ is then:

$$[Q_2]_{\max 2}(\Delta t_2) = b_{30}(\Delta t_2) - b_{31}(\Delta t_2) \cdot e^{-k_{Q3} \cdot (t_{\max 2} - t_{i0})} - b_{32}(\Delta t_2) \cdot e^{-(k_{Q1} + k_{Q2}) \cdot (t_{\max 2} - t_{i0})}. \quad (7)$$

With this equation, $[Q_2]$ can be plotted as a function of Δt_2 for fixed pulse duration and voltages.

In the special case of a single pulse, as well as for the first pulse in a series ($[Q_2]_{s1} = 0$), Eq. 6 is simplified to:

$$t_{\max 1} = t_{10} + \frac{1}{k_{Q3} - k_{Q1} - k_{Q2}} \ln\left[\frac{k_{Q3} - k_{Q1}}{k_{Q2}}\right]. \quad (8)$$

Its maximal value is t_{20}. $[Q_2]_{\max 1}$ is then given by an analogous of Eq. 7:

$$[Q_2]_{\max 1} = b_{10} - b_{11} \cdot e^{-k_{Q3} \cdot (t_{\max 1} - t_{i0})} - b_{12} \cdot e^{-(k_{Q1} + k_{Q2}) \cdot (t_{\max 1} - t_{i0})} \quad (9)$$

RESULTS

Fig. 1 shows a recording of Ca^{2+}_{cyt} in a *Chara* internodal cell before and during electrical excitation. At rest, Ca^{2+}_{cyt} was typically ~400 nM. Stimulation of the cell by a short current pulses (here 100 ms) triggered a transient calcium rise reaching within ~3 s a maximum amplitude of 0.5–1 μM. Kinetics and amplitude of this electrically stimulated transient calcium rise is similar to those reported previously for excursions of Ca^{2+}_{cyt} during membrane excitation in *Chara* (Plieth et al., 1998).

To investigate the variability of electrically triggered transient calcium rises, one *Chara* cell was repetitively stimulated and Ca^{2+}_{cyt} recorded. Fig. 1 illustrates an overlay of transient calcium rises after eight successive stimulations with pulses of the same strength-duration. The plot shows that identical stimulations triggered within one cell transient calcium rises of very similar amplitude and kinetics. This result was confirmed in experiments with five other cells.

To quantify changes in Ca^{2+}_{cyt} during excitation, transient calcium rises were fitted by the sum of two exponentials with the form:

$$Ca^{2+}_{cyt} = A_1 e^{(-\lambda_1 (t - t_0))} + A_2 e^{(-\lambda_2 (t - t_0))}, \quad (10)$$

with a positive amplitude A_1 and a negative amplitude A_2, and time relaxation coefficients λ_1 and λ_2. The start of the fitted interval is given by t_0.

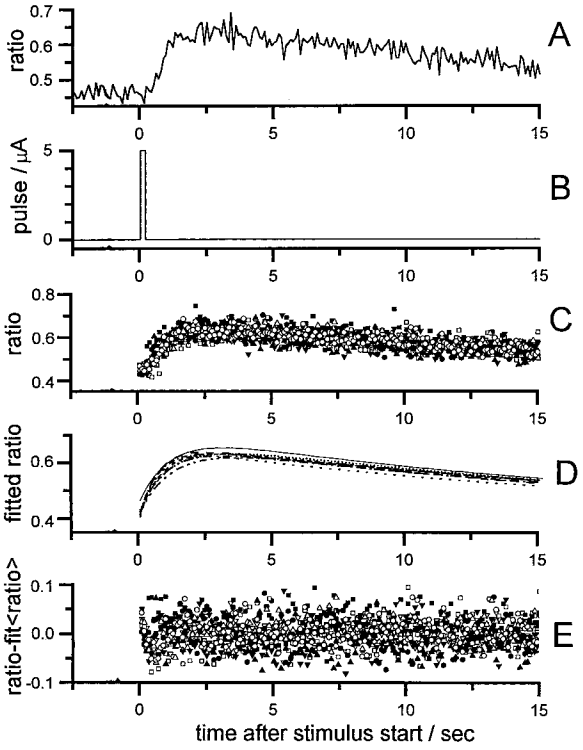

FIGURE 1. Transient Ca^{2+}_{cyt} elevation in response to electrical stimulus. A shows ratiometric measurement of Ca^{2+}_{cyt} in one *Chara* internodal cell loaded with fura-2 upon application of electrical stimulation shown in B. (C) Similarity of transient Ca^{2+}_{cyt} elevation in the same *Chara* cell after repetitive electrical stimulation. Cell stimulated for a period of 1 min using a pulse of 100 ms/5 μA. Overlay of Ca^{2+}_{cyt} recordings from eight successive stimulations. (D) Fit of individual data sets using Eq. 10. (E) Residuals of fits. Calibration: ratio 0.45 = 100 nM Ca^{2+}_{cyt}; ratio 0.7 = 800 nM Ca^{2+}_{cyt}.

As shown in the example in Fig. 2, fitting with two exponentials was adequate for an ad hoc description of transient calcium rises. Therefore, it was used throughout for quantitative description of a transient calcium rise. Notably, in the late phase of transient calcium rises (i.e., at which Ca^{2+}_{cyt} had already decayed back one third of the maximum), the fit often deviated from the data (not shown) indicating that a more complex model is required for description of the real events. However, this did not affect the present analysis.

Transient Calcium Rise Is an All-or-None Response

To determine the relationship between the strength of the stimulation and the evoked elevation of Ca^{2+}_{cyt}, we measured the amplitude of transient calcium rises as a function of stimulus strength. Fig. 2 shows three exemplary transient calcium rises after application of an electrical stimulus (100 ms) with a low (A), medium (B), and (C) high amplitude (Fig. 2 B). The data reveal that a small pulse (now termed subthreshold pulse) caused no detectable change in Ca^{2+}_{cyt} (further details see below). Increasing the pulse amplitude (here by a

