Action Currents Generate Stepwise Intracellular Ca\textsuperscript{2+} Patterns in a Neuroendocrine Cell

(Received for publication, January 29, 1998, and in revised form, May 26, 1998)

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It is believed that specific patterns of changes in the cytosolic-free calcium concentration ([Ca\textsuperscript{2+}]) are used to control cellular processes such as gene transcription, cell proliferation, differentiation, and secretion. We recently showed that the Ca\textsuperscript{2+} oscillations in the neuroendocrine melanotrope cells of Xenopus laevis are built up by a number of discrete Ca\textsuperscript{2+} rises, the Ca\textsuperscript{2+} steps. The origin of the Ca\textsuperscript{2+} steps and their role in the generation of long-lasting Ca\textsuperscript{2+} patterns were unclear. By simultaneous, noninvasive measuring of melanotrope plasma membrane electrical activity and the [Ca\textsuperscript{2+}]i, we show that numbers, amplitude, and frequency of Ca\textsuperscript{2+} steps are variable among individual oscillations and are determined by the firing pattern and shape of the action currents. The general Na\textsuperscript{+} channel blocker tetrodotoxin had no effect on either action currents or the [Ca\textsuperscript{2+}]i. Under Na\textsuperscript{+}-free conditions, a depolarizing pulse of 20 mM K\textsuperscript{+} induced repetitive action currents and stepwise increases in the [Ca\textsuperscript{2+}]i. The Ca\textsuperscript{2+} channel blocker CoCl\textsubscript{2} eliminated action currents and stepwise increases in the [Ca\textsuperscript{2+}]i, in both the absence and presence of high K\textsuperscript{+}. We furthermore demonstrate that the speed of Ca\textsuperscript{2+} removal from the cytoplasm depends on the [Ca\textsuperscript{2+}]i, also between Ca\textsuperscript{2+} steps during the rising phase of an oscillation. It is concluded that Ca\textsuperscript{2+} channels, and not Na\textsuperscript{+} channels, are essential for the generation of specific step patterns and, furthermore, that the frequency and shape of Ca\textsuperscript{2+} action currents in combination with the Ca\textsuperscript{2+} removal rate determine the oscillatory pattern.

Various cellular processes like gene expression, proliferation, contraction, and secretion are regulated by extracellular first-messenger molecules such as hormones, neurotransmitters, and growth factors. Regulation of these processes is often mediated by extracellular second messengers such as cAMP, inositol 1,4,5-trisphosphate, and Ca\textsuperscript{2+}, which convert the extracellular signal into a cellular or subcellular response. Among second messenger-mediated signaling processes, Ca\textsuperscript{2+} signaling is receiving much attention (1–6). This signaling is assumed to be involved in neurotransmitter-controlled biosynthesis of proopiomelanocortin, the precursor of α-melanophore-stimulating hormone (23, 28–30). The Ca\textsuperscript{2+} oscillations depend on the activity of α-conotoxin GVIA-sensitive Ca\textsuperscript{2+} channels in the plasma membrane (25, 26). Spatio-temporal studies using confocal laser-scanning microscopy have shown that each oscillation starts at the plasma membrane and is subsequently propagated as a wave to the nucleus (30, 31). The high temporal resolution of the line-scanning mode of the confocal laser-scanning microscopy has revealed that the rise phase of each oscillation is built up by a number of discrete increases referred to as Ca\textsuperscript{2+} steps (30, 31). It has been suggested that the steps are building blocks for Ca\textsuperscript{2+} signaling in the Xenopus melanotrope cell (31). So far, no detailed information is available on how the steps contribute to the generation of distinct Ca\textsuperscript{2+} patterns. Xenopus melanotrope cells have also been shown to display bursting electrical activity (32, 33). This raises the possibility that the action potentials are the driving force for local Ca\textsuperscript{2+} influxes that give rise to the stepwise build up of Ca\textsuperscript{2+} to form distinct Ca\textsuperscript{2+} patterns. To test this hypothesis we have performed simultaneous measurements of electrical plasma membrane activity (cell-attached patch clamping) and Ca\textsuperscript{2+} signaling (microfluorometry). We show that the membrane action currents are Ca\textsuperscript{2+} currents, that each Ca\textsuperscript{2+} step is created by a single action current, and that the bursting pattern of Ca\textsuperscript{2+} currents, in combination with the Ca\textsuperscript{2+} removal rate, determines the shape of each oscillation.
EXPERIMENTAL PROCEDURES

