THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 266, No. 10, Issue of April 5, pp. 6329-6335, 1991
Printed in U.S.A.

Ionic Events Induced by Epidermal Growth Factor

EVIDENCE THAT HYPERPOLARIZATION AND STIMULATED CATION INFLUX PLAY A ROLE IN THE STIMULATION OF CELL GROWTH*

(Received for publication, June 5, 1990)

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Charybdotoxin, a blocker of K^+ channels, and the imidazole drug SC38249, a blocker of both voltage- and second messenger-operated Ca^{2+} channels, were employed in mouse NIH-3T3 fibroblasts overexpressing the epidermal growth factor (EGF) receptor 1) to characterize the ionic events activated by EGF; and 2) to establish the role of those events in cell growth. The $[Ca^{2+}]_i$ response by EGF was little changed by charybdotoxin while the parallel hyperpolarization was inhibited in a dose-dependent manner. At high toxin concentrations (>3 × 10^{-8} M), the effect of EGF on membrane potential was turned into a persistent depolarization sustained by both Na^+ and Ca^{2+}. Pretreatment with 10 μM SC38249 induced only minor changes of the intracellular Ca^{2+} release by EGF (the process responsible for the initial phase of the $[Ca^{2+}]_i$ and membrane potential responses) and blocked the persistent, second phase $[Ca^{2+}]_i$ and the hyperpolarization responses, both dependent on Ca^{2+} influx, as well as the depolarization in the charybdotoxin-pretreated cells. Long term (up to 2-day) treatment with either charybdotoxin or SC38249 failed to affect the viability and growth of unstimulated EGFR-T17 cells. Moreover, in these cells, the ionic responses to EGF were restored after a 30-min incubation in fresh medium. In contrast, growth stimulated by EGF was inhibited, moderately (−20%) by charybdotoxin and markedly (−60%) by SC38249. These results indicate for the first time that both hyperpolarization and, especially, the persistent increase of $[Ca^{2+}]_i$ sustained by Ca^{2+} influx play a role in the activity of EGF, ultimately cooperating with other intracellular events in mitogenesis.

Work carried out during the last few years has lead to the identification of a series of events elicited within target cells by the activation of the EGF[1] receptor (1–3). The initial step consists in the phosphorylation (by the tyrosine kinase indigenous to the receptor molecule) of a group of substrates among which are various protein kinases and a form of phospholipase C (4–8), designated γ, specific for the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) (1). Tyrosine phosphorylation is believed to activate the enzyme (4–8) and this accounts for the generation of two intracellular second messengers, inositol 1,4,5-trisphosphate and diacylglycerol (9–12). The same messengers result also from the activation of a different class of receptors, *i.e.* those coupled via G proteins to the activation of other phospholipase(s) C (12). An extensive body of evidence suggests PIP_2 hydrolysis to be involved in the stimulation of cell growth triggered by EGF (12–16), although the nature of such an involvement, and the underlying mechanisms, have not been precisely defined yet. Quite a number of processes are affected after PIP_2 hydrolysis, to an extent however which can vary considerably depending on the cell type investigated. Thus, diacylglycerol activates protein kinase C and this in turn induces a variety of effects, including alkalinization of the cytoplasm (1, 12, 17) and, in some cases, cell depolarization (18). Concomitantly, the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, increases due to the activation of both release from intracellular Ca^{2+} stores (triggered by inositol 1,4,5-trisphosphate, Refs. 1, 3, 8–11, 19) and Ca^{2+} influx at the plasma membrane (3, 9–12, 19–21). These ionic events are not limited to the initial few minutes of stimulation but can persist thereafter as rythmic $[Ca^{2+}]_i$ fluctuations accompanied by synchronous hyperpolarizations of the plasma membrane, sustained by the activation of Ca^{2+}-dependent K^+ channels (21).

In order to clarify the physiological importance of these processes, it is necessary to both characterize them in detail and establish conditions under which they are selectively prevented. This has been done in the present work for two interconnected ionic processes stimulated by EGF: $[Ca^{2+}]_i$-dependent hyperpolarization and cation (Ca^{2+}, Na^+) influx at the plasmalemma. These processes were blocked by a scorpion toxin, charybdotoxin (22–24), and by imidazole drugs, SC38249 and analogues (25, 26), respectively, in mouse NIH-3T3 fibroblasts overexpressing the human cloned receptor (the EGFR-T17 clone, Ref. 27). When applied alone, neither agent had a consistent effect on the viability or growth of EGFR-T17 cells. In combination with EGF, however, charybdotoxin and, especially, SC38249 inhibited the growth responses induced by the growth factor. We conclude therefore that at least some of the ionic events triggered by EGF in its target cells participate in the stimulation of cell proliferation, probably acting coordinately with other stimulatory intracellular mechanisms activated in parallel by the growth factor.

* This work was supported in part by grants from the Italian Association for Cancer Research, AIRC, and the Scientific Institute S. Raffaele. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: EGF, epidermal growth factor; $[Ca^{2+}]_i$, cytosolic concentration of free Ca^{2+}; KRH, Krebs-Ringer medium buffered with Hepes; PIP_2, phosphatidylinositol 4,5-bisphosphate; SC38249 and SKF96365, {±-1-2,3-bis-[(4-methoxyphenyl)-methoxy]propyl}-1*H*-imidazole and the 4-methoxyphenetyl analogue; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FCS, fetal calf serum.

