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IONIC SIGNALLING BY GROWTH FACTOR RECEPTORS

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SUMMARY

The proliferation of cells *in vivo* and in culture is regulated by polypeptide growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Growth factors initiate their action by binding to specific cell surface receptors. Receptor occupancy triggers a cascade of physiological changes in the target cell which ultimately lead to DNA synthesis and cell division. Immediate consequences of receptor activation include tyrosine-specific protein phosphorylations, a sustained increase in cytoplasmic pH (pH_i) and a transient rise in free Ca^{2+}. The rise in pH_i has a permissive effect on DNA synthesis and is mediated by an otherwise quiescent Na^+/H^+ exchange mechanism in the plasma membrane, which is turned on by protein kinase C, the cellular receptor for phorbol esters. The rapid Ca^{2+} signal is due to either release from internal stores (PDGF) or net entry *via* a voltage-independent channel in the plasma membrane (EGF). Phorbol esters, acting *via* kinase C, inhibit the growth factor-induced Ca^{2+} signals without affecting resting Ca^{2+} levels.

Monoclonal antibodies against the human EGF receptor can act as partial agonists in that they activate the tyrosine-specific protein kinase without inducing any of the ionic signals. These antibodies fail to induce DNA synthesis when added to quiescent fibroblasts, indicating that the Ca^{2+} and pH_i signals can be dissociated from tyrosine kinase activity and suggesting that these signals are indispensable for the stimulation of cell proliferation.

INTRODUCTION

Growth factors are polypeptide hormones that induce replicative DNA synthesis and cell division in their target cells. Like all polypeptide hormones, growth factors initiate their action by binding to specific, high-affinity receptor molecules on the cell surface. Although much has been learned about growth factor–receptor interaction, the molecular mechanisms of action and the *in vivo* functions of growth factors are poorly understood. A number of growth factors have been purified to homogeneity and their primary structure has been solved, at least in large part. By far the most extensively studied growth factors are epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). EGF is a single polypeptide chain (relative molecular mass, M_r, 6045), routinely isolated from the mouse submaxillary gland,

Key words: epidermal growth factor (EGF), platelet-derived growth factor (PDGF), phorbol esters, protein kinase C, Na^+/H^+ exchange, cytoplasmic free Ca^{2+}, cytoplasmic pH, phosphoinositide hydrolysis, tyrosine kinase, monoclonal antibodies.

that stimulates the proliferation of epithelial cells *in vivo* and of various additional cell types in culture (Carpenter & Cohen, 1979). PDGF is a highly basic glycoprotein (M_r, 30 000–33 000) that is released from platelets during blood clotting and at sites of blood vessel injury, where it may serve to promote wound healing by stimulating the migration and proliferation of fibroblasts, glial cells and smooth muscle cells (Westermark *et al.* 1983). Interest in these mitogens and their receptors has been dramatically intensified by the discovery that at least some of these molecules show a striking structural homology with certain viral oncogene products: the *sis* oncogene encodes a PDGF-like molecule (Doolittle *et al.* 1983; Waterfield *et al.* 1983), while the *erb-B* oncogene product is a truncated form of the EGF receptor (Downward *et al.* 1984). Inappropriate expression of the cellular counterparts of viral oncogenes is thought to be responsible for the initiation and maintenance of malignant growth. Therefore, the study of the mode of action of EGF and PDGF will undoubtedly provide important insights into the mechanisms underlying carcinogenesis.

Understanding the mode of action of growth factors requires the identification of intracellular signals that are essential for the stimulation of DNA synthesis and cell division. The intention of this short review is to focus on the generation of ionic signals by growth factors with emphasis on the activation of Na^+/H^+ exchange and the mobilization of Ca^{2+}. Furthermore, we will describe some of the approaches to assess the interrelationship between the various signal pathways, such as the use of tumour-promoting phorbol esters and monoclonal antibodies against the EGF receptor.

SIGNAL PATHWAYS IN GROWTH FACTOR ACTION

Following growth factor binding, the activated receptor mediates a cascade of rapid biochemical and physiological changes in the cell, which ultimately (after 10–20 h) lead to the stimulation of DNA synthesis. As a rule, the growth factor has to be present throughout the entire 'pre-replicative' phase (usually >8–10 h) for commitment to DNA synthesis to occur. It turns out that there are at least three potential signal pathways in the action of growth factors like EGF and PDGF (Fig. 1).

