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C-*fos* gene activation in murine thymocytes by a mechanism independent of protein kinase C or a Ca^{2+} signal

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The accumulation of c-*fos* mRNA in mouse thymocytes was compared when the cells were stimulated by concanavalin A (Con A), the Ca^{2+} ionophore A23187 or the phorbol ester, TPA, either separately or by combinations of these mitogens. The c-*fos* response to mitogenic concentrations of Con A could not be attributed either to the rise in $[Ca^{2+}]_i$ it induces or to activation of protein kinase C. Thus, although Con A causes the breakdown of phosphatidylinositol 4,5-bisphosphate in these cells, neither of the signals which can be generated by this response was responsible for the activation of the c-*fos* gene by Con A. This implies that some other unidentified signal generated by Con A activates the c-*fos* gene.

C-*fos* gene; Signal transduction; Ionic signal; Protein kinase C; Gene activation; (Lymphocyte)

1. INTRODUCTION

Quiescent lymphocytes can be stimulated to re-enter the cell cycle by a variety of polyclonal mitogens that bind to cell surface receptors. For T cells, antibodies to the T cell receptor complex and to other surface antigens including T11 and *thy* 1.2 have been shown to be mitogenic [1–3]. All of these antibodies cause rapid breakdown of $PtdInsP_2$ to release inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol [4–6]. It is postulated that the signals generated by the two intracellular messengers released by $PtdInsP_2$ breakdown [7], i.e., a rise in $[Ca^{2+}]_i$ mediated by $Ins(1,4,5)P_3$ and the activation of protein kinase C by diacyl glycerol, may be sufficient to drive the cells from G_o through to S phase. This hypothesis is based mainly on the observation that the Ca^{2+} ionophore A23187 in combination with phorbol esters that activate protein kinase C (e.g., 12-*O*-tetradecanoylphorbol 13-acetate (TPA)) can act synergistically as co-mitogens on T cells [8].

The mitogenic action on T cells of lectins such as concanavalin A (Con A) provides an opportunity to test whether there are primary signals other than $PtdInsP_2$ breakdown [9] that are coupled to the mitogenic activation of the cells. Thus Con A binds not only to the T cell receptor but also to many other surface glycoproteins [10], some of which may activate a mitogenic response. If any of these mitogenic receptors operate through primary signals other than $PtdInsP_2$ breakdown, it would be expected that at least a component of the early responses to Con A would not be attributable either to the rise in $[Ca^{2+}]_i$ or to activation of protein kinase C. Previous studies of metabolic responses to Con A in mouse thymocytes (in particular the stimulations of PtdIns synthesis, glycolysis, pH_i increase, and uridine uptake) have provided indirect evidence that these metabolic responses could not be attributed solely to the rise in $[Ca^{2+}]_i$ or to any activation of protein kinase C that may occur [11]. This indirect evidence strongly suggested that unidentified signal(s) from the

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Con A receptors accounted for most if not all of the observed metabolic activation.

Recent studies have indicated that the rapid activation of a set of genes, including the c-*fos* proto-oncogene, may be an obligatory step in the G_0 to S pathway for mitogens acting on lymphocytes and other cells [12–14]. We and others have shown previously that the c-*fos* gene can be activated in mouse thymocytes via an increase in $[Ca^{2+}]_i$ through A23187, or via stimulation of protein kinase C by TPA [15]. In view of the evidence that activation of the gene is important in the early mitogenic pathway, we have compared the activation of the gene by Con A with activation by A23187 or TPA to determine whether Con A is able to stimulate c-*fos* expression by mechanisms other than an increase in $[Ca^{2+}]_i$ or activation of protein kinase C.

2. MATERIALS AND METHODS

2.1. *Cell preparation and $[Ca^{2+}]_i$ measurements*

Murine thymocytes were prepared and incubated in a humidified atmosphere of air/CO_2 (19:1) at 37°C and changes in $[Ca^{2+}]_i$ were determined using quin 2 as described previously [16].