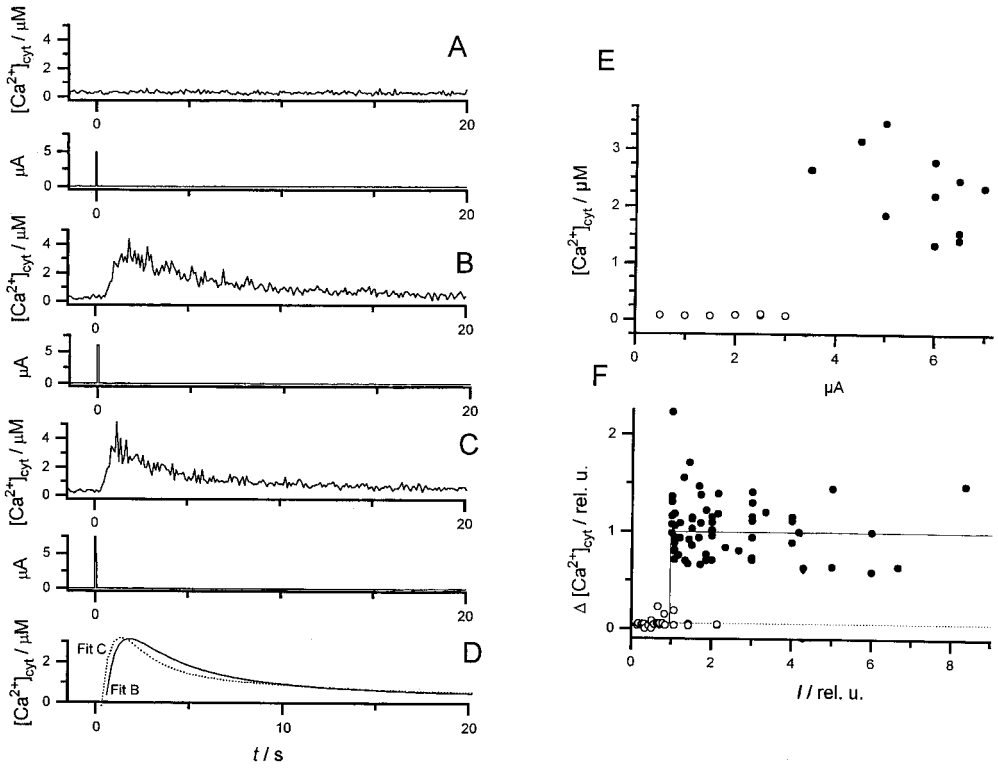

FIGURE 2. Effect of pulse strength on transient Ca^{2+}_{cyt} release in a *Chara* cell. One internodal cell was stimulated using a 100-ms-long pulse of (A) 5, (B) 6, and (C) 7.5 μA. (D) The transient calcium rise induced by pulses with medium and high amplitude were fitted by Eq. 10. (E) The amplitude of Ca^{2+}_{cyt} changes in response to a suprathreshold pulse of 100-ms length was plotted versus the pulse current for cell shown in A–D. (F) Normalized data for experiments with three cells as in E. For normalization, we set the pulse interval obtained by the first successful stimulation and, furthermore, the mean amplitude of Ca^{2+}_{cyt} of all successful stimulations to 1. Stimulating pulses cause either no perceivable change in Ca^{2+}_{cyt} or evoke the maximal response.

factor of 1.2) caused a typical transient calcium rise. Further increase of the pulse amplitude (here by a factor of 1.25) triggered a transient calcium rise of about the same magnitude as that after the medium sized pulse. To illustrate the relationship between pulse strength and transient calcium rise, the amplitudes of transient Ca^{2+}_{cyt} elevations were plotted versus the stimulus strength. Fig. 2 E shows that a narrow threshold exists for the stimulus strength. Below this threshold, Ca^{2+}_{cyt} remains unaffected (further details see below). After passing the threshold, the Ca^{2+}_{cyt} response already approaches its maximum. The same narrow threshold in pulse strength was found in all cells tested. This finding stresses that an all-or-none mechanism is the underlining Ca^{2+} mobilization.

Strength-duration Relationship

To investigate the relationship between pulse strength-duration and transient calcium rises, we monitored Ca^{2+}_{cyt} in response to pulses with different current amplitudes and/or duration. Fig. 3 illustrates a strength-duration curve for the effective stimulation of Ca^{2+}_{cyt} transients as a function of pulse duration and pulse strength. Shown are strength-duration values, which did (filled symbols) and which did not (open symbols) cause transient elevation of Ca^{2+}_{cyt}. The threshold for stimulation follows a hyperbolic function:

$$I = I_0 + q/\Delta t, \qquad (11)$$

that is plotted in Fig. 3. Fitting the data yields a minimum current I_0 of 2.5 μA and a minimal charge q_{min} of 115 nC.

Double Pulse Experiments

In the following experiments, we examined more closely the effect of subthreshold stimulation on Ca^{2+}_{cyt}. At the beginning of an experiment, the strength-duration of a stimulus was adjusted such that it was close to, but still lower than, the stimulation threshold.

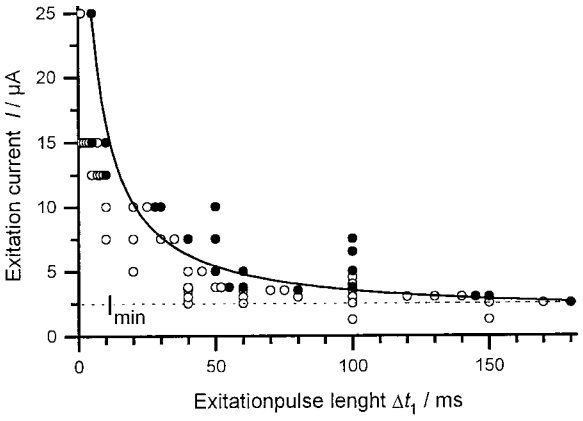

FIGURE 3. Strength-duration curve for relation between the duration of a stimulus current and the current strength needed to stimulate an AP. Shown are strength-duration values that were effective (closed symbols) or not effective (open symbols) for inducing an AP. Fitting the data with a hyperbolic function yielded a minimum current of 2.5 μA and a minimum charge of 115 nC.

