Volume 261, number 1, 35–38 FEBS 08091 February 1990

Mitogen-induced oscillations of membrane potential and Ca^{2+} in human fibroblasts

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Received 6 December 1989

Using the whole-cell technique, we have measured recurring hyperpolarizations induced by fetal calf serum and bradykinin in human fibroblasts. By coupling fura-2 microfluorimetry to electrophysiology, we have also measured directly cytosolic Ca^{2+} and found that Ca^{2+} oscillations occur in synchrony with membrane currents. Mitogen stimulation of cells in which intracellular K^+ had been replaced with Cs^+ resulted in the abolishment of the outward current. We conclude then that the mitogen-induced recurring hyperpolarizations in human fibroblasts are due to the opening of Ca^{2+}-activated K^+ channels.

Mitogen; Cytoplasmic Ca^{2+}; Hyperpolarization; Oscillation; K^+ channel; (Fibroblast)

1. INTRODUCTION

It has been recently observed that serum induces recurring phases of outward membrane current in human fibroblasts [1]. These periodic conductance increases were suggested to be mediated by corresponding changes in cytosolic calcium, because raising the intracellular Ca^{2+} buffering power eliminated the oscillations and also because a similar response could be elicited by application of the Ca^{2+} ionophore A23187 [1,2].

We have now coupled the electrophysiological technique with the measurement of cytosolic Ca^{2+} in a single cell by fura-2 microfluorimetry to directly observe the Ca^{2+} changes. In addition, to match more closely the physiological situation of the cell, we performed some experiments under current-clamp conditions to record the membrane voltage changes induced by mitogens.

2. MATERIALS AND METHODS

Cell cultures and preparation were as described elsewhere [1]. The whole-cell technique was used both in the voltage-clamp and current-clamp mode. Pipettes (2–4 MΩ resistance) were normally filled with (in mM): 140 KCl, 4 $MgCl_2$, 10 Hepes-KOH, 0.1 EGTA (substituted with 0.1 fura-2 for the Ca^{2+} measurements), pH 7.2. The external solution contained (in mM): 125 NaCl, 5 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 25 Hepes-NaOH, 6 glucose, pH 7.3. Bradykinin was added as a drop to a 500-μl volume chamber; temperature was kept at 33–35°C. Current and voltage traces were stored on VCR and subsequently analyzed.

For the measurement of cytosolic Ca^{2+}, the cells were loaded with fura-2 penta-K^+ salt (100 μM) by diffusion from the patch pipette. Excitation wavelength (350 and 380 nm) were alternated by a rotating filter wheel (20 Hz). The excitation light was brought to a Zeiss IM 35 inverted microscope equipped for epifluorescence via a liquid light guide to minimize vibrations coming from the filter wheel. The emitted light was collected by a high numerical aperture objective (Nikon Fluor 40× oil immersion, 1.3 n.a.) and measured by a photomultiplier. Synchronization of the filter wheel and separation of the fluorescence signals excited by the two wavelengths was accomplished by a special spectrophotometer (Cairn Research, Sittingbourne, Kent, England). This allowed us to electronically subtract the background fluorescence signals from each cell while in cell-attached conditions. Calculation of the Ca^{2+} concentration from the F_{350}/F_{380} ratio was performed according to [3]. Calibration of the system was done following the procedure used by Almers and Neher [4].

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3. RESULTS

Fig.1 illustrates the oscillatory response of two quiescent human fibroblasts to the application of fetal calf serum (FCS), measured as recurring membrane hyperpolarizations from a low resting potential (upper trace) or as recurring membrane current transients under voltage-clamp (lower trace).

The membrane voltage record shown in Fig.1 is rather exceptional for the level (–50, –55 mV) reached by the hyperpolarizations: more frequently, recordings would show recurring hyperpolarizations of smaller amplitude (5–20 mV from a resting potential ranging between –5 and –25 mV).

The nature of the membrane changes underlying this behavior was studied previously [1] and experiments with the Ca^{2+} ionophore A23187 suggested that they consist of the activation of a strongly outwardly rectifying cationic conductance by cytoplasmic Ca^{2+}.

