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Two currents activated by epidermal growth factor in EGFR-T17 fibroblasts

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Application of 10 nM Epidermal Growth Factor (EGF) to single EGFR-T17 fibroblasts induced a marked hyperpolarization that could last for tens of minutes; in many cases the first transient was followed by a series of oscillations of the membrane potential. The outward current responsible for the hyperpolarizing response could be recorded simultaneously to an increase in the intracellular calcium concentration, as measured with the fluorescent indicator fura-2. The conductance was nearly linear in the voltage range from −100 to +50 mV. While the EGF-induced current had many characteristics of a K^+ current and was strongly reduced by 50 nM charybdotoxin (ChTx), its reversal potential was apparently more negative than the potassium equilibrium potential (V_K). The application of 2 μM ouabain prior to EGF stimulation produced responses that were similar to those obtained without ouabain; however, under these conditions the EGF-induced current showed a reversal potential of −96.6 ± 3.2 mV, very close to V_K. Simultaneous application of both 2 μM ouabain and 50 nM ChTx completely abolished the response. It can be concluded that the response to EGF stimulation in EGFR-T17 cells consists of two components: the first is a current carried through Ca^{2+}-activated K^+ channels; the second is due to the acceleration of the operation of the Na^+/K^+-ATPase.

Introduction

Among the early events triggered by the binding of growth factors to their receptors, an increase in $[Ca^{2+}]_i$, mainly mediated by phosphoinositide hydrolysis, is a common finding in many cell types [1–4]. This pathway may lead not only to a single transient calcium rise, but to a train of oscillations in $[Ca^{2+}]_i$, for which a role in frequency coding of information relevant to the control of cell growth has been proposed [3].

Another class of mitogen-induced early events that has been the object of considerable interest is represented by the activation of pumps and antiports [2]; more recently, it has been shown that mitogens and growth factors can also directly stimulate ionic fluxes through membrane channels and thus induce electrical responses. Although the role of the latter events as intracellular signals in the transduction of mitogenic agonist action has not yet been clarified, they appear to be quite ubiquitous. Early experiments reported depolarizations induced by epithelial growth factor (EGF) or serum in an epithelial cell line [5] and by serum in human fibroblasts [6]; in contrast, in neuroblastoma cells a more complex response to dialyzed serum and other mitogens was observed [7,8], part of which was attributed to a Ca^{2+}-activated opening of K^+ channels.

Single-channel recordings of currents flowing through Na^+ [9] and Ca^{2+} conducting channels [10,11] in fibroblasts stimulated by growth factors have also been reported. That the $[Ca^{2+}]_i$ increase may be due not only to intracellular release but also to inflow through cation channels of the plasma membrane has been suggested by several experimental findings at least in some cell types [3,12,13].

Recently, Peres and co-workers [14–16] have reported that, in human fibroblasts, foetal calf serum,

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platelet-derived growth factor (PDGF) and bradykinin elicit a series of transient rises in membrane conductance, due to the opening of K^+ channels induced by an increase in internal Ca^{2+}. Combining fluorometric experiments in cuvette with patch clamp recordings on single cells, we have previously reported that, among the early effects of EGF stimulation in a NIH-3T3 mouse fibroblast line overexpressing the transfected human EGF receptor, named EGFR-T17 [19], and in other cell lines, a fast hyperpolarization can be observed, followed in some cases by voltage oscillations that can last for tens of minutes [17]. On the basis of fluorometric cuvette experiments, and of preliminary results with simultaneous patch clamp recordings and fura-2 measurements on single cells [4], the ionic current that causes these effects was labeled as a Ca^{2+}-activated K^+ current. A causal relationship between the $[Ca^{2+}]_i$ rise and the ionic fluxes seen in response to mitogen stimulation seems therefore to be a common finding in different cell types. However, the ionic channels involved appear to belong to different types, and the possibility of other transport mechanisms in addition to passive ionic channels has not been fully explored.

This paper analyzes in detail the main properties of the electrical response induced by EGF in EGFR-T17 cells. The cascade of intracellular events that mediates the early effects of the growth factor is rather well understood for the EGF molecule: the tyrosine kinase activity of the activated EGF-receptor phosphorylates various substrates, among them phospholipase $C\gamma$, thus inducing phosphoinositide hydrolysis and the generation of two well-known second messengers: inositol 1,4,5-trisphosphate (that in turn induces release of Ca^{2+} from intracellular stores) and diacylglycerol, that activates the protein kinase C pathway [18]. Moreover, in the EGFR-T17 cell line the response to EGF stimulation is particularly strong and reproducible.