EXPERIMENTAL PROCEDURES

Cell Culture and Incubation Media—The EGFR-T17 cells (19, 27) used in this study were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) + 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, and used at approximately 90% confluence. The standard incubation medium employed in most experiments was a Krebs-Ringer Hepes (KRH) buffer containing (in millimoles/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. In the Ca^{2+}-free medium, $CaCl_2$ was left out, and 1 mM EGTA was added. In the Na^+-free medium, NaCl was substituted with equimolar choline chloride and Hepes-NaOH with Hepes-Tris. Cl^--Na^+-K^+-free medium contained 270 mM sucrose, 20 mM Hepes-Tris, pH 7.4, and 6 mM glucose.

Measurement of $[Ca^{2+}]_i$—$[Ca^{2+}]_i$ was measured with the fluorescent probe fura-2 (28). 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 EGFR-T17 cells from the dish was complete within 1 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 cells/ml) and loaded by a 30-min incubation period at 37 °C with 5 μM 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 345 nm excitation and 490 nm emission, with slits of 5 nm. Calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was done according to Grynkiewicz *et al.* (28). For additional details see Ref. 19.

Measurement of Membrane Potential—Membrane potential was measured in cell suspensions with the slow response fluorescent dye bis-oxonol (29). The cells (5×10^5/ml) were resuspended as for $[Ca^{2+}]_i$ measurements. One ml of these suspensions was transferred into the spectrofluorimeter cuvette, and 100 nM bis-oxonol was added from a stock solution in dimethyl sulfoxide. Equilibration and redistribution of the dye was allowed to proceed for at least 10 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. In a few experiments, membrane potential measurements were made in cells preloaded with the fluorescent Ca^{2+} chelator, quin-2, by a 45-min incubation (37 °C) with the tetracetoxymethylester, quin-2/AM (90 μM). The final intracellular quin-2 content, measured as in Ref. 30, was 1.2 nmol/10^6 cells. Calibration of signals into membrane potential values was obtained as described by Waggoner (29). To this end, the cells were incubated in the Na^+-free medium described above, and then gramicidin (1.5 μg/ml) was added at different extracellular K^+ concentrations. Intracellular K^+, $[K^+]_i$, was measured by flame spectrophotometry in a IL 943 apparatus (Instrumentation Laboratory, Milano, Italy). For these measurements, cell suspensions were thoroughly washed with K^+-free KRH and then lysed (>99% lysis) with 2% Triton X-100. In a total of five determinations, $[K^+]_i$ was found to be 143 ± 2 mmol/liter. The equation used for the calibration (29) was as follows: membrane potential = $26.7 \ln([K^+]_o/[K^+]_i)$. Under these experimental conditions, the fluorescence membrane potential plots were linear between −80 and +10 mV. The changes in membrane potential are expressed as ΔmV.

Cell Proliferation Assay—The effects of charybdotoxin and SC38249 were investigated as follows. EGFR-T17 cells were plated at 10,000 cells/35-mm Petri dishes and grown in DMEM + 10% fetal calf serum up to ≈30% confluence. Thereafter, the cells were transferred to DMEM supplemented with only 1% fetal calf serum, with or without SC38249 (10 μM) or charybdotoxin (75 nM). Five to seven min later, EGF (10 nM) or fetal calf serum (10%) were added to part of the dishes. After 2 days, the monolayers were washed with phosphate-buffered saline, and the cells were resuspended by a 15-min treatment with 0.05% trypsin, 0.9 mM EDTA. Under these conditions, more than 95% of the cells appeared single upon microscopic inspection. One ml of each cell suspension was mixed with 9 ml of isotonic solution, and the cell number was measured in a Coulter Counter (ZM, Coulter Electronics, Hialeah, FL) using a lower threshold of 8.1 μm. The results are expressed as means ± S.D.

Materials—Culture media and sera were from Flow Laboratories (Milan, Italy). EGF (receptor grade) was purchased from Collaborative Research (Two Oak Park, Bedford, MA); fura-2/AM and quin-2/AM were from Calbiochem, and bis-oxonol from Molecular Probes (Junction City, OR). Purified charybdotoxin and *Leiurus quinquestriatus hebraeus* crude venom were purchased from Latoxan (Rosans, France). SC38249 was the kind gift of Dr. Urs T. Ruegg, Sandoz Ltd., Basel, Switzerland, while tyrphostin RG50810 was the kind gift of Dr. A. Levitzki, the Hebrew University, Jerusalem, Israel. The rest of the chemicals were reagent grade, and their sources were specified elsewhere (9, 19, 21).

