Original Article

Ehrlich Cell Plasma Membrane Redox System Is Modulated Through Signal Transduction Pathways Involving *c*GMP and Ca^{2+} as Second Messengers

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Ehrlich cell plasma membrane ferricyanide reductase activity increased in the presence of mastoparan, a generic activator of G proteins, using either whole cells or isolated plasma membrane fractions. Agents that increase intracellular *c*AMP also increased the rate of ferricyanide reduction by Ehrlich cells. For the first time, evidence is shown on a modulation of plasma membrane redox system by *c*GMP. In fact, permeant analogs of *c*GMP, dibutyryl *c*GMP, and 8-bromo-*c*GMP increased the rate of ferricyanide reduction by the Ehrlich cell plasma membrane redox system. Furthermore, specific inhibition of *c*GMP-phosphodiesterases by dipyridamole was also accompanied by an enhancement in the rate of ferricyanide reduction. On the other hand, treatments expected to increase cytoplasmic Ca^{2+} concentrations were accompanied by a remarkable stimulation of the reductase activity. Taking all these data together, it seems that the Ehrlich cell plasma membrane redox system is under a multiple and complex regulation by different signal transduction pathways involving G proteins, cyclic nucleotides, and Ca^{2+} ions.

INTRODUCTION

Cell membranes are not simple passive barriers delimiting compartments, but they actively participate in the exchange of matter, energy, and information with the cell environment and different cell compartments. Inner mitochondrial and thylakoid membranes have electron transport systems playing key roles in the bioenergetics of essential vital functions, namely, respiration and photosynthesis. A plasma membrane electron transport system, or plasma membrane redox system (PMRS), has been found to be ubiquitous (Crane *et al.*, 1985, 1991; and references therein). However, PMRS has been studied only from the middle seventies on and its actual physiological role and relevance is still not well known; in fact, studies are rather phenomenological. Nonetheless, there is increasing experimental evidence for an important role of PMRS in several vital functions, including nutrient transport (Medina and Núñez de Castro, 1990), and cell growth control (Crane *et al.*, 1985). As a matter of fact, PMRS activities seem to be modified in transformed cells, as compared with those shown by untransformed or differentiated cells (Sun *et al.*, 1983; Löw *et al.*, 1991; Burón *et al.*, 1993; Medina and Núñez de Castro, 1995). Since PMRS seems to play a role in growth control, it has to be somehow integrated in the sequence of events leading to the modulation of cell growth by both external and internal signals. In fact, hormones and growth factors have been demonstrated to modulate PMRS activity (Crane *et al.*, 1985; Navas *et al.*, 1992; Medina *et al.*, 1992). Further, N-*myc* and Ha-*ras* oncogene expressions seem to correlate with PMRS activity (Medina *et al.*, 1992; Crowe

and Crane, 1992). Recently, PMRS has been related to the *ras* signaling pathway (Crowe and Crane, 1992; Mackellar *et al.*, 1994), and *c*AMP and heterotrimeric G proteins have been demonstrated to be involved in the regulation of PMRS (Rodríguez-Aguilera *et al.*, 1993; Morré *et al.*, 1993). In this report, we give evidence for the involvement of two other second messengers, namely, *c*GMP and Ca^{2+}, in the regulation of Ehrlich cell PMRS.

METHODS

Cells

Hyperdiploid Lettré Ehrlich ascites tumor cells were grown in the peritoneal cavity of 2-month-old Swiss female mice, and harvested as described elsewhere (Olavarría *et al.*, 1981). The cells were harvested in the plateau phase of growth (9–11 days after tumor inoculation), and they were washed and suspended in PBS, without or with 5 mM glucose added as the only substrate for long-term (up to 8 h) survival of harvested cells, as demonstrated by Pérez-Rodríguez *et al.* (1987).

Ferricyanide Reductase Assay in Whole Cells

A discontinuous assay was carried out with 5×10^7 cells/ml in the presence of 0.5 mM ferricyanide, as previously described (Medina *et al.*, 1988).