Experimental evidence is given here that this response is due to two components, a Ca^{2+}-activated K^+ current, that has properties that are different from those reported for human fibroblasts [14–16], and a current due to the activation of the Na^+/K^+ pump.

Materials and Methods

Cell culture and incubation protocols

EGFR-T17 cells (mouse NIH 3T3 fibroblasts overexpressing the human EGF receptor cloned from A431 cells; [19]) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C, in a humidified atmosphere of 5% CO_2-air. Exponentially growing cells were shifted to DMEM containing 1% FCS 48–72 h before the electrophysiological experiments. Under such conditions more than 95% of the population is in the G0/G1 state (as measured by DNA flow cytometry).

Electrophysiology

Cells used in most electrophysiological experiments were flat and firmly attached to the dish (35-mm dishes, Nunc, Denmark). Some experiments were performed on suspended cells obtained from the monolayer. Briefly, monolayers were first washed with phosphate-buffered saline (PBS) containing 0.5 mM EDTA, then with PBS containing 0.1% trypsin and 0.5 mM EDTA, detached by gentle scraping with a rubber policeman and resuspended in the conditioned medium. Cells were then incubated at 37°C in an oscillating bath for 2–3 h before the experiment.

The external solutions are listed in Table I. Unless otherwise indicated, standard Tyrode solution (Soln. A, Table I) was superfused at a velocity of 3 ml/min. It was then switched to a Tyrode solution containing 10 nM EGF (Collaborative Research, USA). Since the dish contained about 1 ml of solution, the change of solution was complete in about 1 min. When ion channel or pump blockers were used, the cells were equilibrated for several minutes in the solution containing the blockers before switching to a solution of the same composition with EGF added.

Temperature was 31–33°C. The composition of the intracellular solution used in most experiments was the following (in mM): KCl, 133; $MgCl_2$, 3; EGTA, 0.1; Hepes-KOH, 5; NaGTP, 0.4; Na_2ATP, 5; Na_2PC, 5. pH was 7.3. In some experiments 1 mM EGTA was used, in order to increase the Ca^{2+} buffering capacity; in others, KCl and KOH were replaced equimolarly with CsCl and CsOH.

Whole cell patch clamp recordings were performed using electrodes of 3–8 MOhm impedance connected to a Bio-logic RK 300 patch clamp amplifier. Data were digitized and recorded on a VCR video recorder. Off-line analysis of the data was performed on a Hewlett Packard 9816A computer. Stimulation protocols (square waves and ramps) were generated by a modified Commodore VIC 20 computer.

TABLE I

Composition of external solutions (mM)

All solutions were buffered to pH 7.4.

Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	TEA-Cl	Na-Hepes	Glucose
A	154	4	2	1	–	5	5.5
B	154	4	–	1	–	5	5.5
C	18	140	2	1	–	5	5.5
D	134	4	2	1	20	5	5.5
E	4	4	2	1	150	5	5.5

Capacitance measurements were performed by means of analog compensation.

Charybdotoxin was from Latoxan (France).

Values are given as mean ± S.D.

Ca^{2+} measurements

Single-cell Ca^{2+} measurements with simultaneous electrophysiology were performed as previously described [15]. Briefly, suspended cells were loaded with fura-2 penta-potassium salt through the patch pipette by replacing the EGTA with the dye (100 μM) in the pipette solution; dual excitation (350 and 380 nm) was performed by means of a filter wheel allowing the determination of intracellular Ca^{2+} every 50 ms. A special spectrophotometer (Cairn Research Ltd., UK) provided synchronization and decoding of the emitted light and generation of the $F350/F380$ ratio, according to Grynkievicz et al. [20]. The background fluorescence of each cell was electronically subtracted while in cell-attached conditions. Calibration of the fluorescence ratio in terms of Ca^{2+} concentration was performed as indicated by Almers & Neher [21]. In these experiments the perfusion was halted and EGF was applied focally, i.e. as a small concentrated drop giving, after mixing with the known bath volume, the desired final concentration.

Results

Resting potential and current-voltage curves of EGFR-T17 cells

In 118 EGFR-T17 cells studied in the whole cell configuration with high K^+ intracellular solution the resting membrane potential was -37.2 ± 13.43 mV. This value is consistently more negative than the one reported for human fibroblasts [14], Swiss 3T3 cells [22] and L cells [23], even if it is less negative than the resting potential of Balb/c 3T3 cells [24].