One of the first consequences of growth factor–receptor interaction is the activation of a protein kinase specific for tyrosyl residues (Carpenter, 1984; Heldin & Westermark, 1984; Hunter & Cooper, 1985). In fact, the receptors for growth factors like EGF and PDGF are transmembrane glycoproteins that possess intrinsic tyrosine-specific protein kinase activity (Hunter & Cooper, 1985). Growth factor binding induces a rapid stimulation of the receptor kinase, resulting in autophosphorylation of the receptor itself as well as in the phosphorylation of various substrate proteins. The intrinsic tyrosine-specific kinase activity is shared with several viral oncogene products, such as the transforming protein of Rous sarcoma virus (Bishop, 1985). This suggests that tyrosine-specific protein phosphorylations

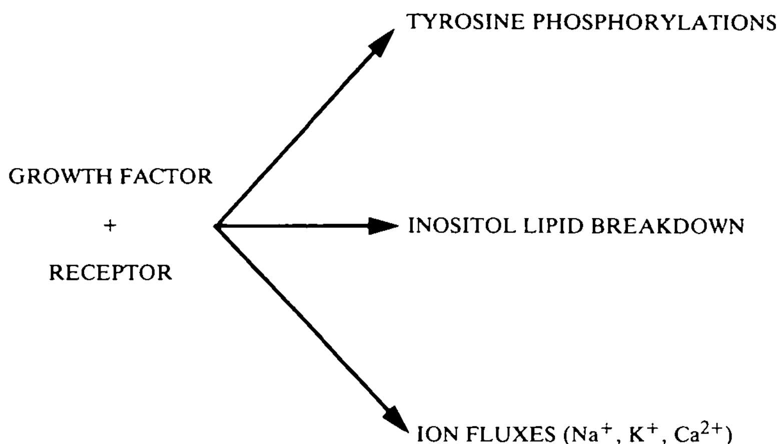

Fig. 1. Potential signalling pathways in the action of growth factors.

may initiate a set of common mitogenic pathways in virus-transformed and growth factor-stimulated cells. However, it is not yet possible to relate increased tyrosine kinase activity to specific metabolic alterations in stimulated cells.

Other immediate consequences of receptor activation (Fig. 1) include the breakdown of inositol phospholipids (Sawyer & Cohen, 1981; Berridge, Heslop, Irvine & Brown, 1984), a transient rise in cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) (Moolenaar, Tertoolen & de Laat, 1984; Moolenaar, Aerts, Tertoolen & de Laat, 1986) and the stimulation of monovalent ion transport across the plasma membrane (Rozengurt, 1981; Moolenaar, 1986*a*). Of the known ionic transport changes in growth factor-stimulated cells, the activation of electroneutral Na^+/H^+ exchange is best characterized (see next section).

A few studies have focused on the electrical membrane properties of stimulated cells. Using conventional electrophysiological techniques, Hülser & Frank (1971) and Moolenaar, Yarden, de Laat & Schlessinger (1982) showed that serum growth factors, when added to quiescent fibroblasts, elicit an immediate but transient membrane depolarization from about $-60\,mV$ to $-15\,mV$, apparently due to a large increase in monovalent ion conductance. A qualitatively similar electrophysiological effect was observed in serum-stimulated neuroblastoma cells (Moolenaar, de Laat & van der Saag, 1979). Whether this sudden ionic 'leakiness' of the plasma membrane is caused by a mitogen-induced phosphorylation reaction is not known; nor is it known which serum component is responsible for this effect, although thrombin is a strong candidate (W. H. Moolenaar, unpublished data).

While EGF can mimic serum in eliciting a transient membrane depolarization in an epithelial cell line (Rothenberg, Reuss & Glaser, 1982), the available evidence nevertheless indicates that rapid changes in ionic conductance and membrane potential are not a general phenomenon accompanying EGF receptor binding (cf. Moolenaar *et al.* 1982, 1986). It therefore appears that changes in ionic conductance,

although interesting in their own right, are not essential for the generation of a mitogenic signal by growth factors.

ACTIVATION OF Na^+/H^+ EXCHANGE

The first direct evidence that growth factors activate an otherwise quiescent Na^+/H^+ exchanger in the plasma membrane came from studies on serum-stimulated neuroblastoma cells (Moolenaar, Boonstra, van der Saag & de Laat, 1981*a*; Moolenaar, Mummery, van der Saag & de Laat, 1981*b*). Re-addition of serum to growth-arrested cells leads to the rapid activation of an electrically silent Na^+ influx pathway, which is sensitive to amiloride and can be stimulated by acidifying the cytoplasm using weak acids. In addition, it was shown that amiloride-sensitive Na^+ uptake in such acid-loaded cells is coupled to the efflux of H^+ with a 1:1 stoichiometry. In a subsequent study it was shown that EGF-induced Na^+ influx in quiescent human fibroblasts is amiloride-sensitive, electroneutral and is enhanced by cytoplasmic acid loads (Moolenaar *et al.* 1982). From these studies it was predicted that stimulation by mitogens would lead to an increase in pH_i, while the accompanying entry of Na^+ would result in a stimulation of the Na^+,K^+-pump. New developments in pH_i-monitoring techniques, particularly the synthesis of fluorescent indicators that can be trapped into the cytoplasm of small cells, have demonstrated that the Na^+/H^+ exchanger is normally involved in the close regulation of pH_i and that activation of the Na^+/H^+ exchanger by growth factors leads to a sustained rise in pH_i of 0·2–0·3 units.

