Journal of Physiology (1989), **416**, *pp.* 589–599
With 6 text-figures
Printed in Great Britain

MEMBRANE CONDUCTANCE OSCILLATIONS INDUCED BY SERUM IN QUIESCENT HUMAN SKIN FIBROBLASTS

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(*Received 13 December 1988*)

SUMMARY

1. Application of fetal calf serum to quiescent human fibroblasts, kept under whole-cell voltage clamp at positive potentials, induced a series of transient rises in membrane conductance.

2. The first transient increase in conductance developed with very short time lag (2–10 s) after serum addition, while the period between successive transients was 30–90 s, being remarkably constant in each particular cell.

3. Raising the Ca^{2+}-buffering capacity of the intracellular solution with 1 mM-EGTA suppressed the appearance of the sustained oscillations.

4. The conductance increase was strongly voltage dependent: voltage ramps applied before, during and after the transients revealed the activation of an outwardly rectifying conductance with variable reversal potentials (between +14 and −55 mV).

5. No significant shifts of the reversal potential were observed when the extracellular K^+ concentration was increased to 126 mM. Substitution of K^+ with Cs^+ as intracellular cation eliminated the outward current in response to serum.

6. External application of the Ca^{2+} ionophore A23187 elicited currents which were very similar in voltage dependence and time course to those triggered by serum.

7. The serum-induced response persisted unaffected by the absence of external Ca^{2+}. The response was also seen in the presence of 1 mM-Cd^{2+} in the external solution.

8. Serum addition caused a rapid morphological rearrangement of the cells.

9. It is concluded that serum triggers a mobilization of Ca^{2+} from intracellular stores which in turn activates cationic channels.

INTRODUCTION

Recent results (Peres, Zippel & Sturani, 1988*b*) have shown that serum application to quiescent human fibroblasts is able to elicit an immediate electrical response in the form of opening of Ca^{2+}-activated membrane channels. Human fibroblasts were previously shown to produce a rapid increase in cytoplasmic Ca^{2+} when stimulated with serum or other growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Moolenaar, Tertoolen & de Laat, 1984). A serum-induced membrane depolarization was reported in human fibroblasts by

Moolenaar, Yarden, de Laat & Schlessinger (1982), who attributed it to an unselective ionic leakiness, and in a monkey epithelial cell line by Rothenberg, Reuss & Glaser (1982).

In cells of nervous origin serum and bradykinin induce more complex electrical responses part of which are due to the development of a Ca^{2+}-activated K^+ conductance (Moolenaar, de Laat & van der Saag, 1979; Moolenaar, Mummery, van der Saag & de Laat, 1981; Higashida & Brown, 1986; Brown & Higashida, 1988*b*).

The activation of Ca^{2+}-mediated conductances, involving inositol lipid hydrolysis (Berridge, 1988; Brown & Higashida, 1988*c*), seems then to be a general feature of the early cellular response to growth factors and mitogens.

In several cases stimulation with the agonist does not result only in a single response but also gives rise to an oscillating behaviour in the target cell which can be revealed as cytoplasmic calcium oscillations (Cuthberson & Cobbold, 1985; Woods, Cuthberson & Cobbold, 1986; Ueda, Oiki & Okada, 1986), membrane potential oscillations (Okada, Doida, Roy, Tsuchiya, Inouye & Inouye, 1977; Nelson & Henkart, 1979; Igusa & Miyazaki, 1986). In some cells the same oscillating behaviour of calcium and membrane potential (or membrane current under voltage clamp) has been shown to occur upon injections of inositol 1,4,5-trisphosphate (Ins1,4,5P_3) (Parker & Miledi, 1986; Berridge, 1988).

This paper is concerned with a detailed study of the early conductance changes brought about by serum in human fibroblasts by means of the whole-cell voltage clamp. A preliminary report concerning this work has appeared recently (Peres *et al.* 1988*b*).

METHODS

Cells. Human skin fibroblasts were cultured and prepared by Dr R. Zippel and Professor E. Sturani. Cells were plated in Dulbecco's modified Eagle's medium (DMEM; Flow) with 10% fetal calf serum (FCS; Flow). After 4–5 days the medium was substituted with DMEM, containing 1% FCS and the cells were used 48 h later. For the whole-cell experiments the cells were detached and kept in suspension as previously described for Swiss 3T3 fibroblasts (Peres, Sturani & Zippel, 1988*a*). Cells were used between the 5th and the 16th passage.