In most cells, the resting potential was rather stable; a decrease of a few mV was observed in some cases during the time (5–10 min) in which the cells were observed under control conditions, before addition of EGF to the bath. In 12 cells the resting potential showed oscillations of a few mV; in three cells these oscillations were of greater amplitude, 20 to 40 mV,

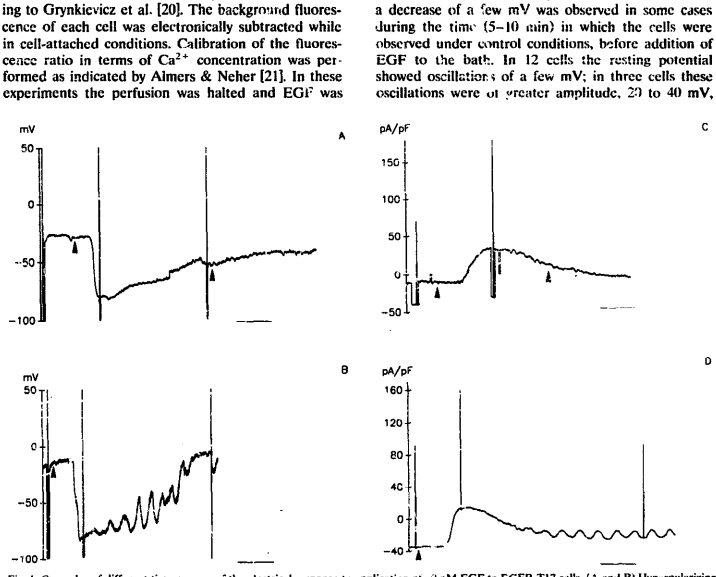

Fig. 1. Examples of different time courses of the electrical response to application of 10 nM EGF to EGFR-T17 cells. (A and B) Hyperpolarizing voltage responses recorded in current clamp mode. (C and D) Outward current responses recorded in voltage clamp mode at $V_h = -50$ mV. Horizontal bar is 2 min in A, 1 min in B, C and D. In B and D oscillations are superimposed on the recovery phase. Large, rapid deflections are voltage ramps applied in order to monitor the I/V relationship. Small deflections in C indicate rapid shifts to the current clamp mode, in order to read the membrane voltage. The first arrow indicates time of application of EGF; the second arrow (when present) indicates time of return to Tyrode solution without EGF.

and after application of EGF no definite response could be observed.

Current-voltage (I/V) curves obtained in control conditions by application of voltage ramps from a holding level of -100 (or -50) mV to $+50$ mV were nearly linear in all cells investigated (see below).

The response to EGF with high internal K^+

The effects of EGF on membrane voltage and currents were studied by switching from the control solution (Soln. A, Table I) to a solution of the same composition to which 10 nM EGF was added.

In 29 EGFR-T17 cells investigated in the whole cell current clamp mode with the 133 mM KCl internal solution, application of 10 nM EGF caused, within 1 min from the change of solution (see Methods), a rather fast hyperpolarization (time to peak, 37.2 ± 14.6 s) from a resting value of -30.5 ± 9.14 mV to -69.76 ± 10.21 mV, followed by a slower decay; the voltage returned to the control level in a time that varied from few minutes to 20–30 min (Fig. 1A). The maximal hyperpolarization observed was to -95 mV. In 15 of these cells, repeated voltage oscillations, that could also last for tens of minutes, were superimposed on the decay phase. Differently from those observed in human fibroblast stimulated with serum or bradykinin [14–16], these oscillations did not have a spike-like appearance, but were in most cases of sinusoidal shape, with amplitudes ranging from few mV to 20–30 mV (Fig. 1B).

The amplitude of the voltage response was also determined in 22 of the cells studied in the voltage clamp mode (see below) by switching for a few seconds to the current clamp mode at the peak of the current response (Fig. 1C). In these cells the average hyperpolarization was from -35.6 ± 12.34 mV to -66.8 ± 10.8 mV.

Thirty cells were studied in the voltage clamp mode, at $V_h = -50$ mV. Some other cells were kept at $V_h = -20$, $+20$ and $+50$ mV. In all these experiments, the EGF-triggered response appeared as an outward current, with roughly the same time course of the voltage response (Fig. 1C).