Besides this indirect evidence of a regulatory role of Ca^{2+} on the membrane conductance, an immediate rise of cytosolic calcium in response to serum (and to other

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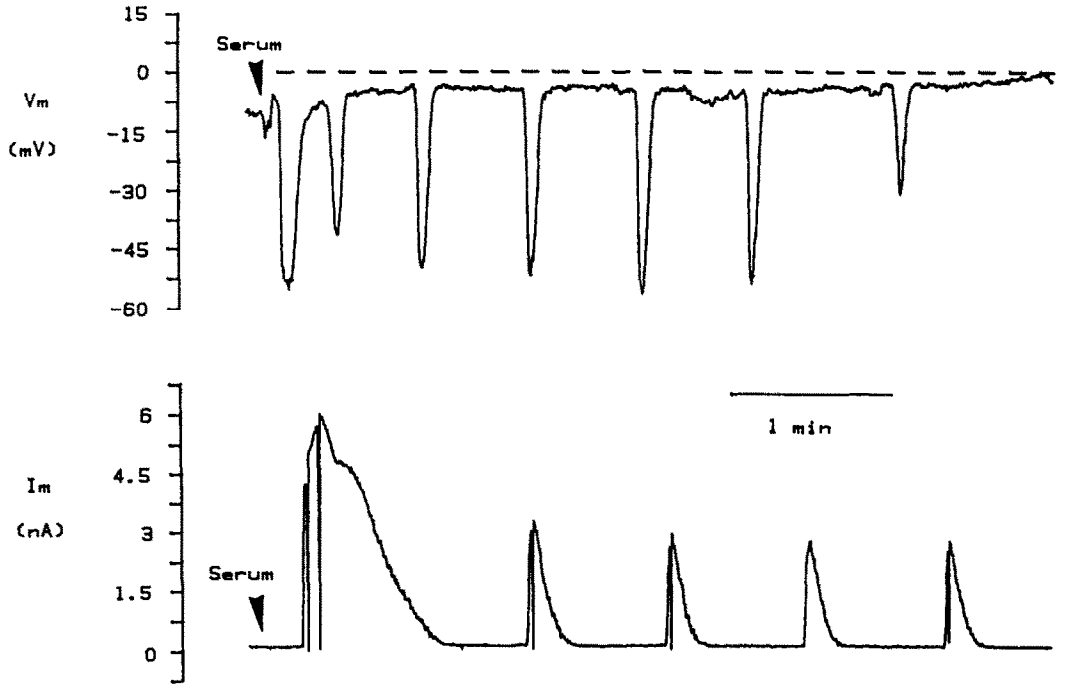

Fig.1. Upper trace: recurring hyperpolarizations in response to serum. The resting potential of the quiescent human fibroblasts was generally between −10 and −20 mV. The repetitive hyperpolarizations induced by FCS were of variable amplitude reaching in a few cases (including the one shown), levels of −50, −60 mV. The interval between oscillations varied from 40 to 150 s. Lower trace: another fibroblast under voltage-clamp (V_h = +50 mV) exhibits periodic phases of outward current with a frequency similar to the hyperpolarizations shown in the upper trace.

growth factors) had been measured directly in human fibroblasts [5,6]. Those results, however, did not show any oscillating behavior, presumably because they were not done on single cells. We tried, therefore, to perform determinations of cytosolic Ca^{2+} by fura-2 microfluorimetry together with the electrophysiological measurements. In these experiments, the mitogenic peptide bradykinin (BK) (10–20 nM) was used to avoid interference from FCS fluorescence. We had already observed that BK is able to activate the same membrane conductance system (Peres, unpublished observations). Fig.2 shows records of cytosolic Ca^{2+} (upper trace) and membrane current (lower trace) after application of 10 nM BK. It is clear that cytosolic Ca^{2+} and the membrane current measured at +50 mV fluctuate synchronously. As expected [1,2], no significant current is observed at -50 mV due to the outward rectifying properties of the conductance.

To investigate the ionic nature of the current, we performed some experiments in which K^+ was substituted with Cs^+ in the pipette (and therefore in the cytoplasm): in these conditions BK still induced the Ca^{2+} increase but this was not accompanied by the development of the outward current. Fig.3 shows one such experiment out of three which gave identical results. In eleven other cells in which K^+ was replaced by Cs^+ (without fura-2), no outward current was seen upon mitogen stimulation. This result clearly shows that the outward current is carried by K^+. In addition, to exclude the already unlikely possibility that the outward current could be due to a Ca^{2+}-activated Cl^- influx, we performed experiments substituting external Cl^- with glutamate–; in these conditions a normal outward current developed upon stimulation (not shown).