Recording. A drop of solution containing the suspended cells was placed in the recording chamber which was then perfused with the desired solution. The whole-cell recording technique was used with pipettes having resistances of 2–5 MΩ as reported in greater detail previously (Peres *et al.* 1988*a*). Once the whole-cell condition was established, the holding potential (V_h) was set (usually at +50 mV).

To rapidly explore a wide voltage range before and during the responses, a triangular command voltage was applied. Ramps were 320 ms in duration and went from +50 to −80 mV (when V_h = +50 mV) or from −80 to +50 mV (when V_h = −80 mV).

Current and voltage traces were stored on a video cassette recorder after pulse code modulation (Sony PCM 601 ESD). Afterwards the recorded traces were appropriately filtered and digitally sampled for analysis.

Noise analysis. A modification of the procedure used by Sigworth (1980) and by Hess & Tsien (1984) for non-stationary noise analysis was applied. This procedure consists basically in relating the mean current $\langle I \rangle$ with the variance σ^2. A 20 s long segment of recorded current trace containing the response was sampled at 1 kHz after filtering at 500 Hz through an 8-pole Bessel filter; subsequently it was divided into twenty elements, each 1 s in duration. In order to depurate the variance from the contribution of the slowly changing current, the best linear fit of each element was substracted from the trace itself leaving only the residuals. From these the variance was calculated and related to the mean current belonging to the same element. Estimates of the number

of channels per cell and of the unitary current were obtained by fitting a parabola to the σ^2 *vs.* $\langle I \rangle$ graph (Sigworth, 1980).

Solutions. The pipettes were normally filled with the following solution (in mM): KCl, 140; $MgCl_2$, 2; EGTA, 0·1; HEPES–KOH, 10; ATP, 2; GTP, 0·5. In some experiments EGTA was 1 mM to increase the Ca^{2+}-buffering capacity; in other experiments, KCl and KOH were replaced with equimolar CsCl and CsOH, to eliminate internal K^+. The pH of the pipette solutions was 7·3.

The external solutions are listed in Table 1. The bath temperature was kept at 34–35 °C (except when otherwise indicated).

TABLE 1. Composition of solutions (mM)

Solution	NaCl	KCl	$MgCl_2$	$CaCl_2$	$CdCl_2$	EGTA	Na^+–HEPES	K^+–HEPES
A	116	5	1·2	8	—	—	25	—
B	—	121	1·2	8	—	—	—	5
C	125	5	2·4	—	—	1	25	—
D	116	5	1·2	8	1	—	25	—

All solutions were buffered to pH 7·3 and contained 6 mM-glucose.

Agonist addition was performed as follows: the perfusion was stopped and a droplet of serum (40 μl) or of A23187 (5 mM in dimethylsulphoxide (DMSO), drop volume 1 μl) was added to the bath. Since the bath volume was approximately 450 μl the final concentrations of serum and of ionophore were 10% and 10 μM respectively.

RESULTS

Membrane current oscillations in response to serum

The recent report from our laboratory (Peres *et al.* 1988*b*) was concerned with the immediate response that serum induces in the membrane of quiescent fibroblasts. This response, which could be better observed at positive potentials, was occasionally followed by a second, smaller phase of outward current as is shown in Fig. 1*A*.

It must be noticed that in that series of experiments the pipette contained no Ca^{2+} and 1 mM-EGTA and therefore the cytoplasmic Ca^{2+}-buffering capacity was rather high. Suspecting a role of intracellular Ca^{2+} in this process, we lowered the EGTA concentration in the pipette to 0·1 mM. Under these conditions the effect of serum application consisted of the large and immediate response previously described (we will call it 'primary response'), usually followed by several periodic phases of outward current rise, as is illustrated in Fig. 1*B*.

The period between successive phases was generally rather constant in each cell, ranging between 30 and 90 s. As will be mentioned later, the morphology of the cell changed drastically after serum exposure, so that eventually the pipette detaches from the membrane. However recordings of up to 10 min including some fifteen periodic responses have been performed.

Sustained oscillations were never seen when the pipette solution contained 1 mM-EGTA. This suggests that periodic oscillations in the concentration of cytoplasmic free calcium may be involved in the long-lasting response of the quiescent cell to stimulation.