As with voltage responses, in 13 cells oscillations in the recovery phase of the current response were observed (Fig. 1D).

In 21 of the cells recorded at $V_h = -50$ mV the cell capacitance was also measured; the current density of the response (peak − control level) was 28.9 ± 16.6 pA/pF.

When the external solution was changed back to normal Tyrode during the recovery phase, no change in the time course of the response could be observed (Figs. 1A,C).

Except for the three cells that showed large resting voltage oscillations (see above), the response to EGF was observed in 100% of the cells investigated.

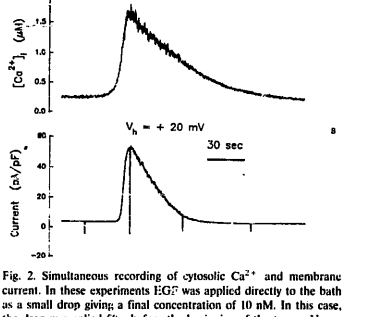

Fig. 2. Simultaneous recording of cytosolic Ca^{2+} and membrane current. In these experiments EGF was applied directly to the bath as a small drop giving a final concentration of 10 nM. In this case, the drop was aplied 50 s before the beginning of the traces. Upper trace: cytosolic Ca^{2+} concentration; lower trace: outward current at $V_h = +20$ mV; rapid deflections in this trace indicate the responses to applications of voltage ramps.

The role of cytosolic Ca^{2+} in mediating the response to EGF was directly verified in some experiments in which the electrophysiological recording was coupled to fura-2 microfluorometry on the same cell. Fig. 2 presents one such experiment: as expected, in addition to the outward current rise, EGF stimulation elicits a simultaneous Ca^{2+} elevation. The same result was observed in four other cells; the average basal Ca^{2+} level was 178 ± 43 nM and the peak level was 1.93 ± 0.76 μM.

Two cells were tested in 0 external Ca^{2+} (Soln. B, Table I). In these cells EGF application induced normal hyperpolarizing responses from -20 to -65 mV and from -12 to -77 mV, respectively.

The oscillations in the recovery phase were observed both with 1 mM (17 cells) and 0.1 mM EGTA (11 cells) in the intracellular solution.

In some cells, I/V relationships at the peak of the response to EGF were obtained by applying ramp voltages from -100 (or -50) mV to $+50$ mV in the voltage clamp experiments, or, in current clamped cells, by temporary switching to voltage clamp, with $V_h = -100$ mV, and applying ramps from -100 to $+50$ mV, before and at the peak of the response to EGF. As can be seen in Fig. 3A, also the EGF-induced current is nearly linear over this voltage range.

The I/V curves in control conditions and at the peak of the response shown in Fig. 3A do not cross within the voltage range tested. This happened in the majority of the cells investigated: only in two cells, out of 18 in which ramps from -100 to $+50$ mV were applied, the curves did cross at voltages between -90

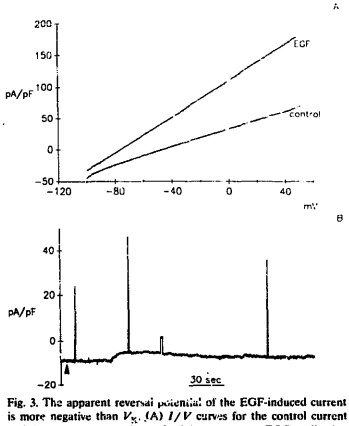

Fig. 3. The apparent reversal potential of the EGF-induced current is more negative than V_K. (A) I/V curves for the control current and the current seen at the peak of the response to EGF application in the cell of Fig. 1C (first and second ramp of Fig. 1C). The two curves do not cross at all in the voltage range tested. (B) The response to EGF stimulation observed at $V_h = -100$ mV. The meaning of the deflections is the same as in Fig. 1

and -100 mV; in all other cases the crossing point was apparently much more negative than -100 mV. Since the crossing point of the two curves indicates the reversal potential for the EGF-induced current, the above finding apparently does not support the hypothesis that this current is carried by K^+ ions, since the calculated V_K in these experiments is -93 mV.

This finding was also confirmed by three additional experiments performed at $V_h = -100$ mV: in all cases an outward current was still observed in response to EGF stimulation, as shown in Fig. 3B.