Animals—Young-adult (8 months of age) male and female specimens of *X. laevis*, raised in our department under standard laboratory conditions, were adapted to a dark background for at least three weeks before the experiments, under continuous illumination, at 22 °C. The animals were fed weekly with beef heart. All experiments have been carried out under the guidelines of Dutch laws concerning animal welfare.

Cell Culture—Animals were anesthetized in a solution containing 0.1% (w/v) MS222 (3-aminobenzoic acid ethyl ester; Sigma). To remove blood cells, the animals were perfused with Xenopus Ringer's solution containing 112 mM NaCl, 2 mM KCl, 2 mM CaCl2, 15 mM Ultral-HEPES (Calbiochem), 10 mM glucose, and 0.025% (w/v) MS222 (pH 7.4). After decapitation, neurointermediate lobes of the pituitary gland were rapidly dissected and rinsed four times in XL L15 culture medium consisting of 76% (v/v) L15 medium (Life Technologies, Inc.), 1% (v/v) kanamycin solution (Life Technologies, Inc.), 1% (v/v) antibiotic/antimycotic solution (Life Technologies, Inc.), 2 mM CaCl2, and 10 mM glucose (pH 7.4). After an incubation period of 45 min in Xenopus Ringer's solution without CaCl2 and with 0.25% (w/v) trypsin (Life Technologies, Inc.), the lobes were dissociated by gentle trituration with a siliconized Pasteur pipette puller (Narishige Scientific Instrument Laboratories, Tokyo, Japan) using a Narishige PP-83 microfluorometric and electrophysiological data was performed with an Apple Macintosh PowerPC 8200/120 with Pulse/Pulsefit software (version 6.07, HEKA). Patch electrodes were pulled from borosilicate glass capillaries (GC150–15; Clark Electromedical Instruments, Pangbourne, UK) using a Narishige PP-83 pipette puller (Narishige Scientific Instrument Laboratories, Tokyo, Japan). They were filled with Xenopus Ringer's solution.

To study the calcium dynamics during loading, cells (n = 4) were loaded with fura-2 via patch pipettes in the whole-cell voltage-clamp configuration. After break-in, Ca2+ influx was triggered every 15 s by depolarizing pulses from −80 to 0 mV with a duration of 100 ms. At the same time fura-2 fluorescence emission intensities were measured at 355 and 380 nm excitation as described above. The ratio of the emission intensities (355 nm/380 nm) was used to determine the decay time constants (τ) and the relative amplitude (A(r)) of the evoked Ca2+ transients at different fura-2 concentrations.

Chemicals—Drug-containing solutions were applied by local perfusion from a wide-mouthed glass pipette (inner diameter 0.8 mm) placed about 100 μm from the recorded cell. The level of the bath solution was kept constant by means of a suction device. In Na+-free conditions, NaCl was replaced by an equiosmotic amount of N-methylglycine. To block Na+ channels, 1 μM tetrodotoxin (TTX) was used, whereas Ca2+ channels were blocked with 2 mM CoCl2. To keep the osmolarity constant, the concentration of NaCl was adjusted when high K+ concentrations (20 mM) were used. All chemicals were from Sigma, unless stated otherwise.