Treatments

Experiments with mastoparan and neomycin sulfate were carried out with no preincubation. In the presence of other compounds, 10^7 cells/ml were preincubated during different times, as follows: 5 min for dibutyryl *c*AMP, dibutyryl *c*GMP, and 8-bromo-*c*GMP; 10 min for thapsigargin and calcium ionophore A23187; and 30 min for dipyridamole. In experiments with sphingosine, cells were incubated for 20 min; then, DMSO or calcium ionophore were added and incubations followed for another 10 min. All the experiments were carried out with parallel controls in which the different drugs were omitted. After preincubation, cells were washed twice and suspended in PBS to carry out ferricyanide reductase assay as described above.

Preparation of Plasma Membrane Vesicles

Highly pure plasma membrane vesicles were isolated from Ehrlich cells by two-phase compartmentation on poly(ethylene glycol)/dextran, as described elsewhere (Luque *et al.*, 1991). The purity of the plasma membranes obtained was greater than 95%, as determined by marker enzyme assays; mitochondrial contamination was less than 0.4%, and endoplasmic reticulum contamination was less than 2%.

Ferricyanide: NADH Oxidoreductase Assay in Vesicles

Ferricyanide reduction was followed in a Shimadzu UV-160 spectrophotometer at 420 nm. The assay medium contained 0.5 mM ferricyanide, 0.5 mM NADH, plasma membrane vesicles (50 μg proteins), and different amount of mastoparan in 5 mM Hepes (pH 7.4) in a final volume of 0.3 ml.

RESULTS

The involvement of PMRS in important vital functions points to its role as a component of signal transduction pathways. Thus, PMRS can be involved in downstream second messenger function by direct action on cytosolic components or by modification of other membrane enzymes directly involved in messenger formation (Crane, 1989). In this context, PMRS has been shown to activate certain protein kinases (Malviya and Anglard, 1986; Harrison *et al.*, 1991). On the other hand, PMRS seems to respond to upstream modulatory signals. The modulatory effects of extracellular signals, namely hormones and growth factors, on PMRS are well documented (Crane *et al.*, 1985; Navas *et al.*, 1992; Medina *et al.*, 1991). Much less is known concerning the role of intracellular signals and signal transducers on the modulation of PMRS activity. Ehrlich cells could be a convenient model for this kind of studies.

Mastoparan, a generic activator of animal G proteins (Higashijima *et al.*, 1988), stimulated Ehrlich cell ferricyanide reductase of both intact cells (Fig. 1) and plasma membrane vesicles (Fig. 2). This stimulatory effect was maximum during the first 2 min of incubation with intact cells. In vesicles, mastoparan stimulated ferricyanide reductase in a dose-response manner in the range 1–20 μM (see Fig. 2). On the other hand,
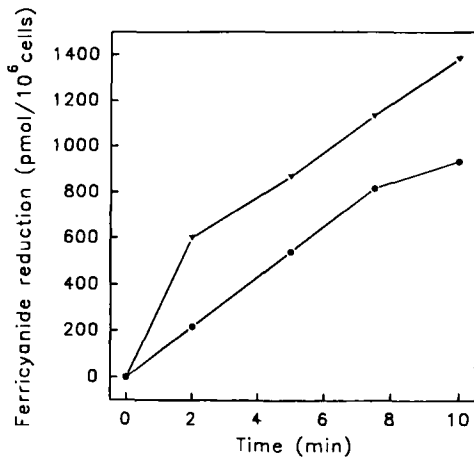
Fig. 1. Effect of mastoparan on ferricyanide reduction by intact Ehrlich cells. Cells were incubated either in the absence (circles) or the presence (triangles) of 10 μM mastoparan, as described in the experimental procedures, and the time courses of ferricyanide reduction were followed. Experiments were carried out several times and in all of them the tendencies were similar. A representative result is depicted.

the presence of neomycin sulfate (100 μM) in the incubation medium prevented the stimulatory effect of mastoparan on ferricyanide reductase in whole cells (results not shown).

