Epidermal Growth Factor-activated Calcium and Potassium Channels*

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The earliest responses to activation of the epidermal growth factor (EGF) receptor include a transient increase in calcium influx and a transient membrane hyperpolarization. The underlying mechanisms are, however, not well understood as yet. In the present study, we have applied patch clamp recording in the cell-attached and the outside-out mode, and fluorimetric cytosolic Ca²⁺ determinations, to identify the nature of the ion channels involved, to characterize their properties at the level of single channels, and to unravel their mechanism of activation. We provide evidence that activation of the EGF receptor results initially in the activation of voltage-independent Ca²⁺ channels that can be defined as direct receptor-operated channels. This in turn causes the activation of Ca²⁺-dependent K⁺ channels, which results in a (delayed) membrane hyperpolarization and then leads to the activation of a second class of Ca²⁺ channels that are sensitive to hyperpolarization. An autocatalytic generation of further hyperpolarization and Ca²⁺ influx is the predicted outcome of this ionic cascade. Based on the observed inhibitory effects of protein kinase C activity on the Ca²⁺ influx (9), we propose that protein kinase C is involved in the negative regulation of this cascade, which explains the transient nature of these responses.

Epidermal growth factor (EGF) is an important regulator of cell proliferation in a variety of cell types. The human A431 carcinoma cell line has been widely used as a model system for the characterization of the structure and function of the EGF-R, since these cells express an unusually high number of these receptors (1). The human EGF-R is a 170-kDa plasma membrane-spanning glycoprotein. Characteristically, its cytoplasmic domain contains a tyrosine kinase moiety (2). It is now generally believed that binding of EGF to its receptor results in enhanced receptor-receptor affinity, thereby causing receptor dimerization. In turn, this initiates the activation of the EGF-R tyrosine kinase (3, 4), which is essential for further signal transduction (5, 6). Most cells, including A431 cells, express at least two classes of EGF-R based on the determination of EGF binding affinity. Recent studies, in which monoclonal antibodies that can interfere specifically with the binding of EGF to either the high or low affinity class were used, have shown that the minor class of high affinity EGF-R (5-10% in A431 cells) is primarily involved in signal transduction (7).

The earliest cellular responses to the activation of the EGF-R include a number of ionic changes, such as a transient plasma membrane hyperpolarization and increase in [Ca²⁺]i, and a sustained rise in pH (8-10). Some of these responses have been linked to the hydrolysis of phospholipids (14) by phospholipase C, which is probably activated as a direct consequence of phosphorylation of tyrosines residues by the intrinsic EGF-R tyrosine kinase (11). As a result, the second messengers 1,2-diacylglycerol and Ins(1,4,5)P³ are released. 1,2-Diacylglycerol activates protein kinase C (12) that in turn is able to phosphorylate the Na⁺/H⁺ exchanger (13), causing a stimulation of this antipporter and thus a rise in pH (14). It has been well established that Ins(1,4,5)P³ production is responsible for the release of Ca²⁺ from intracellular stores (15).

In A431 cells, the Ca²⁺ release constitutes only a minor component of the total increase in [Ca²⁺]i, since it has been shown that the major part originates from EGF-induced Ca²⁺ influx (8, 9, 16-18). In accordance, EGF causes only a minor production of Ins(1,4,5)P³ in these cells (19). This is in contrast with the effect of other agonists, like bradykinin. Addition of bradykinin to A431 cells results in rapid elevation of Ins(1,4,5)P³ levels and subsequent release of Ca²⁺ from intracellular stores (20). As yet, no convincing clues as to the mechanisms responsible for the initiation of Ca²⁺ influx have been provided, EGF does not employ depolarization-activated Ca²⁺ channels to raise [Ca²⁺]i, because depolarization of A431 cells does not induce Ca²⁺ influx. Furthermore, the EGF-induced Ca²⁺ signal is not affected by nifedipine, a potent antagonist of depolarization-activated Ca²⁺ channels (9). The nature of the Ca²⁺ channel responsible for the EGF-induced Ca²⁺ influx has thus still to be established. In this context, it is of interest to note that of all cellular responses to EGF checked for, Ca²⁺ influx is the only one that is impaired in A431 cells when EGF binding to the major class of low affinity receptors is prevented by pretreatment of the cells with monoclonal antibody 2E9 (7).