RESULTS

Calcium Dynamics and Fura-2-loading—Because it is known that the concentration of exogenous Ca2+ buffers like fura-2 can alter the amplitude and kinetics of Ca2+ transients (36, 37) we checked whether this also holds for *Xenopus* melanotropes. Cells (n = 4) were loaded with fura-2 via patch pipettes in the whole-cell voltage-clamp configuration. After break-in, Ca2+ influx was triggered every 15 s by depolarizing pulses from −80 to 0 mV with a duration of 100 ms (Fig. 1A, circles). The Ca2+ -insensitive fluorescence (F355) was used to monitor the diffusion of fura-2 into the cell (Fig. 1A). We assumed that the concentration of fura-2 in the cell was equal to the fura-2 pipette concentration when F355 reached a plateau. As F355 followed an exponential time course, the fura-2 concentration during loading could be calculated at any given time. The ratio of the emission intensities (355 nm/380 nm; e.g., Fig. 1A, inset) was used to determine the decay time constants (Fig. 1B, τ(peak)) and relative amplitude (Ar = Rpeak/Rbasal; Fig. 1C) of the evoked transients at different fura-2 concentrations. The inset in Fig. 1A shows a transient directly measured after break-in (Fig. 1A; black circle). To determine r, single exponentials were fitted to the decays of the Ca2+ transients with a fit range that started within 20 ms after the peak and extended to 5 s after the peak. The dependence of r on the [fura-2] was well described by a linear relationship according to r = A(B + [fura-2]) with A = 2.5 ± 0.37, B = 4.5 × 10−3 ± 8 × 10−3, and a linear correlation coefficient (Pearson's r; rp) of 0.0024. This almost horizontal line indicates that r was not dependent on the [fura-2]. The relationship between Ar and the [fura-2] was described by the line Ar = C + D × [fura-2] (Fig. 1C) with C = 1.38 ± 0.027, D = −0.002 ± 0.0006, and rp = 0.67. This means that there was only a small decrease (ΔA = −0.002 ± 0.0006) in amplitude during loading. However, no relationship between Ar and τ was found (Fig. 1D; horizontal line, τ = E + F × Ar with E = 2.5 ± 2.93, F = 0.019 ± 2.27, and rp = 0.0024).

Spontaneous Ca2+ Patterns and Electrical Activity—About 80% of the single melanotrope cells derived from the pituitary gland of *X. laevis* appear to display spontaneous Ca2+ oscillations (26). In the present study, a total of 42 oscillating cells were studied to investigate the detailed nature and origin of the Ca2+ patterns in individual cells loaded with the Ca2+ indicator fura-2/AM. When recording the [Ca2+]i at a sampling rate of 6 s, smooth oscillations with a fixed frequency and amplitude were observed (e.g., Fig. 2A). With a much higher temporal resolution of 20 ms, cells showed highly complex Ca2+ oscillation patterns with strong inter- and intracellular differences (Fig. 2, B–D). In most cells (37 of 42), oscillations did not appear smooth but showed stepwise increases, the Ca2+ steps (Fig. 2, B and C). In only a few cases (5 of 42), the oscillations...
reached the peak amplitude after one discrete rise in the [Ca\(^{2+}\)], (Fig. 2D). Oscillations of different cells not only varied in frequency and relative amplitude but also in the number of steps building up an oscillation, which ranged from 1 (Fig. 2D) to 17 (Fig. 8A). Within a given cell, the number of Ca\(^{2+}\) steps building up a Ca\(^{2+}\) oscillation can also vary (Fig. 2E). Fig. 2E shows that the amplitude of the oscillatory pattern may not necessarily be determined by the number of steps building up an oscillation. For example, the first and second oscillation shown in Fig. 2E have the same relative amplitude (Ar = 1.39), whereas in the first oscillation two more steps are required to reach this amplitude. The amplitude of the oscillation displayed in Fig. 2D is even bigger (Ar = 1.69) than that shown in