2.2. *RNA preparation and analysis*

RNA was isolated by the guanidinium isothiocyanate/$CsCl_2$ method described in [15] and was fractionated by electrophoresis in 1% agarose/16.7% formaldehyde gels containing 1 μg/ml ethidium bromide. Loading of equal amounts of total RNA into each lane was confirmed by inspection of the gels under 260 nm illumination. RNA was transferred to nitrocellulose and the filters were hybridized for 12 h at 42°C with nick-translated c-*fos* or c-*myc* probes [15] (5×10^8 dpm/μg) in 50% formamide, 1 × Denhardt's solution, 10% dextran sulphate, 4 × SSC, 20 μg/ml sonicated herring sperm DNA. The filters were washed twice for 30 min at room temperature in 250 ml of 2 × SSC, 0.1% SDS and twice for 30 min at 65°C in 250 ml of 0.1 × SSC, 0.1% SDS, and exposed to pre-flashed X-ray film at −70°C. A range of exposures of each filter was obtained to ensure all lanes were in the linear range for the film, and the intensities of c-*fos* or c-*myc* bands were quantitated by scanning densitometry.

3. RESULTS AND DISCUSSION

3.1. *Ca^{2+} dependence of* c-fos *response to A23187, TPA and Con A*

A23187, TPA and Con A caused detectable increases in c-*fos* mRNA in thymocytes within 10 min and maximal responses between 20 and 30 min (shown for A23187 in fig.1A), consistent with previous data [15]. The average values of c-*fos* responses relative to the stimulation caused by 10 μg/ml Con A (100%) were: 1 μg/ml Con A, 22 ± 2%; 10 nM TPA, 94 ± 9%; 10 nM A23187, 110 ± 21%; 30 nM A23187, 460 ± 72%. The dose-response curves showed that stimulation of c-*fos* after 30 min was maximal at 30–50 nM A23187 and above 10 nM TPA (fig.2A,B), similar to the optimal concentrations of these agents for costimulation of DNA synthesis. The c-*fos* response increased progressively with concentration from 0.1 μg/ml to 10 μg/ml Con A (fig.2C) although the optimal mitogenic concentration is about 1 μg/ml. Concentrations of 10 μg/ml Con A are toxic to the cells after several hours [17].

The c-*fos* responses to the three mitogens were compared with the corresponding $[Ca^{2+}]_i$ responses in normal and low Ca^{2+} medium ($[Ca^{2+}]_o = 0.1$ μM) to determine the extent to which the c-*fos* responses might depend on an increase in $[Ca^{2+}]_i$. In low Ca^{2+} medium A23187 at concentrations up to 100 nM caused negligible stimulation of c-*fos* mRNA (fig.2A). The maximal c-*fos* response to A23187 required a free concentration of greater than 0.3 mM in the medium (fig.1B). Re-addition of Ca^{2+} to low Ca^{2+} medium fully restored the c-*fos* response to A23187 (fig.1A) indicating that block of the response was due to the low external Ca^{2+} concentration rather than a toxic effect of the ionophore in this medium. The large, transient $[Ca^{2+}]_i$ response generated by 30 nM A23187 in low Ca^{2+} medium (fig.3A) was insufficient by itself to stimulate significant c-*fos* expression.

The removal of Ca^{2+} from the medium did not significantly affect (< 10%) the response to TPA at concentrations up to 100 nM (fig.2B) or to Con A over the mitogenic concentration range up to 1 μg/ml (fig.3C). However there was a variable decrease in the response (35 ± 15%) at the supramitogenic concentration of 10 μg/ml Con A. These data suggested that at 1 μg/ml of Con A, the $[Ca^{2+}]_i$ response (fig.3B) was too small to contribute significantly to the stimulation of c-*fos* expression but that there may be a minor contribution caused by the increase in $[Ca^{2+}]_i$ at higher Con A concentrations. This is consistent with the negligible c-*fos* response to A23187 at concentrations of about 5 nM (fig.2A) which gave