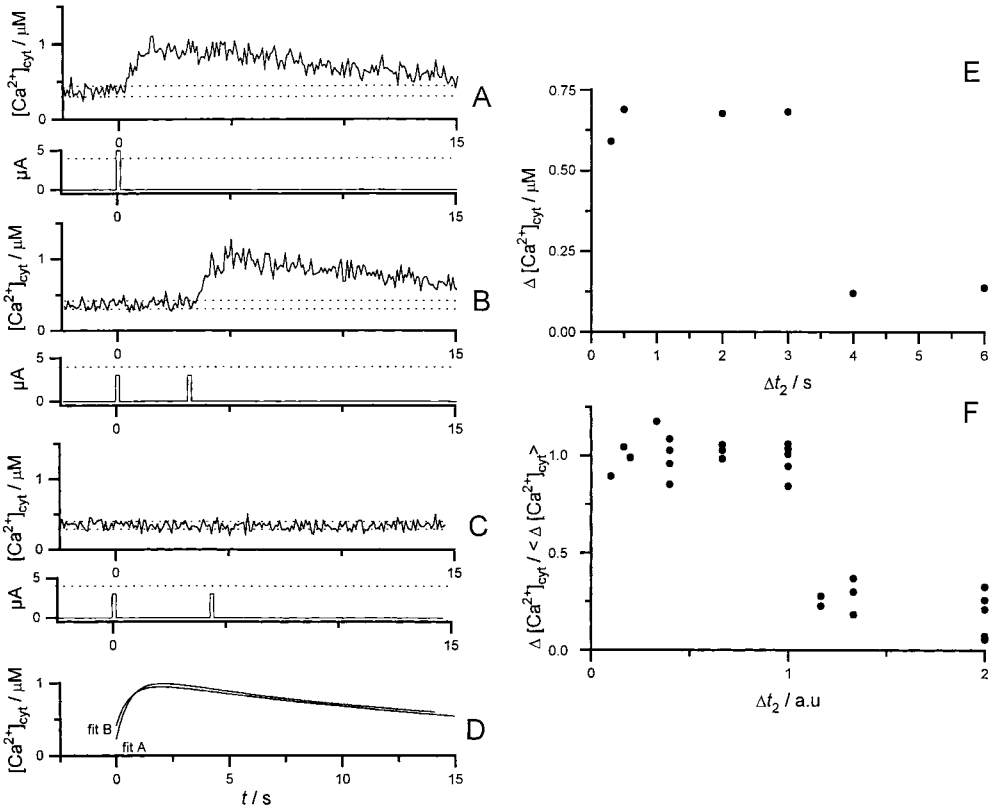

FIGURE 4. Summation of subthreshold pulses for induction of transient calcium rise. (A) One *Chara* internodal cell was stimulated by a suprathreshold pulse to induce a transient calcium rise. (B) A similar transient calcium rise was obtained in response to the second pulse in a series of two subthreshold pulses with an interval of 3 s. (C) Increasing the interval between two subthreshold pulses renders them ineffective for inducing a transient calcium rise. All pulses were 200 ms long. (D) The transient calcium rise induced by a single suprathreshold pulse and by dual subthreshold pulses were fitted by Eq. 10. (E) Amplitudes of Ca^{2+}_{cyt} changes in response to dual subthreshold pulses as a function of the pulse interval for cell shown in A–D. (F) Normalized data for experiments with five cells as in E. For normalization, we set the pulse interval obtained by the last successful stimulation and the mean amplitude of Ca^{2+}_{cyt} of all successful stimulations to 1.

Fig. 4 shows representative recordings from an experiment in one *Chara* cell with the response of Ca^{2+}_{cyt} to a single or a series of subthreshold pulses. The data in Fig. 4 (A and B) show that Ca^{2+}_{cyt} is not affected appreciably by subthreshold pulses. To examine the possibility that such changes might be small and therefore unresolved, we analyzed the noise in Ca^{2+}_{cyt} recording in response to subthreshold pulses. Fig. 5 reports the variance of Ca^{2+}_{cyt} from the mean Ca^{2+}_{cyt} data obtained before, during and after subthreshold pulses. The absence of any appreciable change in variance in correlation with the pulses further shows that Ca^{2+}_{cyt} is not affected by subthreshold pulses.

Fig. 4 B shows that the effect of subthreshold pulses can be additive. In the present case, a subthreshold pulse was followed after 3 s by a second pulse of the same strength and duration. In this case, the second pulse triggered a transient calcium rise (Fig. 4 B), and this was similar in magnitude and shape to the transient calcium rise obtained by a large pulse in the same cell (Fig. 4 A). This shows that the stimulation encoded in any pulse is additive.

Fig. 4 C further shows that the additive effect of multiple subthreshold pulses is only effective for triggering a transient calcium rise if the interval between two pulses is not too long. In the case reported in Fig. 4 C, the two subthreshold pulses were separated by 4 s. In this case, Ca^{2+}_{cyt} remained entirely unaffected. Fig. 4 E summarizes the effect of dual pulses on transient calcium rises in one cell. Plotted are the amplitudes of Ca^{2+}_{cyt} changes as a function of the pulse interval. It is apparent that intervals must be shorter than ~4 s to assure an additive effect of subthreshold pulses. The same pattern for stimulation of a transient calcium rise by subthreshold pulses was observed in five other cells tested. This result was independent on whether the experiment was started with a short or a long interval. This renders an endogenous decrease in excitability of the cell unlikely as explanation for the results.

To further test the hypothesis that two subthreshold pulses could be additive in their ability to stimulate Ca^{2+} mobilization, we compared the minimum charge (q_{min}) required for stimulation with single and double pulses. Therefore, one cell was stimulated (as in Fig. 4) with a double pulse protocol. However, in this case, strength and duration of the second pulse were varied, whereas the parameters of the first pulse as well as Δt_2 were kept constant. For comparison, the same cell was also stimulated with single pulses of variable strength and duration. The plot in Fig. 6 illustrates the strength-duration

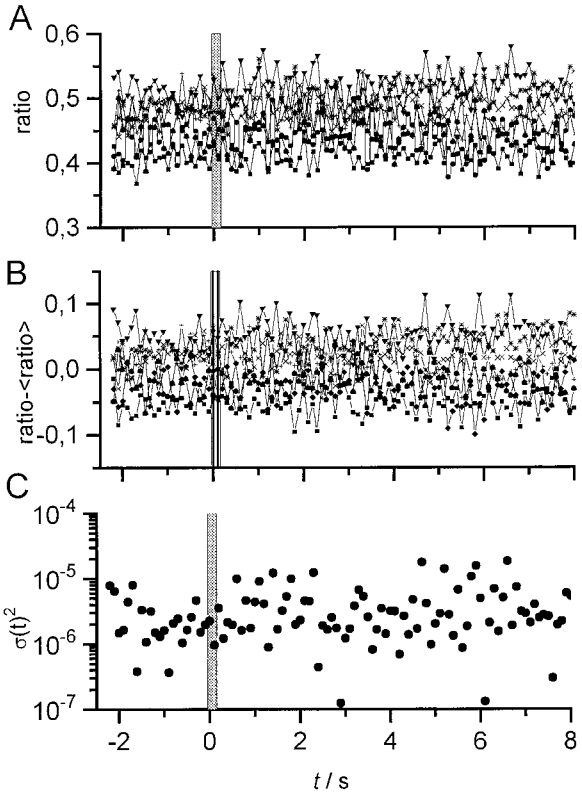

FIGURE 5. Analysis of variance in Ca^{2+}_{cyt} recordings before, during, and after stimulation with subthreshold pulses. (A) Ca^{2+}_{cyt} data from eight measurements. (B) Deviation of individual data from mean. (C) Variance calculated from B. Bar indicates duration of electrical stimulus.