**Fig. 1. Calcium dynamics and fura-2 loading.** *Xenopus melano- tropes* were loaded with 100 μM fura-2 via patch pipettes in the whole-cell voltage-clamp configuration. A, the Ca\(^{2+}\)-insensitive isosbestic fluorescence (F_{iso}) was used to monitor the loading of cells with 100 μM fura-2. Background fluorescence intensity was determined in the cell-attached mode (dashed line). After break-in, Ca\(^{2+}\) influx was triggered every 15 s by depolarizing pulses from -80 to 0 mV with a duration of 100 ms (indicated by circles). The inset shows the ratiometric Ca\(^{2+}\)-transient [F_{380}/F_{355}] measured 15 s after break-in (black circle in F_{355} trace). τ is obtained from the single exponential function describing the decay phase of the transient. The Ar in arbitrary units (AU) is given as $R_{sec}/R_{basal}$. B, the decay time constants (±S.D.) of the ratiometric Ca\(^{2+}\) transients as a function of the [fura-2]. The line $\tau = A + B \times [\text{fura-2}]$, with $A = 2.5 \pm 0.37, B = 4.5 \times 10^{-5} \pm 8 \times 10^{-3}$, and $r_p = 0.0024$, describes the relationship between $\tau$ and the [fura-2], C, the Ar of the ratiometric Ca\(^{2+}\) transients as a function of the [fura-2]. The line $Ar = C + D \times [\text{fura-2}]$, with $C = 1.38 \pm 0.027, D = -0.002 \pm 0.0098$, and $r_p = -0.67$, describes the relationship between Ar and the [fura-2]. D, the decay time constants (±S.D.) of the ratiometric Ca\(^{2+}\) transients as a function of Ar. The line $\tau = E + F \times Ar$, with $E = 2.5 \pm 2.93, F = 0.0019 \pm 2.27$, and $r_p = 0.0024$, describes the relationship between $\tau$ and Ar (for details, see "Results").
tropes (Fig. 2F; n = 6). These bursts were similar to the bursts observed during combined measurements with fura-2-loaded cells (see Fig. 3A), indicating that loading the cells with fura-2 does not alter the firing behavior.

Relation between Ca$^{2+}$ Patterns and Action Potential Firing—To study the relation between the electrical activity and Ca$^{2+}$ oscillations of the same cell, electrophysiological measurements were combined with simultaneous measurements of changes in the [Ca$^{2+}$], (Fig. 3A; n = 42). To check for possible pipette-induced changes in the original Ca$^{2+}$ signal, each combined experiment was preceded by a Ca$^{2+}$ measurement alone. Fig. 3A shows that the bursts of electrical activity are directly related to the Ca$^{2+}$ oscillations. From looking in detail (Fig. 3B), it is evident that each action current is accompanied by a discrete rise in the [Ca$^{2+}$]. After a burst of action currents, the [Ca$^{2+}$] smoothly returns to the basal level.

During some measurements, action current firing changed from a bursting mode into continuous firing (Fig. 4A), resulting in the disappearance of Ca$^{2+}$ oscillations and a steady high [Ca$^{2+}$]. Because this phenomenon was observed during the Ca$^{2+}$ measurements preceding the combined measurements, we conclude that these particular changes in the firing pattern could have been induced by the pipette. Therefore, such recordings were discarded. Nonetheless, it is interesting to note that even at this high Ca$^{2+}$ level, the tight relationship between action current firing and the occurrence of Ca$^{2+}$ steps was maintained (Fig. 4B).

Involvement of Na$^{+}$ and Ca$^{2+}$ in Step Generation—To determine the nature of the inward currents, the Na$^{+}$ channel blocker TTX and the inorganic Ca$^{2+}$ channel blocker Co$^{2+}$ were added. 1 μM TTX did not have an effect on either action currents or Ca$^{2+}$ oscillations in any of the cells measured (Fig. 5A; n = 11). On the other hand, applying 2 mM CoCl$_2$ clearly abolished both action currents and Ca$^{2+}$ oscillations in every cell studied (Fig. 5B; n = 15). In the complete absence of extracellular Na$^{+}$ (Na$^{+}$ replaced by N-methyl-d-glucamine), no action currents or Ca$^{2+}$ oscillations were observed (Fig. 6A; n = 4). Under this Na$^{+}$-free condition, action currents and a rise in the [Ca$^{2+}$], could still be induced by a depolarizing K$^+$ (20 mM) pulse (Fig. 6A; n = 4). The rise in the [Ca$^{2+}$], was clearly built up by a number of Ca$^{2+}$ steps, each accompanied by an action current (Fig. 6B).