Also, the mechanism underlying the observed hyperpolarization is not well understood. A431 cells hyperpolarize transiently upon addition of EGF from their normal Vm of −65 mV to −85 mV (8, 21). It was shown that this hyperpolarization was carried by K⁺ ions. Since it occurs concomitantly with the increase in [Ca²⁺], and can be mimicked by raising [Ca²⁺], it has been assumed that Ca²⁺-activated K⁺ channels are responsible for this response (8, 21).

In the present study, we have applied patch clamp recording and fluorometric Ca²⁺ measurements on A431 cells to study the regulation of Ca²⁺ influx and hyperpolarization at the level of single ion channels. We show that activation of the EGF-R triggers an autocatalytic ionic cascade, in which a
voltage-independent activation of Ca\textsuperscript{2+} channels is the primary response.

The resulting Ca\textsuperscript{2+} influx causes increased activity of (Ca\textsuperscript{2+}-dependent) K\textsuperscript{+} channels, thus leading to a hyperpolarization which in turn results in further Ca\textsuperscript{2+} influx due to activation of hypopolarization-dependent Ca\textsuperscript{2+} channels. In addition, we provide evidence that protein kinase C activation serves as a negative feedback mechanism by a specific decreasing effect on K\textsuperscript{+} channel activity. This could explain the observed transient nature of the EGF-induced hyperpolarization and Ca\textsuperscript{2+} influx.

**EXPERIMENTAL PROCEDURES**

**Materials**—EGF was purchased from Collaborative Research. Bradykinin was obtained from Sigma. In all experiments, the final concentrations of EGF and bradykinin were 100 ng/ml and 1 \( \mu \text{M} \), respectively. These concentrations of EGF and bradykinin elicit maximum responses concerning both hyperpolarization and Ca\textsuperscript{2+} influx (e.g. Refs. 8 and 19).

**Cell Culture**—Human carcinoma A431 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal calf serum and buffered with 40 mM NaHCO\textsubscript{3} under a 7.5% CO\textsubscript{2} atmosphere. For patch clamp recordings in the cell-attached configuration, the cells were grown to 50–90% confluency on 35-mm dishes (Costar). Cells were switched to Dulbecco's modified Eagle's medium without fetal calf serum 4 h before the onset of experiments. Composition of the external medium was (in mmol/liter) NaCl, 140; KCl, 5; CaCl\textsubscript{2}, 2; MgCl\textsubscript{2}, 1; glucose, 10; HEPES, 10; adjusted to pH 7.4 with NaOH at 33 °C.

For experiments with the outside-out configuration of the patch clamp technique, other bath and pipette solutions were used. The external medium (Table I) was used for these channels (25–26). Composition of the pipette pipette solution was (in mmol/liter) BaCl\textsubscript{2}, 96; HEPES, 10; adjusted to pH 7.4 with KOH (approximately 6 mmol/liter needed) at 33 °C.

**Electrical Recording**—For the patch clamp analysis (22) of calcium channels in the cell-attached configuration, Ba\textsuperscript{2+} was used as charge carrier to improve signal resolution, since this ion is a better permeant for A431 cells, not exposed to EGF, reveal multiple channel openings. Their nature can be identified on the basis of their reversal potentials and on the effects of specific channel blockers. For studies of Ca\textsuperscript{2+} channels, Ba\textsuperscript{2+} was used routinely as a charge carrier in the pipette solution. Under these conditions, two types of ion channels were detectable (Figs. 1 and 2).