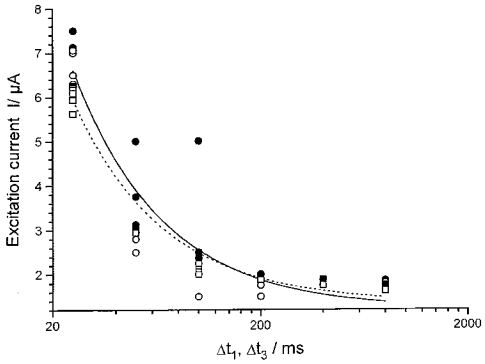

FIGURE 6. Strength-duration relationship for electrical stimulation of transient calcium rise. One cell was stimulated with two different protocols. Stimulation was either performed with a single pulse (circles) or as in Fig. 4 with double subthreshold pulses (squares). In the latter case, only the trailing pulse was varied in duration and strength while the parameters of the leading pulse (I = 2 µA, t = 200 ms) as well as Δt_2 (500 ms) were kept constant. Shown are strength-duration values that were effective (closed symbols) or ineffective (open symbols) for inducing a transient calcium rise. In the case of double pulse stimulation, only the strength-duration values of the trailing pulse are considered in the plot. Fitting the data with a hyperbolic function yielded a minimum current I_0 = 1.2 µA and a minimum charge q_{min} = 135 nC for single pulse stimulation. For the trailing pulse in the double pulse protocol, the fit yielded I_0 = 1.3 µA and q_{min} = 114 nC.

relationship in one cell for the two different modes of stimulation, i.e., stimulation with a single pulse (closed symbols) and stimulation with a variable second pulse after a leading constant pulse (open symbols). Fitting of both data sets with Eq. 11 yielded very similar values for I_0. However, the q_{min} value from the double pulse stimulation was 1.19 times lower than that for single pulse stimulation. The same result was confirmed in three similar experiments showing that the q_{min} required for effective stimulation was on average 1.2 ± 0.06 times smaller, when the stimulating pulse was preceded by a subthreshold pulse. These data and the finding that the strength-duration plot for the second pulse shows the same hyperbolic relationship as that obtained for single pulse stimulation is best explained by the fact that individual pulses are indeed additive.

Fig. 7 shows another surprising observation with respect to a minimum interval between two effective subthreshold stimuli. In this case, pulses with low strength and long duration were chosen. As a single pulse, these were not able to stimulate a transient calcium rise (not shown). When two pulses of the same kind were applied in series with an interval of 300 ms, a transient calcium rise was stimulated. Subsequently, the interval between the two stimuli was shortened and the effect on Ca^{2+}_{cyt} was monitored. Fig. 7 (B and C) shows that also a reduction in the interval between two subthreshold pulses resulted in a loss of the additive effect of subthreshold pulses as trigger for Ca^{2+}_{cyt} mobilization. Fig. 7 D summarizes the effects of dual pulses on transient calcium rises tested in the same cell. The plot shows the amplitudes of Ca^{2+}_{cyt} changes as a function of the pulse interval. It is apparent that intervals must be longer than ~200 ms to assure an additive effect of subthreshold pulses. The same pattern for stimulation of transient calcium rises by subthreshold was observed in four other cells tested. The result was independent on whether the experiment was started with a short or a long interval. This renders an endogenous decrease in excitability of the cell unlikely as explanation for the results.

In conclusion, the present data show that two subthreshold pulses have an additive effect on the stimulation of a transient calcium rise. Summation of the effect of single subthreshold pulses is only possible if the intervals are neither too long nor to short.

DISCUSSION

It has long been known that electrical excitation in *Chara* is associated with a transient calcium rise (Williamson and Ashley, 1982; Kikuyama and Tazawa, 1983). The present data now provide information on the mechanisms linking electrical stimulation and cytoplasmic Ca^{2+} mobilization.