Most cells maintain their pH_i at 7·0–7·4. This is well above the electrochemical equilibrium value of 6·0–6·4 that is predicted by the Nernst equation from a transmembrane potential of approximately −60 mV. In vertebrate cells, the specific H^+-extruding mechanism which raises pH_i appears to be a Na^+/H^+ exchange (Roos & Boron, 1981; Moolenaar, 1986*b*). The functioning of the Na^+/H^+ exchanger in the plasma membrane and its role in pH_i homeostasis is most easily assessed by continuously monitoring the rapid recovery of pH_i to its resting level after a sudden acidification of the cytoplasm, as induced by a NH_4^+-prepulse or by weak acids (Roos & Boron, 1981). In most cells, this pH_i recovery process follows an exponential time course and is entirely due to net H^+ extrusion *via* the Na^+/H^+ exchanger, which utilizes the energy stored in the transmembrane Na^+ gradient. The major determinant of the rate of the Na^+/H^+ exchanger is pH_i. At normal pH_i values (near 7·0) the exchanger is relatively inactive, although the steep transmembrane Na^+ gradient could theoretically raise pH_i by about one unit.

As pH_i falls below a certain 'threshold', the Na^+/H^+ exchanger is increasingly stimulated. Aronson and coworkers (Aronson, Nee & Suhm, 1982) were the first to point out that the Na^+/H^+ exchanger is apparently set in motion through an allosteric activation by cytoplasmic H^+ at a regulatory site which is distinct from the internal H^+ transport binding site. The relatively strong pH_i sensitivity of the exchanger is, of course, a crucial property for an H^+-extruding system to maintain pH_i at a critical level. Furthermore, a change in the apparent affinity of the exchanger

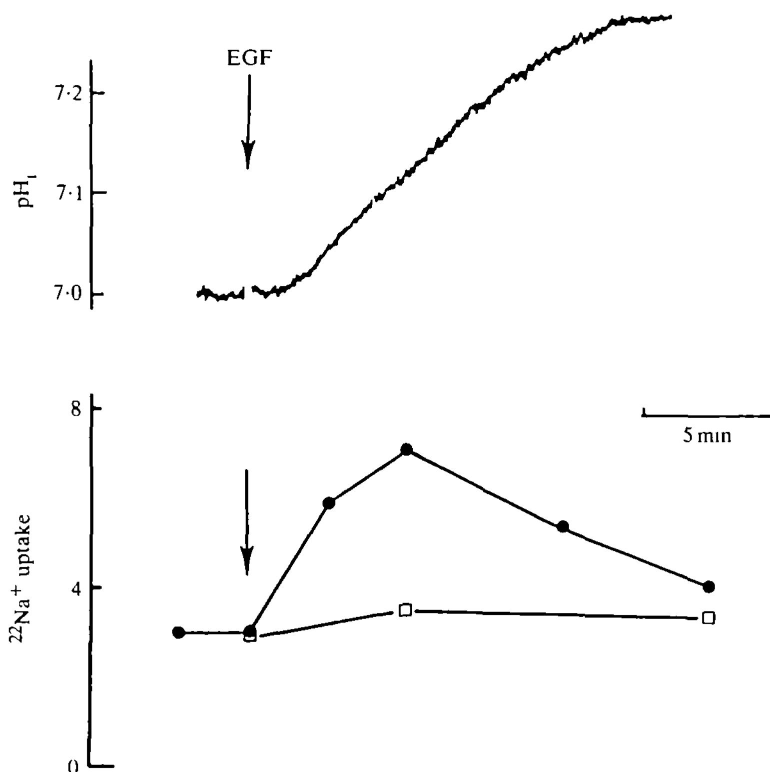

Fig. 2. Activation of Na^+/H^+ exchange by epidermal growth factor (EGF) in human fibroblasts. Upper panel: shift in pH_i induced by EGF ($10\,ng\,ml^{-1}$). Quiescent monolayers were loaded with bis(carboxyethyl)carboxyfluorescein (BCECF) as described (Moolenaar, Tsien, van der Saag & de Laat, 1983). Lower panel: time course of EGF-induced $^{22}Na^+$ uptake in the presence (□) and absence (●) of $1\,mmol\,l^{-1}$ amiloride. Initial rates of $^{22}Na^+$ uptake were measured over 3-min intervals in the presence of $0{\cdot}1\,mmol\,l^{-1}$ ouabain to block active Na^+ efflux.

for internal H^+ would provide a powerful mechanism by which external stimuli could regulate the physiological state of the exchanger.

The diuretic amiloride reversibly inhibits Na^+/H^+ exchange, apparently by competing with Na^+ for binding to the same external site (K_i: 2–5 $\mu mol\,l^{-1}$). Several amiloride analogues have been found to be 10–100 times more potent than amiloride in blocking Na^+/H^+ exchange (l'Allemain, Franchi, Cragoe & Pouysségur, 1984; Zhuang *et al.* 1984). It should be noted, however, that amiloride and its potent analogues are taken up by most cells in culture and thereby may affect cellular functions such as protein synthesis and kinase activity (Zhuang *et al.* 1984; Besterman *et al.* 1985; Davis & Czech, 1985).

