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The Effects of Epidermal Growth Factor on Membrane Potential

RAPID HYPERPOLARIZATION FOLLOWED BY PERSISTENT FLUCTUATIONS*

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The effects of epidermal growth factor (EGF) on membrane potential were investigated in suspensions of the following three cell types endowed with a large complement of specific receptors: EGFR-T17 (a clone of mouse NIH-3T3 fibroblasts overexpressing EGF receptors); A431 and KB (two human carcinoma lines). In all these lines EGF induced a rapid and marked hyperpolarization constituted by an initial peak (in all three cell lines) and a subsequent sustained plateau phase, concomitant with the well-known increase of $[Ca^{2+}]_i$. The time course and phorbol ester inhibitability of the membrane potential effects were the same as for the $[Ca^{2+}]_i$ response. Experiments with Na^+-free and chloride-free media excluded a major role of the latter ions in the EGF-induced hyperpolarization. In contrast, experiments with high K^+ media, with the monovalent cation ionophore gramicidin and with Ca^{2+}-free media together with either a Ca^{2+} ionophore (ionomycin, in A431 and EGFR-T17), or an agonist (bradykinin, in A431) addressed to a receptor coupled to phosphoinositide hydrolysis, were consistent with the involvement of Ca^{2+}-activated K^+ channels. The EGF-induced hyperpolarization was completely blocked by the K^+ channel blocker, quinidine, and unaffected by a variety of other drugs. Patch clamping of individual EGFR-T17 cells confirmed the initial hyperpolarization (from ≈ −30 mV, the resting potential, to −60, −80 mV) was due to the activation of an outward current. This initial hyperpolarization was followed by fluctuations (period ≈1 min) persisting as long as the cells could be analyzed. Thus, the changes of membrane potential appear to be not only novel members of the group of early events triggered by EGF in target cells but also long-lasting effects of the growth factor, which continue for unexpectedly long periods of time after EGF application.

Activation of the EGF[1] receptor (EGFR) is known to elicit a complex array of intracellular signals which can ultimately lead to DNA synthesis and cell division. The receptor molecule is endowed with a tyrosine kinase activity localized in the cytoplasmic domain, whose activation upon EGF binding (1, 2) leads to the phosphorylation of the receptor itself and other protein substrates (1–3). Concomitantly, membrane polyphosphoinositides are hydrolyzed, with generation of inositol-1,4,5-trisphosphate ((1,4,5)IP_3) (4–7), and 1,2-*sn*-diacylglycerol (8), release of Ca^{2+} from intracellular stores, and activation of Ca^{2+} influx from the extracellular medium (4, 9). The latter two events induce the concentration of cytosolic Ca^{2+}, $[Ca^{2+}]_i$, to increase. Finally, the cytosolic pH increases, mainly due to the activation of an electroneutral Na^+/H^+ exchange reaction (10).

Although quite variable in size, depending on the number of specific receptors expressed by different cell types, the reactions mentioned so far are believed to be triggered by EGF in all target cells. In contrast, the effect of the growth factor on the membrane potential (V_m) has been reported to change not only in size but also in quality. In one cell type (BSC-1) EGF has in fact been reported to cause a small and brief depolarization (11), and in another (A431 epidermoid carcinoma, which expresses over 10^6 receptors/cell) to induce no detectable effect (12). Finally, in mammary epithelial cells the growth factor was found to induce hyperpolarizing V_m fluctuations beginning, however, not immediately but only after several hours of treatment (13). In order to solve these apparent discrepancies and to clarify the relationship (if any) between the EGF effects on V_m and the other ionic events induced by the growth factor (in particular, the $[Ca^{2+}]_i$ changes), the whole field needed to be reinvestigated. This has been done by employing three cell types of various origin (EGFR-T17, which are mouse NIH-3T3 fibroblasts overexpressing the human EGF receptor cloned from A431 cells (14, 15), A431 and KB human carcinoma lines). The study of V_m was carried out on both cell suspensions and single cells by the use of a fluorescent dye (bis-oxonol) and by electrophysiology (whole cell configuration of the patch clamping technique), respectively, and was paralleled by the study of $[Ca^{2+}]_i$. The results obtained unambiguously demonstrate that the initial $[Ca^{2+}]_i$ increase triggered by EGF is accompanied by marked hyperpolarization due to the activation of Ca^{2+}-dependent K^+ channels, and that these events can be followed by V_m fluctuations which persist for considerable periods of time.

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[1] The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; (1,4,5)IP_3, inositol-1,4,5-trisphosphate (Ins-P_3 in "Results"); $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; V_m, membrane potential; KRH, Krebs-Ringer Hepes buffer; PMA, phorbol 12-myristate,13-acetate; TEA, tetraethylammonium; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; fura-2/AM, fura-2 pentacetoxymethylester; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

EXPERIMENTAL PROCEDURES

Cell Culture and Incubation Media—The cell lines used in this study (A431, EGFR-T17, and KB) were maintained at 37 °C in Dulbecco's modified Eagle's medium + 10% fetal calf serum supplemented with antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml), under a humidified atmosphere of 95% air, 5% CO_2. All the cells were used at approximately 90% confluence except for the patch clamp studies, in which lower densities (50–70%) were used for technical reasons. The standard solution employed in most experiments was a Krebs-Ringer Hepes buffer (KRH) containing (in mmol/liter): NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgSO_4$, 1.2; KH_2PO_4, 1.2; glucose, 6; Hepes-NaOH, 25, pH 7.4. The Na^+-free solution (referred as choline-KRH) was made by substituting NaCl with equimolar choline chloride, and Hepes-NaOH by Hepes-Tris. Cl^--Na^+-K^+-free solution contained 270 mM sucrose, 20 mM Hepes-Tris, pH 7.4, and 6 mM glucose. The Ca^{2+}-free solution ($[Ca^{2+}]_o$, $\approx 10^{-5}$ M) contained all the original components of KRH with the exception of $CaCl_2$.

