Analysis of Calcium Activated Chloride Current Fluctuations in *Xenopus laevis* Oocytes

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Abstract. Fluctuactions of calcium activated chloride currents were investigated in oocytes of *Xenopus laevis*. The method of noise analysis and the model of chloride channels activation by calcium ions were used to estimate the chloride channels lifetime and the average frequency of current fluctuations, which depends on changes of cytoplasmic calcium concentration. This current fluctuations can be evoked by activation of cholinergic receptors or inhibition by Na_3VO_4 of plasma membrane Ca^{2+}—ATPase. The average opening lifetime of chloride channels was $\approx$ 100 ms. The frequency of fluctuations increased with the increasing extracellular calcium concentrations and external ACh concentrations. Caffeine in 2 mmol/l concentration changed the current fluctuations into oscillations with a period of about 18—20 s. Ten mmol/l caffeine fully inhibited the oscillation activity.

Key words: *Xenopus laevis* oocyte — Noise analysis — Calcium oscillations

Introduction

The *Xenopus laevis* oocyte is a convenient model system for the investigation of neurotransmitter-induced membrane electrical responses. The acetylcholine (ACh)-induced Cl^- current was first reported by Kusano et al. (1977), and subsequently studied in detail (Kusano et al. 1982; Dascal and Landau 1980, 1982; Dascal et al. 1984). The muscarinic response of the oocyte comprise large inward current fluctuations. These membrane currents are activated by intracellular Ca^{2+} (Dascal at al. 1985;) and can be mimicked by intracellular injection of inositol 1, 4, 5-trisphosphate (Insl, 4, $5P_3$) (Oron et al. 1985; Parker

and Miledy 1986). Insl, 4, $5P_3$ has been identified as a second messenger that can mobilize Ca^{2+} from the endoplasmatic reticulum (Berridge and Irvine 1984).

Many cells display oscillations of intracellular calcium resulting from periodic calcium releases from intracellular reservoirs. Such oscillations of calcium have einther been measured directly, as in mammalian eggs (Cuthbertson and Cobbold 1985; Igusa and Miyazaki 1986), in hepatocytes (Woods et al. 1986) and in cultured cells (Ueda et al. 1986; Yada et al. 1986), or they have been inferred from indirect measurements of membrane potential or current, as in secretory cells (Rapp and Berridge 1981) and *Xenopus* oocytes (Kusano et al. 1982; Miledi et al. 1982; Oron et al. 1985; Berridge 1988).

In the case of *Xenopus* oocytes, which are known to have muscarinic receptors coupled to the hydrolysis of phosphatidylinositol 4,5— bisphosphate (Oron et al. 1985, Nomura et al. 1987), the calcium oscillatory activity induced by acetylchloline is apparently triggered by the formation of Ins1,4,$5P_3$, which controls the periodic release of calcium from the endoplasmatic reticulum. The oocyte plasma membrane has calcium-sensitive chloride channels opening and closing of which are responsible for chloride current fluctuations. Injection of cells with EGTA blocks these fluctuations induced by either ACh or Ins1,4,$5P_3$ (Parker et al. 1985; Dascal et al. 1985; Hirono et al. 1987). On the other hand, removal of external calcium or the addition of calcium antagonists has little effect on oscillatory activity in *Xenopus* oocytes (Parker et al. 1985; Zachar et al. 1988).

All this evidence suggests that the inward current fluctuations depend upon the oscillations in intracellular Ca^{2+}, which appear to arise as a result of a periodic release of stored calcium. In an attempt to uncover the cellular basis of the periodic release of calcium and the inward current fluctuations, we developed a model of calcium dependence of the chloride current. The parameters of the model were obtained by fitting of experimental data. Oscillatory activity was studied in *Xenopus* oocytes following addition of ACh or inhibition of ATPase by Na_3VO_4.

Materials and Methods

Preparation: *Xenopus laevis* females were from the breeding station of the Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences.