Fig. 2 shows a typical example of an alkaline pH_i shift after addition of EGF to quiescent cells loaded with the pH-sensitive dye bis(carboxyethyl)carboxyfluorescein (BCECF). The shift in pH_i is initiated within 20–30 s and is complete by 10–15 min. The elevated pH_i persists for as long as the growth factor is present. In general, the induced alkalinizations range from 0·1–0·3 pH units; they are inhibited

by amiloride and by Na^+ removal and are accompanied by a transient increase in amiloride-sensitive $^{22}Na^+$ uptake (Fig. 1; for review see Moolenaar, 1986*a*). Furthermore, the rise in pH_i is converted into a fall in pH_i when the direction of the transmembrane Na^+ gradient is reversed (Moolenaar, Tsien, van der Saag & de Laat, 1983). When taken together, these data convincingly demonstrate that the mitogen-induced pH_i rise is mediated by the Na^+/H^+ exchanger.

MECHANISM OF ACTIVATION OF Na^+/H^+ EXCHANGE

How does receptor occupancy lead to activation of the Na^+/H^+ exchanger? Recent studies have shown that the activation is attributable to an alkaline shift in the pH_i sensitivity of the exchanger (Moolenaar *et al.* 1983; Paris & Pouysségur, 1984; Grinstein *et al.* 1985). As mentioned above, this pH_i sensitivity is determined by an allosteric H^+-binding site on the cytoplasmic face of the exchanger. It thus seems plausible to assume that the altered pH_i sensitivity of the exchanger is due to some conformational change resulting in an increased pK_a of the regulatory H^+-binding site. Thus, the physiological effect of growth factors on the Na^+/H^+ exchanger is to increase its pH_i threshold, that is the level to which pH_i must rise before the exchanger virtually shuts off. Indeed, the Na^+/H^+ exchanger is only transiently stimulated by external stimuli and its activity returns to the control level once pH_i has attained its new stable value (Fig. 2; Moolenaar, 1986*a*).

The obvious next question is by which route growth factors modify the pH_i sensitivity of the exchanger to raise pH_i. An important key comes from studies using the tumour promotor 12-*O*-tetradecanoyl-13-acetate (TPA), which binds to and directly activates the phospholipid-dependent protein kinase C (Nishizuka, 1984). Under normal conditions, kinase C is activated by endogenous diacylglycerol (DG) derived from the breakdown of inositol phospholipids. Both TPA and synthetic DG are capable of mimicking the effects of growth factors on Na^+/H^+ exchange and pH_i in various cell types (reviewed by Moolenaar, 1986*a*). The simplest explanation for these findings is that kinase C directly phosphorylates the exchanger, but it cannot, of course, be excluded that kinase C acts in a more indirect way to activate Na^+/H^+ exchange. Information concerning the molecular structure of the exchange carrier may become available in the near future, and this should greatly facilitate further study of the role of kinase C and other kinases in the activation process.

The apparent involvement of kinase C strongly suggests that an alkaline shift in pH_i is not uniquely induced by growth factors and phorbol esters but may be a common cellular response in the action of those hormones and neurotransmitters that trigger the hydrolysis of inositol phospholipids and thereby generate diacylglycerol. There is some evidence to support this notion: in typical model systems of hormone-induced phosphoinositide turnover, such as platelets and neutrophils, the pH_i rises rapidly after activation by physiological stimuli (Molski *et al.* 1980; Horne, Norman, Schwartz & Simons, 1981).

Finally, it is noteworthy that there is evidence, albeit circumstantial, that pathways other than kinase C may be involved in Na^+/H^+ exchange activation (Vara &

Rozengurt, 1985; Paris & Pouysségur, 1986). The biochemical nature of these pathways remains to be elucidated.

Ca^{2+} MOBILIZATION BY GROWTH FACTORS

In addition to activating Na^+/H^+ exchange, mitogens like EGF and PDGF induce a rapid but transient rise in $[Ca^{2+}]_i$ in their target cells, as measured by quin-2 fluorescence (Moolenaar *et al.* 1983, 1986; Hesketh *et al.* 1985). The best characterized example of a mitogen-induced $[Ca^{2+}]_i$ rise comes from PDGF-treated fibroblasts (Berridge *et al.* 1984; Moolenaar *et al.* 1984). Addition of PDGF to quiescent fibroblasts elicits an approximately twofold rise in $[Ca^{2+}]_i$ which is initiated without a detectable lag period and is usually complete within 30–60 s. Thereafter, $[Ca^{2+}]_i$ gradually returns to near-basal levels over a 10- to 15-min period. Fig. 3 schematically illustrates the time courses of both the $[Ca^{2+}]_i$ transient and the rise in pH_i as induced by EGF in responsive cells.