Measurement of $[Ca^{2+}]_i$—$[Ca^{2+}]_i$ was measured with the fluorescent probe fura-2 (16). Monolayers of three to five dishes (100-mm diameter) were washed twice with phosphate-buffered saline and then treated at 37 °C with 2 ml of the same buffer containing trypsin (0.01%) and EDTA (0.9 mM). Detachment of the cells from the dish was complete within 1 (EGFR-T17 or KB cells) or 10 (A431 cells) min. Trypsin was neutralized by washing the cells in growth medium followed by two washes with KRH (centrifugation at 1000 rpm for 5 min). The pellets were resuspended in KRH (cell concentration, 5×10^6 cell/ml) and loaded with fura-2 by a 30-min incubation period at 37 °C with 5 μM of fura-2 pentacetoxymethylester (fura-2/AM). For the fluorimetric measurement of $[Ca^{2+}]_i$, 10^6 cells were placed in the cuvette of a thermostatically controlled (37 ± 1 °C) cell holder. Fluorescence records were taken at an excitation of 345 nm and an emission of 490 nm, with slits of 5 nm. The calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was done according to Grynkiewicz *et al.* (16).

Measurement of Membrane Potential with Bis-oxonol—V_m was measured in cell suspensions with the slow response fluorescent dye bis-oxonol (17, 18). The cells (5×10^5/ml) were resuspended as for cuvette $[Ca^{2+}]_i$ measurements. One ml of these suspensions was transferred into the spectrofluorimeter cuvette, and 100 nM bis-oxonol added from a stock solution in dimethyl sulfoxide. Equilibration and redistribution of the dye was allowed to proceed for at least 8 min before any addition was made. Fluorescence measurements were taken at excitation and emission wavelengths of 540 and 580 nm with slits of 5 nm. Downward or upward deflections of the fluorescence tracings represent hyper or depolarizations, respectively. For the calibration of signals into V_m values, the cells were incubated in Na^+-free medium (choline-KRH, which has effect on neither basal nor stimulated fluorescence) and then gramicidin (1.5 μg/ml) (19) was added at different extracellular K^+ concentrations. The equation used for the calibration was as follows: $V_m = 26.7 \ln ([K^+]_o [K^+]_i)$ (17, 20, 21). Under these experimental conditions the fluorescence-V_m plots were linear between −80 and +10 mV. The changes in V_m are expressed as mV or fluorescence arbitrary units.

Electrophysiology—EGFR-T17 fibroblasts attached to 35-mm Petri dishes and kept in Dulbecco's modified Eagle's medium containing 1% fetal calf serum for 24–48 h were investigated with the whole-cell version of the patch clamp technique as described in detail elsewhere (22). Composition of the external medium was (in mmol/liter) NaCl, 154; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 5.5; Hepes, 5; pH 7.4; except in the experiments in zero $[Ca]_o$ in which $CaCl_2$ was omitted from the solution. Composition of internal solution was (in mmol/liter): KCl, 133; $MgCl_2$, 3; GTP, 4; ATP, 1; phosphocreatine, 1; EGTA, 0.1; Hepes, 5; pH, 7.3.

Unless otherwise indicated, all statistical values are given as mean ± S. D.

Materials—Culture media and sera were from Flow Labs and Biochrom. EGF (receptor grade) was purchased from Collaborative Research. Fura-2/AM and ionomycin were from Behring Diagnostics. Phorbol esters, gramicidin, bradykinin, quinidine, *d*-tubocurarine, 4-aminopyridine, apamin, and tetraethylammonium were from Sigma. Bis-oxonol was purchased from Molecular Probes. The rest of the chemicals were reagent grade, and their specific sources have been described elsewhere (4, 15).

RESULTS

EGF Induces Parallel Changes of Cytosolic Ca^{2+} and Membrane Potential—Previous studies had already shown that addition of EGF to target cells endowed with a high number of specific receptors induces an increase of $[Ca^{2+}]_i$ which is the consequence of both the Ins-P_3-induced release of Ca^{2+} from an intracellular store(s) and the activation of Ca^{2+} transport at the plasma membrane (4, 7, 9). In cell suspensions prepared from the three lines investigated in the present work, the $[Ca^{2+}]_i$ increase triggered by a maximally effective EGF concentration (10 nM) was found to begin without appreciable lag and to reach a peak within 10–20 s (Fig. 1, *A–C*) from the application of the growth factor. Thereafter, $[Ca^{2+}]_i$ declined progressively toward basal values but with slightly different kinetics in each cell line: longer-lasting in A431 than in KB and, especially, in EGFR-T17 cells. As far as V_m (measured by bis-oxonol fluorescence), a marked hyperpolarization was