RESULTS

The ionic events induced by EGF in mouse EGFR-T17 fibroblasts were investigated by measuring the changes of both $[Ca^{2+}]_i$ and membrane potential, as revealed by specific fluorescent dyes, fura-2 and bis-oxonol, respectively. The $[Ca^{2+}]_i$ effect of EGF consists of an initial peak increase, which depends primarily on Ca^{2+} release from intracellular stores, followed by a prolonged plateau phase entirely dependent on Ca^{2+} influx (Fig. 1*A*; Refs. 1, 3, 7–9, 16, 18). When the optimal EGF concentration (10 nM) was used, the peak increase was on the average from 221 ± 10 to 884 ± 97 nM (n = 7), and the plateau was maintained at 306 ± 10 nM (after 5 min, n = 9). The effect on membrane potential, on the other hand, consists of a hyperpolarization which evolves in parallel to the $[Ca^{2+}]_i$ increase (Fig. 1*C*, Ref. 21). With 10 nM EGF, the average hyperpolarization above resting (−22 ± 6 mV) was −36 ± 6 mV at the peak and −14 mV during the plateau, measured 5 min after application of the growth factor. All these effects of EGF were completely wiped off by a long term (24-h) pretreatment of the cells with a tyrosine kinase blocker, the tyrphostin RG50810 (60 μM, Ref. 31, not shown). Since this drug is an inhibitor of the tyrosine kinase indigenous to the receptor, this result confirms that the ionic effects of EGF are a consequence of the receptor enzyme activation (1–8, 31).

Ionic Effects of Charybdotoxin—In marked contrast to tyrphostin, the inhibitory action of the scorpion toxin, charybdotoxin, on the EGF-induced trasmembrane signals was not general. In EGFR-T17 fibroblasts, the $[Ca^{2+}]_i$ responses were not significantly modified by the toxin, both in their initial peak and also in the subsequent plateau phase (Fig. 1*B*; n = 10; $p > 0.1$ in either case). Moreover, the toxin alone had no effect on the resting membrane potential but markedly inhibited the hyperpolarization response to EGF. At low concentrations, the latter effect consisted essentially of a decrease of the response size with no major changes of the kinetics, while at suboptimal concentrations, the toxin was able to reveal a new effect of EGF, *i.e.* a depolarization appearing after the residual hyperpolarizing phase had subsided (Fig. 1*D*). At the highest toxin concentrations used (0.06–0.1 μM), hyperpolarization by EGF was almost completely blocked, and the size and kinetics of the depolarization became fully evident (Fig. 3*A*). Blockade of hyperpolarization developed within a few seconds and persisted apparently unchanged as long as the toxin was maintained in contact with the cells (incubations up to 24 h). These effects of charybdotoxin were not limited to EGFR T17 cells only since they were obtained also in another EGF target cell, the human epidermoid carcinoma line A431, which required, however, slightly higher concentrations (not shown).

Fig. 2 illustrates the concentration dependence of the charybdotoxin inhibition of the EGF-induced hyperpolarization in EGFR-T17 fibroblasts. IC_{50} was approximately 20 nM. Results parallel to those with the pure toxin were obtained with the crude scorpion venom, using, however, ≈20-fold greater protein concentrations. With A431 cells, the IC_{50} of pure charybdotoxin was around 30 mM (not shown).

The depolarization induced by EGF in EGFR-T17 cells pretreated with charybdotoxin was characterized in detail.

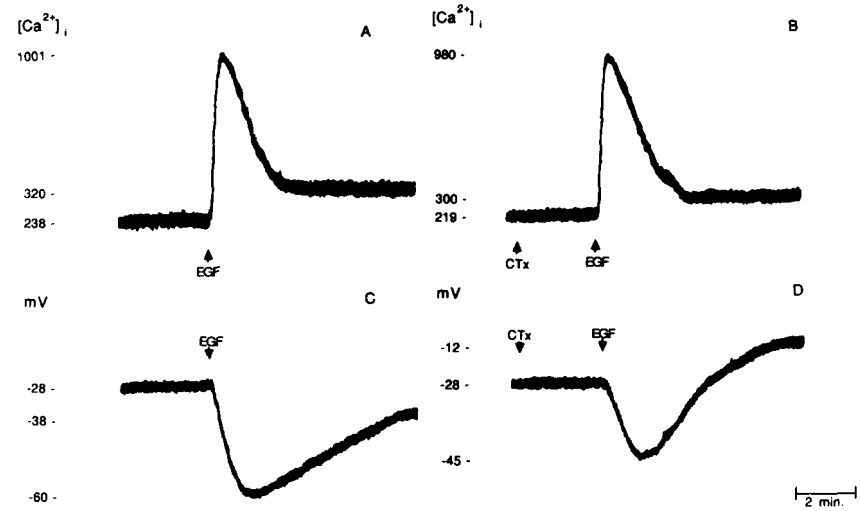

FIG. 1. **$[Ca^{2+}]_i$ increase (A and B) and plasma membrane hyperpolarization (C and D) induced by EGF in control and charybdotoxin-pretreated EGFR-T17 cells.** Administration of EGF (10 nM) and charybdotoxin (*CTx*, 30 nM) to cells loaded with fura-2 (*A* and *B*) and bis-oxonol (*C* and *D*) are marked by *arrows*. *Numbers* to the *left* of the *A–B* traces indicate calibration of $[Ca^{2+}]_i$ results, expressed in nanomolars. Membrane potential traces (*panels C* and *D*) are calibrated in millivolts.