How does growth factor binding increase $[Ca^{2+}]_i$? The Ca^{2+} signal in response to PDGF is not prevented by removal of external Ca^{2+}, indicating that the Ca^{2+} is released from intracellular stores, presumably the endoplasmic reticulum (Moolenaar *et al.* 1984). Indeed, PDGF provokes the rapid formation of inositol-1,4,5-trisphosphate (IP_3), the key messenger for mobilizing Ca^{2+} from non-mitochondrial stores (Berridge *et al.* 1984; Berridge & Irvine, 1984). Surprisingly, the Ca^{2+} signal in response to EGF shows no contribution from intracellular stores but seems to result from net Ca^{2+} entry through a voltage-independent Ca^{2+} channel in the plasma membrane (Moolenaar *et al.* 1986). This interpretation is based on the finding that the EGF-induced $[Ca^{2+}]_i$ rise in human A431 carcinoma cells is critically dependent on the extracellular Ca^{2+} concentration, is accompanied by enhanced $^{45}Ca^{2+}$ uptake (Sawyer & Cohen, 1981), is blocked by Ca^{2+} entry blockers like La^{3+} and Mn^{2+}, while it is not accompanied by changes in transmembrane potential. Since EGF is known to stimulate phosphoinositide turnover and diacylglycerol formation in A431 cells (Sawyer & Cohen, 1981; Sahai, Smith, Pannerselvam &

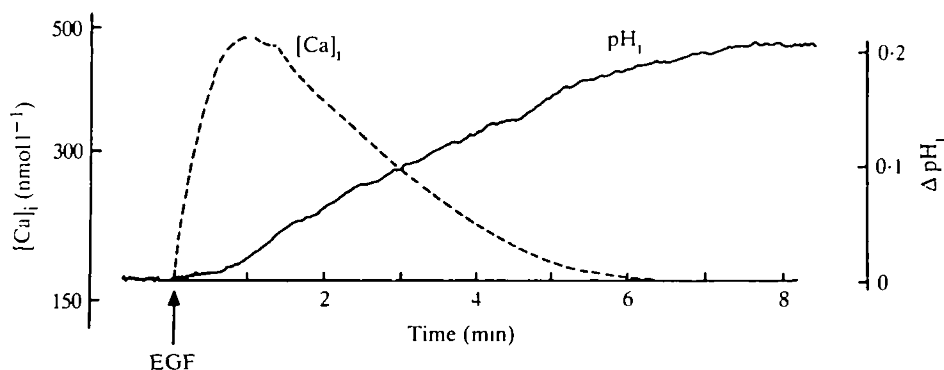

Fig. 3. Changes in $[Ca]_i$ and pH_i in human A431 cells following the addition of epidermal growth factor (EGF) (100 ng ml^{-1}). For methods see Moolenaar, Tsien, van der Saag & de Laat (1983) and Moolenaar, Aerts, Tertoolen & de Laat (1986).

Salomon, 1982), it is intriguing that EGF does not seem to act on intracellular stores to raise $[Ca^{2+}]_i$.

What could be the molecular nature of the putative Ca^{2+} channel that is activated when EGF binds to its receptor? An attractive candidate for mediating Ca^{2+} entry is phosphatidic acid, an anionic phospholipid that is rapidly formed from diacylglycerol in EGF-treated A431 cells (Sawyer & Cohen, 1981) and that may function as a Ca^{2+} ionophore in model systems (Serhan *et al.* 1982) and in intact cells from various tissues (Putney, Weiss, van der Walle & Haddas, 1980; Ohsako & Deguchi, 1981). Our preliminary experiments (unpublished) indeed indicate that exogenous phosphatidate can transiently elevate $[Ca^{2+}]_i$ in A431 cells. On the other hand, the absolute requirement for external Ca^{2+} could, of course, simply reflect a Ca^{2+}-dependent coupling of individual EGF receptors to the IP_3-generating process. Further characterization of phosphoinositide metabolism in EGF-treated A431 cells is therefore eagerly awaited.

INHIBITORY ACTIONS OF PHORBOL ESTER

Unlike the mitogen-induced pH_i shift, the rise in $[Ca^{2+}]_i$ cannot be mimicked by phorbols ester like TPA (Moolenaar *et al.* 1984, 1986), consistent with the notion that TPA acts at a point (kinase C) distal from Ca^{2+} mobilization. This result demonstrates that both ionic signals are dissociable and can occur independently of each other.