Preincubations for 5 min in the presence of the permeant cyclic nucleotide analogs, dibutyryl cGMP and dibutyryl cGMP, resulted in an enhanced ferricya-
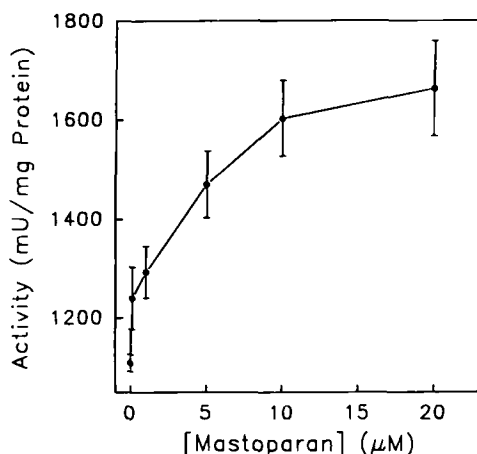

Fig. 2. Stimulation of Ehrlich cell plasma membrane ferricyanide reductase activity by mastoparan. Pure plasma membrane fractions were isolated and their ferricyanide reductase activity were continuously followed for 2 min in the presence of different concentrations of mastoparan, as described in the experimental procedures. Results are given as means ± S.D. of three different experiments.

nide reductase activity (Fig. 3). Furthermore, Ehrlich cell ferricyanide reductase was stimulated by dibutyryl cGMP in a dose-response manner in the micromolar range (see Fig. 4). The use of another permeant cyclic nucleotide analog, namely 8-bromo-cGMP, also resulted in an increased ferricyanide reductase activity (results not shown). It is noteworthy that dipyridamole, a selective inhibitor of cGMP-phosphodiesterase (Nawy and Jahr, 1991), also stimulated Ehrlich cell ferricyanide reductase activity (see Fig. 5).

In order to test a possible involvement of cytosolic Ca²⁺ concentrations on the modulation of PMRS activity, Ehrlich cells were treated with several compunds which increase cytosolic Ca²⁺ concentration in different ways. All of these treatments gave rise to an apparent increase of Ehrlich cell ferricyanide reductase activity (Table I). This effect was abolished by the calcium sequestrating agent EGTA.

DISCUSSION

Involvement of G Proteins

Heterotrimeric G proteins mediate the effects of agonists by working through specific receptors and
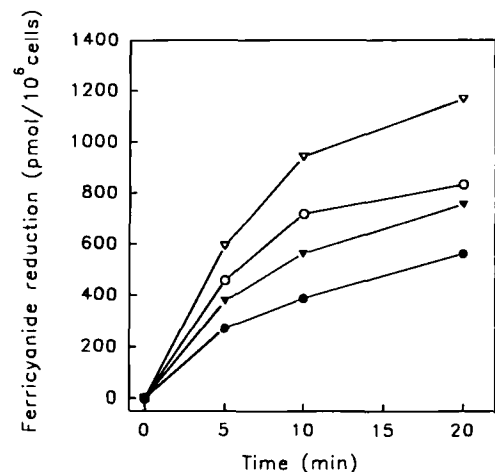

Fig. 3. Stimulatory effect of dibutyryl-cAMP and dibutyryl-cGMP on ferricyanide reduction by intact Ehrlich cells. Cells were preincubated in the absence (circles) or the presence (triangles) of 10 μM dibutyryl-cAMP (open symbols) or 10 μM dibutyryl-cGMP (closed symbols) as described in the experimental procedures. Afterwards, the time courses of ferricyanide reduction were followed. Results are given as means of two different experiments for dibutyryl-cAMP, and three different experiments for dibutyryl-cGMP. S.D. are omitted for the sake of clarity. At all the different times measured, the differences between cells preincubated with cyclic nucleotide analogs and their respective controls were significant.
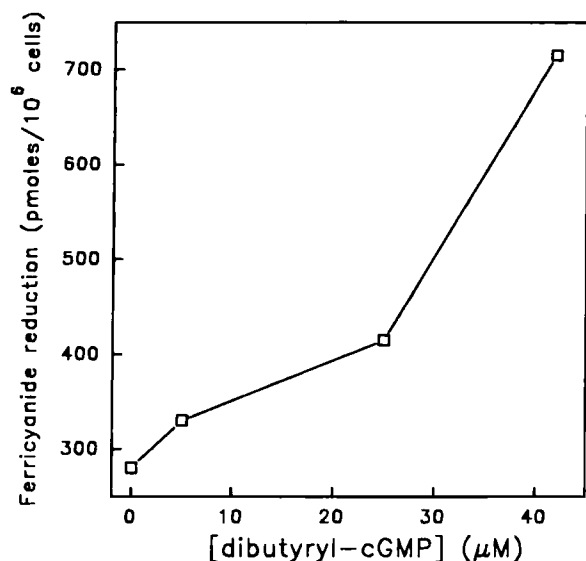
Fig. 4. Dose-dependent stimulation of dibutyryl-*c*GMP on ferricyanide reduction by intact Ehrlich cells. Cells were preincubated in the presence of different concentrations of dibutyryl-*c*GMP as described in the experimental procedures. Afterwards, cells were incubated in the presence of ferricyanide for 5 min and ferricyanide reduction was determined. Results are means of two different experiments for each dibutyryl-*c*GMP concentration tested and four different control determinations. Differences with the control values were significant even for the lower dibutyryl-*c*GMP concentration tested.