Relation between the Shape of an Action Current and the Amplitude of a Ca$^{2+}$ Step—Because action current measurements were performed simultaneously with Ca$^{2+}$ measurements, a link between the shape of an action current and the amplitude of a Ca$^{2+}$ step could be demonstrated. The upper trace of Fig. 7A shows the action currents reflecting the Ca$^{2+}$ steps shown in the lower trace of Fig. 7A. Whereas the amplitude of the successive action currents decreased, the amplitude of the accompanying Ca$^{2+}$ steps increased. The relative difference in the ratio values between the start and the top of a Ca$^{2+}$ step ($Ar = R_{top}/R_{basal}$; lower trace of Fig. 7A) was taken as the step amplitude. To determine the amount of charge entering
FIG. 5. Effect of Na\(^+\) channel blocker TTX and Ca\(^{2+}\) channel blocker Co\(^{2+}\) on action current firing and the [Ca\(^{2+}\)]. The figure shows simultaneous measurements of action currents (upper trace) and [Ca\(^{2+}\)], changes (lower trace). A, 1 \(\mu\)M TTX was applied to the cells as indicated by the horizontal bar. TTX did not have an effect on either action current firing or on Ca\(^{2+}\) oscillations. B, by adding 2 \(\mu\)M CoCl\(_2\) in the extracellular solution, Co\(^{2+}\) was applied to the cells as indicated by the horizontal bar. Co\(^{2+}\) reversibly blocked both action current firing (upper trace) and Ca\(^{2+}\) oscillations (lower trace).

the cell during an action current, the peak areas of the downward action currents were integrated. In Fig. 7B the peak area of each action current was plotted against the relative amplitude of the Ca\(^{2+}\) steps in Fig. 7A. The peak areas of the three successive action currents increased, and the amplitude of the Ca\(^{2+}\) steps increased. A clear linear relation was found between the current peak area and the Ca\(^{2+}\) step size.

Kinetics of Ca\(^{2+}\) Removal during a Ca\(^{2+}\) Oscillation—The calcium removal kinetics have been studied to investigate the role of this removal in shaping the calcium oscillations. The presence of a discontinuous (i.e. stepping) Ca\(^{2+}\) oscillation presents a unique opportunity to analyze the speed of removal of Ca\(^{2+}\) from the cytoplasm \((V_d)\) during the rising phase of a Ca\(^{2+}\) oscillation. The speed of Ca\(^{2+}\) removal was analyzed both during the rising phase following each Ca\(^{2+}\) step and during the declining phase, bringing Ca\(^{2+}\) back to basal level. Fig. 8A shows an example of a Ca\(^{2+}\) oscillation built up by steps. In total, 27 steps can be distinguished distributed over three individual oscillations (numbered I, II, and III).

Curve fitting was applied to model the kinetics during different phases of the Ca\(^{2+}\) oscillation. An exponential function can be used to adequately describe first order processes. By using a standard nonlinear regression algorithm (38), the decline after the top of an oscillation was modeled with a first order exponential function,

\[
R_x = A + B \times e^{-(t-x_0)/\tau}
\]  
(Eq. 1)

where \(R_x\) is the fura-2 emission ratio at time \(x\), and \(A, B, x_0\), and \(\tau\) are constants obtained from the fit.