There is growing evidence that TPA, acting *via* protein kinase C, not only stimulates cellular functions but also has a negative feedback role in that it can shut off certain intracellular signal pathways in the action of growth factors and hormones. For example, phosphorylation of the EGF receptor by kinase C appears to decrease its EGF-stimulated tyrosine kinase activity (Hunter & Cooper, 1985). Furthermore, TPA has been shown to inhibit both the EGF-stimulated Na^+/H^+ exchange (Whiteley, Cassel, Zhuang & Glaser, 1984) and the $[Ca^{2+}]_i$ rise in A431 cells (Moolenaar *et al.* 1986). In TPA-treated 3T3 cells, the Ca^{2+} signal in response to EGF is also completely blocked (Hesketh *et al.* 1985). TPA also causes the closure of antigen-activated Ca^{2+} channels in the plasma membrane of basophils (Sagi-Eisenberg, Lieman & Pecht, 1985). The molecular mechanism underlying these inhibitory effects of TPA is not precisely known, although it is becoming increasingly apparent that the receptor-linked breakdown of phosphoinositides is strongly suppressed once kinase C is activated.

By which mechanism does kinase C inhibit phosphoinositide hydrolysis? It has been proposed that a novel GTP-binding protein (G_p or N_p; see Cockroft & Gomperts, 1985) is involved in coupling hormone receptors to phospholipase C and phosphoinositide breakdown. Interestingly, it has been reported that kinase C can phosphorylate a GTP-binding protein (G_i/N_i) and thereby inhibit its regulatory function (Katada *et al.* 1985). It is an attractive hypothesis that G_p may also serve as a physiological substrate for protein kinase C and that its presumed function in phospholipase C stimulation is impaired by its phosphorylation. Whatever the nature

Table 1. *Comparison of the biological effects of EGF and anti-EGF receptor monoclonal antibodies on human A431 cells and fibroblasts*

	EGF	2E9	2D11
Precipitation of EGF receptor		+	+
EGF binding competition	+	+	−
Stimulation of tyrosine kinase	+	+	+
Morphological changes	+	−	+
Rise in $[Ca^{2+}]_i$	+	−	−
Rise in pH_i	+	−	−
Stimulation of DNA synthesis	+	−	−

Stimulation of DNA synthesis was tested on quiescent fibroblasts; all other effects on A431 cells. For further details see Defize, Moolenaar, van der Saag & de Laat (1986).

of the phosphorylated substrate(s), it appears that protein kinase C functions to uncouple certain growth factor receptors from their second-messenger generating systems. Perhaps this represents a mechanism whereby phorbol esters like TPA exert their tumour-promoting effects.

DISSOCIATION OF SIGNAL PATHWAYS BY ANTI-RECEPTOR MONOCLONAL ANTIBODIES

The partial activation of post-receptor signalling pathways by phorbol esters has proved to be a fruitful approach in elucidating interrelationships among these pathways. An alternative tool for the dissociation of molecular events in the signalling cascade is provided by the availability of monoclonal antibodies to the EGF receptor. We have used three different anti-EGF receptor monoclonal IgGs, directed against distinct epitopes of the extracellular domain of the human EGF receptor (Defize, Moolenaar, van der Saag & de Laat, 1986), to test their ability to act as partial or full agonists of the EGF receptor. All three antibodies (named 2E9, 2D11 and 2G5, respectively) are able to immunoprecipitate a functional EGF receptor showing EGF-dependent tyrosine kinase activity. Monoclonal 2E9 is unique in that it recognizes a peptide determinant at or close to the EGF binding domain of the receptor. As a consequence, 2E9 competitively inhibits EGF receptor binding. In contrast, the other monoclonals (2D11 and 2G5) are directed to bloodgroup A-specific carbohydrate structures on the EGF receptor and fail to affect EGF binding. We have tested these antibodies for their EGF-like properties in stimulating the receptor-mediated tyrosine phosphorylations, cytoplasmic alkalinization, Ca^{2+} mobilization and DNA synthesis. As summarized in Table 1, all three monoclonal IgGs can stimulate the tyrosine-specific autophosphorylation of the 170kD EGF receptor both in isolated A431 membranes and in intact cells (Defize, Moolenaar, van der Saag & de Laat, 1986). Interestingly, none of these antibodies is capable of triggering a rise in $[Ca^{2+}]_i$ and inducing cytoplasmic alkalinization (Table 1), even after addition of a second cross-linking anti-IgG. Finally, the anti-receptor antibodies fail to stimulate DNA synthesis in quiescent human fibroblasts. Stimulation of the receptor's intrinsic tyrosine kinase is apparently not sufficient, by itself, to

elicit a mitogenic response (for further details see Defize, Moolenaar, van der Saag & de Laat, 1986). Another important conclusion from those results is that stimulation of the EGF receptor kinase does not necessarily activate the post-receptor pathway that leads to an increase in $[Ca^{2+}]_i$ and in pH_i. These findings further support the view that the two major ionic signals are indispensable for the stimulation of DNA synthesis and cell proliferation.