4. DISCUSSION

The main finding of this work is the observation of recurring membrane potential changes in single human fibroblasts exposed to mitogenic stimulation. These fluctuations of the membrane potential are due to periodic permeabilizations of the membrane to K^+. We also directly show that these periods of enhanced K^+ conductance occur concurrently with elevations of cytosolic calcium levels.

The ability to generate recurring hyperpolarizations through Ca^{2+}-activated K^+ channels was already described in detail in the case of mouse fibroblasts [7,8]. Several other cases have been reported recently showing pulsatile calcium rises with associated membrane conductance changes [9–12].

It is important to remember here that not all the tested cells responded to stimulation [2], the proportion of non-responsive cells being about 35%. Also, the pattern of the response was quite variable in terms of lag time, period and number of oscillations; in some cells dumped oscillations were seen. However, in all cases tested with simultaneous Ca^{2+} and electrophysiological

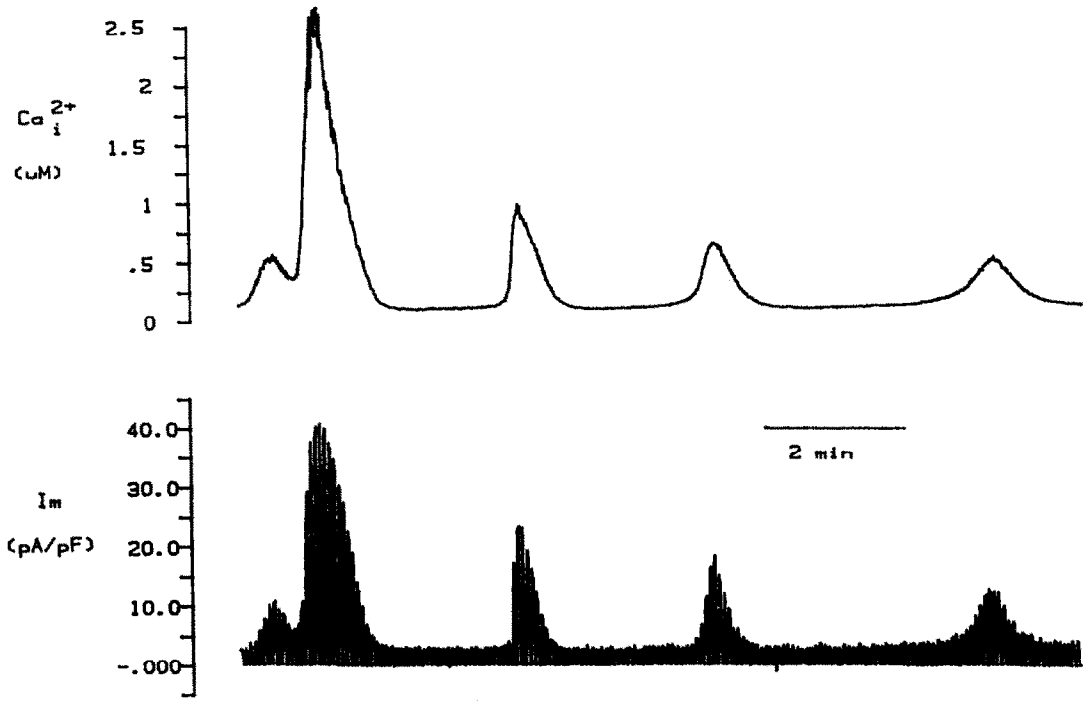

Fig.2. Upper trace: oscillations of cytosolic Ca^{2+} concentration following BK application. The lower trace shows the membrane current under voltage-clamp. The membrane potential was alternated every 2 s between −50 and +50 mV. It is seen that at −50 mV there is no obvious change in membrane current, while at +50 mV the membrane current fluctuates according to the cytoplasmic Ca^{2+} level. BK (10 nM final concentration) was added 15 s before the beginning of the traces. To compare results from different cells, the currents in this and in the subsequent figure have been normalized to the membrane capacitance.