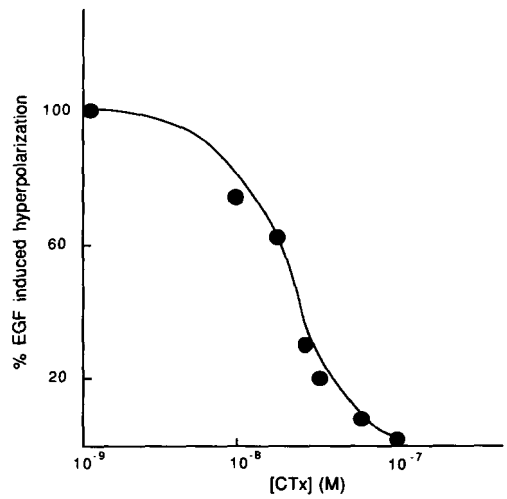

FIG. 2. **Charybdotoxin inhibition of the EGF-induced hyperpolarization of EGFR-T17 cells.** Values shown are averages of the peak values obtained in two to five experiments in which parallel samples of bis-oxonol-loaded cells with or without charybdotoxin pretreatment (concentrations on *abscissa*) were exposed to 10 nM EGF.

The concentration dependence of this effect resembled that of the $[Ca^{2+}]_i$ response. In fact, it was appreciable already at EGF concentrations ≈0.2 nM and maximal at 10 nM and above. Calibration of the fluorescence signal (21) revealed maximal depolarization to be on the average ≈15 mV. The time course of the voltage drop was slow (maximum reached in 1–2 min, over twice the time needed to complete the much larger hyperpolarization response). Once reached, however, the depolarized potential was maintained apparently indefinitely (see Fig. 3, A; longest experiment carried out: 1 h).

The membrane potential effects of EGF were compared also to those of ATP, an agent that in EGFR-T17 cells causes a shorter lived $[Ca^{2+}]_i$ increase (not shown). Also, the parallel hyperpolarization response induced by ATP was more transient than that by EGF (compare *trace C* in Fig. 3 to *C* in Fig. 1). Pretreatment with charybdotoxin inhibited the ATP-induced hyperpolarization and revealed a depolarization initially similar to that by EGF but markedly shorter lived (back to resting potential within 15 min, Fig. 3*B*). Experiments were also carried out by administering in sequence EGF and ATP to charybdotoxin-pretreated cells. *Trace D* in Fig. 3 shows the responses in cells pretreated with a suboptimal concentration (20 nM) of the toxin. ATP administered during the depolarization phase of the EGF response induced a transient hyperpolarization followed by return of the membrane potential to the depolarized values sustained by EGF.

Fig. 4 illustrates the cation dependence of the EGF-induced depolarization, investigated in charybdotoxin-poisoned EGFR-T17 cells. As can be seen, removal from the medium of either Ca^{2+} (by addition of excess EGTA) or Na^+ (replaced by the nonpermeant cation $choline^+$) failed to modify the response substantially (*traces A* and *B*) whereas replacement of Na^+ combined with Ca^{2+} chelation prevented the response (Fig. 4*C*). These results appear consistent with the involvement of a channel activated by EGF and permeable to both Na^+ and Ca^{2+} (32, 33). Indeed, when these two cations were used at submaximal concentration (15 and 1 mM, respectively), their combination in the medium yielded additive depolarization in charybdotoxin-pretreated cells exposed to EGF (Fig. 4*D*). In order to investigate the possible dependence of such a channel on $[Ca^{2+}]_i$, experiments were carried out with EGFR-T17 cells preloaded with high (millimolar) concentrations of the Ca^{2+} chelator quin-2. Such a treatment induced the almost complete blockade of the $[Ca^{2+}]_i$ response to EGF, whereas that induced by the Ca^{2+} ionophore ionomycin was still appreciable (Fig. 4*E*, *inset*). As far as the membrane potential responses, neither the hyperpolarization (21) nor the depolarization induced by the growth factor in charybdotoxin-pretreated cells remained visible in the quin-2 hyperloaded cells (Fig. 4*E*).

Ionic Effects of SC38249—The imidazole derivative, SC38249, and its analogue SKF96365, have recently been described (25, 26) as blockers of two types of Ca^{2+} channels: L-type channels operated by voltage and the cationic channels that open following the activation of receptors coupled to PIP_2 hydrolysis via G proteins (second messenger-operated Ca^{2+} channels, Refs. 12 and 33). The present experiments were carried out to investigate whether the cationic channels activated by EGF, responsible for the $[Ca^{2+}]_i$ plateau and the depolarization responses, are sensitive to SC38249 blockade. Since EGFR-T17 cells express no voltage-operated channels, an effect of SC38249 on these latter channels could be excluded in our experiments. An additional effect of the drug was, however, revealed in preliminary experiments, *i.e.* a transient rise of $[Ca^{2+}]_i$. Such an effect was due to release from intracellular stores because it was maintained in the Ca^{2+}-free medium (not shown). In order to exclude any major influence of this latter effect on the $[Ca^{2+}]_i$ responses induced