Effects of ion substitutions and of blockers

In order to clarify the ionic nature of the EGF-induced current, experiments were performed in which the electrochemical gradient for K^+ was inverted, and the Cl^- gradient was unchanged. In three experiments the standard external Tyrode solution was substituted with a solution containing 140 mM KCl (Soln. C, Table I). After the membrane potential had reached a value of about 0 mV, a V_h of -50 mV was imposed and Soln. B with 10 nM EGF added was applied. In all three cases, the response appeared in the form of an inward current (Fig. 4A), with a time course similar to that of the outward current responses described above in standard Tyrode solution.

This observation is qualitatively in agreement with the hypothesis of a response due to a K^+ current (in these conditions V_h is more negative than V_K) and seems to exclude the involvement of a Cl^- current, since the electrochemical gradient for Cl^- is the same as in the experiments with $V_h = -50$ mV in Tyrode solution.

Complete substitution of internal K^+ with Cs^+ did not abolish the voltage response recorded in current clamp mode (one cell), nor the outward current response seen in voltage-clamped cells (15 cells; $V_h = -50$ mV; Fig. 4B). In nine of the latter cells, the current density was measured and the average peak value of the response to EGF was 16.0 ± 10.4 pA/pF. This value was compared with that obtained in the same experimental conditions from cells internally perfused with high K^+ (Soln. F): 28.9 ± 16.6 pA/pF (21 cells, see above). The reduction in the amplitude of the response observed in the Cs^+-perfused cells was statistically significant (t-test, $P < 0.05$). The effect of Cs^+ on this current is less marked than the effect observed on serum-induced K^+ currents in human fibroblasts [14] and in other Ca^{2+}-activated K^+ channels [25,26].

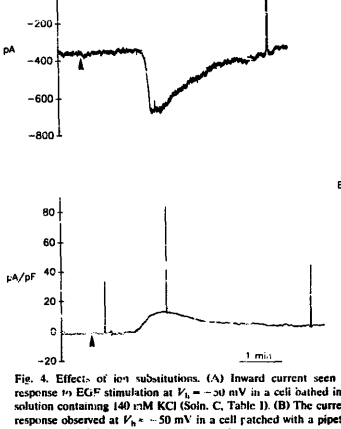

Fig. 4. Effects of ion substitutions. (A) Inward current seen in response to EGF stimulation at $V_h = -50$ mV in a cell bathed in a solution containing 140 mM KCl (Soln. C, Table I). (B) The current response observed at $V_h = -50$ mV in a cell patched with a pipette in which all K^+ was substituted with Cs^+. Deflections are responses to ramps from -50 to $+50$ mV.

Some putative K^+-channel blockers were tested. Substitution of the standard Tyrode solution with a solution containing 20 mM TEA-Cl (Soln. D, Table I) had no effect on the response: in two cells tested ($V_h = -50$ mV) the current densities were of the same order of magnitude of those recorded in normal solution (Fig. 5A). In one cell bathed in 150 mM TEA-Cl, however (Soln. E, Table I), the response was completely abolished (not shown), in accordance with previous observations in cuvette experiments [17].

Charybdotoxin (ChTx), a selective blocker of some types of high-conductance Ca^{2+}-activated K^+ channels [27], had a blocking effect on the EGF-induced electric events. Application of 50 nM ChTx strongly reduced the response to EGF (3–6 pA/pF at $V_h = -50$ mV; five cells, Fig. 5B). Accordingly, in current-clamp mode, a hyperpolarization of only few mV was observed (not shown).

The response to EGF stimulation recorded from suspended cells

The data presented above, while being in qualitative accordance with the hypothesis that EGF elicits a Ca^{2+}-activated K^+ current in EGFR-T17 cells, show, however, several discrepancies from this assumption. Two possible ways of solving this problem were tested.

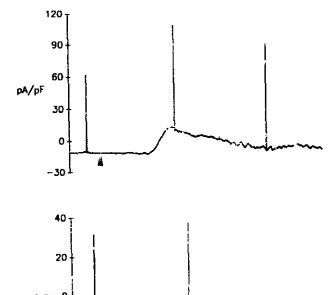

Fig. 5. Effects of K^+ channel blockers. (A) Outward EGF-induced current seen at $V_h = -50$ mV with 20 mM TEA-Cl in the external solution. (B) The response to EGF application observed at $V_h = -50$ mV in the presence of 50 nM charybdotoxin (ChTx) in the external solution. Deflections: responses to ramps from −50 to +50 Mv in A, to ramps from −100 to +50 mV in B.