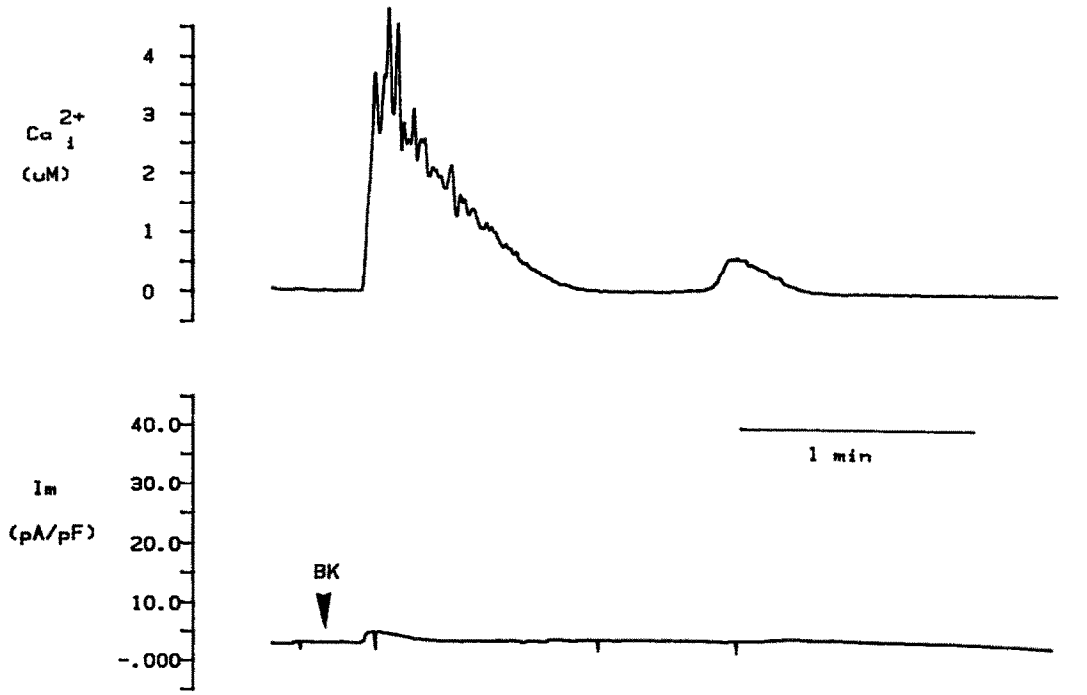

Fig.3. Response to BK from a cell loaded with Cs^+ (replacing K^+): in the upper trace, the Ca^{2+} concentration raises normally to micromolar levels, while the current (V_h = +50 mV) shows only a very small increase (lower trace).

measurements (47 cells) there was a strict correspondence between Ca^{2+} rises and membrane current, as shown in fig.2.

In the light of our results then, the smooth and prolonged elevation of cytosolic Ca^{2+} reported by Moolenaar et al. [5], in human fibroblasts stimulated

by serum and other growth factors, probably represents the collective behavior of a large number of cells, each of which generates its individual oscillating response.

Our results suggest that the membrane potential is not involved in the generation of the pulsatile Ca^{2+} changes since the response occurs both in voltage-clamp and in current-clamp conditions. This is in agreement also with the intracellular nature of the Ca^{2+} source [1.5].

The strong rectification of the Ca^{2+}-activated current prevented an accurate determinaton of the reversal potential [1], however, the almost complete disappearance of the outward current in cells in which internal K^+ was replaced by Cs^+, in spite of a normal Ca^{2+} increase after stimulation, confirms that the main carrier of the current is K^+. As it is shown in fig.3, no inward current could be seen in these conditions, making the participation of other external cations (i.e. Na^+) to the current rather unlikely.

The advantages and the possible meanings of a pulsatile calcium increase have been already proposed by others [10,13,14]. In the case of quiescent human fibroblasts exposed to mitogens, the observation of oscillatory responses that may last in time is particularly interesting: in fact these pulsatile Ca^{2+} changes may constitute a link between the early events that take place immediately after the mitogen stimulation and DNA duplication that begins about 12 h later. In particular, sustained Ca^{2+} oscillations may have a role in regulating processes that have slow 'off' rates and that are related to the commitment to enter the proliferative cycle. As an example, periodic Ca^{2+} elevations could cause pulsatile activation of Ca^{2+}-dependent kinases; in the case of slow dephosphorylation rates [15], intermittent Ca^{2+} rises would produce a continuous state of phosphorylation without the necessity of maintaining high Ca^{2+} levels for very long times [10].

Acknowledgements: We are grateful to Dr R. Zippel and Prof. E. Sturani for the culture and preparation of the cells as well as for continuous and helpful discussion. The skillful assistance of Mr G. Mostacciuolo was much appreciated. This work was partially funded by an AIRC grant to Prof. L. Alberghina and by an MPI 40% grant to Prof. E. Wanke.

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