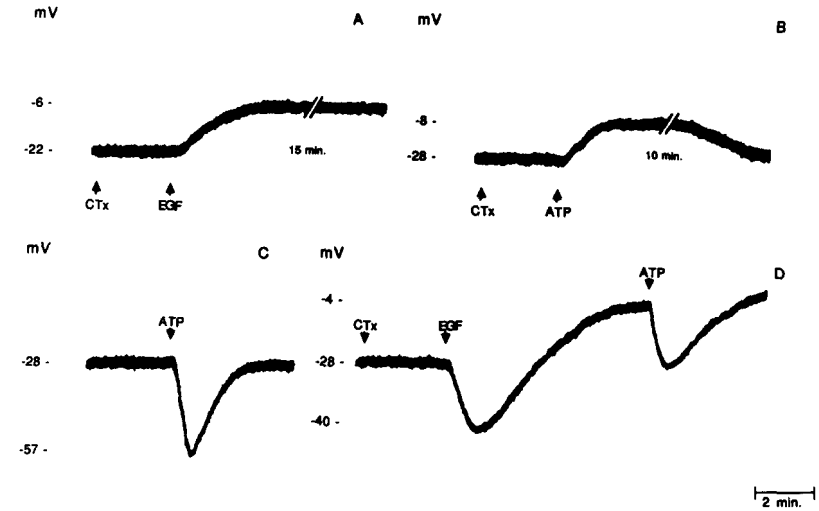

FIG. 3. **Changes of membrane potential induced by EGF and ATP in EGFR-T17 cells with and without charybdotoxin pretreatment.** EGF and ATP were administered at the final concentrations of 10 nM and 100 μM, respectively; charybdotoxin at 75 (*traces A* and *B*) and 20 (*trace D*) nM. At the interruptions shown in *A* and *B*, portions of the traces (corresponding to the time periods indicated) were cut out. Other details are as in Fig. 1, *C* and *D*.

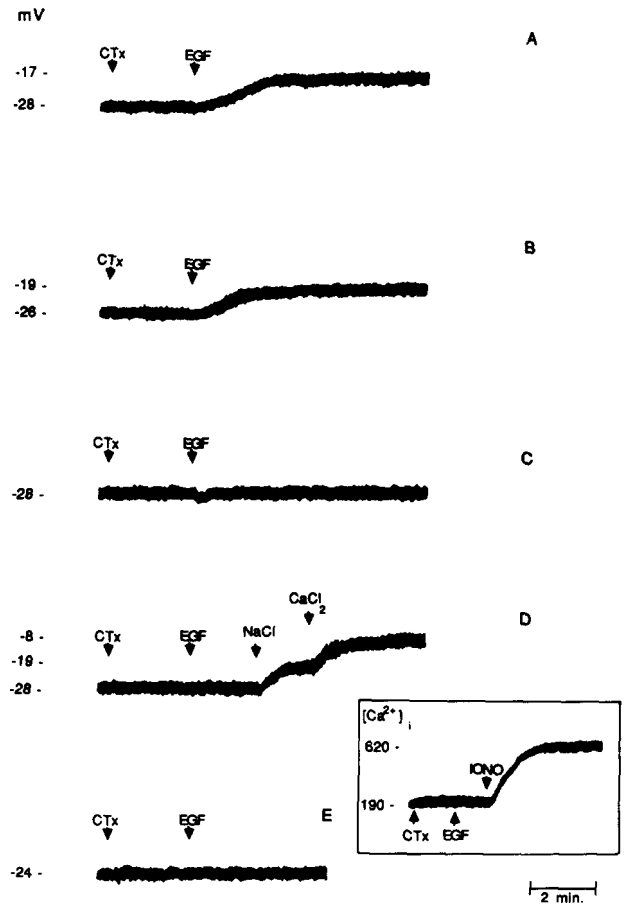

FIG. 4. **Cation dependency of the EGF-induced depolarization.** *Traces A–D*, bis-oxonol-loaded EGFR-T17 cells were treated with charybdotoxin (*CTx*, 70 nM) followed by EGF (10 nM) when incubated in: *A*, KRH incubation medium supplemented with excess (3 mM) EGTA; *B*, Na^+-free incubation medium; *C*, as in *B*, however, with excess EGTA added; *D*, as in *C*, however, with readmission of 15 mM NaCl and 1 mM $CaCl_2$ where indicated. In the experiment illustrated in *E*, the cells were preloaded with a high concentration of the fluorescent Ca^{2+} chelator, quin-2 (1.2 nmol/10^6 cells), before loading with bis-oxonol and treatment in the KRH incubation medium. The *inset* shows the lack of $[Ca^{2+}]_i$ response to EGF in quin-2 hyperloaded cells; ionomycin was added at the final concentration of 1 μM.