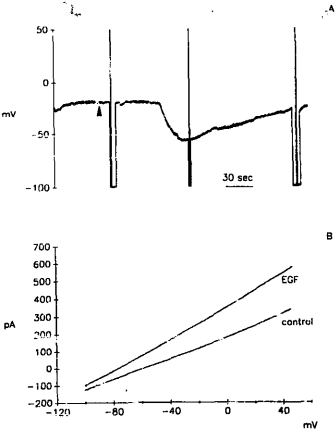

Fig. 6. The response to EGF stimulation recorded from a suspended cell. (A) Hyperpolarization observed in current clamp mode. (B) I/V curves from the first two ramps of A (under resting conditions and at the peak of the response).

The first arises from the fact that, in flat attached cells like the ones used in the above experiments, the control of membrane voltage in voltage clamp mode and the degree of internal perfusion may not be complete: this could account for the residual outward current observed at V_h around −100 mV, and for the relatively weak blocking effect of internal Cs^+.

To test this possibility, three experiments were performed with suspended cells; in these conditions, a better voltage control can be achieved. Fig. 6A shows the hyperpolarizing response observed in one of these experiments, while in Fig. 6B the I/V curves in control conditions and at the peak of the response are plotted; in this case, too, they do not cross within the spanned voltage range. Similar results were obtained from the other two cells. They do not differ from those obtained with flat cells; therefore, the exceptionally negative reversal potential of the EGF-induced current is not an artifact arising from poor voltage control.

The second possible explanation assumes that the ionic current elicited in EGFR-T17 cells in response to EGF is made of two components: a Ca^{2+}-activated K^+ current and a second one, with a more negative reversal potential.

Effects of ouabain on the response to EGF

It is known that one of the early events triggered by growth factors in many cells types is the activation of the Na^+/K^+ pump [2,6], and that the reversal potential of this pump is to be placed at voltages markedly more negative than V_K [28]. Therefore, in a series of experiments 2 μM ouabain was added to the standard Tyrode solution.

In five cells kept at $V_h = -50$ mV, an outward current was observed after application of EGF (Fig. 7A), with amplitude and time course similar to those seen in the absence of ouabain; in these conditions, however, the I/V curves obtained from ramps from -100 to $+50$ mV crossed at voltages between -90 and -100 mV (Fig. 7B). Average reversal potential for the five cells investigated was -96.6 ± 3.2 mV

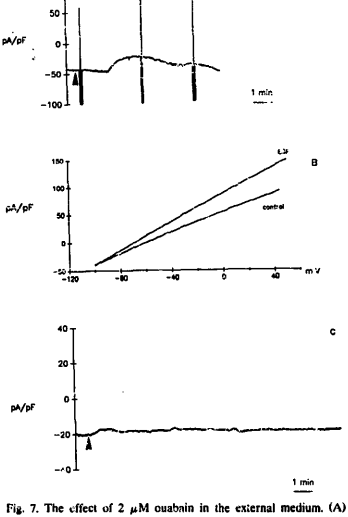

Fig. 7. The effect of 2 μM ouabain in the external medium. (A) Outward current recorded at $V_h = -50$ mV in response to EGF stimulation. Large deflections are responses to ramps from -100 to $+50$ mV. (B) I/V curves from A under control conditions and at the peak of the response (first two ramps in A). The reversal potential of the EGF-induced current (given by the crossing point of the two curves) is -95 mV. (C) The recording from one experiment at $V_h = -50$ mV in which EGF was added after 30 nM ChTx + 2 μM ouabain. Note the absence of a detectable response in terms of an outward current.

In five experiments performed with the addition of 2 μM ouabain together with 50 nM ChTx, no response could be observed after application of EGF (Fig. 7C), differently from the finding of a small residual response in 50 nM ChTx alone.

Discussion

The data presented in this paper, obtained from single cell recordings combined with fura-2 measurements of $[Ca^{2+}]_i$ changes, while confirming the previous observations [17] that EGF stimulation induces a strong hyperpolarizing response in EGFR-T17 fibroblasts, show that this response can be ascribed to the activation of two currents: a Ca^{2+}-activated K^+ current, and a second component, carried by the Na^+/K^+-ATPase, whose activity is known to be elevated, as a secondary effect of mitogen stimulation, through the activation of the Na^+/H^+ antiporter [2]. This is the first direct experimental evidence, at least at the single cell level, of the involvement of a carrier-mediated current in the electrical response of fibroblasts and other cell types to growth factor stimulation. The conductance changes can be observed at voltages (e.g. -50 mV) that are close to the resting potential of these cells.