Fig. 5. Stimulatory effect of dipyridamole on ferricyanide reduction by intact Ehrlich cells. Cells were preincubated in the absence (open circles) or the presence (closed circles) of 1 μM dipyridamole as described in the experimental procedures. Afterwards, the time courses of ferricyanide reduction were followed. Results are given as means of three different experiments. At all the different times measured, the differences between cells preincubated with cyclic nucleotide analogs and their respective controls were significant.

giving rise to a modulation in the activity of some effector proteins as a final response (Gilman, 1987). Recently, evidence has been shown for the involvement of heterotrimeric G proteins in the activation of NADH oxidase activity of soybean hypocotyl plasma membrane (Morré *et al.,* 1993).

Mastoparan, a peptide toxin from wasp venom, constitutively activates G proteins by mimicking receptor-G protein interactions (Higashijima *et al.,* 1990). For this reason, mastoparan is used to study the possible involvement of G proteins in different processes. Furthermore, since mastoparan is amphiphilic, it is possible to use it in whole cell experiments, in contrast to GTPγS (Ross and Higashijima, 1994). The stimulatory effect of mastoparan on Ehrlich cell ferricyanide reductase detected even at short-term incubations (2 min) seems to reinforce the previous observation of a role for G proteins in PMRS activation (Morré *et al.,* 1993).

However, since mastoparan has been described to activate other proteins, a cellular response to mastoparan should not be taken as a firm indication that the response is controlled by a G-protein signaling pathway. Nonetheless, in highly pure isolated plasma membrane vesicles mastoparan also produced a clear
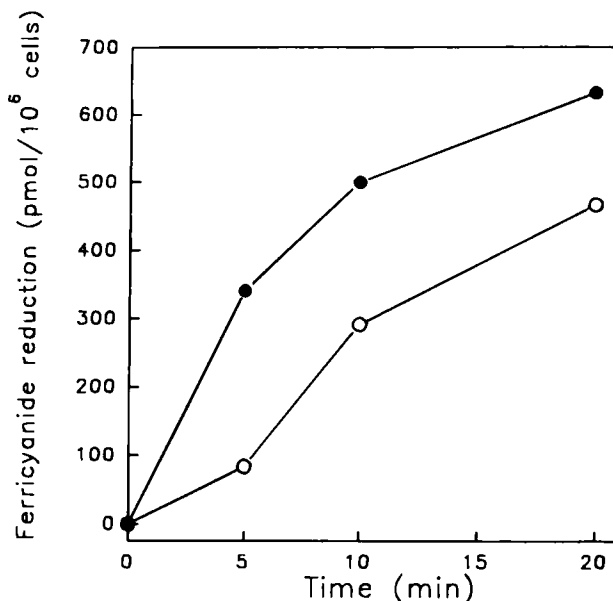
stimulatory effect in a dose-dependent manner, as depicted in Fig. 2. These data and some additional experimental data discussed below concerning the role of intermediates downstream of G proteins considered together seem to indicate that G proteins are indeed involved in the control of PMRS functions.