by EGF, in most experiments, the cells were first exposed to a concentration of SC38249 (10 μM) capable on its own of inducing only a small $[Ca^{2+}]_i$ increase (Fig. 5*C*). EGF was added 2 min later, after the drug-induced $[Ca^{2+}]_i$ rise had largely subsided. $[Ca^{2+}]_i$ results obtained by the application of EGF to EGFR-T17 cells pretreated with SC38249 are shown in Fig. 5*C*. Compared to the transients elicited by EGF in control cells (Fig. 5*A*), those after SC38249 pretreatment were distinctly shorter lived. Specifically, the initial peak phase was unchanged, whereas the subsequent plateau phase was almost completely abolished. Thus, the $[Ca^{2+}]_i$ responses to EGF in SC38249-pretreated cells closely resembled those observed in the cells tested after an excess of EGTA was added to chelate extracellular Ca^{2+} (Fig. 5, compare *B* and *C*). Results similar to those with EGFR-T17 were obtained using A431 cells. Taken together, these results clearly document an inhibition by the drug of the EGF-induced Ca^{2+} influx. This problem was further investigated in cells by measuring membrane potential changes. Administration of SC38249 induced an initial, small hyperpolarization (Fig. 6*A*), which in most cells subsided within 1–2 min, most likely the consequence (via activation of Ca^{2+}-dependent K^+ channels) of the Ca^{2+} release effect of the drug. This response was in fact inhibited by charybdotoxin (not shown). Pretreatment with the drug, on the other hand, failed to prevent the initial hyperpolarization by EGF (compare Fig. 6*A* with Fig. 1*C*), again in agreement with the $[Ca^{2+}]_i$ results (Fig. 5*C*). Finally, in the cells treated also with a high concentration of charybdotoxin to inhibit hyperpolarization, SC38249 prevented (when administered before, Fig. 6*B*) or gradually dissipated (when administered after, Fig. 6*C*) the depolarization induced by EGF (see Fig. 3*A*). Notice that the effect of SC38249 illustrated in Fig. 6*C* occurs in charybdotoxin-pretreated cells and is persistent. Thus, it cannot be due to the hyperpolarization induced by the drug because the latter is transient (Fig. 6, *A* and *B*) and is blocked by the toxin.

Effects of Prolonged Treatments with Charybdotoxin and SC38249—This series of experiments was carried out to investigate the possible effect of our two experimental tools on the stimulated EGFR-T17 cell proliferation. Since the protocol employed requires long term exposure of the cells to the blockers, with or without EGF, it was first important to establish whether or not these treatments induce unspecific toxicity or other effects incompatible with our experimental aims. Neither charybdotoxin (and even the crude *Leiurus quinquestriatus hebraeus* scorpion venom) nor SC38249, at the concentrations used (75 nM and 10 μM, respectively),

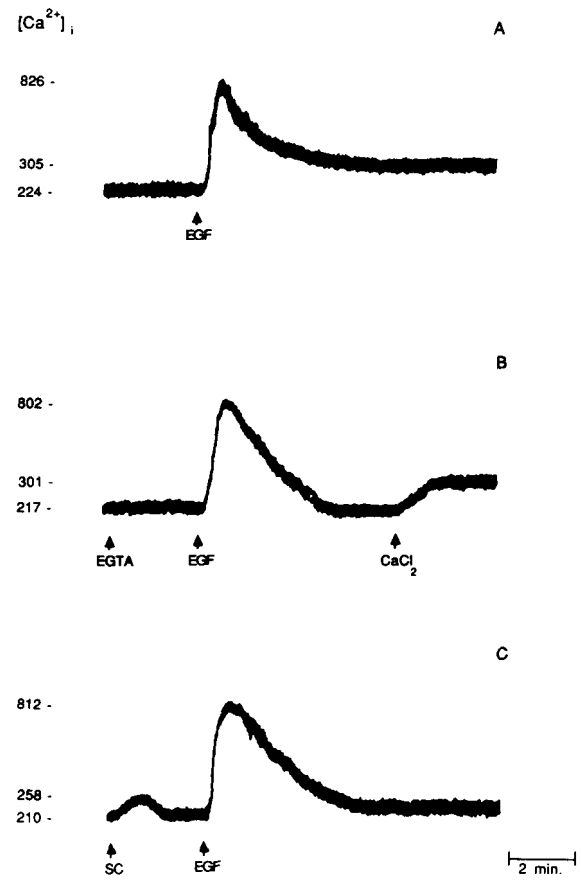

FIG. 5. **EGF-induced $[Ca^{2+}]_i$ increase in EGFR-T17 cells: effect of pretreatment with either excess EGTA or SC38249.** EGTA, SC38249 (*SC*), and EGF were administered where indicated at 3 mM, 10 μM, and 10 nM final concentration, respectively. In *B*, Ca^{2+} was reintroduced into the EGTA-containing medium (2 mM $CaCl_2$ added, corresponding to ≃1 mM free Ca^{2+}).