In order to check whether the response was due to low molecular weight substances

or to macromolecular serum factors we performed some experiments using dialysed serum (containing only substances with molecular weight > 3500). This dialysed fraction is able to elicit the response, indicating that the agonist(s) must be searched for among macromolecules. The response could also be induced by application of serum boiled at 100 °C for 5 min. This result will restrict the identification of the agonist to thermally stable molecules such as platelet-derived growth factor (Antoniades, Scher & Stiles, 1979).

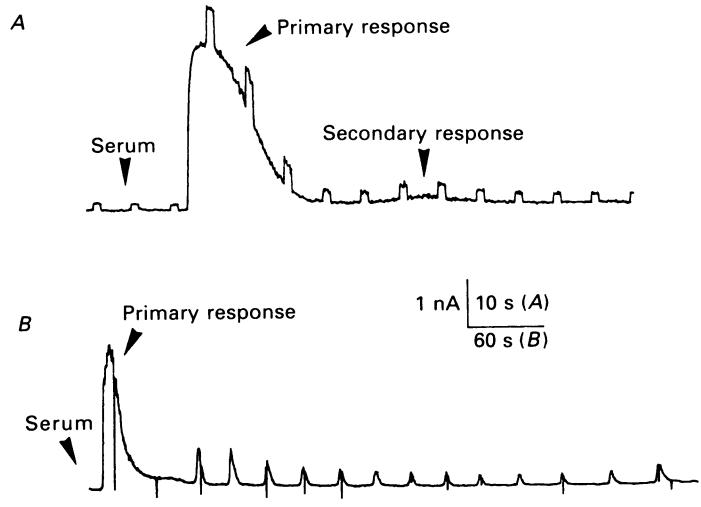

Fig. 1. Responses to serum application. In *A* the intracellular EGTA concentration was 1 mM. Under these conditions the first large outward current rise was sometimes followed by a second, much smaller, phase of outward current; in this experiment 1 s long, 10 mV pulses were superimposed every 5 s on the +50 mV holding potential. In *B* the intracellular EGTA concentration was 0·1 mM; the reduced calcium-buffering capacity uncovered several periodic phases of outward current following the first larger event. Rapid deflections in the trace are responses to fast voltage ramps.

Outward rectification of the serum-induced conductance

Both the primary response as well as the successive periodic responses are due to the activation of a strongly outward-rectifying conductance. This is illustrated in Fig. 2 where the membrane currents elicited by serum in two cells kept at $V_h = +50$ and -80 mV respectively are shown. In contrast to the large outward current seen at $V_h = +50$ mV (Fig. 2*A*), the response at $V_h = -80$ mV is a small inward current (Fig. 2*B*).

However, in both cases the same conductance system has been activated, as may be revealed by applying brief voltage ramps from +50 to −80 mV (Fig. 2*A*) or from −80 to +50 mV (Fig. 2*B*). It can be seen that the current levels reached at −80 mV at rest and at the peak of the serum-induced response in Fig. 2*A* are very similar. On the contrary, the current levels reached at +50 mV in Fig. 2*B* increase rapidly and transiently after serum application.

This strong non-linearity of the serum-induced current can be recognized better when the currents elicited by the voltage ramps are plotted against voltage (Fig. 2*C* and *D*).

The outward rectification is also a characteristic of successive responses: in Fig. 3 the *I–V* curves from several voltage ramps during the primary and the first of the subsequent responses are shown, suggesting that the same kind of permeability change underlies the oscillatory responses.