One of the possible roles of activated G proteins is the activation of phospholipases C, which in turn give rise to a transient increase in the levels of inositol phosphates (Rana and Hokin, 1990), which represent

Table I. Effect of Modification in Intracellular Ca^{2+} Concentrations on Ferricyanide Reductase Activity[a]

Additions	Activity (%)
Thapsigargin (10 μM)	213
Thapsigargin (10 μM) + EGTA (5mM)	94
A23187 (10 μM)	137
Sphingosine (15 mM)	447
A23187 (10 μM) + sphingosine (15 μM)	187

[a] Cell incubations with each compound and ferricyanide reductase measurement were carried out as described in the experimental procedures. 2 mM Ca^{2+} was added in all determinations. Results are presented as a percentage of controls taken as 100%.

critical steps in the transduction of a multitude of signals across animal cell plasma membranes (Berridge, 1993). Neomycin sulfate is described as an inhibitor of phospholipases C (Legendre *et al.*, 1993). Our results confirm that the presence of neomycin sulfate avoids the stimulatory effect of mastoparan on ferricyanide reductase activity.

Evidence for PMRS Regulation by Cyclic Nucleotides

Activation of certain heterotrimeric G proteins results in an activation of adenylate cyclase, and a consequent increase in cytosolic concentration of cAMP, one of the first described second messengers that is required for activation of many cellular events (Bourne and De Franco, 1989).

It has been shown very recently that HL-60 cell PMRS is controlled by cAMP, by using agonists and antagonists of cAMP (Rodríguez-Aguilera *et al.*, 1993). Our results with Ehrlich cells preincubated with dibutyryl cAMP for 5 min to allow intracellular cAMP accumulation (Yoshikawa *et al.*, 1991), confirm those results previously reported. In fact, we detected a 43% activation of Ehrlich cell PMRS activity in the presence of 10 μM dibutyryl cAMP, a value analogous to that obtained in other cell type with a different experimental enzyme assay as an estimation of PMRS activity (Rodríguez-Aguilera *et al.*, 1993).

Although much less is known on the role of cGMP as a second messenger, there is little doubt that it should play an important role in signal transduction (Chinkers and Garbers, 1991). To our knowledge, the present report shows for the first time evidence for a role of cGMP in the control of PMRS. As indicated in Fig. 3, the stimulatory effect of 10 μM dibutyryl cGMP on ferricyanide reductase is quantitatively very similar to that produced by 10 μM dibutyryl cAMP. On the other hand, there is a clear dose–response effect in such a way that 42 μM dibutyryl cGMP produced a 2.5-fold increase in the amount of ferricyanide reduced by Ehrlich cells for 5 min (see Fig. 4).

Complementary data reinforce the arguments in favor of a role for cGMP in the activation of PMRS. Firstly, 8-bromo-cGMP, another relatively permeant analog of cGMP (Perreault and De Marte, 1993), also stimulated PMRS activity. On the other hand, treatment with dipyridamole, a selective inhibitor of cGMP-phosphodiesterase (Nawy and Jahr, 1991), also produced an increase in ferricyanide reductase activity.

A Role for Ca²⁺ as a Second Messenger

Inositol phosphate signaling pathways involve transient increases in cytosolic Ca²⁺ concentrations from 0.1 μM to 0.5–1 μM (Bourne and De Franco, 1989; Irvine, 1992). Data obtained with neomycin sulfate suggested a putative role for Ca²⁺ as a second messanger in the control of PMRS. To test this possiblity, transient increases in cytosolic Ca²⁺ levels were induced through three different ways: (i) by using the Ca²⁺ ionophore A23187 in the presence of extracellular Ca²⁺ ions; (ii) by treatment with thapsigargin, which inhibits the transport of Ca²⁺ into the endoplasmic reticulum (Mendoza and Tesarik, 1993); and (iii) by incubations with sphingosine, which can be transformed by sphingosine kinases in the newly described lipid second messenger sphingosine-1-phosphate, involved in the transient opening of reticulum Ca²⁺ channels (Gosh *et al.*, 1994). As summarized in Table I, the three procedures produced significant increases in PMRS activity. The very high activation induced by sphingosine is remarkable. It is also interesting to remark that the addition of A23187 decreased this activation to almost a fifth of the values obtained in the absence of the Ca²⁺ ionophore. In this context, Chakraburti *et al.* (1990) have described antagonic effects of A23187 and sphingosine. On the other hand, it is noteworthy that the presence of EGTA in the incubation medium abolished the activation produced by thapsigargin.