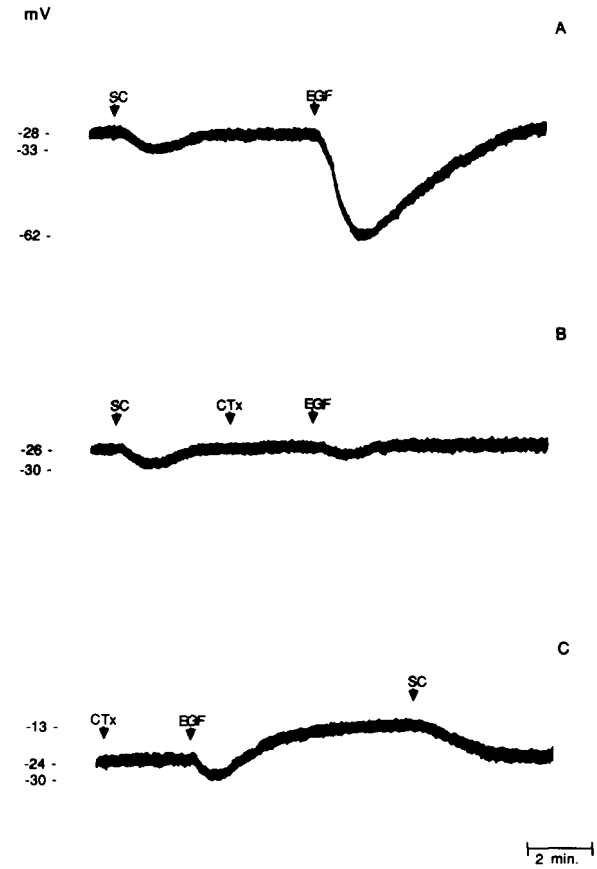

FIG. 6. **Effects of SC38249 on the membrane voltage changes induced by EGF in EGFR-T17 cells with and without charybdotoxin pretreatment.** In *A*, SC38249 (*SC*, 10 μM) is shown to induce a small, transient hyperpolarization on its own and to shorten the hyperpolarization induced by a subsequent administration of EGF (10 μM). In *B*, the drug prevents and in *C* relieves the depolarization triggered by EGF (10 μM) in charybdotoxin (*CTx*, 60 nM)-pretreated cells (compare also with Figs. 3 and 4).

increased cytotoxicity in the cultures, as revealed by both the vital dye test with trypan blue (not shown) and the direct counting of cells cultured in 1% fetal calf serum ± either blocking agent (Tables I and II). Moreover, cell monolayers cultured for 24 h with either the toxin or the drug exhibited unchanged EGF binding (not shown). Cells from these monolayers, dissociated in the presence of the blockers and then washed for 30 min with fresh medium, were found to respond normally to EGF in terms of both $[Ca^{2+}]_i$ and membrane potential (not shown). In contrast, consistent effects of the two agents were observed when the cells were stimulated to proliferate. Treatment with charybdotoxin induced a moderate (−20%) but highly reproducible inhibition of the mitogenic effect of 10 nM EGF, whereas the effect of 10% serum remained unchanged (Table I). A much larger inhibition (−60%) was observed in the cells stimulated with EGF in the presence of SC38249, and the latter drug was even more effective (−80%) in the cells stimulated with high serum (Table II).

TABLE I

Effect of charybdotoxin (CTx) on EGF- and serum-stimulated EGFR-T17 cell growth

Values shown are averages of six experiments ± S.D. EGFR-T17 cell monolayers were exposed to the indicated treatments for 2 days, then dissociated and counted.

Addition	Cell number
FCS, 1%	274,813 ± 17,768
FCS, 10%	729,346 ± 17,205
EGF, 10 nM	749,806 ± 24,185
FCS, 1% + CTx, 75 nM	265,683 ± 20,671
FCS, 10% + CTx, 75 nM	729,356 ± 26,711
EGF, 10 nM + CTx 75, nM	643,203 ± 25,782

TABLE II

Effect of SC38249 on EGF- and serum-stimulated EGFR-T17 cell growth

Values shown are averages of six experiments ± S.D. EGFR-T17 cell monolayers were exposed to the indicated treatments for 2 days, then dissociated and counted.

Addition	Cell number
FCS, 1%	274,290 ± 5,925
FCS, 10%	708,930 ± 33,191
EGF, 10 nM	781,800 ± 20,336
FCS, 1% + SC38249, 10 μM	232,620 ± 990
FCS, 10% + SC38249, 10 μM	308,710 ± 3,040
EGF, 10 nM + SC38249, 10 μM	432,120 ± 20,364

DISCUSSION

The results that we have reported characterize the ionic responses triggered in target cells by the administration of EGF. Hyperpolarization had already been described in our previous studies (21) and shown to be the consequence of the $[Ca^{2+}]_i$ changes induced by the growth factor. The latter are due initially (first 1–2 min) to the release of Ca^{2+} from intracellular stores; later, to the stimulation of Ca^{2+} influx and the establishment of rythmic $[Ca^{2+}]_i$ fluctuations which