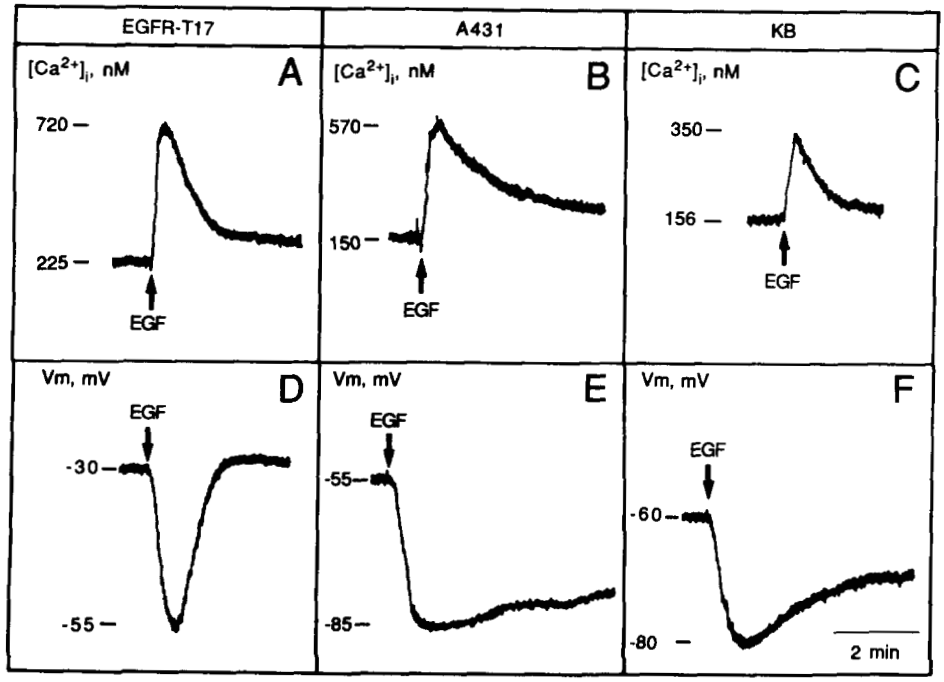

FIG. 1. **$[Ca^{2+}]_i$ and V_m changes induced by maximal concentrations of EGF (10 nM) administered to EGFR-T17 (*panels A* and *D*), A431 (*panels B* and *E*), and KB cells (*panels C* and *F*).** In this and the following two to eight figures, $[Ca^{2+}]_i$ results (*panels A–C*) and V_m results (*panels D–F*) were obtained by the fura-2 and by the bis-oxonol fluorescence techniques, as described in detail under "Experimental Procedures." Notice that $[Ca^{2+}]_i$ increase causes an upward movement and hyperpolarization a downward movement in the traces. The *numbers* to the left of each trace refer to either the $[Ca^{2+}]_i$ or the V_m calibration, and are expressed as nM or mV, respectively.

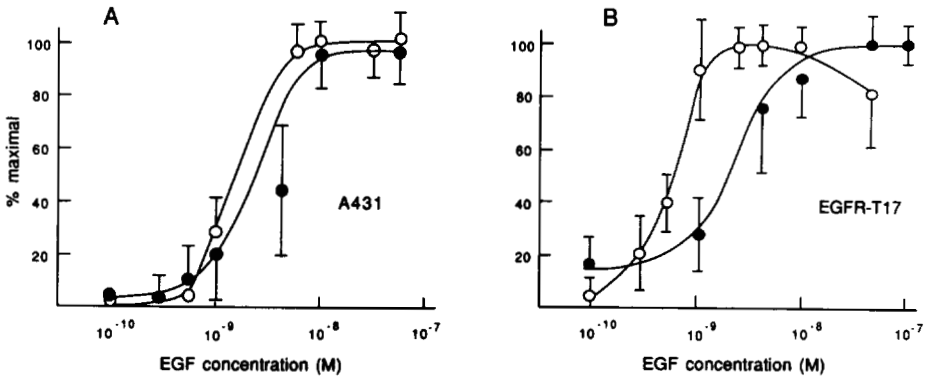

FIG. 2. **EGF concentration dependence of the $[Ca^{2+}]_i$ (●) and V_m (○) increase in A431 (*panel A*) and EGFR-T17 (*panel B*) cells.** Values (means of three experiments ± S.D.) are expressed as percentage of the peak increases observed after administration of maximal concentrations of the growth factor.

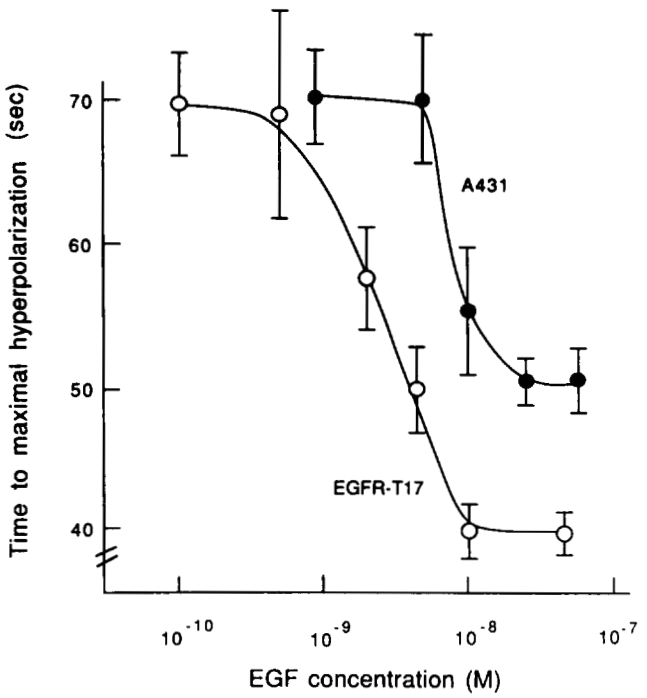

FIG. 3. **EGF concentration dependence of the V_m increase time course in EGFR-T17 (○) and A431 (●) cells.** Values shown (means of two experiments ± S.D.) are the times (s) elapsed between the administration of the growth factor and the reaching of the peak hyperpolarization in the two cell types.

found to occur after EGF treatment. Basal V_m of A431, EGFR-T17, and KB cells was −57 ± 7, −31 ± 11, and −63 ± 14 (mean ± S. D. of 12, 7, and 4 measurements, respectively). The EGF-induced effect consisted in an initial, rapid (no appreciable lag) hyperpolarization, (ranging from 15 to 50 mV in the three cell lines, Fig. 1, *D–F*) concomitant with the $[Ca^{2+}]_i$ peak, followed by a phase consisting in a progressive, slow return toward basal values. As with the $[Ca^{2+}]_i$, this second phase was longer lasting in A431 and KB (16 ± 2.8 and 12 ± 4 min, $n = 5$ and 2, respectively) than in EGFR-T17 (104 ± 18 s, $n = 17$) cells.

The V_m effect of EGF was further characterized in terms of concentration dependence and time course. Fig. 2, *A* and *B* shows that in A431 cells the initial hyperpolarization occurs at EGF concentrations similar to those required for the $[Ca^{2+}]_i$ increase (Fig. 2*A*). In contrast, in EGFR-T17 the V_m concentration dependence was shifted to the left (of about half a log) with respect to $[Ca^{2+}]_i$ (Fig. 2*B*). The rate of hyperpolarization in response to EGF was found to be strictly dependent on the concentration used (Fig. 3). In the $3 \times 10^{-10} - 3 \times 10^{-9}$ (EGFR-T17) and $3 \times 10^{-9} - 3 \times 10^{-8}$ (A431) M ranges the time to reach maximal hyperpolarization increased with decreasing concentrations of EGF.