For peak I, the parameters in this equation were estimated from a fit of 628 data points: \(A = 1.04 \pm 0.000166, B = 0.737 \pm 0.00151, x_0 = 25.08\), and \(\tau = 4.75 \pm 0.0312\). Given the quality of the fit \((\chi^2 = 0.000008, \text{S.D.} < 10\%)\) we considered the exponential model to be valid (38). However, between steps, each decline consists of a limited amount of data points (varying between 24 and 104 for Fig. 8A), and a reliable exponential fit could not be obtained. Because over a small interval an exponential function can be approximated by a straight line, we used a linear approximation for quantifying the calcium re-
Ca\textsuperscript{2+} Action Currents and Stepwise Ca\textsuperscript{2+} Oscillations

produce the line shown in Fig. 8C. For the analyzed trace, \(V_{d,\text{linear}}\) was dynamically up- and down-regulated during the different phases of the Ca\textsuperscript{2+} oscillation, such that steps near the top of the oscillation were associated with a higher \(V_{d,\text{linear}}\).

To determine whether \(V_d\) is a function of \(R_{t,n}\) (i.e. if the removal speed of Ca\textsuperscript{2+} is a function of \([\text{Ca}^{2+}])\), the decline speed \(V_{d,\text{linear}}\) was plotted against \(R_{t,n}\) (Fig. 8D). A clear linear correlation between \(R_{t,n}\) and \(V_{d,\text{linear}}\) was found (\(A = -0.23 \pm 0.020, B = 0.22 \pm 0.013, r_{\text{value}} = 0.92\)), reflecting a linear increase of \(V_{d,\text{linear}}\) with \(R_{t,n}\). A saturation of \(V_{d,\text{linear}}\) was not observed during analysis (Fig. 8D, no deviation from the linear model at the highest \(R_{t,n}\) values).

To compare Ca\textsuperscript{2+} removal kinetics during the rising phase (after each step) with the Ca\textsuperscript{2+} removal kinetics during the declining phase of the oscillation, \(V_{d,\text{linear}}\) was compared with \(V_{d,\text{exp}}\), the velocity of Ca\textsuperscript{2+} removal given by a first order exponential function (Eq. 1). By substituting \(R_{t,n}\) and \(R_{b,n+1}\) (which were directly estimated from the original recording) in Eq. 1, \(x_{t,n}\) and \(x_{b,n+1}\) can be calculated by writing

\[
x = x_0 - \mu \times \ln(R_t - A)/B \quad \text{(Eq. 3)}
\]

In this way, substituting \(R_{t,n}\) gives \(x_{t,n}\), and \(R_{b,n+1}\) gives \(x_{b,n+1}\). By calculating \((R_{t,n} - R_{b,n+1})/(x_{b,n+1} - x_{t,n})\), \(V_{d,\text{exp}}\) can be obtained for each \(n\). Fig. 8E clearly shows that \(V_{d,\text{linear}}\) and \(V_{d,\text{exp}}\) are linearly correlated (\(A = 0.0016 \pm 0.00064, B = 0.87 \pm 0.054, r_{\text{value}} = 0.92\)). The low value of \(A\) and a value of \(B\) near unity show that the linear correlation is almost ideal (for a 45° line, consistent with a perfect linear correlation, \(A = 0\) and \(B = \tan 45° = 1\)). In general, the correlation between \(V_d\) and \(R_{t,n}\) was not affected at all by occurrence of steps during the rising phase of the oscillation.

**DISCUSSION**

**Calcium Dynamics and Fura-2 Loading**—Because it is known that the concentration of exogeneous Ca\textsuperscript{2+} buffers like fura-2 can alter the amplitude and kinetics of Ca\textsuperscript{2+} transients in presynaptic terminals of neurons (36, 37), we checked whether this holds also for the neuroendocrine melanotrope cells of *Xenopus*. The \(\tau\) values of the decays of the Ca\textsuperscript{2+} transients are independent of the [fura-2] and are in the range of 2–5 s. Only a very small decrease in relative amplitude (\(Ar\)) was found during loading. However, absolutely no relationship between \(\tau\) and \(Ar\) is present. Therefore, we conclude that the intrinsic Ca\textsuperscript{2+} removal in *Xenopus* melanotropes is rather slow and not altered by the loading with fura-2.