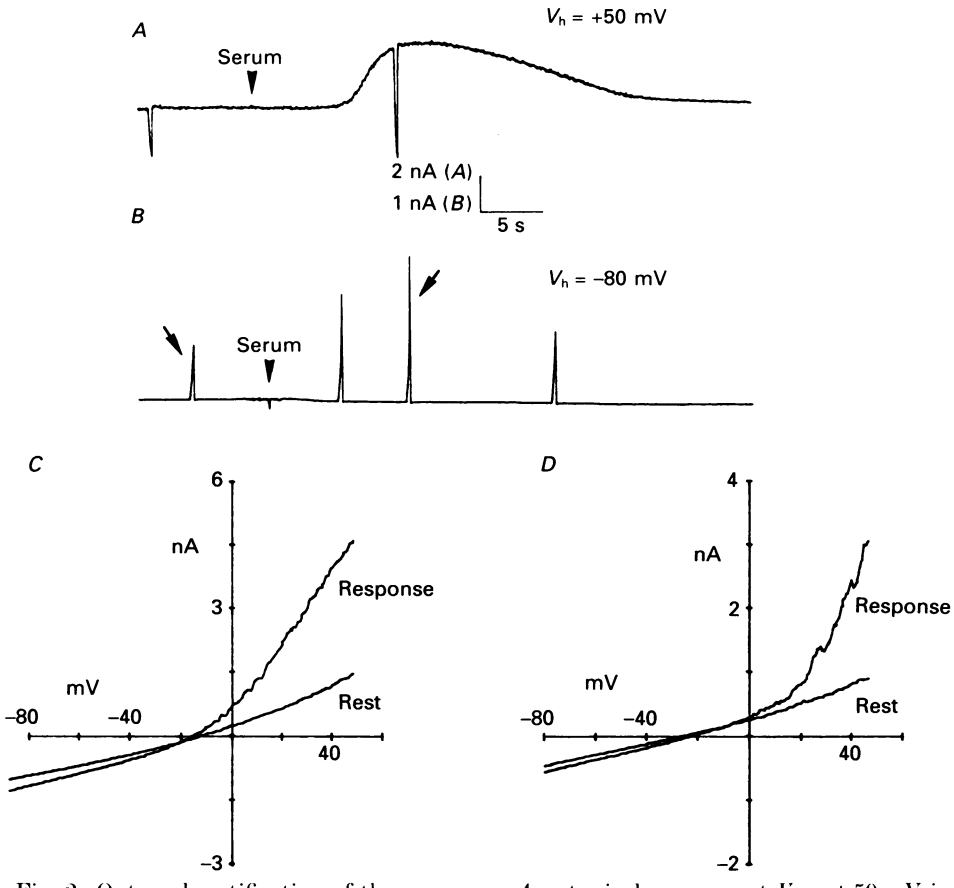

Fig. 2. Outward rectification of the responses. *A*, a typical response at $V_h = +50$ mV is shown. Voltage ramps from +50 to −80 mV were applied before and during the response. These are plotted as *I–V* relationships in *C* showing a marked outward rectification. In *B* the cell was kept at $V_h = -80$ mV. Only a small inward current develops after serum addition, but the response is revealed by various voltage ramps from −80 to +50 mV. The *I–V* plot of the ramps indicated by the arrows in *B* is shown in *D*. The reversal potential (measured as the voltage at which response and rest *I–V* curves cross each other) was −18 mV in *C* and −8 mV in *D*.

Ionic nature of the serum-induced conductance

The reversal potential (E_{rev}) of the serum-induced response is, in principle, the voltage at which the *I–V* relationships at rest and during the response cross each other.

The value of E_{rev} that can be measured under control conditions (solution C in Table 1) by the ramp method is very variable, ranging between −55 and +14 mV

(mean ± S.D., −10·1 ± 18·7 mV, fifteen cells). This variability is due to the low conductance at negative potentials and to possible changes in leakage current due to the morphological rearrangements induced by serum. These estimates, however, do not favour the possibility that K^+ alone could be the carrier of the current, as has

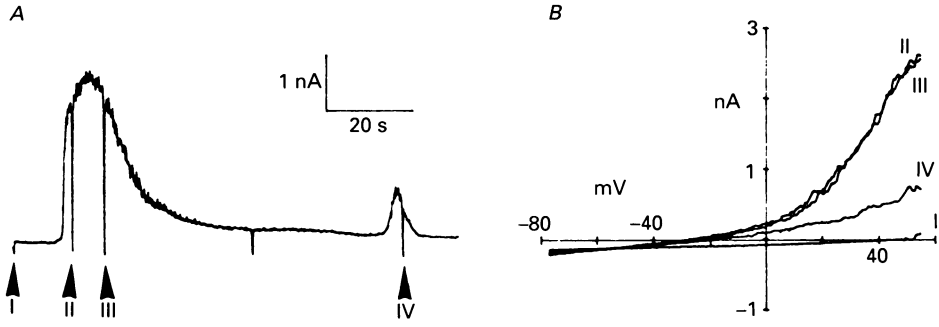

Fig. 3. The voltage dependence of the secondary responses is also outwardly rectifying. *A*, a primary response induced by serum at $V_h = +50$ mV followed by the first of several periodic current phases is shown. Roman numerals indicate the ramps plotted in *B*. *B*: *I–V* plots obtained from the ramps indicated in *A* showing a linear relationship at rest (I) and outward rectification during the primary (II and III) and subsequent (IV) response.

been shown in a similar case (Brown & Higashida, 1988*b*). None the less we did some experiments in high external K^+ (solution B, Table 1) in order to detect possible shifts in E_{rev}. No significant differences could be found either in E_{rev}, which was −5·1 ± 10·2 mV (mean ± S.D., seven cells), or in the shape of the *I–V* relationship, as is illustrated in Fig. 4*A*.