Phorbol esters are tumor promoters known to induce marked inhibition of various signals generated at the EGF receptor (autophosphorylation of the receptor; $[Ca^{2+}]_i$ increase, Refs. 23 and 24) via the activation of protein kinase C. Here we have also found that the V_m changes induced by the growth factor are inhibited by a short pretreatment of the cells with phorbol ester (Fig. 4, *A–C*) and that this inhibition is both time and concentration dependent (Fig. 4, *D* and *E*). In A431 cells half-maximal inhibition of the EGF-induced hyperpolarization was observed to require 40 s of preincubation with saturating concentrations (100 nM) of phorbol 12-myristate,13-acetate (PMA) (Fig. 4*D*). With long preincubations (4 min), the same effect was induced by a concentration of PMA as low as 240 pM (Fig. 4*E*). In A431 cells phorbol esters induced a modest depolarization, particularly when applied in the Ca^{2+}-containing medium (compare Fig. 4, *A* and *B*). This event, however, cannot account by itself for the inhibition of the EGF effects on V_m because 1) no phorbol ester-induced depolarization was observed in EGFR-T17 cells (Fig. 4*C*), and 2) depolarization of the same magnitude by $[K^+]_o$ (15 mM) caused only a relatively small inhibition of the EGF effects (see below Fig. 6).

Hyperpolarization Mechanisms: Involvement of a K^+ Conductance—Hyperpolarization of plasma membrane could be due to changes in the permeability to Na^+, K^+, Cl^-, or Ca^{2+}. In order to investigate the ion conductance(s) involved in the EGF-induced hyperpolarizing response, experiments were carried out by using appropriately modified extracellular buffers. First, the role of extracellular Na^+ was investigated. Replacement of medium Na^+ with choline failed to modify significantly the resting $[Ca^{2+}]_i$ in A431 cells (not shown) suggesting that, if present, the Na^+/Ca^{2+} exchange system does not play an important role in $[Ca^{2+}]_i$ control. Similarly, resting V_m of EGFR-T17 (−29 ± 7.2, $n = 3$) and A431 (−52 ± 9, $n = 3$) cells was not significantly modified by the same Na^+ replacement in the medium. Moreover, under these conditions the ability of EGF to induce plasma membrane hyperpolarization in both cell lines was unaffected (compare tracings *A* and *B*, *D* and *E*, in Fig. 5). On the other hand, substitution of all chloride-containing salts in KRH with sucrose did not block completely, but only slightly attenuate, the hyperpolarizing effect of the growth factor (Fig. 5*C*). In contrast, a progressive inhibition of the EGF-induced hyperpolarization was observed, in parallel to basal depolarization, when the extracellular concentration of K^+, $[K^+]_o$, was increased step by step (Fig. 6*A*). Complete blockade of the EGF-induced hyperpolarization occurred at 40 or 60 mM $[K^+]_o$ for EGFR-T17 or A431 cells, respectively (Fig. 6*B*). Similarly, hyperpolarization was blocked by the dissipation of the K^+

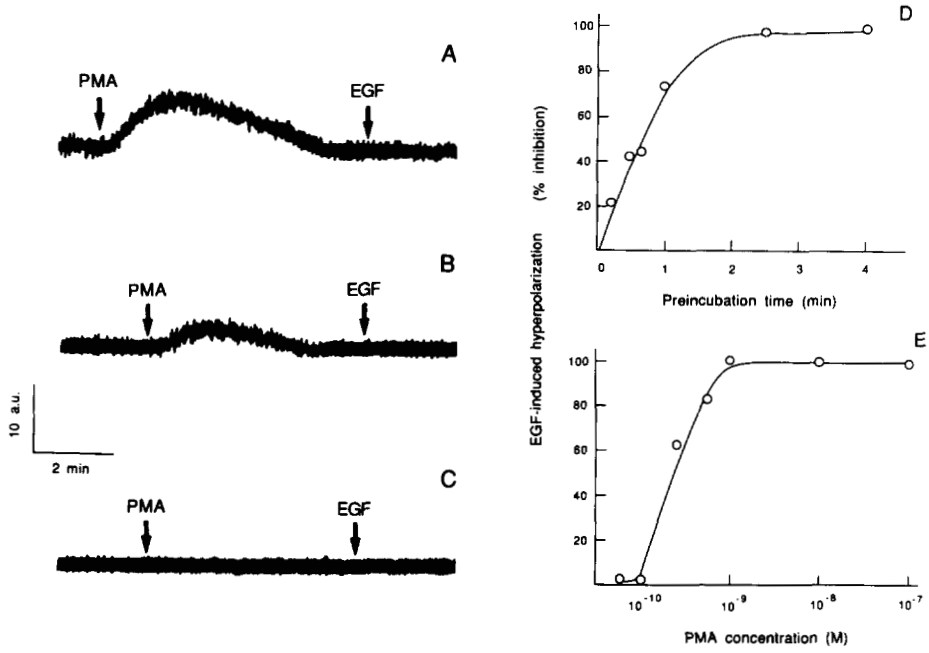

FIG. 4. **Phorbol ester inhibition of the hyperpolarization response induced by EGF in A431 and EGFR-T17 cells.** *Panels A–C* illustrate the effects of maximal concentrations of phorbol 12-myristate,13-acetate (100 nM, *PMA*) administered 6 min before 10 nM EGF. In A431 cells PMA causes a transient cell depolarization, which is greater with Ca^{2+}-containing (*A*) than with Ca^{2+}-free (*B*) medium. No such an effect is visible in EGFR-T17 cells (*C*). In both cell types the EGF response on V_m is blocked by the previous administration of the phorbol ester (*A–C*). *Panels D* and *E* illustrate the time course and concentration dependence of the inhibition by phorbol ester of the EGF-induced hyperpolarization in A431 cells. Preincubation with PMA for the experiments in *panel E* was 4 min. 100% inhibition refers to complete abolishment of the EGF response. Here and in Figs. 5 and 7, 10 arbitary units = 10 absorbance units (excitation fluorescence at 540 nm).