The predominant channel was identified as a K\textsuperscript{+} conducting channel on the basis of its reversal potential (85.6 mV) (Table I), the theoretical reversal potential being 86.4 mV in our configuration, as calculated from the Nernst equation (intracellular [K\textsuperscript{+}] = 166 mM, (9), pipette [K\textsuperscript{+}] = 6 mM). Furthermore, the reversal potential of this channel could be shifted to more positive values by adding K\textsuperscript{+}-aspartate or KCl to the pipette medium (Table I). The K\textsuperscript{+} channel showed burst-like gating kinetics (Fig. 2, d and f), its conductance was 18 pS.

The minor class of ion channels represented Ca\textsuperscript{2+} conducting channels, their conductance being 1–3 pS. We determined a reversal potential of 162 mV (Table I) for these channels, which is close to its theoretical reversal potential of 178 mV ([Ca\textsuperscript{2+}]\textsubscript{e} = 200 nM, pipette [Ba\textsuperscript{2+}] = 96 mM). These currents disappeared completely from our recordings when 10 mM Co\textsuperscript{2+} (a potent inhibitor of Ca\textsuperscript{2+} currents) was added to the pipette solution (Fig. 2b). The gating kinetics of this Ca\textsuperscript{2+} channel are shown in Fig. 2, c and e.

**K\textsuperscript{+} and Ca\textsuperscript{2+} Channel Activity Is Dependent on Membrane Potential**—The membrane potential is a common denominator of the activity of many types of ion channels. Under our conditions, the \( V_{m} \) of A431 cells averaged −65 mV. An imposed depolarization of the patch significantly increased the \( P_{\text{open}} \) of the K\textsuperscript{+} channels (Fig. 3b). This property of the K\textsuperscript{+} channel provides these cells with an efficient \( V_{m} \) stabilizing mechanism. The Ca\textsuperscript{2+} channels could not be activated by depolarizing the patch. On the contrary, it appeared that depolarization abolished completely all Ca\textsuperscript{2+} openings. However, at hyperpolarizing patch potentials, ranging from −70 mV to −85 mV, the \( P_{\text{open}} \) of the Ca\textsuperscript{2+} channel was markedly increased. This increase was due to (on average) shorter time intervals between successive Ca\textsuperscript{2+} channel openings. The duration of the openings stayed the same. When the patch was hyperpolarized to −90 mV or even more negative values, the \( P_{\text{open}} \) decreased again. The \( V_{m} \) dependence of the Ca\textsuperscript{2+} channel is shown in Fig. 3a. These results demonstrate that a hyperpolarization of A431 cells will result in an activation of Ca\textsuperscript{2+} channels, while depolarization inhibits Ca\textsuperscript{2+} channels.

**EGF Induces Increased Activity of Both Ca\textsuperscript{2+} Channels and K\textsuperscript{+} Channels**—Earlier studies have shown that adding EGF to A431 cells results in a hyperpolarization (8) as well as a Ca\textsuperscript{2+} influx (e.g. Refs. 9 and 29). Our measurements confirm these observations at the level of single ion channels. The bath application of EGF increased the \( P_{\text{open}} \) of both K\textsuperscript{+} and

**RESULTS**

**Characterization of K\textsuperscript{+} and Ca\textsuperscript{2+} Ion Channels**—Cell-attached patch clamp recordings on A431 cells, not exposed to EGF, reveal multiple channel openings. Their nature can be identified on the basis of their reversal potentials and on the effects of specific channel blockers. For studies of Ca\textsuperscript{2+} channels, Ca\textsuperscript{2+} was used routinely as a charge carrier in the pipette solution. Under these conditions, two types of ion channels were detectable (Figs. 1 and 2).