Another condition which has been tested in a number of experiments has been the substitution of internal K^+ with Cs^+. In this situation no responses were seen after serum application (Fig. 4*B*). An identical result was seen in other twelve cells.

Two known inhibitors of Ca^{2+}-activated K^+ channels, apamin (1 μM) and *d*-tubocurarine (1 mM) (Brown & Higashida, 1988*a*) were not effective in blocking the serum-induced response.

The serum-induced conductance is mediated by a rise in intracellular calcium

As mentioned in the Introduction, serum and other specific growth factors are known to induce a quick increase of cytoplasmic calcium in many cell types, including human fibroblasts (Moolenaar *et al.* 1984; Hepler, Nakahata, Lovenberg, Di Guiseppi, Herman, Earp & Harden, 1987; Pandiella, Malgaroli, Meldolesi & Vicentini, 1987).

In order to assess the role of cytoplasmic Ca^{2+} in mediating the response we exposed various cells to the action of the Ca^{2+} ionophore A23187. Figure 5*A* illustrates the response of a fibroblast to the addition of a drop of A23187 (dissolved in DMSO, final concentration 10 μM); in Fig. 5*B* the *I–V* relationships before and during the response are plotted, showing the same kind of outward rectification described for the serum-induced response. Application of DMSO alone was ineffective.

This result suggests that an elevation of cytoplasmic Ca^{2+} is able to elicit the same

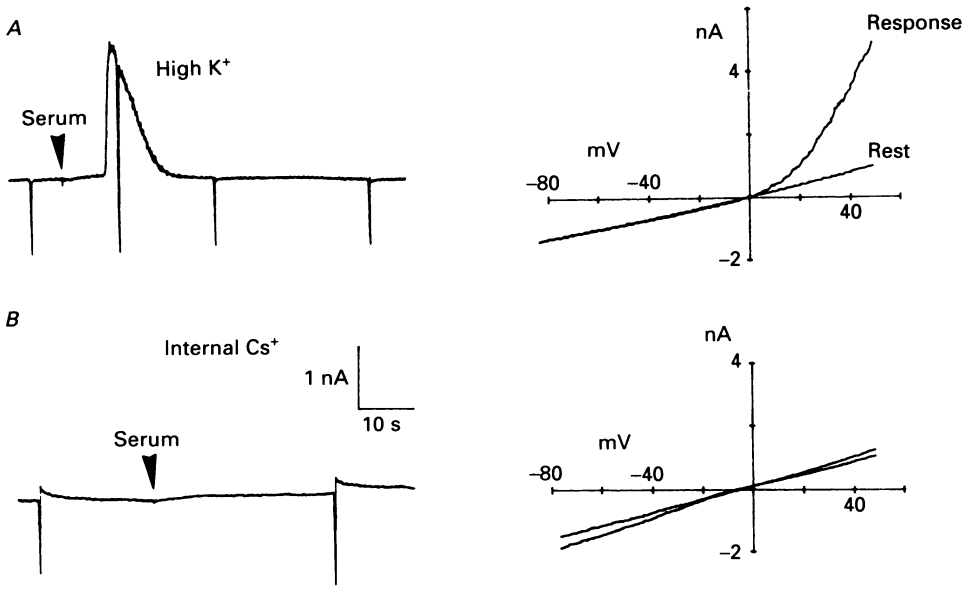

Fig. 4. Serum-induced responses at $V_h = +50$ mV in high external K^+ (*A*, solution B) and in 0 internal K^+ (*B*, K^+ in the pipette was substituted with Cs^+ as explained in the Methods). In the right-hand column the *I–V* relationships from ramps before and after serum application (at the peak of the response for *A*) are shown. In *A* E_{rev} was 0 mV.