Hyperpolarizing responses, generated by outward currents through Ca^{2+}-activated K^+ channels, have been observed in several cell lines stimulated with different mitogens, following the cytosolic Ca^{2+} rise due to receptor-mediated phosphoinositide hydrolysis and the consequent inositol trisphosphate-induced Ca^{2+} release from internal stores. In some cases [4,14,15,17] these responses were the only electrical event that could be recorded following mitogen stimulation. In others, however, [7,8,29] they were associated with other electrical events (a depolarizing response or an inward current). In our case, too, two components can be separated: however, differently from the observations reported above, the two components converge in producing a hyperpolarizing response.

The first component, that can be isolated by blocking the second one with ouabain, can be ascribed to a Ca^{2+}-activated K^+ current on the basis of the following evidence: (i) a conspicuous $[Ca^{2+}]_i$ rise can be observed simultaneously to the current; (ii) it is blocked by ChTx, a potent blocker of several types of Ca^{2+}-activated K^+ channels [25,27]; (iii) its reversal potential is very close to V_K; (iv) reversing the K^+ electrochemical gradient at $V_h = -50$ mV (without modifying the Cl^- gradient) causes the current to change direction, from outward to inward.

An unexpected finding of our investigation is that this current has properties that are quite different from

the current, induced by several agonists, observed in human fibroblasts [14–16]: while the current observed in these cells shows a high degree of outward rectification, in our case the I/V relationship for the EGF-induced K^+ current is nearly linear in the voltage range tested. − 100 mV to +50 mV. Moreover, its reversal potential is very close to V_K, differently from the reversal potential of the current reported in human fibroblasts [14], that appears to be less well defined, and in any case more positive, implying an involvement of other ions in addition to K^+. Complete substitution of K^+ with Cs^+ in the intracellular solution completely abolished the mitogen-induced response in human fibroblasts [14], but only reduced it in our experiments. It is known that Ca^{2+}-activated K^+ channels show different degrees of permeability to Cs^+ ions [25]; alternatively, this difference could be due to the fact that our experiments with high $[Cs^+]_i$ have been performed on very flat, dish-attached cells, in which substitution of cytosolic K^+ with pipette Cs^+ may not be complete. It must be noted that this possibility should not impair the estimation of the reversal potential obtained in high internal K^+, since the K^+ concentration of the intracellular solution (see Materials and Methods) is very close to the normal $[K^+]_i$ of many fibroblast cells [30].

It is known [25] that high-conductance Ca^{2+}-activated K^+ channels (BK channels) show voltage dependence, while intermediate conductance (IK) channels may be voltage-insensitive. Moreover, ChTx sensitivity is different in the two classes: a 50% block can be obtained in some BK channels with 3 nM ChTx, while a 10-fold higher concentration is needed to obtain the same effect on IK channels [25]. On the basis of these observations, the current described in this paper can be ascribed to the activation of a class of IK-type Ca^{2+}-activated K^+ channels. The EGF-induced Ca^{2+}-activated K^+ current described in our experiments appears therefore to flow through an ionic channel quite different from the PDGF- and bradykinin-activated channel of human fibroblasts [14–16]. This difference could be due to the different mitogens used, or, more likely, reflect a difference in membrane conductance properties between human and mouse fibroblasts. A more detailed comparative analysis on different cell lines could help to clarify this issue.

The second component of the hyperpolarizing response appears to be due to the activation of the Na^+/K^+ pump. It has been known for a long time, since the experiments of Rozengurt and Heppel [31], that growth factors accelerate the operation of this pump. This activation is an indirect one, in response to the Na^+ influx carried by the Na^+/H^+ antiporter, activated by growth factors through the diacylglycerol-protein kinase C pathway [2]. In our experiments, 2 μM ouabain blocks the outward current that is still present at $V_h = -100$ mV, i.e. at voltages slightly more negative than V_K, and cannot therefore be attributed to outward charge flux through K^+ channels. The contribution of this mechanism to the hyperpolarizing response, albeit small, may be relevant to its maintenance for rather long times (see below). No clear evidence for the presence of such a current has been found in whole-cell recordings from human fibroblasts [14–16]: it must be remembered that EGFR-T17 cells have a high density of receptors ($3 \cdot 10^5$/cell; [19]) for the specific growth factor used in these experiments, EGF, and that all the responses in these cells ($[Ca^{2+}]_i$ rise, amplitude of the hyperpolarizing response) appear to be quite strong.