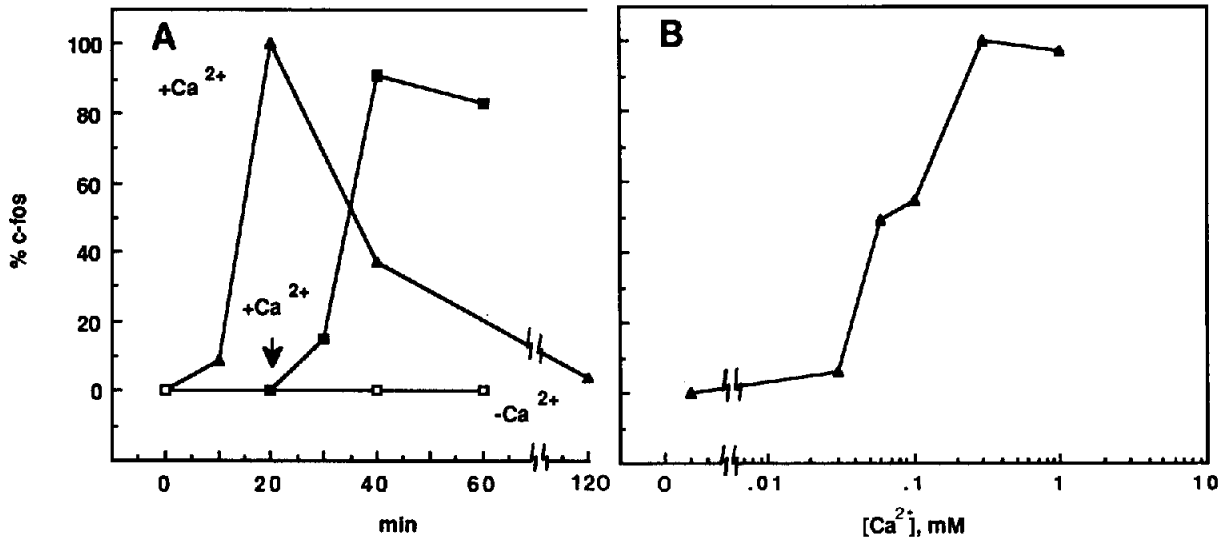

Fig.1. c-*fos* mRNA responses to A23187. The amounts of mRNA are expressed relative to the maximal response in all experiments. (A) Time course of response to 30 nM A23187 in (▲) normal medium, (□) low Ca^{2+} medium ($[Ca^{2+}]_o$ adjusted to 0.1 μM with EGTA), (■) $CaCl_2$ re-added to give 0.43 mM free Ca^{2+} after 20 min in low Ca^{2+} medium. (B) $[Ca^{2+}]_o$ dependence of the response to A23187.

similar $[Ca^{2+}]_i$ responses to 1 μg/ml of Con A (fig.3B,C).

3.2. c-fos *responses to combinations of mitogens*

The conclusion that there is no significant $[Ca^{2+}]_i$-dependent component of the c-*fos* response to mitogenic concentrations of Con A depends on the assumption that the overall c-*fos* response is simply the sum of the separate components that contribute to it. It is possible, however, that the $[Ca^{2+}]_o$ independence of the overall c-*fos* response to Con A is fortuitous if one component of the c-*fos* response is enhanced in low Ca^{2+} medium and compensates exactly for the component of the response which is caused by the $[Ca^{2+}]_i$ increase in normal Ca^{2+} medium. Experiments were therefore performed to determine whether the c-*fos* response to various mitogens were additive in normal and low Ca^{2+} medium.

When combinations of TPA and A23187 were examined, titration of the ionophore concentration in the presence of 10 nM TPA showed that the effects of the two mitogens on the amount of c-*fos* mRNA were approximately additive, although the response to TPA was small relative to the stimulation by 50 nM A23187 (fig.2D). When the experiment was repeated in low Ca^{2+} medium, the response to TPA was unaffected but the component due to A23187 was abolished (fig.2D). These data therefore imply that the c-*fos* responses to activation of protein kinase C and an increase in $[Ca^{2+}]_i$ are approximately additive. To determine whether additive c-*fos* responses were also obtained with the $[Ca^{2+}]_o$-independent response to Con A and an increase in $[Ca^{2+}]_i$, the cells were stimulated with a combination of Con A and A23187 (10 nM). The c-*fos* response to these mitogens was approximately additive in normal Ca^{2+} medium, but reduced to the same level as for Con A alone in Ca^{2+}-free medium (cf. fig.2E and C). These data confirm the conclusion that the c-*fos* response to mitogenic concentrations of Con A does not depend significantly on the $[Ca^{2+}]_i$ response to Con A.