The animals were maintained as described elsewhere (Dascal et al. 1984, 1985). The frogs were first anesthetized by immersion in a 0.2 % solution of ethyl m-aminobenzoate (MS-222). A small incision was made in the abdomen and several ovarian lobes were removed. Individual oocytes were freed from the surrounding epithelial cells, but the follicle cells were not removed as this has been shown to lead to a marked decline in the responsiveness to ACh. The oocytes were maintained at 16°C in modified Barth solution (MBS) containing 50 μg/ml gentamycin and 50 μg/ml nystatin.

Table 1. Composition of solutions (in mmol/l)

No	Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	Tris-HCl
1	Normal Ringer	116	2	1.8	1.0	5
2	Higher Ca	106	2	6.8	1.0	5
3	High Ca	96	2	11.8	1.0	5
4	Ca free	82.5	2	—	20.0	5

Electrophysiology: The oocytes were held in a 2 ml bath at 20 °C constantly perfused with one of the solutions listed in Table 1. The electrophysiological recording was made with a two microelectrode-voltage clamp amplifier (Kristian and Poledna 1990). The cell was impaled with two conventional 3 mol/l KCl electrodes. The microelectrode for membrane potential measurement had a resistance of 2—5MΩ, the tip of the other one for current injection was gently broken to give resistances of 0.5 — 1 MΩ. The mictroelectrodes were connected to the voltage clamp amplifier with an Ag-AgCl silver wire. The extracellular medium was connected to the current-voltage convector via an Ag--AgCl agar-3 mol/l KCl bridge.

The experiments were started 20 min after the impalement of both microelectrodes to allow the membrane potential to stabilize. Muscarine responses were elicited by 3—4 min applications of acetylcholine (ACh) every 40 min. The long interval was required to warrant reproducibility of the responses. At least two identical ACh responses were obtained before the beginning of the experiment. In intervals between the drug applications, the voltage clamp circuit was usually disconnected, so that no current was applied. Thus, the cells were voltage clamped only during a few minutes before and during the drug application.

Solutions: The solutions used are listed in Table 1. The experiments were performed at room temperature (20—22 °C). The pH of the solutions was 7.2. All the solutions used in a single experiment had the same pH (within 0.05 units) and the same osmolality.
The Barth solution had the following composition (in mmol/l): 88 NaCl, 1 KCl, 0.41 $CaCl_2$, 0.3 $CaNO_3$, 0.82 $MgSO_4$, 2.37 $NaHCO_3$, 5 Tris-HCl (pH 7.4)

Signal recording and analysis: Membrane potential and current were recorded on a dual-channel potentiometric chart recorder and a Tektronix 5103N dual beam oscilloscope. The signal from the current-voltage transducer (membrane current) was amplified and filtered at 5 Hz by a low-pass filter (-3 dB, 4-pole Bessel type), then digitized at a 14 Hz sampling rate in a 12-bits analog-digital convertor of a SM 50/50 computer. The data were stored on diskettes for further analysis. The records of 3072 samples were divided into six sections containing 512 samples each. From each section the DC level was subtracted, the spectral densities were calculated, and subsequently averaged to obtain the mean power spectrum.

Control noise records were subtracted from agonist-evoked fluctuations. An attempt to fit the resulting power spectrum with a single Lorentzian function was unsuccessful (Fig. 1.*a*). The power spectral density function of Poisson wave with unit events equal to the difference between two exponential functions successfully fitted the resulting power spectral density calculated from the recorded data (Fig. 1. b). The function of an unit event (unit current) is