POSSIBLE PHYSIOLOGICAL ROLE OF THE IONIC SIGNALS

Cytoplasmic alkalinization

An early rise in pH_i appears to be a fairly common response of metabolically dormant cells to appropriate surface stimuli. Several lines of evidence support the hypothesis that a sustained increase in pH_i, mediated by the Na^+/H^+ exchanger, may be required for mitogenesis. Perhaps the clearest demonstration of a role for cytoplasmic alkalinization in the initiation of a mitogenic response comes from studies on fertilized sea urchin eggs, where it has been shown that pH_i must rise from 6·8 to above 7·0 to permit DNA synthesis to begin (Whitaker & Steinhardt, 1982). One of the critical pH_i-dependent steps in the fertilized eggs appears to be the stimulation of protein synthesis. From studies on somatic cells it is known that pH_i may participate in the regulation of such diverse processes as glycolosis, cell-to-cell communication and cytoskeletal reorganization (reviewed by Busa & Nuccitelli, 1984).

Fig. 4 shows the effects of artificially shifting pH_i on the ability of quiescent human fibroblasts to reinitiate DNA synthesis in the presence of serum. It is seen that below a certain threshold value (near 7·0) pH_i becomes limiting for DNA synthesis, but that thymidine incorporation dramatically increases between pH_i 7·0–7·2. It is also observed that an artificially induced rise in pH_i is not sufficient, in itself, to stimulate DNA synthesis in serum-deprived cells (Fig. 4). These results agree, at least qualitatively, with earlier observations in thrombin-stimulated hamster fibroblasts: using mutant cells that lack a functional Na^+/H^+ exchanger, Pouysségur *et al.* (1984) and Pouysségur, Franchi, l'Allemain & Paris (1985) elegantly showed that the initiation of DNA synthesis in mitogen-stimulated cells is extremely sensitive to pH_i. At $pH_i < 7{\cdot}2$, DNA synthesis is completely blocked, but the mutant cells resume growth as soon as their pH_i is artificially elevated, either by raising external pH or incubating them in bicarbonate-buffered media. Addition of bicarbonate apparently raises pH_i into the 'permissive range' due to the operation of a Na^+-dependent HCO_3^-/Cl^- exchanger (l'Allemain, Paris & Pouysségur, 1985).

Increases in pH_i during the cell cycle have been observed in a few different cell types. In the slime mould, *Physarum*, pH_i rises rapidly just before mitosis whereas in *Tetrahymena* there are two alkaline pH_i transients per cell cycle (Busa & Nuccitelli, 1984). In the cellular slime mould *Dictyostelium discoideum* there is a cycle of pH_i that closely corresponds to the DNA replication cycle, with a peak during S phase and mitosis (Aerts, Durston & Moolenaar, 1985). Interestingly, when protein and DNA synthesis are blocked, pH_i continues to oscillate with a similar period as in the

uninhibited division cycle. Conversely, an artificial increase in pH_i by only 0·1 unit causes a several-fold increase in the rates of protein and DNA synthesis. It was concluded that this autonomous 'pH_i oscillator' may have an important role in the timing and regulation of the cell cycle (Aerts *et al.* 1985). It was further hypothesized that the observed pH_i cycles are due to periodic alterations in the activity of a plasma membrane H^+ pump, probably an H^+-ATPase. Obviously, it would be of great interest to determine whether pH_i regulated by Na^+/H^+ exchange exhibits similar oscillations during the cell cycle of higher eukaryotes.

In summary, the available evidence provides strong support for the view that pH_i plays an important role in the regulation of cellular metabolism in general and in the regulation of DNA synthesis and cell proliferation in particular.

Rise in $[Ca^{2+}]_i$

Another key question is what the physiological role of the $[Ca^{2+}]_i$ transient is in the action of growth factors. Numerous cellular functions are regulated by $[Ca^{2+}]_i$ and many of them through the formation of Ca^{2+}-calmodulin complexes. A discussion on this topic is beyond the scope of this brief review. Of particular relevance to mitogen action is the finding that artificially raising $[Ca^{2+}]_i$ using the Ca^{2+} ionophore A23187, can mimic the effects of EGF and PDGF on the rapid induction of the c-*fos* and c-*myc* proto-oncogenes (Bravo, Burckhardt, Curran & Müller, 1985; Tsuda,