**Ca\textsuperscript{2+} Patterns**—Ca\textsuperscript{2+} oscillations in *Xenopus* melanotropes have a frequency of about 1–3/min, and within each cell, have a rather stable amplitude (24–27). They have a smooth appearance when observed during Ca\textsuperscript{2+} measurements using a sampling interval of 6 s (see Fig. 1A). In the present study, these oscillations have been studied in detail, with particular attention to their shape and the mechanisms that generate this shape. By performing continuous measurements with a high temporal resolution (20 ms) clear inter- and intracellular variations in Ca\textsuperscript{2+} patterns were observed. The Ca\textsuperscript{2+} oscillations did not appear smooth anymore but were built up by discrete changes in the [Ca\textsuperscript{2+}], the Ca\textsuperscript{2+} steps. These findings extend recent confocal line scanning experiments (30, 31), which suggested that the rising phase of oscillations are built up by three to four of these discrete steps. The present study, where the microfluorometric method allows continuous Ca\textsuperscript{2+} measurements with a high temporal resolution, shows that the number of steps during an oscillation is variable and can be as high as 17. The oscillations had discrete rises not only during the rising phase of an oscillation but often also on top of the oscillation. This has not been reported for melanotropes or other secretory cells before.
The Role of Action Potentials in the Generation of Specific Ca²⁺ Oscillation Patterns—To study the ability of a cell to transfer information in the form of complex Ca²⁺ patterns, it is important to understand how the Ca²⁺ patterns are generated. From fast line scanning experiments, it is known that the Ca²⁺ oscillations in Xenopus melanotropes can travel through the cell as multiple waves from the plasma membrane into the nucleus (30, 31). The Ca²⁺ oscillations are inhibited by the N-type Ca²⁺ channel blocker ω-conotoxin GVIA (26) and not by the L-type Ca²⁺ channel blocker nifedipine (26). This indicates that they originate at the membrane by Ca²⁺ influx through N-type Ca²⁺ channels. It has been hypothesized that action potentials cause the opening of the voltage-operated N-type Ca²⁺ channels (32, 33, 39, 40). However, a direct relation between action potentials and Ca²⁺ oscillations has never been shown in pituitary melanotropes. Until now, the action potentials in Xenopus melanotropes observed have been induced by depolarizing pulses using the whole-cell patch-clamp technique (32, 33), or they have been measured using the perforated-patch configuration (33). In the present study, the cell-attached configuration of the patch-clamp technique was used to prevent any disturbances of the intracellular environment. In this