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**Fig. 1.** Cell-attached single channel currents from A431 cells. The membrane potential ranges from −65 to +40 mV (shown at the left of the trace). At −65 mV, unitary inward Ba\textsuperscript{2+} currents are observed. At the other membrane potentials, predominantly unitary outward K\textsuperscript{+} currents are seen. Closed and open states are indicated at the top right.
**EGF-controlled Ion Channels**

**Fig. 2.** The characteristics of the single channel currents. a, amplitude distributions of A431 cell-attached patches at several holding potentials. The Ca²⁺ and K⁺ channels are indicated by the black lines. Bin width, 0.075 pA. b, the amplitude distributions with 10 mM Co²⁺ in the pipette medium show complete inhibition of the Ca²⁺ channel, with only minor effects on K⁺ currents. Bin width, 0.04 pA. c, distribution of open times of the Ca²⁺ channel. Bin width, 1 ms; time constant, 0.9 ms. d, open times distribution (bin width, 1 ms) of the K⁺ channel, open time constant, 5.8 ms. e, distribution of closed times of the Ca²⁺ channel (bin width, 50 ms), fitted by one exponent. Time constant, 260 ms. f, the closed times distribution (bin width, 10 ms) of the K⁺ channel is fitted by 2 exponents, the fast component has a time constant of 12 ms, time constant of the slow component is 90 ms.

Ba²⁺ unitary currents in 29 out of 30 patches (Fig. 4a, Table I). A lag period of variable duration (10–100 s) before the onset of these effects was observed consistently. After this lag period, the p_open of the K⁺ channels increased 2–8-fold. Unitary Ba²⁺ currents showed a 1.5–10-fold increase in p_open. The p_open of both channels decreased again after 30–60 s and returned eventually to initial values (Fig. 4a). EGF did not affect the amplitude of the unitary K⁺ or Ba²⁺ currents (data not shown).

The effect of EGF on the p_open of the Ca²⁺ channels could be attributed mainly to a marked increase in the number of openings that showed a 2–10-fold increase per 10-s period at maximum EGF effect. The EGF-induced increase in the p_open of the K⁺ channels was, however, of a more complex nature. Firstly, EGF induced a (on average) longer duration of K⁺ channel openings (Table I). Secondly, EGF increased the time that K⁺ channels are bursting (we noted that the bursts lasted approximately 3 times longer). No effect on the time intervals between two successive K⁺ channel openings or bursts could be detected.

The increased p_open of the K⁺ and Ca²⁺ channel evidently reflect the earlier observed EGF-induced hyperpolarization and Ca²⁺ influx, respectively. Subsequent experiments were carried out to establish the possible cause-effect relationships.
EGF-controlled Ion Channels

**Table 1**

Summary of the channel characteristics, obtained in the cell-attached configuration

|                        | K+ Channel                  | Ca2+ Channel               |
|------------------------|-----------------------------|----------------------------|
| **General channel characteristics, average ± S.E., n = 21** |                            |                            |
| Conductance            | 18 ± 1.1 pS                 | 2.8 ± 0.35 pS              |
| Reversal potential     | −85 ± 0.26 mV               | 160 ± 5.0 mV               |
| 𝜏, open                | 5.7 ± 0.27 ms               | 2.2 ± 0.30 ms              |
| 𝜏, closed, fast        | 17 ± 3.2 ms                 | 400 ± 66 ms                |
| 𝜏, closed, slow        | 190 ± 37 ms                 |                            |

**Effects of 56 mM potassium (pipette medium) on channel characteristics, n = 7**

|                        | K+ Channel                  | Ca2+ Channel               |
|------------------------|-----------------------------|----------------------------|
| Conductance            | 15 ± 2.6 pS                 | 2.0 ± 0.33 pS              |
| Reversal potential     | −52 ± 3.7 mV               | 170 ± 26 mV               |
| 𝜏, open                | 10 ± 3.0 ms                 | 1.4 ± 0.04 ms              |
| 𝜏, closed, fast        | 17 ± 2.8 ms                 | 200 ± 130 ms               |
| 𝜏, closed, slow        | 340 ± 64 ms                 |                            |

**Effects of 10 mM cobalt (pipette medium) on channel characteristics, n = 6**

|                        | K+ Channel                  | Ca2+ Channel               |
|------------------------|-----------------------------|----------------------------|
| Conductance            | 13 ± 2.2 pS                 |                            |
| Reversal potential     | −75 ± 14 mV                |                            |
| 𝜏, open                | 11 ± 3.5 ms                 |                            |
| 𝜏, closed, fast        | 13 ± 2.8 ms                 |                            |
| 𝜏, closed, slow        | 140 ± 76 ms                 |                            |