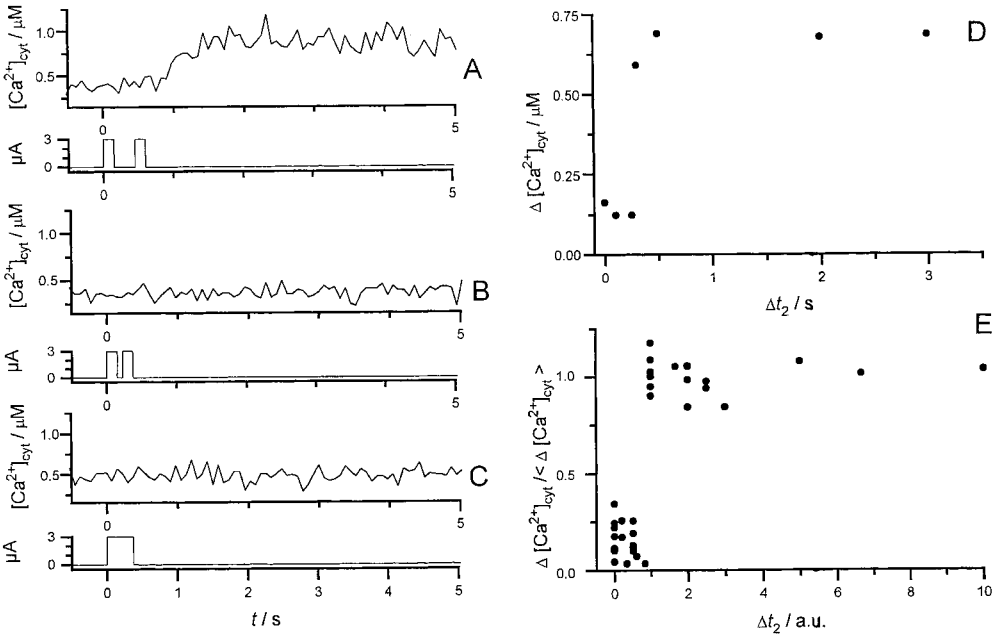

FIGURE 7. Summation of subthreshold pulses for induction of a transient calcium rise. (A) One *Chara* internodal cell was stimulated by two subthreshold pulses to induce a transient calcium rise. (B) After reduction of the pulse interval to 100 ms (B) and 0 ms (C) the stimulation failed to induce a transient calcium rise. All pulses were 200 ms long. (D) Amplitudes of $Ca^{2+}{}_{cyt}$ changes in response to dual subthreshold pulses as a function of the pulse interval for the cell shown in A–C. (E) Normalized data for experiments with four cells as in D. Normalization as in Fig. 5.

One key finding is that the mechanism of Ca^{2+} elevation has a very steep dependency on the strength of the stimulation pulse. Over a very narrow range of pulse strength $\Delta Ca^{2+}{}_{cyt}$ varies between zero and the maximal amplitude. This threshold-like dependency of $Ca^{2+}{}_{cyt}$ on pulse strength fosters the view that the electrical stimulation causes elevation of $Ca^{2+}{}_{cyt}$ by an all-or-none type mechanism.

Previously it had been suggested that the transient calcium rise during excitation is due to an influx of Ca^{2+} via voltage-sensitive Ca^{2+} channels in the plasma membrane (Kikuyama and Tazawa, 1998). However, the steep dependency of $Ca^{2+}{}_{cyt}$ on the stimulating pulse as well as the all-or-none type behavior of transient calcium rises is not in accordance with the operation mode of any known voltage-dependent channels (Hille, 1992). This excludes Ca^{2+} influx via voltage-sensitive Ca^{2+} channels in the plasma membrane as the source of the bulk rise in calcium during excitation. The present data are better explained by a second messenger–operated release of Ca^{2+} from internal stores. This is in accordance with previous reports stressing that Ca^{2+} is mobilized from internal stores in the course of an AP (Beilby, 1984; Thiel et al., 1993; Plieth et al., 1998; Thiel and Dityatev, 1998).

The double pulse experiments show that the stimulation by a pulse is longer lived than the duration of the pulse itself. Furthermore the information encoded by individual subthreshold pulses is additive. The best explanation for these data is that membrane depolarization causes production of an intermediate second messenger with a lifetime in the order of seconds. This cannot be Ca^{2+} since we did not even detect minor changes in global $Ca^{2+}{}_{cyt}$ upon subthreshold pulses. Furthermore the life time of Ca^{2+} in the cytoplasm is at least one order of magnitude shorter (Lipp and Niggli, 1996) stressing that Ca^{2+} is not the intermittent messenger.

Previously it has been observed that elevation of IP_3 is effective in triggering membrane excitation in *Chara* (Thiel et al., 1990). Also, inhibition of IP_3 production by inhibitors of PLC was reported to suppress membrane excitation (Biskup et al., 1999). This fostered the hypothesis that membrane depolarization causes production of the second messenger IP_3 and consequent mobilization of Ca^{2+} from internal stores. Thus, the best candidate for the second messenger Q_2 linking electrical stimulation and Ca^{2+} mobilization is IP_3. In this context, it can now be assumed that membrane depolarization causes, by a yet unknown mechanism, a rapid production of IP_3 drawing from the PIP_2 pool. The latter would be equivalent to the pool Q_1 in our model. If the IP_3 level remains below a threshold, no Ca^{2+} is mobilized from the stores. Above this critical value, IP_3 causes complete mobilization from the internal stores. This view of IP_3 action is consistent with the finding, that IP_3 is indeed known to cause an all-or-none type calcium liberation from internal stores of animal cells (Parker and Ivorra, 1990).

Upon elevation in the cytoplasm, IP_3 is known to be subjected to degradation to the inactive IP_2 (Berridge, 1987), equivalent to pool Q_3 in our model. The lifetime of IP_3 was determined in animal cells and was found to be of the order of ~1 s (Wang et al., 1995; Fink et al., 1999). From this long lifetime of IP_3 it can be assumed that any further mobilization of IP_3 during this decay time will add to the IP_3 remaining from the first stimulation. By summation, the cytoplasmic concentration of IP_3 could then exceed the threshold. In the present experiments, we found that double pulses were only effective if the pulse intervals were not longer than ~3 s.