**FIG. 8.** Kinetics of Ca²⁺ removal from the cytoplasm changes during a Ca²⁺ oscillation. The velocity of Ca²⁺ removal from the cytoplasm (V_d) was analyzed both during the rising phase and the declining phase of oscillations. A, three oscillations (I, II, and III) containing 27 steps (see numbers) were used to study V_d. Dotted lines represent the linear fits of the interval [Rt,n, Rb,n+1] (see panel B). Step marked by an asterisk (*) was omitted from analysis (bad fit). The box indicates the part that is enlarged to form panel B. B, parameters used to describe the step kinetics. Rₘ,n, the resting fura-2 emission ratio; Rₜ,n, the fura-2 emission ratio at the top of step n; Rₘ,n, the fura-2 emission ratio just before occurrence of step n; and dxₙ, the time needed for the fura-2 emission ratio to drop from Rₜ,n to Rₘ,n+1. An asterisk (*) marks the time needed for the fura-2 emission ratio to rise from Rₘ,n to Rₜ,n. C, V_d(linear), the velocity of Ca²⁺ removal after a Ca²⁺ step determined by a linear fit (for details, see “Results”), plotted against the step number. D, a linear correlation was found between V_d(linear) and Rₜ,n, the fura-2 emission ratio at the top of step n. E, a clear linear correlation was found between V_d(linear) and V_d(exp). V_d(exp) is the velocity of Ca²⁺ removal during the declining phase of oscillations determined using a first order exponential function (see Eq. 1 under “Results”).
patch configuration, bursts of action currents representing bursts of action potentials were observed. The bursting behavior was not induced by fura-2 loading as both loaded and unloaded cells displayed this firing behavior. The combined patch-clamp and Ca\(^{2+}\) microfluorometry showed that each action current is generating a discrete, stepwise increase in the [Ca\(^{2+}\)]\(_i\). In this way, a burst of action currents builds up a full-sized Ca\(^{2+}\) oscillation. Also the Ca\(^{2+}\) steps on top of an oscillation appeared to be generated by action currents. After each burst of action currents, the [Ca\(^{2+}\)]\(_i\) smoothly returned to the basal level. In some combined experiments, the Ca\(^{2+}\) signal went up and stayed high. At the same time, action current firing changed from a bursting pattern into continuous firing, as observed previously in Xenopus melanotropes (32, 33). These changes may have been induced by the patch pipette because such sustained Ca\(^{2+}\) elevations were never observed in the control Ca\(^{2+}\) measurements preceding the combined experiments.

A relation between action potentials and Ca\(^{2+}\) transients has been established in other pituitary cells (41–44), but in none of these studies have such complex Ca\(^{2+}\) patterns been seen to be generated spontaneously as in the Xenopus melanotropes. In the former studies, either single, small amplitude Ca\(^{2+}\) transients were generated by single action potentials firing at a low frequency (41–43), or large transients lacking discrete rises (44) were generated by bursts of action potentials. In rat corticotrophs stimulated by vasopressin, a huge transient rise in [Ca\(^{2+}\)]\(_i\) was generated by bursts of action potentials. If the Ca\(^{2+}\) removal speed linearly increases, reaching a maximum near the end of a burst of action potentials. If the Ca\(^{2+}\) removal is very fast, each action potential gives rise to a single Ca\(^{2+}\) transient. In these models fixed values for the speed of Ca\(^{2+}\) removal were used. However, we have now demonstrated that the Ca\(^{2+}\) removal rate is not fixed during an oscillation.

When a burst of action currents is terminated, the [Ca\(^{2+}\)]\(_i\) smoothly declines in a single exponential way, indicating the presence of one Ca\(^{2+}\) removal process. The mechanism of termination of a burst may involve Ca\(^{2+}\)-dependent K\(^+\) channels (50, 51) or voltage- or Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels (50, 52, 53). The single exponential function describing the decay after the top of an oscillation exactly fits the recorded phases of the Ca\(^{2+}\) steps during the rising phase of the oscillation. This indicates that the same Ca\(^{2+}\) removal process is active during the rising phase and the decay phase of an oscillation.

**Conclusion**—This study shows that bursting firing in combination with the calcium removal rate determine the shape of a Ca\(^{2+}\) oscillation. In this way individual Xenopus melanotrope cells can generate different Ca\(^{2+}\) oscillatory patterns. We propose that not only the frequency and amplitude of the Ca\(^{2+}\) oscillations serve to encode cellular regulatory information but also other oscillation pattern variables such as number of Ca\(^{2+}\) steps, the step amplitude, the step frequency, and the speed of Ca\(^{2+}\) removal. Therefore, the information storage capacity of the Ca\(^{2+}\) signaling seems much more complex than previously thought. Currently, studies are being carried out on the modulation of the Ca\(^{2+}\) patterns in Xenopus melanotropes by various regulatory neurotransmitters and under different conditions of adaptation of the animal to background light intensity.

**Acknowledgments**—We thank P. M. J. M. Crijnsen for technical assistance and R. J. C. Engels for animal care.

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\(^{1}\)J. R. Lieste, unpublished observation.
\(^{2}\)W. J. H. Koopman, unpublished observation.