$$i(t) = h(e^{-t/T1} - e^{-t/T2}) \quad (1)$$

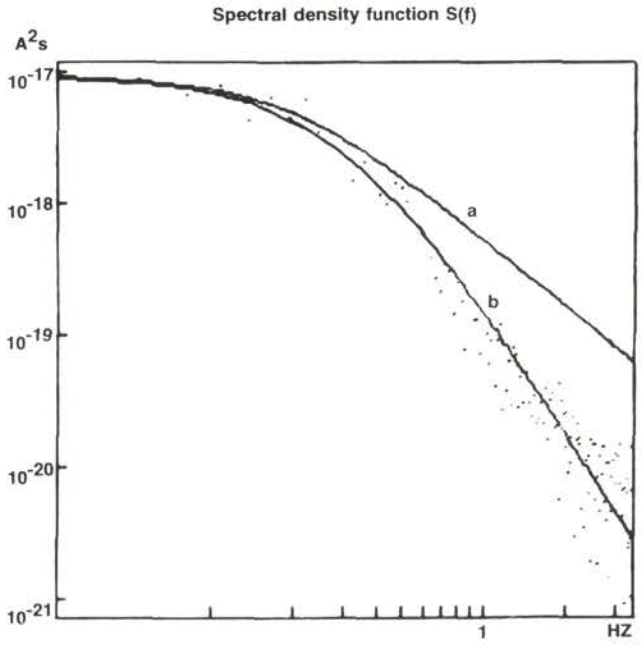

Fig. 1. The power spectrum of 10^{-5} mol/l ACh induced chloride current fluctuations and their fits to Lorentz function (a) or to function (4) (b). Holding potential -50 mV. The values of $T1$ and $T2$ are 1.2 s and 0.105 s.

The average current I is given by

$$I(t) = \nu \int i(t)\mathrm{d}t = \nu h(T1 - T2) \tag{2}$$

where ν is the average frequency of the events occurrence. The variance is given by

$$\sigma^2 = \nu \int i(t)^2 \mathrm{d}t = \frac{1}{2} \nu h^2 \frac{(T1 - T2)^2}{T1 + T2} \tag{3}$$

and the power spectral density function is given by

$$\mathrm{S}(f) = \frac{2.\nu h^2 . (T1 - T2)^2}{1 + \omega^2(T1^2 + T2^2) + \omega^4 T1^2 T2^2} \tag{4}$$

$\omega = 2\pi f$

The values of $T1$, $T2$ and S(0), estimated by a direct fit of the resulting power spectra, were used to calculate the variance σ^2 given by

$$\sigma^2 = \frac{\mathrm{S}(0)}{4(T1 + T2)} \tag{5}$$

The variance of a record was calculated also by

$$\sigma^2 = \int S(f)\mathrm{d}f \tag{6}$$

and by

$$\sigma^2 = \frac{\sum_{k=1}^{n} (I - i_k)^2}{n - 1} \tag{7}$$

where I is the average current of the record, i_k are the recorded values, to compare with the variance obtained by equation (5). The three estimates of the variance gave similar (within an accuracy of 10 %) values, and either of them could be used for the average frequency calculation.

The average frequency of current fluctuations is given by equation

$$\nu = \frac{I^2}{2\sigma^2(T1 + T2)} \tag{8}$$

Fluctuating chloride currents are activated by increasing intracellular calcium concentratios. The release of Ca^{2+} from intracellular stores leads to a step change of Ca^{2+} concentration beneath the cell membrane and activates the chloride channels. Then, the calcium concentration begins rapidly decreasing due to sequestration of Ca^{2+} ions into the intracelular stores. The intensity of the channel activation is given by

$$\mathrm{d}x/\mathrm{d}t = -\alpha x \tag{9}$$

where α is the rate constant of the channel activation changes.