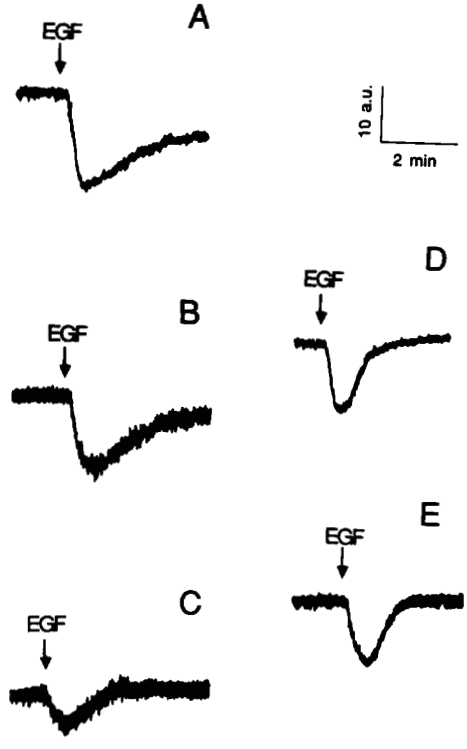

FIG. 5. **Effects of extracellular ion replacements on the EGF-induced V_m increase in A431 (*traces A–C*) and EGFR-T17 (*traces D* and *E*) cells.** *Traces A* and *D* were obtained in complete KRH medium, *traces B* and *E* in the medium (choline-KRH, see under "Experimental Procedures") where NaCl had been replaced with choline-chloride, *trace C* with the medium with salts replaced by sucrose. EGF concentration used, 10 nM.

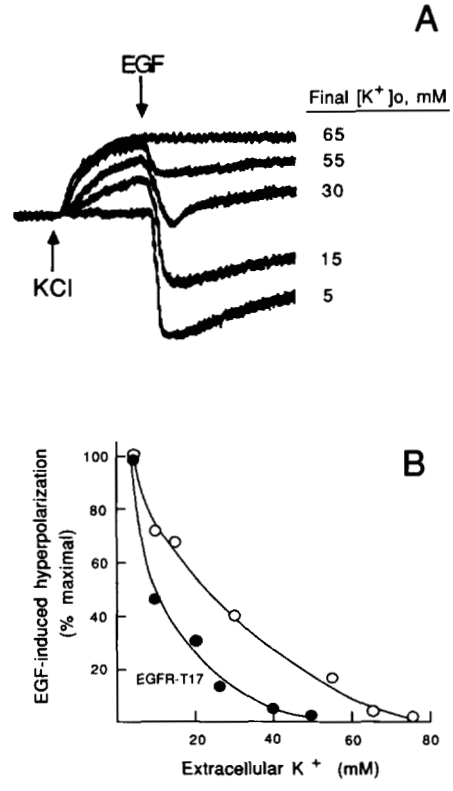

FIG. 6. **Effect of $[K^+]_o$ increase on the EGF-induced hyperpolarizaton.** *Panel A* illustrates the progressive disappearance of the V_m response induced by 10 nM EGF in A431 cells investigated while suspended in incubation media containing increasing $[K^+]_o$. The initial increase of the signal observed after KCl addition is due to the depolarization induced by the cation. *Panel B* shows the concentration dependence of the K^+ inhibitory effect in A431 (○) and EGFR-T17 (●) cells (means of two experiments).

electrochemical gradient obtained by incubating the cells with the monovalent cation ionophore gramicidin (1 μg/ml, in choline-KRH). Under these conditions the V_m of the treated cells is shifted to the E_K (≈ −80 mV). Taken together, these results strongly suggest that the activation of one (or more) K^+ current(s) is (are) directly responsible of the hyperpolarization induced by EGF.

The EGF-induced Hyperpolarization Is a Consequence of $[Ca^{2+}]_i$ Rise: Involvement of Ca^{2+}-activated K^+ Channels—Different types of K^+ channels have been characterized depending on their physiological and pharmacological properties and their modulation by receptors and second messengers (25). Since, as already mentioned, the growth factor is known to induce an increase in the $[Ca^{2+}]_i$ in its target cells, we next

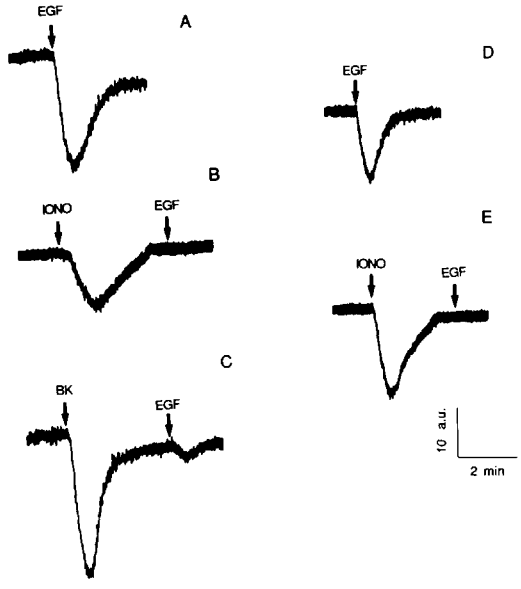

FIG. 7. **V_m changes induced by EGF, ionomycin, and bradykinin administered in Ca^{2+}-free medium to A431 (*panels A–C*) and EGFR-T17 (*panels D* and *E*) cells.** Notice that, in the condition of the experiment preadministration of ionomycin (*IONO*, 100 nM) causes the complete disappearance of the effect of EGF (10 nM), whereas with bradykinin (*BK*, 50 nM) a small fraction of the EGF response is maintained.