can persist for long periods of time (tens of minutes) after the application of the growth factor (21). In these previous studies, we had identified Ca^{2+}-dependent K^+ channels as responsible for hyperpolarization. At the time, however, we were unable to adequately block this response which proved to be insensitive to apamine and inhibitable by tetraethylammonium and quinidine only at high concentrations (10^{-1} and 10^{-3}M, respectively) (21). Since prolonged treatments with these high blocker concentrations induced unspecific cell lesions, their use in cell proliferation experiments was precluded. We have now found that the hyperpolarization response after EGF can be selectively and profoundly inhibited by charybdotoxin (a known blocker of the high conductance, Ca^{2+}-dependent K^+ channels (19–21), purified from the *Leiurus quinquestriatus hebraeus* scorpion venom) with no apparent sign of cell toxicity or effect on EGF receptor functioning. Charybdotoxin also revealed an effect of EGF, a depolarization response, which had not yet been identified. Without the toxin, this effect remains in fact masked by the large, K^+-dependent increase in membrane potential. By the use of modified media, the EGF-induced depolarization was shown to be sustained by both Na^+ and Ca^{2+}. This is a property typical of unspecific cation channels which turn on in response to the activation of receptors coupled to PIP_2 hydrolysis (12, 32–35). Most of the receptors competent for the activation of these channels are believed to operate via the interaction with G protein(s) (12, 32). Together with a recently reported patch clamp study in A431 cells (36), the present results demonstrate that this property also concerns a growth factor receptor, which is structurally very dissimilar from its G protein-operated counterparts and is believed to activate PIP_2 hydrolysis by a direct, tyrosine phosphorylation of phospholipase Cγ (3–8). These considerations appear consistent with the possibility discussed elsewhere (12, 32–36) that these channels are activated by messenger(s) generated following PIP_2 hydrolysis (second messenger-operated channels, Refs. 12 and 32). As far as the nature of the second messenger, the lack of depolarization observed in cells overloaded with the fluorescent Ca^{2+} chelater, quin-2, to quench the response depending on Ca^{2+} release from intracellular stores (see Ref. 37), suggests an involvement of the initial $[Ca^{2+}]_i$ peak increase induced by EGF. A similar conclusion was obtained previously in leukocytes exposed to the chemotactic peptide, Fmet-Leu-Phe, investigated by patch-clamping (38). In a variety of other cell types, however, a $[Ca^{2+}]_i$ increase appears not to be needed for channel opening. The result that we have observed could thus be a property of NIH-3T3 cells and not necessarily a general property of the channels sensitive to EGF when expressed by other cell types.

Because of their permeability to Ca^{2+}, the second messenger-operated channels discussed above are likely to be responsible for Ca^{2+} influx, an effect of EGF recognized already in 1981 (39) and believed until recently to be the only channel activity induced by the growth factor (20). Such a conclusion is supported, on the one hand, by the results of our $[Ca^{2+}]_i$ experiments which revealed Ca^{2+} influx occurring in parallel to depolarization; on the other hand, by the fact that both Ca^{2+} influx and depolarization were blocked by the imidazole derivative, SC38249. The latter drug and its analogue SKF96365 have been shown recently to be blockers of both second messenger- and voltage-operated Ca^{2+} channels (25, 26). In a few cell types, these drugs are known to stimulate also Ca^{2+} release from intracellular stores (25). SC38249 is therefore far from being a highly specific drug. In our cells, however, voltage-operated channels are not expressed[2] and therefore cannot be responsible for the results we have obtained. Although the lowest SC38249 concentration inducing marked channel inhibition (10 μM) was still able to stimulate intracellular Ca^{2+} release, this latter effect was relatively small and transient, apparently inadequate to seriously affect general Ca^{2+} homeostasis. Even prolonged (2-day) treatment with SC38249, at the concentration specified above, failed to induce frank signs of cell toxicity. Moreover, when long term treated cells were washed free of the drug, they quickly recovered normal responses to EGF, both in terms of $[Ca^{2+}]_i$ and membrane potential (with and without charybdotoxin). Based on these findings, we believe that SC38249, although not a specific second messenger-operated channel blocker, is an appropriate tool to investigate the possible role of the prolonged increase of $[Ca^{2+}]_i$ and of the ensuing processes, including hyperpolarization, in the cell proliferation response induced by EGF in EGFR-T17 cells.

The possibility that at least some of the events elicited by PIP_2 hydrolysis, including the increase of $[Ca^{2+}]_i$, cooperate in growth stimulation by EGF and other factors had already been considered. This hypothesis was based primarily on the fact that other receptors coupled to PIP_2 hydrolysis (however via G protein interaction), such as the angiotensin II, bombesin, and even some serotonin and muscarinic receptors, induce cell growth on their own (12–16, 40, 41) or, even better, in combination with typical growth factor receptors (41–43). So far, however, direct cell proliferation experiments in which individual events initiated by PIP_2 hydrolysis were inhibited had never been reported. The results that we have obtained document for the first time that both the stimulation of cation influx induced by EGF and one of the ensuing events, hyperpolarization, play a role in the mitogenic action of the growth factor. At the moment, no information is available about the mechanisms by which these processes could be involved in growth regulation. Hyperpolarization can be hypothesized to facilitate the transport of ions and metabolites across the membrane while the persistent (and possibly oscillatory, Ref. 21) increase of $[Ca^{2+}]_i$ resulting from stimulated influx could contribute more directly to the cascade of intracellular processes (such as activation of enzymes, especially protein kinases, expression of oncogenes, and activation of their products) interposed between cell stimulation and DNA duplication. Because of their limited specificity, the role of the processes that we have investigated is expected to be mainly helper, or at the most permissive, with respect to that of other events also activated by the growth factor. Future, more specific studies might ultimately clarify the sites and mechanisms of these hypothesized interactions among intracellular stimulatory events.

Acknowledgments—The kind gift of tyrphostin RG50810 (from A. Levitzki) and SC38249 (from U. T. Ruegg) is gratefully acknowledged. We thank S. Monti and L. Di Giorgio for secretarial assistance.

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