Channels from the open state return to the closed state. Channels in open state are described by

$$\mathrm{d}y/\mathrm{d}t = x - \beta y \tag{10}$$

where β is the rate constant of channel closing. For the initial conditions $x(\mathrm{O} = a$, $y(\mathrm{O}) = \mathrm{O}$, the solution to these equations is

$$x = a \cdot \exp(-t\alpha) \tag{11}$$

$$y = (a/(\alpha - \beta))\,(\exp(-t\alpha) - \exp(t\beta)) \tag{12}$$

There expression for the average current I is

$$I = ipN \tag{13}$$

where i is the unit current of one channel, p is the probability of open state, N is the number of channels. Then, pN is the number of open channels, and

$$pN = y \tag{14}$$

$$I = iy \tag{15}$$

Using equations (1) and (15),

$$T1 = 1/\alpha$$

$$T2 = 1/\beta$$

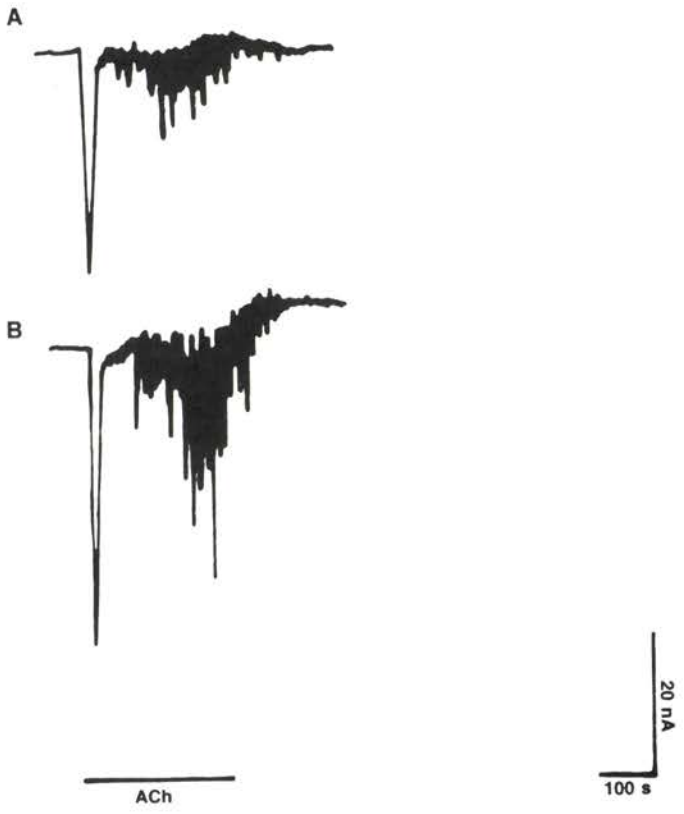

Fig. 2. Membrane current recorded in a *Xenopus* oocyte stimulated with 10^{-7} (A) and 10^{-6} (B) mmol l ACh. The oocyte was voltage clamped at a potential of -50 mV.

where $T1$ is the time constant of the activation intensity, and $T2$ is the lifetime of a channel in the open state.

Results

A typical response of *Xenopus* oocytes to ACh is shown in Fig. 2. Following stimulation with ACh there was a burst of spikes followed by a return to the resting level. The computed power spectrum from these fluctuations was fitted with function (4). By this fit we estimated values of the constants $T1$, $T2$, and then computed the average frequency ν. The calculated value of the average lifetime of chloride channels ($T2$) was 100 ms (the same value was obtained by Takahashi et al. (1987)).

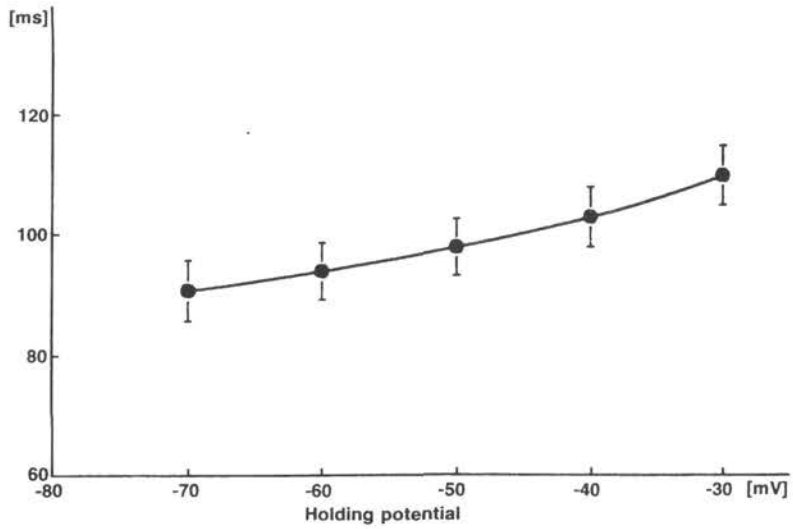

Fig. 3. The average channel lifetime as a function of membrane potential. Values are means ± S.E.M. ($n = 6$).