TABLE I

Effect of K^+ channel blockers on EGF-induced hyperpolarization

A431 or EGFR-T17 cells were preincubated with the different treatments for >4 min before the addition of EGF and V_m changes monitored by measuring bis-oxonol fluorescence changes. The high concentration tetraethylammonium (TEA) solution (150 mM TEA) contained all the original components of KRH, but NaCl was replaced by 150 mM TEA-Cl. The results are expressed as % of inhibition of maximal fluorescence decreases caused by EGF in the two target cell lines analyzed (means of at least two separate determinations). ND = not determined.

	% Inhibition	
	A431	EGFR-T17
Apamin (1 μM)	0	0
4-Aminopyridine (5 mM)	27	71
d-Tubocurarine (500 μM)	12	60
TEA		
10 mM	0	0
150 mM	56	81
TEA 10 mM		
+ KCl 5 mM	ND	52
+ KCl 10 mM	ND	70
+ KCl 20 mM	ND	90
KCl 10 mM		40

investigated the possible involvement of Ca^{2+}-activated K^+ channels. Such an involvement is suggested by the results shown in Fig. 7. Exposure of the cells to EGF in KRH without Ca^{2+} added ($[Ca^{2+}]_o \approx 10^{-5}$ M) was found to markedly abbreviate the V_m responses (especially in A431 cells, compare Fig. 1*E* with Fig. 7*A*), similar to what is known to occur for the corresponding $[Ca^{2+}]_i$ responses (4, 15). In order to investigate whether the remaining initial hyperpolarization was the consequence of the intracellular Ca^{2+} release induced by the growth factor, experiments were carried out in which A431 cells were pretreated with either bradykinin (a peptide that stimulates a vigorous phosphatidylinositol-4,5-bisphosphate hydrolysis response[2] or ionomycin (a Ca^{2+} ionophore). When

[2] A. Pandiella, M. Magni, and J. Meldolesi, manuscript in preparation.

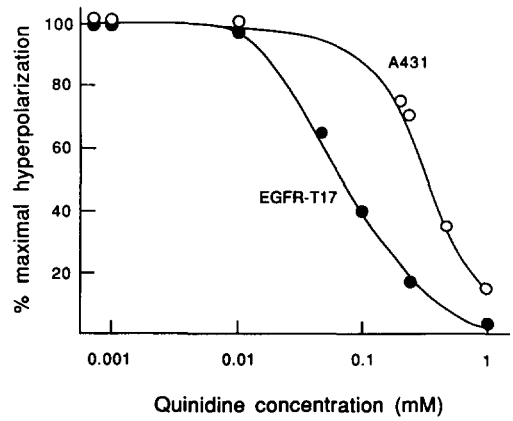

FIG. 8. **Concentration dependence of the quinidine inhibition of the EGF-induced hyperpolarization in A431 (○) and EGFR-T17 cells (●).** Cells were pretreated with quinidine 4 min before the growth factor (10 nM). Values shown are percentages of the peak hyperpolarization effects observed in the two cell types after administration of EGF to unpretreated cells (means of two experiments).

administered in Ca^{2+}-free medium both these substances are expected to cause the depletion of the (1,4,5)IP_3-sensitive internal Ca^{2+} store(s). As can be seen in Fig. 7, *B* and *C*, under these circumstances both ionomycin (100 nM) and bradykinin (100 nM) induced rapid hyperpolarizations, while the V_m effect of a subsequent administration of EGF was almost completely eliminated, consistently with a major role of $[Ca^{2+}]_i$ in the generation of the K^+ current in A431 cells. Analogously, ionomycin pretreatment in the absence of $[Ca^{2+}]_o$ was found to block the EGF-induced hyperpolarization in EGFR-T17 cells (compare Fig. 7, *D* and *E*). The results obtained with ionomycin appear consistent with a major role of $[Ca^{2+}]_i$ in the generation of the K^+ current. The same appears to be true for bradykinin. In fact, the possibility that a considerable part of the inhibition by the peptide was due to activation of protein kinase C (occurring via the generation of 1,2-*sn*-diacylglycerol at the B_2 receptor level) was excluded by experiments with A431 cells treated long term with phorbol esters (24 h, 500 nM PMA). Under these conditions protein kinase C is known to be markedly down-regulated (24), yet the bradykinin inhibition of the EGF-induced V_m response was not significantly different from that observed in cells not pretreated with the phorbol ester (results not shown).

Studies in different cell types have revealed the existence of at least two Ca^{2+}-activated K^+-currents characterized by their pharmacological properties (25). In order to analyze the drug sensitivity of the EGF-induced hyperpolarization, different treatments were made 5–10 min before EGF. The results of such experiments are summarized in Table I and Fig. 8. Apamin (100–1000 nM), which has been shown to inhibit a type of Ca^{2+}-activated K^+ conductance (25–27), was unable to block the effect of the GF. *d*-Tubocurarine (500 μM) and 4-aminopyridine (up to 5 mM) induced a small hyperpolarization of A431 cells and had almost no effect on EGF-induced changes in V_m. The last two drugs, however, blocked more efficiently the EGF-induced effect on V_m in EGFR-T17 cells. High concentrations of tetraethylammonium (>50 mM) were able to block the EGF effect on V_m. Lower concentrations of tetraethylammonium (10 mM) were markedly effective only when the cells were partially depolarized by increasing $[K^+]_o$. The most potent and efficient inhibitor was quinidine (Fig. 8), a known K^+ channel blocker. Quinidine induced only a small depolarization in unstimulated A431 cells and almost no change in V_m in EGFR T17 cells (not shown) and readily