In our experiments, no evidence has been obtained that EGF stimulation induces an inward, depolarizing current in addition to the outward current, carried by K^+ ions, even when the latter is partially or totally abolished by ChTx or ChTx plus ouabain. This finding differs from the observations by Magni et al. [29], obtained in cell suspension experiments with voltage-sensitive dyes on the same cell line. In those experiments, when the hyperpolarizing component was abolished or greatly reduced, a stable depolarization could be revealed. On the basis of ion substitution experiments it was concluded that this depolarization was due to the opening of cation channels permeable to both Na^+ and Ca^{2+}. It has been shown by several authors using non-electrophysiological techniques (e.g. Refs. 3, 12 and 13) that the $[Ca^{2+}]_i$ rise seen in response to mitogen stimulation is also caused by Ca^{2+} influx through membrane channels, in addition to release from internal stores; on the other hand, the opening of Na^+- and Ca^{2+}-permeable cation channels in response to mitogen stimulation has been reported in several cell lines [9–11]. One possible explanation of the discrepancies between our data and the findings of Magni et al. [29] is that some unknown internal messenger may be involved in the activation of the depolarizing current: in whole-cell patch clamp experiments, internal perfusion of the cells with the pipette solution may wash away this substance.

As reported before [4,17], the hyperpolarizing response, and the ionic currents that generate it, can last as long as the longest recordings that have been performed (from 20 to 30 min, in our conditions), and show in many cases an oscillating behavior. These oscillations are linked to oscillations of $[Ca^{2+}]_i$, in analogy with what has been reported for human fibroblasts [15]. The data presented in this paper, obtained from a much larger number of cells, provide clear experimental evidence for a heterogeneity in the response: only about 50% of the cells tested showed an oscillating response. This finding could be due to the presence of subpopulations of cells, or to other unknown factors; further investigation is needed to solve

this issue. It must be noted that, differently from the observation by Berridge [32], calcium oscillations in non excitable cells are usually spike-like, while those observed in excitable cells have a sinusoidal behavior, in our experiments the voltage or current oscillations observed in response to EGF stimulation are usually of sinusoidal shape.

In some cells we have observed oscillations in membrane voltage (or current) in control, unstimulated conditions. Generally, these oscillations were of small amplitude (a few mV), but in three cases they were of the same order of magnitude as the response to EGF, even if slower. The observation that fibroblast cells in culture may show spontaneous voltage oscillation is not new [33], and it has been the object of controversies (e.g. Refs. 34 and 35). Our data, together with previous experiments on Balb/c 3T3 cells [24] indicate that in the great majority of the cells investigated the membrane potential in control conditions is rather stable; the voltage oscillations seen in some cells may arise from spontaneous oscillations in $[Ca^{2+}]_i$, due to some unknown mechanism. This finding points again to the problem of the possible heterogeneity of cell populations in culture. Usually, data obtained from experiments on the whole dish or cuvette are considered as arising from an uniform cell population; however, subpopulations may be present, with different behaviour and properties, thus complicating the picture.

Finally, an important question to be asked is if these electrical responses may have any role in the progression of cells from a quiescent state to mitosis. It has been observed [4] that up to now no definite proof exists for a role of these 'early' events in the progression of the cell cycle; however, as reported before, in EGFR-T17 cells [4,17] and human fibroblasts [14] the hyperpolarizing response and the ionic currents that generate it can last as long as the longest recordings that have been performed (20 to 40 min) and show in many cases an oscillating behavior, linked to $[Ca^{2+}]_i$ oscillations. In other preparations [36] it has been shown that $[Ca^{2+}]_i$ oscillations can last for several hours. Thus these events can cover a time span comparable with the time scale of the processes that lead to DNA synthesis and cell division. Moreover, it has been proposed [3] that the calcium oscillations seen in many cell types in response to agonist stimulation may have a role in frequency coding of information relevant to the cell metabolism and growth; electrical events could play a similar role.

Apart from other possible roles, a strong and sustained hyperpolarization could be important in increasing the electrochemical gradient for ion exchangers, like the Na^+/H^+ antiporter, crucial in the signal cascade that leads to cell proliferation after mitogen stimulation [2].

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