**Effects of EGF on channel parameters, n = 30**

|                              |                         |                          |
|------------------------------|-------------------------|--------------------------|
| **Open time probability:**    |                         |                          |
| Before EGF                   | 0.042 ± 0.0087          | 0.0094 ± 0.0010          |
| After (maximal effect)       | 0.18 ± 0.04             | 0.0080 ± 0.0016          |
| 𝜏, open                      | 9.54 ± 2.6              | 2.5 ± 0.61 ms            |

**Effects of TPA on channel open time probability, n = 8**

|                              |                         |                          |
|------------------------------|-------------------------|--------------------------|
| Before TPA                   | 0.12 ± 0.07             | 0.0012 ± 0.00054         |
| After (maximal effect)       | 0.034 ± 0.014           | 0.0013 ± 0.00075         |

**Fig. 3.** Membrane potential dependency of the Ca2+ and the K+ channel. a, 𝑝_\text{open} of the Ca2+ channel as a function of the holding potential. b, probability of K+ channel opening with respect to the holding potential. (Values are corrected for V_m).

**Fig. 4.** Action of EGF and Ca2+ on ion channel activity. a, EGF (applied at the dotted line) increases 𝑝_\text{open} of both K+ and Ca2+ channels. b, the EGF-induced activation of K+ channels can be mimicked by raising [Ca2+]. Application of 10 mM Ca2+ (dotted line) results in a transient increase of K+ channel activity.

**Fig. 5.** EGF-induced K+ channel activity is dependent on [Ca2+]. Elimination of extracellular Ca2+ (EGTA applied at the dotted line) has little effect on K+ channel activity. Subsequent addition of EGF (dotted line) does not result in an effect on K+ channel 𝑝_\text{open} until normal extracellular Ca2+ levels are restored (dotted line) after which a response to EGF is still present.

EGF-induced K+ Channel Activity Is Dependent on [Ca2+].—It has been suggested that the EGF-induced hyperpolarization results from Ca2+-activated K+ channels (8, 21). To test this hypothesis, we determined whether the K+ channels could be activated by an increased [Ca2+], in the absence of EGF. To this end, we elevated [Ca2+], by a stepwise increase of the extracellular Ca2+ concentration to 10–20 mM, or, alternatively, by adding bradykinin to the cells. Both stimuli transiently raised [Ca2+], as was confirmed in fluorimetric studies. Both conditions resulted in an approximately 10-fold increase of the 𝑝_\text{open} of K+ channels (Fig. 4b), demonstrating that the EGF-induced hyperpolarization can indeed be caused by Ca2+-activated K+ channels.

Our experimental set-up enabled us to see whether the activation of K+ channels is solely mediated by the rise in [Ca2+]i or whether other factors are involved. To this end, the effects of adding EGTA (3 mM) to the bath medium were studied. EGTA was applied after formation of the gigaseal. The addition of EGTA did not produce any effect on our recordings (Fig. 5). Importantly, subsequent addition of EGF did not activate K+ channels unless the normal extracellular Ca2+ concentration was restored. After such a restoration, a response to EGF was still present (n = 6; Fig. 5). The response to bradykinin, which is not influx-dependent, is not affected by extracellular EGTA (n = 8). In control experiments, no significant effect on the 𝑝_\text{open} of the K+ channels was noted when EGTA was added nor when, subsequently, the original extracellular Ca2+ levels were restored (data not shown). These experiments show that the increase in the 𝑝_\text{open} of the
K⁺ channels induced by EGF is completely mediated by an elevation of [Ca²⁺].