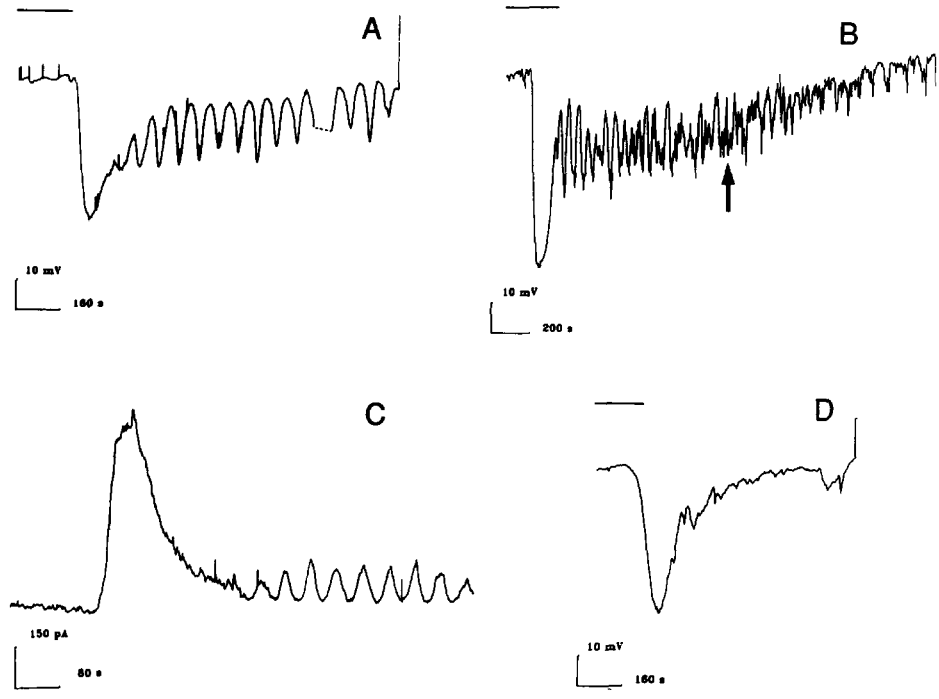

FIG. 9. **Patch clamping of individual EGFR-T17 cells.** *Panels A, B,* and *D* illustrate the V_m changes (hyperpolarization downwards) induced by application of EGF (10 nM) under current clamp conditions. (*Panels A* and *B*, Ca^{2+}-containing; *panel D*, Ca^{2+}-free incubation medium. *Bars* over the beginning of the traces mark 0 mV potential. The interruption in A (*broken line*) was used to generate I/V plots. EGF was administered a few seconds before the beginning of the hyperpolarization response and, in *panel B*, removed when indicated by an *arrow*. *Panel C* illustrates the current changes (outwards) induced by EGF under voltage clamp conditions.

blocked the EGF induced hyperpolarization (EC_{50} = 350 and 70 μM for A431 and EGFR-T17 cells, Fig. 8).

Electrophysiology—A total of 13 attached EGFR-T17 cells was investigated by the whole cell patch clamping technique. The results obtained have been extremely consistent. Under current clamp conditions (Fig. 9, *A* and *B*), basal voltage was found to be stable (fluctuations well below 5 mV). Application of EGF to cells (internally perfused with a buffer containing low EGTA, 100 μM, and therefore expected to develop intracellular $[Ca^{2+}]_i$ transients) was found to induce a rapid, major hyperpolarization, corresponding to a change of the V_m from the basal -28.1 ± 9.1 to -71.3 ± 8.1 mV (n = 9). During hyperpolarization the membrane conductance was markedly increased. Moreover, voltage clamp studies carried out in two cells with resting potential, of -45 mV revealed the current reverse potential after EGF to have moved to approximately -75 mV, *i.e.* a clear shift approaching the K^+ reverse potential. About 30 s after the beginning of the hyperpolarization response, V_m begun to move back toward the resting level. This, however, was not reached rapidly. V_m fluctuations did in fact become evident, beginning 1–2 min from the initial V_m peak, and continuing in all cells investigated up to the interruption of the measurements (in one case, Fig. 9*B*, 32 min). The period of these fluctuations was initially similar ($\approx$ 60 s) in all cells, but their development could vary. In some cells fluctuations, although initially ordered, became progressively more frequent and irregular (Fig. 9*B*). Removal of EGF from the bathing medium failed to induce an immediate stop of the fluctuations (2 cells) (*arrow* in Fig. 9*B*). When EGF was administered to cells bathed in a Ca^{2+}-free medium (Fig. 9*D*) the initial hyperpolarization was unchanged. V_m fluctuations, however, failed to appear except for the first two to three which in addition were greatly reduced in size. In a few additional cells, the effect of EGF was investigated under voltage-clamp conditions (-30 mV). As can be seen in Fig. 9*C*, a large initial outward current followed by smaller fluctuations was revealed. Both these processes appeared to be strictly parallel (and therefore interdependent) with the V_m changes previously described.

DISCUSSION

The results we have obtained clearly demonstrate that, in the three target cell types investigated, hyperpolarization is a novel member of the group of early signals triggered by the activation of the EGF receptor. The mechanisms responsible for the EGF-induced hyperpolarization have been clarified, at least in part, by a number of consistent observations. On the one hand electrophysiology demonstrated that hyperpolarization is due to an outward current, with a shift of the reverse potential toward more negative values. This excludes that the process is due to decreased influx of cations, such as Na^+, a possibility which appears incompatible also with the negative results obtained, by using fluorimetric techniques, with the Na^+-free medium. The same Na^+-free results exclude the participation of the Na^+/Ca^+ and Na^+/H^+ exchange systems in the V_m effects of EGF. Likewise, the data with Cl^--free medium exclude a major involvement of this anion. In contrast, the results obtained with the high K^+ media and the monovalent cation ionophore gramicidin appear entirely compatible with the involvement of a stimulated K^+ efflux, and this possibility is supported also by the electrophysiological and pharmacological studies, where the EGF-effect on V_m was found to be inhibited by quinidine,[3] a known blocker of K^+ channels (25), and unaffected by a variety of other drugs. The role of Ca^{2+} as the intracellular trigger of the K^+ channel activation is supported by the results with the Ca^{2+} ionophore, ionomycin, and the Ca^{2+} chelator, EGTA, and by the correlation observed between the EGF-induced V_m increases investigated here and the $[Ca^{2+}]_i$ increases investigated in this and in previous studies (4, 15). Such a correlation concerns the time course of the two responses as well as their parallel inhibitability both by phorbol esters (24) and (in cells incubated in Ca^{2+}-free medium) by the discharge of the intracellular Ins-P_3-sensitive Ca^{2+} stores. As far as the concentration dependence of the two EGF-induced responses, the correlation was good in A431 cells, while in EGFR-T17 cells we observed a shift, with maximal V_m increases occurring at