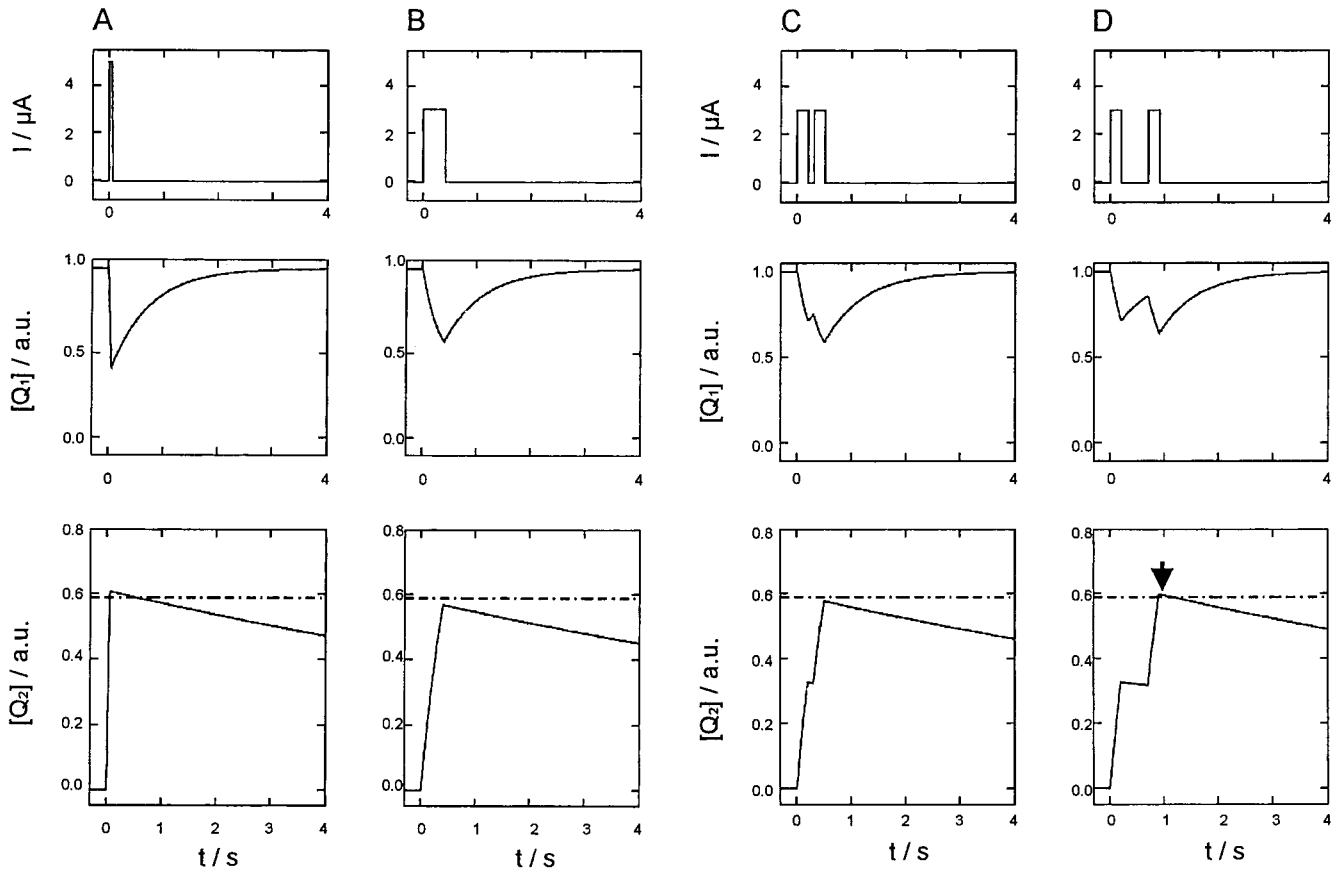

FIGURE 8. Calculation of the content of the pool Q_1 (middle) and concentration of Q_2 (bottom) in response to single and double pulses illustrated in top panel. The threshold in concentration of Q_2 required for Ca^{2+} mobilization is shown as dotted line. Time courses of the concentrations of Q_1 and Q_2 underlying effective stimulation (A and D, arrow) and ineffective stimulation (B and C) are calculated using the following parameters: $k_{Q1} = 1.4\ s^{-1}$, $k_{Q2} = 0.064\ s^{-1}$, $q = 117$ nC, $I_0 = 2.77$ µA, and $c_2 = 1$ in all four examples. Pulse strength/duration are 5 µA/100 ms in A, 3 µA/400 ms (B), and 3 µA/200 ms (C and D). Pulse intervals are 100 ms (C) and 500 ms (D).

This time is within the lifetime of IP_3 and, thus, supports our notion that IP_3 can act as the intermittent second messenger in question.

The view of IP_3 production as second messenger is also consistent with the observation that two low amplitude subthreshold pulses must have a minimum interval to be effective as trigger. This experimental result can be explained by the fact that IP_3 production during a subthreshold pulse draws on the pool of PIP_2. If the refilling of the PIP_2 pool from phosphatidylinositolphosphate is not too fast relative to the decay of IP_3, a second pulse can meet the system in a situation in which the PIP_2 pool is so far depleted that the second pulse is unable to generate enough IP_3 to exceed the threshold required for Ca^{2+} mobilization.

Simulation

Here, we described the present experimental data in the context of a second messenger, Q_2, linking electrical stimulation and Ca^{2+} mobilization. On the basis of the aforementioned evidence, we assume that IP_3 is the second messenger in question. But in principle the model is valid for any other chemical second messenger with a metabolism similar to IP_3. We assume that electrical stimulation causes a graded production of Q_2 and that the concentration of Q_2 needs to exceed a threshold for complete mobilization of Ca^{2+} from internal stores. The concentration of Q_2 upon stimulation is given by the rate of production from Q_1 and by the rate of decay to Q_3 (see MATERIALS AND METHODS). For estimation of the relative magnitude of the rate constants, it is important to note that transient calcium rises can be elicited by single pulses as short as 10 ms. The interval between two stimulating subthreshold pulses, on the other hand, can be in the range of seconds. This means that the rate of Q_2 production is much larger than the rate of decay ($k_{Q2} \gg k_{Q3}$). The existence of a minimum interval between subthreshold pulses can be explained with the fact that pool Q_1 needs refilling to allow sufficient production of Q_2 during the second pulse. Under the condition that $k_{Q1}$is larger than k_{Q3}, two pulses can be additive.

Fig. 8 (A–D) shows that this model is able to explain

the body of the present data. A pair of subthreshold pulses is unable to stimulate a transient calcium rise if the interval is too long. The reason for the failure is that the concentration of Q_1 has decreased so far that production of Q_2 during the second pulse is not able to add sufficient new Q_2 required for exceeding the threshold. A pair of subthreshold pulses is also not able to stimulate a transient calcium rise when the pulses are too close together (Fig. 8 C). In this case, stimulation fails because pool Q_1 is so far empty that the second pulse is not able to produce sufficient new Q_2 for exceeding the threshold. Only an intermediate spacing of the two pulses guarantees that the second pulse can produce enough Q_2 to propel it over the threshold.

To examine the dependency of the concentration of Q_2 on pulse intervals, we calculated with the appropriate rate constants the maximal concentration of Q_2 achieved at the second pulse as a function of the pulse intervals. The plot in Fig. 9 shows that only pulse intervals between 0.3 and 3 s cause elevation of Q_2 over the threshold and, thus, are able to trigger a transient calcium rise. Shorter and longer intervals are predicted to not stimulate a transient calcium rise. This features are in good agreement with the experimental data.

In double pulse experiments, we found that summation of stimulation by long single pulses with low strength (p_l with strength i_l, duration $\Delta t_{1,l}$) was within a single cell only possible if the pulse intervals were neither too long (interval $\Delta t_{2,max}$) nor too short (interval $\Delta t_{2,min}$) (Figs. 4 and 6, respectively). Moreover, we found for each cell also short single suprathreshold pulse (p_h with i_h, $\Delta t_{1,h}$).