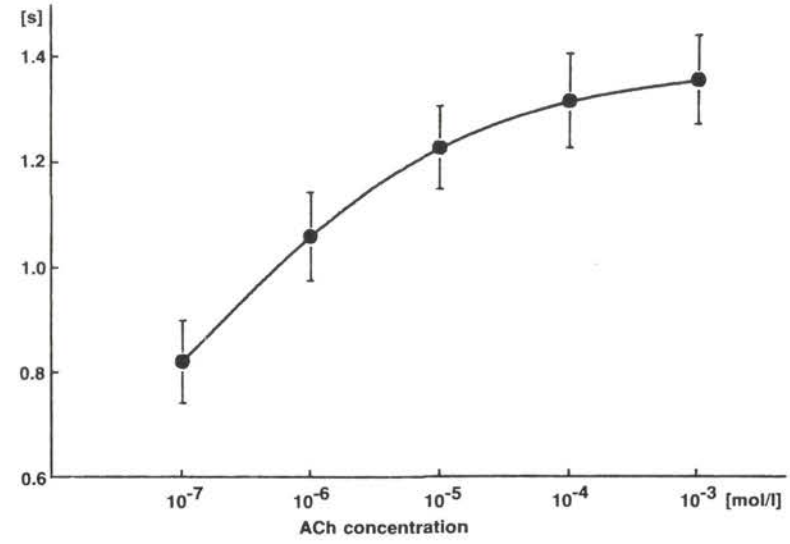

Fig. 4. The influence of ACh concentration on the activation intensity time constant $T1$. Holding potential −50 mV. The points are means ± S. E. M. ($n = 6$).

The average lifetime of the channel was independent of the ACh concentration applied, and on the extracellular calcium concentration. The holding

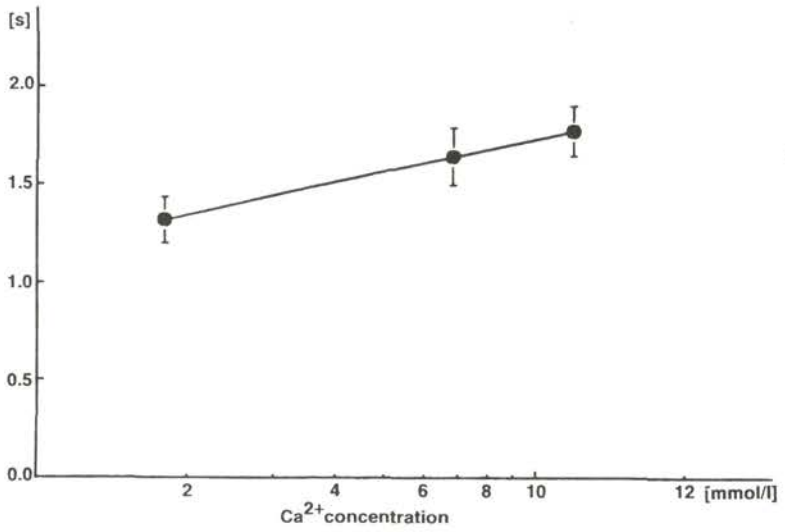

Fig. 5. The influence of extracellular Ca^{2+} concentration on the activation intensity time constant $T1$. Holding potential -50 mV. Experiments performed at 10^{-4} mol/l ACh. Values are means $\pm$ S.E.M. ($n = 6$).

potential altered the average lifetime (Fig. 3). This dependence agrees well with the single-dipole conformation model of the gating molecule.