CONCLUDING REMARKS

The use of activators and inhibitors of intermediary steps in a signal transduction pathway can clarify the role of these steps in the sequence of events leading to a concrete response. Though no individual result can be considered as conclusive evidence for the involvement of certain signal transduction pathways, the cummulative and coincident results shown here offer a congruent and consistent picture of a very complex and tightly regulated process. According to the key processes that seem to play a role in cell physiology (Crane *et al.*, 1985), PMRS should be expected to be accurately regulated by more than one regulatory pathway. In fact, this is the main conclusion that could be obtained from the data given in this report.

Our data confirm those previously published, pointing to a role for G protein (Morré *et al.*, 1993) and cAMP (Rodríguez-Aguilera *et al.*, 1993) on the control of PMRS. The cytosolic concentrations of Ca²⁺

seem to play an important role as second messengers. For the first time evidence is shown for a role of cGMP in the control of PMRS. Our data seem to indicate that extracellular signals could modulate PMRS at least by two different types of signaling pathways involving receptors linked to G proteins, one of them through cyclic nucleotides as second messengers, and the other through a change in cytosolic Ca^{2+} concentrations. The first pathway should involve the action of protein kinases A, as suggested previously (Rodríguez-Aguilera et al., 1993), and/or that of protein kinases activated by cGMP. The second pathway could be started by activation of phospholipases C by G proteins and could give rise to an activation of specific protein kinases C. Several clues indicate a role for certain protein kinases C in the control of PMRS. These kinases are dependent on cytosolic Ca^{2+} and diacylglycerol. As discussed above, neomycin sulfate, an inhibitor of phospholipase C activity, abolishes the activating effect of G proteins. In the context of protein kinase C activities, it should be kept in mind that phospholipase C produce diacylglycerols and inositol triphosphate, the latter releasing intracellular Ca^{2+} from storage. The stimulatory effect of an increase of cytosolic Ca^{2+} has also been discussed above. Thus, the actual involvement of different protein kinases in the modulation of PMRS should be studied in order to clarify the scheme of signal transduction pathways implicated in the control of PMRS.

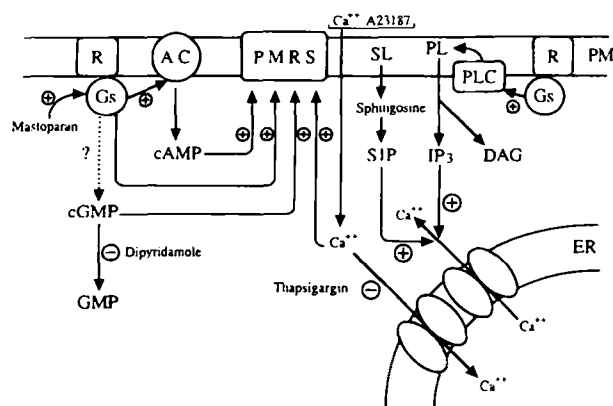Figure 6 summarizes the data presented and discussed in this work.

Fig. 6. Summary of the interactions discussed in the present paper. PM: plasma membrane; R: receptor; AC: adenylate cyclase; Gs: heterotrimeric Gs protein; PMRS: plasma membrane redox system; SL: sphingolipid; PL: phospholipid; PLC: phospholipase C; S1P: sphingosine-1-phosphate; IP3: inositol triphosphate; DAG: diacylglycerol; ER: endoplasmic reticulum.

Finally, consideration should be given to the fact that our results have been obtained in a model system, which is a transformed, neoplastic cell. This fact raises the question whether similar mechanisms of control operate on PMRS of normal, nontransformed cells.

ACKNOWLEDGMENTS

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