EGF-induced Ca²⁺ influx is dependent on hyperpolarization—A prediction from the observed inhibitory effects of an imposed depolarization on Ca²⁺ channel activity would be that such a depolarization is able to inhibit the EGF-induced Ca²⁺ influx in A431 cells. That this is indeed the case was confirmed by comparing the effect of EGF on [Ca²⁺]ᵢ in normal bath medium versus a depolarizing bath medium (Fig. 6a). Under depolarizing conditions, the amplitude of the EGF-induced elevation of [Ca²⁺]ᵢ was greatly reduced. Moreover, the time course of the EGF effect became markedly slower. This corresponded with our electrophysiological measurements (Fig. 6b) under these conditions. Addition of EGF did still induce a higher p_open of the (Ca²⁺-activated) K⁺ channels (1.5–3-fold, n = 6), but with a significantly increased lag time (range 200–300 s versus 10–100 s in normal medium). The absence of a hyperpolarization did apparently block [Ca²⁺]ᵢ influx partially. These results indicate that most of the EGF-induced Ca²⁺ influx is dependent on a hyperpolarization.

EGF activates initially voltage-independent Ca²⁺ channels—the interdependent activation of Ca²⁺-dependent K⁺ channels and hyperpolarization-dependent Ca²⁺ channels in response to EGF provides an attractive autocatalytic mechanism to explain the generation of the observed hyperpolarization and Ca²⁺ influx; yet leaves the mechanism of initiation of these events unresolved. To obtain insight into this mechanism, we have applied the patch clamp method in the outside-out configuration. In this configuration, recordings are made from an isolated patch of plasma membrane of which the original outside is exposed to the bathing medium. This abolishes the possible effects of second messengers to a large extent, and thus allows the detection of possible receptor-operated ion channels (30). It appeared that bath application of EGF caused the immediate activation of a novel 10-pS conductance that could be identified as a Ca²⁺ channel. The observed reversal potential was 33 mV, as is its theoretical reversal potential (pipette [Ca²⁺] = 25 mM, bath [Ca²⁺] = 2 mM). Subsequent addition of 25 mM CaCl₂ to the bath solution shifted the reversal potential to 0 mV (Fig. 7), as can be predicted from the Nernst equation. This Ca²⁺ channel showed no Vₛ-dependent behavior. This type of ion channel could not be detected in the cell-attached recordings after bath application of EGF and may thus represent an EGF-R-operated Ca²⁺ channel. These findings indicate that EGF-R activation results initially in the induction of Vₛ-independent receptor-operated Ca²⁺ channels. As a consequence, the activity of Ca²⁺-dependent K⁺ channels will increase, which will lead to a hyperpolarization. This in turn will act on hyperpolarization-activated Ca²⁺ channels, and a further autocatalytic Ca²⁺ influx and hyperpolarization will result.

Involvement of EGF-R tyrosine kinase and protein kinase C—In the experiments in the outside-out configuration of the patch clamp technique, it was observed that EGF regulates Ca²⁺ channel activity in the absence of Mg²⁺ and ATP in the pipette solution. This might indicate that the regulation of ion channel activity occurs in the absence of phosphorylation. To establish further the relationship between EGF-R activation and the increases in ion channel activity, we have determined the effect of the EGF-R tyrosine kinase inhibitor tyrphostin AG 213 (a kind gift of Dr. A. Levitski, Hebrew University, Jerusalem, Israel). Tyrphostin AG 213 (100 μM) antagonizes specifically tyrosine kinase activity of the EGF-R, but does not influence receptor dimerization (4). Preincubation of the A431 cells for 24 h with this inhibitor completely blocked all responses of EGF with respect to K⁺ and Ca²⁺ channel activity. This demonstrates that the ion fluxes of EGF are mediated by the tyrosine kinase of the EGF-R. Apparently, in the process of making outside-out patches, sufficient Mg²⁺ and ATP remains associated with the patch membrane to sustain the kinase reactions involved.