As the time constant $T1$ expresses the change of channel activation by calcium ions, it depends on the cytoplasmic Ca^{2+} concentration. Higher agonist concentrations raised the value of $T1$ (Fig. 4.), since the higher the agonist concentration, the more calcium is released and the more chloride channels are activated. A similar effect was observed upon perfusing the oocyte with high Ca^{2+} solutions during ACh application (Fig. 5.).

The relationship between the agonist concentration and the frequency of oscillations at concentrations of 10^{-9}—5.10^{-7} mol/l was studied by Berridge (1988). The individual inward current spikes were induced by application of up to 10^{-7} mol/l ACh. At higher concentrations the spikes were larger and began to overlap resulting in the inward current fluctuations (Berridge 1988). These fluctuations do not allow direct estimating the average frequency of oscilations. In our experiments the fluctuation activity appeared at 10^{-7} mol/l ACh. Fig. 6 shows the relationship between the agonist concentration and the average-oscillation frequency. Within the concentration range from 10^{-7} to 10^{-3} mol/l, the average frequency of oscillations ranged between 0.16 Hz and 1.3 Hz. At higher concentrations (10^{-4} mol/l and 10^{-3} mol/l), the range of oscillation

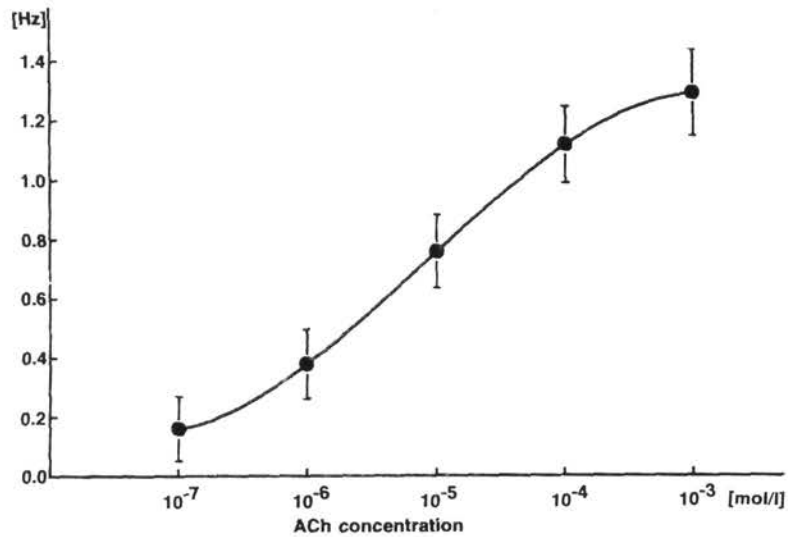

Fig. 6. Frequency of membrane current fluctuations as a function of ACh concentration. Holding potential -50 mV. Values are means $\pm$ S.E.M. ($n = 6$).