To examine whether the $[Ca^{2+}]_o$-independent c-*fos* response to Con A was due to activation of protein kinase C the effect of combinations of Con A and TPA in normal or low Ca^{2+} medium was examined. The data in fig.2F show that there was a significant c-*fos* response attributable to 1 μg/ml Con A in the presence of 10 nM TPA which was unaffected by the presence of Ca^{2+} in the medium. This component of the c-*fos* response to Con A in low Ca^{2+} medium was very unlikely to be due to the transient $[Ca^{2+}]_i$ increase caused by Con A (fig.3B), since the substantially larger

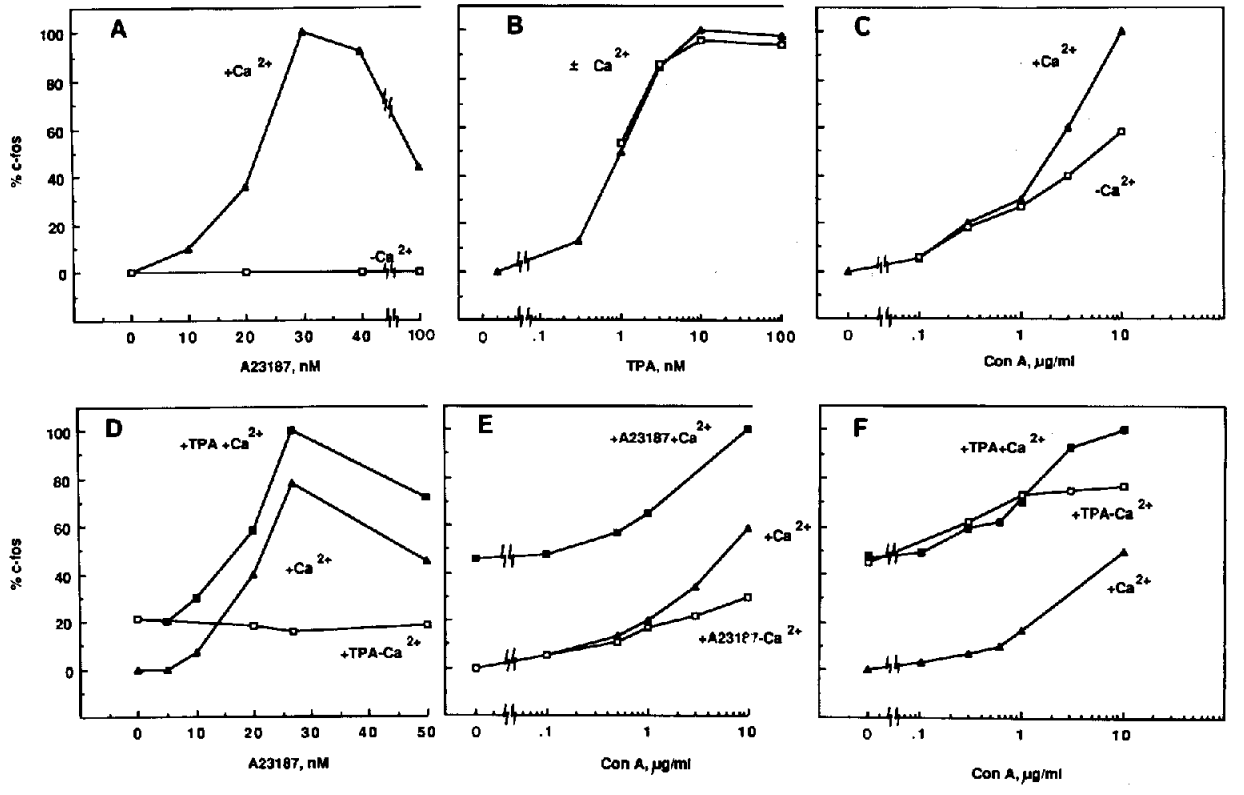

Fig.2. Effect of $[Ca^{2+}]_o$ on the c-*fos* mRNA responses to mitogens after 30 min. Responses to single mitogens: (A) A23187, (B) TPA, (C) Con A; (▲) $[Ca^{2+}]_o$ = 0.43 mM, (□) $[Ca^{2+}]_o$ = 0.1 µM. Responses to combinations of mitogens: (D) 10 nM TPA + A23187, (E) 10 nM A23187 + Con A, (F) 10 nM TPA + Con A; (▲) single mitogen, $[Ca^{2+}]_o$ = 0.43 mM, (■) two mitogens, $[Ca^{2+}]_o$ = 0.43 mM, (□) two mitogens, $[Ca^{2+}]_o$ = 0.1 µM.

$[Ca^{2+}]_i$ transient from A23187 in low Ca^{2+} medium (fig.3A) did not cause a c-*fos* response in combination with 10 nM TPA (see fig.2A). It is therefore concluded that Con A generates a $[Ca^{2+}]_i$-independent signal, other than activation of protein kinase C, which stimulates c-*fos* expression. The approximately additive c-*fos* responses to Con A and TPA also implied that it was unlikely that activation of protein kinase C was a prior requirement for stimulation of c-*fos* by the unidentified $[Ca^{2+}]_o$-independent mechanisms operated by Con A, but suggested that the two mitogens activated the c-*fos* response independently.

The additive pattern of c-*fos* responses to the