The transient character of the EGF-induced hyperpolarization and Ca²⁺ influx requires the existence of an efficient negative feedback mechanism. Activation of protein kinase C is a possible candidate to exert such an effect, since such activation by phorbol esters is known to inhibit various responses to EGF, including the rise in [Ca²⁺], and the hyperpolarization (8, 9, 16, 17). For that reason, we investigated the influence of 100 ng/ml TPA on the properties of the ion channels present in our preparation. TPA did decrease the p_open of the K⁺ channels, without affecting the Ca²⁺ channels. Within 2 min after the addition of TPA, we observed that the p_open of the K⁺ channels was diminished to 20–40% of its initial value (Fig. 8 and Table 1). These results show that the K⁺ channels can be inhibited by the activation of protein kinase C.
kinase C in A431 cells. Protein kinase C could thus play a role as a negative feedback mechanism in regulating the transient nature of the EGF-induced hyperpolarization and Ca\textsuperscript{2+} influx.

**DISCUSSION**

Earlier studies have categorized a transient hyperpolarization and Ca\textsuperscript{2+} influx among the first detectable responses of the activation of the EGF-R in A431 cells (8, 9, 16–18). The underlying mechanisms are, however, not well understood as yet. In the present study, we have applied patch clamp recording in the cell-attached and the outside-out mode, and fluorometric [Ca\textsuperscript{2+}]\textsubscript{i}, determinations, to identify the nature of the ion channels involved, to characterize their properties at the level of single channels, and to unravel the mechanism of activation. We provide evidence that activation of the EGF-R results initially in the activation of voltage-independent Ca\textsuperscript{2+} channels that can be defined as direct receptor-operated channels. This in turn causes activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels, which results in a (delayed) membrane hyperpolarization and then leads to the activation of a second class of Ca\textsuperscript{2+} channels that are sensitive to hyperpolarization. An autocatalytic generation of further hyperpolarization and Ca\textsuperscript{2+} influx is the predicted outcome of this ionic cascade. Based on the observed inhibitory effects of protein kinase C activation on the activity of the Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels, we propose that protein kinase C is involved in the negative regulation of this cascade, which explains the transient nature of these responses (see also Fig. 9). These responses provide a satisfying explanation for the well known EGF-induced hyperpolarization and Ca\textsuperscript{2+} influx in A431 cells (8, 9, 16–18) and confirm the suggested involvement of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels in these responses (8, 21).

An important outcome of our experiments is the identification of the primary target of EGF-R activation. While cell-attached patch clamp recordings showed the involvement of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels and hyperpolarization-activated Ca\textsuperscript{2+} channels in the ionic responses to EGF, they could not resolve their sequential activation. The observation by Pandiella et al. (8) that EGF fails to induce a hyperpolarization after depletion of the intracellular Ca\textsuperscript{2+} stores and in the presence of EGTA, together with our finding that the K\textsuperscript{+} channel activity is strictly dependent on [Ca\textsuperscript{2+}], renders it unlikely that K\textsuperscript{+} channels are directly activated by the EGF-R. On the other hand, we observed still a slow rise of [Ca\textsuperscript{2+}], after EGF addition under conditions that prevented the induction of a hyperpolarization. This indicated the existence of a voltage-independent Ca\textsuperscript{2+} influx. Measurements in the

**Fig. 9.** Proposed mechanism underlying the ionic changes in A431 cells after addition of EGF. Activation of the receptor first induces a small Ca\textsuperscript{2+} influx, which initiates the autocatalytic process. This ultimately leads to an elevated [Ca\textsuperscript{2+}], and a hyperpolarization of the cell. The transient nature of these ion fluxes is explained by inhibition of K\textsuperscript{+} channels by protein kinase C activation.

![Diagram of proposed mechanism](image_url)
(e.g. T lymphoblasts (33), hepatocytes (34), parotid acinar cells (35), neutrophils (36), basophilic leukemia cells (37), and platelets (38)). Moreover, for some of these cell types, it was found that phorbol esters inhibited a rise of [Ca\textsuperscript{2+}]. We speculate that in these cell types the receptor-operated Ca\textsuperscript{2+} influx is mediated by a similar transactivation of hyperpolarization-activated Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels. It could well be that such an autocatalytic cascade stands as a model for receptor-operated ionic responses (including [Ca\textsuperscript{2+}], oscillations) in many nonexcitable cell types. The mechanism of ion fluxes proposed here may thus have a physiological relevance which exceeds the EGF response in A431 cells.

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