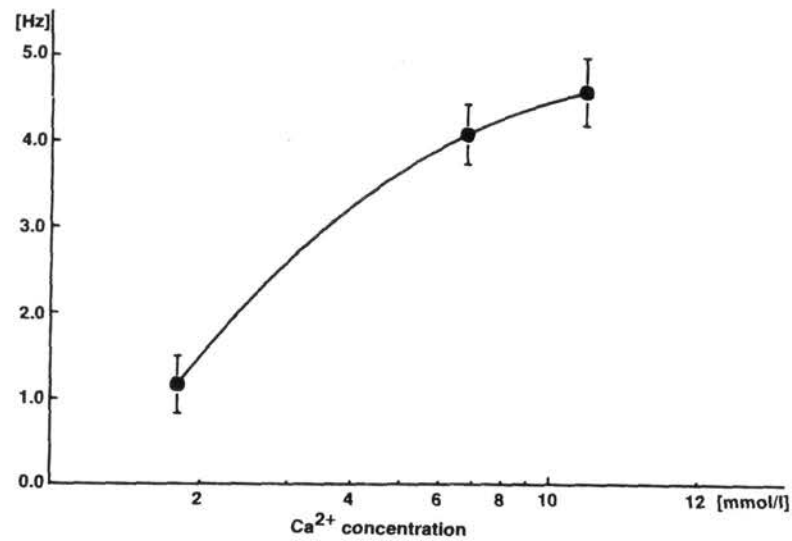

Fig. 7. The dependence of fluctuation frequency on extracellular Ca^{2+} concentration. The oocytes were stimulated with 10^{-4} mol/l ACh and voltage clamped at a potential of -50 mV. Values are means $\pm$ S.E.M. ($n = 6$).

frequencies was narrower. A model pressented by Berridge(1988) explains inward current oscillations activated by calcium based on the phenomenon of

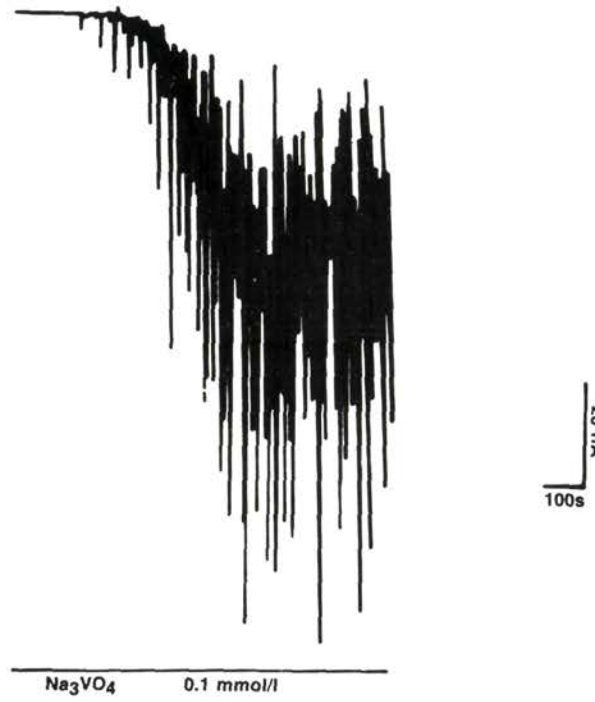

Fig. 10. The current fluctuations induced by 0.1 mmol/l vanadate. After 2—3 minutes of treatment of the oocyte with vanadate current spikes occurred, which gradually grew larger and changed into fluctuation currents.

observation was up to 30 min). At a higher concentration (10 mmol/l), the fluctuation currents were gradually inhibited after 2—5 min, and the current returned to the resting level within 7—8 min (Fig. 11). The cytoplasmic concentrations of vanadate may reach levels sufficient to inhibit ATPases in the endoplasmatic reticulum (ER) thus resulting in discontinuation of the CICR process. Another explanation for caffeine and vanadate effects is that the intracellular calcium concentration is increased to a level at which oscillation activity cannost arise.

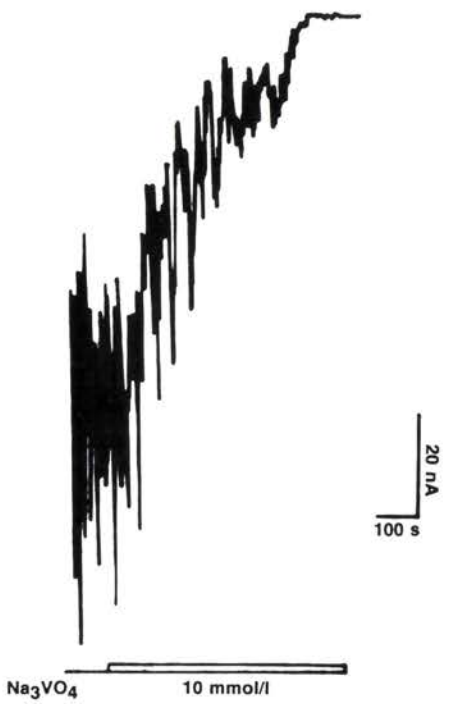

Fig. 11. The suppression of fluctuations by vanadate. After 2—5 minutes of treatment of an oocyte during fluctuation activity with a higher vanadate concentration (10 mmol/l), the fluctuation activity was inhibited. The application of 10 mmol/l vanadate during 7—8 min. fully blocked the fluctuation current.