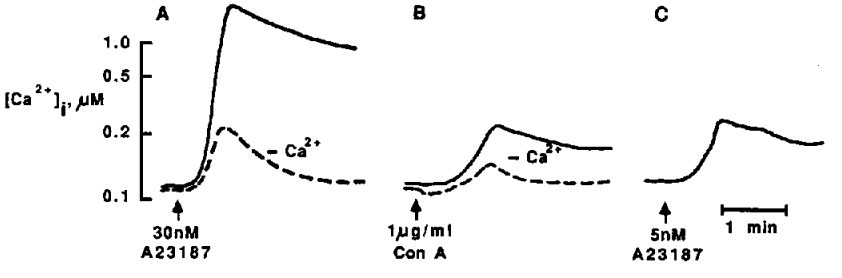

Fig.3. $[Ca^{2+}]_i$ responses to mitogens in normal medium or in low Ca^{2+} medium in thymocytes loaded with quin 2. $[Ca^{2+}]_o$ was adjusted to 0.1 µM by addition of EGTA immediately prior to mitogen addition. (A) 30 nM A23187 ± EGTA; (B) 1 µg/ml Con A ± EGTA; (C) 5 nM A23187.

mitogens and their resolution into $[Ca^{2+}]_o$-dependent and $[Ca^{2+}]_o$-independent components can be rationalised by assuming that mitogenic concentrations of Con A generate a signal that is independent of the $[Ca^{2+}]_i$ increase or of any activation of protein kinase C. This signal accounts for virtually all of the c-*fos* response to Con A at 1 μg/ml. The generation of an unidentified signal is consistent with recent phosphorylation studies showing that Con A causes extensive phosphorylation in these cells independently of any $[Ca^{2+}]_i$ increase or activation of protein kinase C [18]. A plausible candidate for this signal is the activation of a tyrosine kinase, leading directly or otherwise to activation of the c-*fos* gene independently of $[Ca^{2+}]_i$ or protein kinase C. Very rapid tyrosine phosphorylation of a p21 peptide associated with the T cell receptor has been reported for murine hybridoma cells [19], although it remains to be determined whether this response is dependent on $PtdInsP_2$ breakdown.

The implication of the present studies that the c-*fos* gene activation by Con A does not depend on the classical signals generated by $PtdInsP_2$ breakdown should help to focus attention on defining the coupling mechanisms between the putative primary signal from the T cell receptor ($PtdInsP_2$ breakdown) and the activation of early genes believed to be obligatory for the G_o to S transition.

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