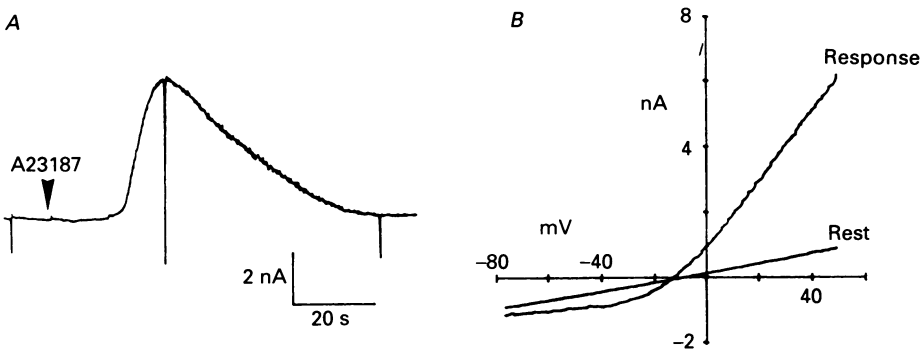

Fig. 5. *A*, response induced by the Ca^{2+} ionophore A23187. V_h was +50 mV. Application of the ionophore to the bath caused a response similar in time course and voltage dependence to those induced by serum. *B*, *I–V* plots of the ramp applied at rest (before ionophore addition) and at the peak of the response.

kind of membrane conductance increase as that caused by serum and therefore points to Ca^{2+} as a second messenger mediating the observed response.

Extracellular calcium is not necessary for the serum response

We then investigated the role of Ca^{2+} influx *vs.* Ca^{2+} mobilization from intracellular stores in our system. The simplest way to address this point was to add serum to cells

bathed in zero-Ca^{2+} (solution C in Table 1). A typical result is presented in Fig. 6 where it is evident that external Ca^{2+} is unnecessary both for the primary and for the subsequent periodic serum-induced responses to occur. A similar result was obtained in eight other cells.

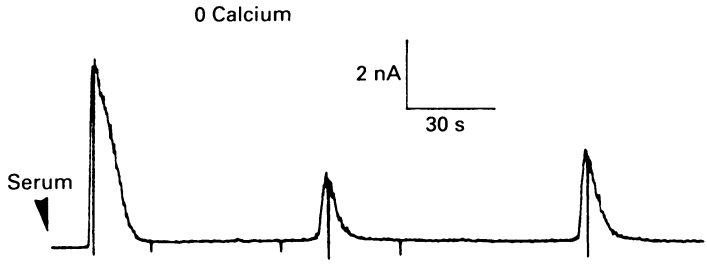

Fig. 6. Periodic responses to serum observed in 0 external Ca^{2+} (solution C); V_h = +50 mV. This result shows that neither the primary nor the subsequent outward current transients are dependent on Ca^{2+} influx. In this particular experiment the bath temperature was 29·5 °C.

We also performed experiments in the presence of 1 mM-Cd^{2+} (solution D in Table 1), a well-known blocker of voltage-dependent Ca^{2+} channels (Miller, 1987), as well as of voltage-independent Ca^{2+} channels in some instances (Kuno, Goronzy, Weyand & Gardner, 1986). All the three cells tested in this experimental condition responded normally to serum (not shown).

Estimation of single-channel conductance from noise analysis

In the current traces there is an obvious increase in noise during the serum-stimulated response. This observation prompted us to perform a non-stationary noise analysis, based on the method used by Sigworth (1980) and by Hess & Tsien (1984). This allowed us to calculate values for single-channel current of 5·1 ± 1·9 pA (mean ± S.D.) at +50 mV and for a number of channels per cell of 822 ± 384 (mean ± S.D.; n = 6). Considering that the mean E_{rev} in this group of cells was −10·5 mV, the estimated single-channel chord conductance (at V = +50 mV) was 83 pS. However, this value is affected by the uncertainty of the E_{rev} determination.

Morphological changes of the cell upon serum stimulation

As already mentioned, a morphological effect of serum was apparent in our experiments: soon after serum addition, but with some delay compared to the electrical events, the cell started to extrude what appeared as bubbles of various dimensions which could be reabsorbed in a few minutes. Often this phenomenon caused the detachment of the pipette leading to the end of the experiment. This event may be similar to the 'ruffling' described by others (Rothenberg *et al.* 1982).