[3] Unfortunately, the toxicity of quinidine, the most efficient hyperpolarization blocker in our studies, precludes its use in cell proliferation experiments.

concentrations of the growth factor which induce only moderate $[Ca^{2+}]_i$ changes. It should be emphasized, however, that our measurements reflect average $[Ca^{2+}]_i$ of the cells while the activation of K^+ channels occurs at the inner face of the plasma membrane, where $[Ca^{2+}]_i$ is expected to be higher than average due to EGF-induced activation of Ca^{2+} influx. Even the concentration dependence data in EGFR-T17 cells may thus be consistent with the conclusion that hyperpolarization responses induced by EGF are most probably due to the activation of one or more Ca^{2+}-dependent K^+ channel type(s). The nature of the channels involved (whether of the large, intermediate, or small conductance) has not been directly investigated by patch clamping. The negative results with apamin tend to exclude the small conductance channel types. Since, however, those data were obtained in cell suspensions prepared by trypsin treatment, a possible injury of the apamin-binding site by the enzyme cannot be excluded (28).

A question to be asked is whether hyperpolarization occurs only in the three cell types we have investigated or is more widespread among the various EGF targets. The strict correlation we have observed between the increases of V_m and $[Ca^{2+}]_i$ seems to exclude the possibility of hyperpolarization taking place in the cell types where EGF (because of the small number of its specific receptors) fails to induce any detectable $[Ca^{2+}]_i$ increases (5, 29). In this respect it is interesting to mention that in at least some of the latter cell types EGF has no mitogenic effect unless it is administered in combination with another agent (such as bradykinin) capable of inducing phosphatidylinositol-4,5-bisphosphate hydrolysis (29), and thus $[Ca^{2+}]_i$ (and possibly V_m) increases. In addition, in order to develop EGF-induced hyperpolarization a cell needs to express Ca^{2+}-dependent K^+ channels. These channels are known to be common to many cell types but not ubiquitous (30). Lack of expression of these channels could explain the depolarization (instead of hyperpolarization) observed in BSC-1 cells (11). Such a depolarization could be due to the activation of Ca^{2+} influx, which is also triggered by EGF (4, 9). In contrast, the lack of any V_m effect, previously reported in A431 cells (12), remains entirely incompatible with the present positive data in the same (and other) cell type(s).

Up to now, the initial $[Ca^{2+}]_i$ spike increase, triggered by EGF as a consequence of both intracellular Ca^{2+} release and Ca^{2+} influx, was believed to be followed by a persistent plateau phase, largely dependent on a slowly inactivating Ca^{2+} influx. The results obtained by whole cell patch clamp electrophysiology in single EGFR-T17 cells suggest, however, the existence of $[Ca^{2+}]_i$ fluctuations occurring approximately every minute, capable of triggering V_m fluctuations and persisting for considerable periods of time. $[Ca^{2+}]_i$ fluctuations had been observed previously in a variety of cell types shortly after stimulation with agonists of many receptors (31), never, however, with typical growth factors except for a recent observation in human fibroblasts treated with whole serum (32), which contains a mixture of growth and other stimulating factors. The mechanism(s) responsible for these events is(are) still debated. In some cells fluctuations were shown to originate from Ca^{2+} influx, via the activation of voltage-gated Ca^{2+} channels in the plasma membrane (33), in others from intracellular Ca^{2+} release (31). The cytoplasmic stores responsible for the release process have not been identified with certainty. Stores sensitive to (1,4,5)IP_3 and to increased $[Ca^{2+}]_i$ are suspected to be both implicated (31). In EGFR-T17 cells some V_m fluctuations were observed also in the Ca^{2+}-free medium, suggesting an at least partial intracellular origin. Moreover, the slow rate of fluctuation disappearance after removal of the growth factor speaks in favor of a mechanism initiated by EGF, but then dependent on other processes, such as another intracellular messenger or simply the change in the equilibrium between the different rapidly exchanging Ca^{2+} stores. Unfortunately, technical problems have prevented the single cell study of other cell types, such as A431. As a consequence, we do not know yet whether or not fluctuations occur in EGFR-T17 cells only or, as it appears likely, in other EGF cell targets as well. In any event, two considerations need to be made. The first concerns the similarity between our fluctuations of V_m and those observed previously in mammary gland cells beginning, however, a few hours after the start of the EGF treatment (13). Because of this delay, those fluctuations were believed to depend on "some complex metabolic processes" initiated by EGF (13). A possible explanation based on the combination of the previous and present data is that at the beginning of the treatment with EGF the primary cultures of mammary epithelial cells may lack a component already present in EGFR-T17 cells (for example the Ca^{2+}-dependent K^+ channel) and need to synthesize it in order to initiate V_m fluctuations. The observation that the expression of voltage-gated K^+ channels in lymphocytes varies consistently with the cell's developmental state (34) supports this hypothesis. The second consideration concerns the duration of the oscillating responses. We have observed fluctuations persisting for over 30 min from the application of EGF, *i.e.* our longest time of analysis by patch clamping. It is thus possible that in intact, undisturbed cells these events continue for hours, as suggested also by the studies in mammary epithelial cells (13). These events therefore approach and may even possibly reach the time when the events directly related to cell proliferation (for example, DNA synthesis) take place. Clearly, at the moment no proof exists of a direct connection between V_m fluctuations and cell proliferation. However, one of the main objections against the physiological relevance of ionic events induced by growth factors, *i.e.* the marked difference in duration with respect to the growth factor stimulation times required for the cells to enter the proliferation program, may be not as strong now as it appeared up to very recently.

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