Discussion

Inward chloride current fluctuations can be evoked by injecting larger doses of Ca^{2+} (Miledi and Parker 1984; Dascal et al. 1985), IP_3 (Oron et al. 1985; Parker and Miledi 1987; Berridge 1988; 1989). Similar current fluctuations are induced by treating oocytes with Na-free solution (Dascal et al. 1984). All these processes induced an elevation of cytosolic calcium which apparently is one of the crucial requirements to trigger the oscillations.

The chloride current fluctuations reflect changes of intracellular calcium concentration. An analysis of the information obtained from chloride current measurements required the development of a model relating the chloride channel activation and intracellular calcium changes. This approach allowed the quantitative description of the processes studied.

In *Xenopus* oocytes, which are known to have muscarinic receptors coupled to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Oron et al. 1985; Nomura et al. 1987), the oscillation activity induced by acetylcholine apparently is triggered by the formation of Ins $1,4,5P_3$ which controls the periodic release of calcium from the endoplasmic reticulum. The ER is divided into an Ins $1,4,5P_3$- sensitive and an Ins $1,4,5P_3$-insensitive pool (Biden et al. 1986) both of which could contribute to the oscillations. It seems unlikely that the Ins$1,4,5P_3$-sensitive pool will contribute directly to oscillatory activity because it was rapidly desensitized after repeated injections of Ins$1,4,5P_3$ (Berridge 1988, 1989). Probably, the function of Ins$1,4,5P_3$ is to enhance the cytosolic calcium level and to induce tehe oscillations.

A support to the CICR model of cytosolic calcium oscillations in *Xenopus* oocytes are observations that a change of cytosolic calcium concentration changes the oscillation frequency. Caffeine decreases the amplitude of oscillations and, at higher cocentrations, it fully inhibits the oscillation activity.

Cells display characteristic regular oscillatory patterns. If the individual calcium stores lie close to each other in an orderly manner, an initial increase in calcium concentration at one point may trigger progressive release of calcium from one store to the next, thus allowing the initial signal at the cell surface to spread as a wave throughout the cell. The existence of calcium waves has been described for eggs (Miyazaki et al. 1986; Busa and Nuccitelli 1985) and rat myocytes (Kort et al. 1985). The spreading of such calcium waves throughout the cell occurs when the discharges of the internal pools are locally synchronous. The pools must have a suitable spatial arrangement, and the rate constants of the calcium transport processes are appropriate for synchronous release of calcium from a part of the individual stores and to spatial coordination of this process. *Xenopus* oocytes are large cells, and the release of calcium cannot be synchronous in the entire volume. Upon treating oocytes with caffeine, the random nonsynchronous fluctuations were changed into regular oscillations. Caffeine probably alters the oscillatory conditions, which results in synchronous triggering of calcium release from internal stores.

The function of the oscillatory system in *Xenopus* oocytes is unknown, but it may play a functional role in cell signalling (Rapp and Berridge 1981). The idea is that calcium might mediate its second messenger action through a frequency-encoded rather than an amplitude-dependent mechanism. The encoding signal, in terms of the frequency of calcium oscillations, provides a more preciss modulation system. Moreover, calcium oscillations allow for selective differential stimulation of cellular processes (Woods et al. 1987), since the occurrence of a particular response will depend on the rates of the phosphorylation and dephosphorylation reactions controlled by calcium transients (Goldbeter et al. 1990).

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