DISCUSSION

Long-lasting membrane conductance oscillations

One of the most significant observations of this work is the long-lasting nature of the serum-induced response. The observation of an oscillating behaviour in cells

stimulated by various agonists is being reported more and more frequently in the literature (Igusa & Miyazaki, 1986; Parker & Miledi, 1986; Ueda *et al.* 1986; Woods *et al.* 1986; Berridge, 1988). In our case the ability to record the oscillatory events is limited (up to about 20 min) because of the morphological changes of the cell leading to pipette detachment. However, using less invasive techniques such as the measurement of cytoplasmic Ca^{2+} concentration with aequorin, the oscillatory behaviour can be shown to last hours in the case, for example, of the fertilized mouse oocyte (Cuthberson & Cobbold, 1985).

An oscillatory behaviour in membrane potential can also be generated by injections of $Ins1,4,5P_3$ (Capiod, Field, Ogden & Sandford, 1987; Berridge, 1988), and recently Thompson, Proctor, Grant & Thomas (1988) have shown that stimulation of phosphatidylinositol turnover by epidermal growth factor in A431 cells may last up to 10 h. All these findings begin to fill the gap between short- and long-term cellular responses to the action of growth factors.

The conductance oscillations can best be seen at positive potentials which do not occur physiologically. However we have shown in Fig. 2 that the primary response can also be revealed when V_h is kept at -80 mV and serum-induced oscillations of membrane potential may be observed when working in current-clamp mode (A. Peres, unpublished observation).

Ca^{2+} as intracellular messenger

Our evidence for an important role of Ca^{2+} in mediating the serum-induced membrane conductance changes resides in the fact that both serum and the calcium ionophore A23187 trigger a response with the same time course and voltage dependence. Moolenaar *et al.* (1984) measured directly, using Quin-2 fluorescence, Ca^{2+} rises induced by serum, EGF and PDGF; in their traces the onset of the Ca^{2+} rise is immediate and its time course correlates favourably with our conductance changes. Moolenaar *et al.* (1984) did not mention subsequent increases or oscillatory responses; however Quin-2 may have acted as a rather strong buffer in their experiments preventing the successive Ca^{2+} rises.

Influx of Ca^{2+} from the outside appears to be unnecessary for the serum-induced response. This is again in agreement with the results of Moolenaar *et al.* (1984) and also coincides with the situation in many other systems (Dascal, Gillo & Lass, 1985; Neher & Almers, 1986; Yada, Oiki, Ueda & Okada, 1986; Brown & Higashida, 1988*b*).

Buffering the intracellular Ca^{2+} with EGTA eliminates the periodic oscillations but leaves undisturbed the primary response. This result, while confirming the role of calcium in mediating the conductance oscillations, raises the problem of the source of Ca^{2+} for the primary response. The idea of different intracellular pools from which Ca^{2+} could be released has been put forward already, at least for *Xenopus* oocytes (Dascal *et al.* 1985; Berridge, 1988). Our data support this idea, suggesting that the store that delivers Ca^{2+} for the periodic oscillations is more sensitive to the chelating action of EGTA than the store involved in the primary release. A similar observation has been made by Dascal *et al.* (1985) who found a greater sensitivity to EGTA of the second phase of their response.

Ionic nature of the serum-induced current

The reversal potential of the serum-induced conductance appears difficult to measure because of the strong non-linearity of the *I–V* relationship shown in Figs 2 and 3. As already mentioned, serum also induces morphological changes of the cell and even small leakage currents due to membrane rearrangements near the pipette may strongly affect the determination of E_{rev}.

Yet the calculated K^+ equilibrium potential ($E_K = -90$ mV, in solution A) is too far from the measured E_{rev} to attribute the current to K^+ alone. However, the finding that substituting internal K^+ with Cs^+ causes the disappearance of the outward current indicates that K^+ participates in the current. This experiment also seems to exclude the possibility that the current might be carried by Cl^-, as has been shown to be the case in mast cells stimulated by secretagogues (Penner, Matthews & Neher, 1988).

Summarizing all the results we can conclude, for the moment, that the serum-activated conductance is due to non-specific cationic channels. Ca^{2+}-activated channels with such non-specific permeability have been described, for instance by Yellen (1982) in neuroblastoma and by Colquhoun, Neher, Reuter & Stevens (1981) in cultured heart cells. However, these do not appear to possess the same kind of strong outward-going rectification as the conductance described in the present paper.

It is a pleasure to thank Mr G. Mostacciuolo for expert technical help, Dr R. Zippel and Professor E. Sturani for preparing the cells and for useful discussions during the course of this work. This work was supported by a grant from Associazione Italiana per la Ricerca sul Cancro to Professor L. Alberghina and a 60% grant from MPI to Professor E. Wanke.

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