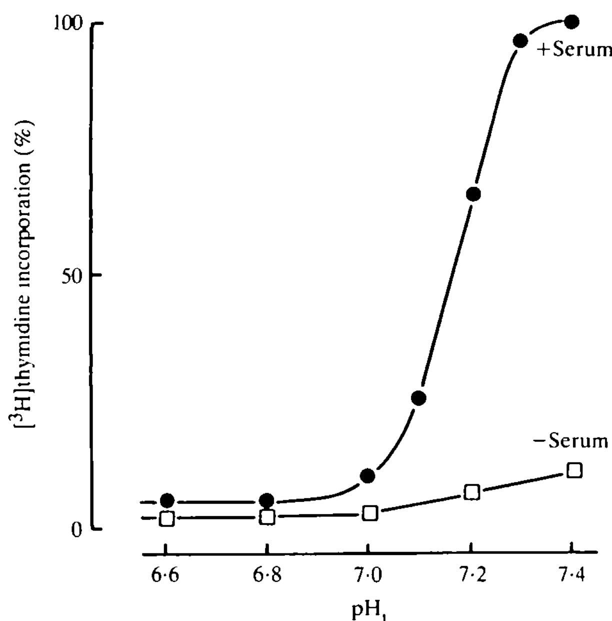

Fig. 4. Dependence of DNA synthesis on pH_i in human fibroblasts. Growth-arrested fibroblasts were incubated in Hepes-buffered media in the presence (●) or absence (□) of serum (10% v/v) and their pH_i was adjusted to the indicated values (by changing extracellular pH) for a period of 20 h. Thereafter, [^{3}H]thymidine (1 μCi ml^{-1}) was added at physiological external pH (7·4) and acid-insoluble counts were determined after 24 h.

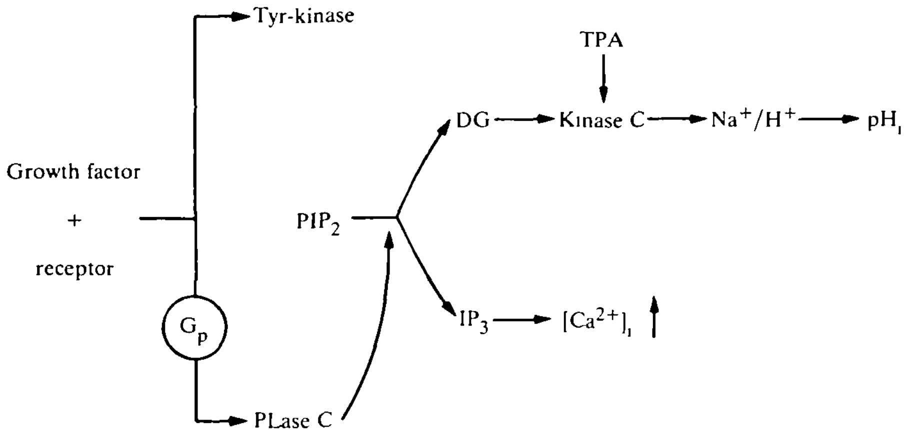

Fig. 5. Proposed sequence of events leading from growth factor binding to ionic signals. G_p is the putative GTP-binding protein; Tyr-kinase, tyrosine-specific protein kinase; PLase C, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DG, 1,2-diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate. Note that there is a fundamental difference between the $[Ca^{2+}]_i$-raising mechanism of platelet-derived growth factor (PDGH) (*via* IP_3) and that of epidermal growth factor (EGF) (*via* Ca^{2+} entry). See text for details.

Kaibuchi, West & Takai, 1985). We recently found that Ca^{2+}-mobilizing neurohormones like bradykinin and histamine similarly induce c-*fos* and c-*myc* expression as well as cell division in their target cells (W. Kruijer & W. H. Moolenaar, unpublished data). These results raise the intriguing possibility that Ca^{2+} could function as a second messenger that mediates, either directly or indirectly, the early transcriptional effects of growth factors and some hormones. In addition, they provide a framework for future studies on the role of intracellular Ca^{2+} in growth control.

CONCLUDING REMARKS

In recent years much has been learned about the various molecular events that are induced by growth factor–receptor interaction. These include tyrosine-specific protein phosphorylations, inositol phospholipid breakdown and the two major ionic events discussed above, namely, transient elevation of $[Ca^{2+}]_i$ and activation of Na^+/H^+ exchange resulting in a sustained rise in pH_i. There is good evidence that both ionic events are the direct consequence of the receptor-mediated hydrolysis of inositol phospholipids, while tyrosine kinase activity seems to initiate a distinct pathway, as summarized in the scheme of Fig. 5. Although uncertainty still exists about the biological significance of each of these 'early' steps in the eventual initiation of DNA synthesis, occurring many hours later, it seems likely that the various events evoked by inositol phospholipid breakdown act in concert with tyrosine-specific protein phosphorylations to stimulate cell proliferation. Selective pharmacological inhibitors without non-specific side effects could greatly assist in elucidating the relevance of each of the signal pathways, but such agents are lacking at present. It is obvious that mutant cells defective in Na^+/H^+ exchange activity (Pouysségur *et al.*

1984, 1985) or other functions, as well as anti-receptor monoclonal antibodies that act as partial agonists (Defize, Moolenaar, van der Saag & de Laat, 1986) are of enormous help in analysing the role of the various signal pathways in growth control.

Another challenge for future studies is to examine whether the ionic signals generated by growth factor receptors have their correlates in the action of certain transforming oncogene products, some of which are known to be tyrosine-specific protein kinases. It is reasonable to speculate that malignant growth, induced by inappropriate expression of cellular proto-oncogenes, is partially the result of the continual production of second messengers like IP_3 and diacylglycerol. This might lead to altered cellular Ca^{2+} pools and to constitutive activation of kinase C and, hence, of the Na^+/H^+ exchanger.

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