To quantify k_{Q1}, k_{Q3}, and the factor determining k_{Q2}, c_2 from such a set of experimental data (p_l, p_h, I_0, q, $\Delta t_{2,min}$, and $\Delta t_{2,max}$), we derived some conditions that have to be complied with by Eqs. 6 and 8, respectively, for different pulse intervals assuming that Eqs. 6–9 gives a good description of the kinetics of Q_2. The exclamation marks above the (equal) signs in the following equations shows the condition/character of the equations. They are not fulfilled a priori.

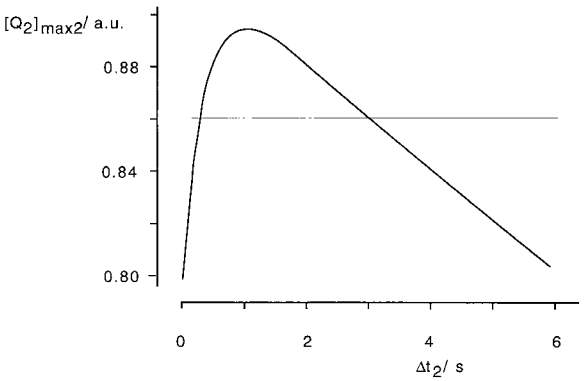

FIGURE 9. Maximal concentrations of Q_2 as a function of pulse interval. The maximal concentration of Q_2 (solid line) achieved during electrical stimulation with pulses of constant strength an duration was calculated from Eq. 7 for $k_{Q1} = 2.5\ s^{-1}$, $k_{Q3} = 0.05\ s^{-1}$, and $c_2 = 1.8$. They were derived from the following data set: $p_l = 3$ μA; 200 ms, $p_h = 5$ μA; 50 ms, $I_0 = 2.8$ μA, $q = 109$ nC, $\Delta t_{2,min} = 300$ ms, and $\Delta t_{2,max} = 3{,}000$ ms. The thin line indicates the threshold, which needs to be exceeded for effective Ca^{2+} mobilization.

For a given subthreshold pulse p_l, the maximal concentration of Q_2 ($[Q_2]_{max2}$) induced by the minimal pulse interval $\Delta t_{2,min}$ has to be equal to $[Q_2]_{max2}$ evoked by the maximal stimulating pulse interval $\Delta t_{2,max}$. This must be the case because shorter (for $\Delta t_{2,min}$) and longer (for $\Delta t_{2,max}$) pulse intervals evoked no transient calcium rise. Hence,

$$[Q_2]_{max2}(\Delta t_{2,max}, p_l) - [Q_2]_{max2}(\Delta t_{2,min}, p_l) \overset{!}{=} 0. \quad (12a)$$

The concentration $[Q_2]_{max2}(\Delta t_{2,min}, p_l)$ is then assumed as the threshold concentration $[Q_2]_{thres}$.

The function described by Eq. 7 is steady for $\Delta t_2 \geq 0$. For Δt_2 longer than $\Delta t_{2,min}$ but shorter than $\Delta t_{2,max}$, $[Q_2]_{max2}(\Delta t_2)$ must consequently be higher than $[Q_2]_{thres}$ because all $\Delta t_2 \in [\Delta t_{2,min}, \Delta t_{2,max}]$ induce a transient calcium rise.

$$[Q_2]_{max2}(\Delta t_2, p_l) - [Q_2]_{thres} \overset{!}{>} 0, \quad \text{for } \Delta t_2 \in [\Delta t_{2,min}, \Delta t_{2,max}]. \quad (12b)$$

Moreover, for pulse intervals $\Delta t_2 < \Delta t_{2,min}$ and for pulse intervals longer than $\Delta t_{2,max}$, $[Q_2]_{max2}$ has to be lower than $[Q_2]_{thres}$. This is because pulse intervals with these lengths do not induce a transient calcium rise.

$$Q_{2max2}(\Delta t_2, p_l) - Q_{2thres} \overset{!}{<} 0, \quad \text{for } \Delta t_2 < \Delta t_{2,min} \text{ or } \Delta t_2 > \Delta t_{2,max}. \quad (12c)$$

Finally, $[Q_2]_{max}$ induced by a single suprathreshold pulse p_h has to reach at least $[Q_2]_{thres}$.

$$[Q_2]_{max1}(p_h) - [Q_2]_{thres} \overset{!}{\geq} 0. \quad (12d)$$

We use this system of (in)equations to construct an error function (see APPENDIX) in which only the three parameters k_{Q1}, k_{Q3}, and c_2 are variables. By minimization of the error function through variation of these three parameters, we found for a given set of experimental data appropriate values for which Eq. 12 a–d are fulfilled. The results are listed in Table I.

Conclusion

The bulk of the experimental data on electrically stimulated elevation of Ca^{2+}_{cyt} can be simulated with a model, which is based on the voltage-dependent production of a second messenger. The distinct relationship between strength/duration of electrical stimuli and an all-or none mobilization of Ca^{2+} from internal stores can be explained in context of the velocity for

TABLE I

Model Fitting Parameters

	Data set 1	Data set 2	Data set 3
High pulse (p_h)	5 μA; 50 ms	5 μA; 20 ms	5 μA; 50 ms
Low pulse (p_l)	3 μA; 200 ms	2.5 μA; 200 ms	2 μA; 200 ms
min;	(250/300);	(50/100);	(250/500);
max Δt_2[a]/ms	(3,000/4,000)	(500/1,000)	(1,500/1,750)
q/nc	109	51.5	160
I_0/μA	2.8	2.4	1.75
k_{Q1}/s^{-1}	2.45 ± 0.19	3.27 ± 0.06	2.45 ± 0.48
k_{Q3}/s^{-1}	0.051 ± 0.005	0.24 ± 0.03	0.09 ± 0.02
c_2	1.83 ± 0.03	1.82 ± 0.11	1.96 ± 0.07
$k_{Q2}(p_h)/s^{-1}$	36.5 ± 0.63	97 ± 2	36.4 ± 2.3
$k_{Q2}(p_l)/s^{-1}$	3.04 ± 0.05	3.24 ± 0.05	3.03 ± 0.19
τ_{Q3}/s	19.8 ± 1.8	4.15 ± 0.45	11.0 ± 2.29

Parameters obtained from fitting data from experiments with both supra- and subthreshold pulses with error function to the model. Top two lines denote experimental conditions, third line gives experimentally determined boundary values for interval of subthreshold pulses. Remaining lines report fitted parameters. The mean lifetime of Q_3 is given by τ_{Q3}.
[a]Boundary values.

second messenger production and decomposition as well as the availability of the precursor for the second messenger production. The data further allow approximation of the major rate constants k_{Q1}, k_{Q2}, and k_{Q3}, which are relevant for production and decay of the second messenger. Assuming that IP_3 is this second messenger in question (Thiel et al., 1990; Biskup et al., 1999), the present data provide some quantitative information on the metabolism of this second messenger.

APPENDIX

Error Function

This section describes the algorithms that were used to obtain values for k_{Q1}, k_{Q3}, and $[Q_2]_{thres}$ as well as the constant c_2 (Eq. 3). The rate constant k_{Q2} was calculated from c_2, q, and I. I_0 was determined from Eq. 10, and the parameters q and I_0 were obtained from a fit of the measured strength duration plots (Fig. 3). I was set in the experiment. Under these circumstances, c_2 is the only free parameter for determining k_{Q2} and, hence, is used as a fitting parameter.

As limiting conditions, the following experimentally determined values were used. (a) A single superthreshold pulse p_h (with duration Δt_{1h} and amplitude i_h), which is only just sufficient to stimulate an AP, provides the value $[Q_2]_{max1}(p_h)$. This unknown value is considered as the threshold. (b) A pair of subthreshold pulses p_l with identical duration Δt_{1l} and Δt_{3l} and intensity i_l, for which the dynamics of $[Q_2]_{max2}(\Delta t_2)$ were calculated in relation to Δt_2. (c) The rheobase I_0 from the strength duration plot of an individual cell. (d) The integral q of the pulse strength over time of stimulation (Δt) used in the strength duration experiments. (e) The minimal distance Δt_{2min} required for a second pulse to stimulate a transient rise in Ca^{2+}. (f) The maximal distance Δt_{2max} allowed between two subthreshold pulses without the second pulse losing its ability to stimulate a transient rise in Ca^{2+}.

In principle Eqs. 3 and 4 allow us to derive the temporal variation of the pool sizes $[Q_1](t)$ and $[Q_2](t)$ in the model. However, the problem is that the dynamics of the pool sizes cannot be determined, because the experiments only provide data in a situation, in which Ca^{2+} is released. Thus, the fitting algorithm has no reference to a continuous function. The only guides for the improvement of the fit are the above stated conditions.

To write Eq. 6, which should fulfil numerically conditions a–c, in such a way that it depends explicitly on Δt_2, the coefficients b_{31} and b_{32} have to be calculated, because their values depend on $[Q_2]_{30}$ and $[Q_1]_{30}$ respectively. Both these values could be written (with Eqs. 3 and 4 for i = 2) as functions of Δt_2.

In the double pulse experiments, in which only the parameter Δt_2 was varied, $[Q_2]_{max2}(\Delta t_2)$ (Eq. 6) could then be written (with the assumption $t_{max2} = t_0 + \Delta t_1 + \Delta t_2 + \Delta t_3$) as a sum of two exponentials with constant coefficients. This function depends only on Δt_2. With these constraints, the trajectory of the function is more or less determined because the sum of two exponents can have only one extreme. In the present case, this extreme must be, because of conditions a and d, between the pulse intervals Δt_{2min} and Δt_{2max}.

In this way the four conditions for fitting $[Q_2]_{max2}(\Delta t_2, p_l)$ and $[Q_2]_{max1}(p_h)$ can be reduced to the following condition:

$$[Q_2]_{max2}(\Delta t_{2,max}, p_l) \overset{!}{=} [Q_2]_{max2}(\Delta t_{2,min}, p_l) \overset{!}{=} [Q_2]_{max1}(p_h) \overset{!}{=} [Q_2]_{thres}.$$

This equation has more than one solution because it can be solved for different values of $[Q_2]_{thres}$, k_{Q1}, k_{Q3}, and c_2.

The possible solutions for k_{Q1}, k_{Q3} and c_2 depend strongly on the threshold $[Q_2]_{thres}$. To obtain a criterion for a unique solution, the following extra criterion was considered in the fitting. The relative deviation between the threshold $[Q_2]_{thres}$ and the calculated concentration $[Q_2]_{max2}(0)$ in response to an experimentally measured subthreshold double pulse with the distance 0,

$$\frac{[Q_2]_{max2}(0) - [Q_2]_{thres}}{[Q_2]_{thres}},$$

should be maximal. The rational behind this is that a signal transduction system should produce a signal which is large enough to be recognized by the next downstream step in the cascade to avoid a false alarm.

On the background of these considerations, the error function that should be minimized here for obtaining the values in Table I can be written as Eq. 13:

$$f_{err}(k1, k3, c2) = \Delta t_{2max}\left(\begin{array}{c}([Q_2]_{max2}(\Delta t_{2max}) - ([Q_2]_{max2}(\Delta t_{2min})))^2 \\ + ([Q_2]_{max2}(\Delta t_{2max}) - [Q_2]_{max2}(p_h))^2\end{array}\right) + ([Q_2]_{max2}(\Delta t_{2min}) - [Q_2]_{max1}(p_h))^2 + 1/([Q_2]_{max2}(\Delta t_{2min}) - [Q_2]_{max2}(0))^2. \quad (13)$$

For evaluation of the variable parameters in question, f_{err} was minimized by a downhill-simplex algorithm (Press et al., 1989).

Because the number of APs that can be induced in an experiment on a single cell is limited, it is in practice not possible to determine the exact values of $\Delta t_{2,min}$ and $\Delta t_{2,max}$. To nonetheless approximate the kinetic parameters from our experiments, we used the following boundary values: for $\Delta t_{2,min}$ the longest subthreshold interval $<\Delta t_{2,min}$ and the shortest suprathreshold interval $>\Delta t_{2,min}$ and for $\Delta t_{2,max}$ the shortest subthreshold interval $>\Delta t_{2,max}$ and the longest suprathreshold interval $<\Delta t_{2,max}$.

For calculation of the parameters in question, we assumed these boundary values to be $\Delta t_{2,min}$ and $\Delta t_{2,max}$ and fitted the parameters for each of the four possible combinations of the boundary-values. The averaged solutions of the four data sets were used as the approximation for the kinetic parameters.

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