Phosphoinositide metabolism and the calcium response to concanavalin A in S49 T-lymphoma cells

A comparison with thymocytes

Michael V. TAYLOR,* T. Robin HESKETH and James C. METCALFE

Department of Biochemistry, Tennis Court Road, Cambridge CB2 1QW, U.K.

Comparisons were made between transformed S49 T-lymphoma cells and normal murine thymocytes in their polyphosphoinositides, inositol polyphosphates and cytosolic free calcium concentrations ($[Ca^{2+}]_i$), and the effects of the T-cell mitogen concanavalin A (Con A) on these properties. 1. The ratios of the polyphosphoinositides to phosphatidylinositol in both exponential-phase S49 cells and mitogen-stimulated thymocytes (G_1 phase) were greater than in quiescent (G_0 -phase) thymocytes. 2. In response to Con A, the amount of phosphatidylinositol 4,5-bisphosphate (PtdIns P_2) in S49 cells decreased slightly (17% in 30 min), and this was sufficient to account for the small amounts of inositol phosphates that accumulated. In contrast, it has been shown previously that Con A stimulates a rapid resynthesis of PtdIns P_2 in thymocytes and the amounts of inositol phosphates released rapidly exceed the steady-state amount of the PtdIns P_2 precursor [Taylor, Metcalfe, Hesketh, Smith & Moore (1984) *Nature* (London) **312**, 462–465]. 3. The $[Ca^{2+}]_i$ did not differ significantly in S49 cells and thymocytes before the addition of Con A, and the increases in $[Ca^{2+}]_i$ in response to Con A were similar in both types of cell. 4. The $[Ca^{2+}]_i$ increase in response to Con A was inhibited by similar concentrations of intracellular cyclic AMP (2–10 μ M) in S49 cells and thymocytes, suggesting that similar regulatory mechanisms act on this response in both types of cell. The data demonstrate that the basal $[Ca^{2+}]_i$ and phosphoinositide metabolism is similar in both the normal cells and their transformed counterparts. In addition, they suggest that the activated Con A receptors generate very similar signals in the two cell types, and that any perturbations of primary signal transduction to the secondary phosphoinositide and $[Ca^{2+}]_i$ responses in the S49 phenotype are quantitative rather than qualitative.

INTRODUCTION

Normal growth-arrested eukaryotic cells, including thymocytes and Swiss 3T3 fibroblasts, respond to stimulation by some mitogens with a rapid breakdown in phosphatidylinositol 4,5-bisphosphate (PtdIns P_2) and a sequential increase in the cytosolic free calcium concentration ($[Ca^{2+}]_i$) and the cytosolic pH [1]. It has been proposed that the two signals generated by PtdIns P_2 breakdown, a rise in $[Ca^{2+}]_i$ through the release of inositol 1,4,5-trisphosphate (Ins P_3) and activation of protein kinase C by diacylglycerol, act synergistically to initiate the mitogenic pathway and drive the cells through to DNA synthesis [2]. However, an analysis of the activation of murine thymocytes by the polyclonal mitogen concanavalin A (Con A) strongly implied that mitogenic signals in addition to those generated by PtdIns P_2 breakdown were required for progression to S phase [3]. Although the ‘dual signal’ hypothesis is very probably an over-simplification, it remains likely that the PtdIns P_2 breakdown activated by the receptors for Con A is necessary, if not sufficient, to activate the mitogenic pathway by this mitogen. Perturbations of polyphospho-

inositide metabolism and the PtdIns P_2 response to mitogenic stimulation are therefore potential mechanisms for oncogenic transformation, as suggested by evidence from fibroblasts transformed by *ras* oncogenes [4–6].

Here we have used the S49 lymphoma cell line as a transformed counterpart of murine thymocytes. The S49 cells are of mouse T-cell origin, are tumorigenic when injected into mice and grow indefinitely in suspension culture [7], where they require stimulation by serum or components of a complex defined medium to proliferate [8]. The S49 cells are similar to normal thymocytes in that their growth is inhibited by a sustained increase in cyclic AMP [9,10], but differ from their normal counterparts in that Con A does not stimulate DNA synthesis [11]. Indeed, their growth is inhibited by relatively high concentrations of Con A [11] that are also supra-mitogenic for murine thymocytes [10]. In general, comparisons of the early responses to growth factors and mitogens in normal cells with their transformed counterparts remain poorly characterized, although such comparisons are relevant to the mechanism of autocrine stimulation of the growth of some transformed cells [12,13]. To determine the similarity of the early responses

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; [cAMP] $_i$, intracellular cyclic AMP concentration; Con A, concanavalin A; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PGE $_1$, prostaglandin E $_1$; PtdIns, phosphatidylinositol; PtdIns P_4 , phosphatidylinositol 4-phosphate; PtdIns P_2 , phosphatidylinositol 4,5-bisphosphate; Ins P , inositol monophosphate; Ins P_2 , inositol bisphosphate; Ins P_3 , inositol trisphosphate; Ins P_4 , inositol 1,3,4,5-tetrakisphosphate.

* To whom reprint requests should be sent. Present address: CRC Molecular Embryology Group, Department of Zoology, Downing Street, Cambridge CB2 3EJ, U.K.

to a mitogen in transformed S49 cells and normal thymocytes, we have studied the early $[Ca^{2+}]_i$ and phosphoinositide responses to Con A in the two cell types, and in particular the modulation by cyclic AMP of the early $[Ca^{2+}]_i$ response. Additionally, these responses and the basal $[Ca^{2+}]_i$ and polyphosphoinositide metabolism were compared to determine whether perturbations of these properties are associated with the S49 cell transformed phenotype.

MATERIALS AND METHODS

Cell cultures and loading with quin2

S49 cells (from the Cell Culture Facility, University of California, San Francisco, CA, U.S.A., and Dr. B. R. Martin, Department of Biochemistry, University of Cambridge) were grown in suspension culture in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% heat-inactivated horse serum (Gibco), 19.4 mM-glucose and 44 mM- $NaHCO_3$ at 37 °C in a humidified atmosphere of CO_2 /air (1:9). Cells in culture medium were loaded with quin2 after centrifugation (5 min, 65 g) and resuspension at 2×10^7 cells/ml in DMEM supplemented with 19.4 mM-glucose and 25 mM-Hepes, pH 7.4. The cells were incubated with 15–20 μ M- $[^3H]$ quin2 acetoxymethyl ester in dimethyl sulphoxide (<0.25% v/v) for 45 min at 37 °C and washed by two centrifugations (3 s in Microfuge) separated by a 15 min incubation. The first resuspension was in DMEM/25 mM-Hepes, pH 7.4, at 2×10^7 cells/ml, and the second in a simplified medium (1 mM- $CaCl_2$, 4.8 mM-KCl, 0.5 mM- $MgCl_2$, 145 mM- $NaCl$, 10 mM-glucose, 10 mM-Hepes, pH 7.4) at 10^7 cells/ml for fluorescence measurements. For experiments at low extracellular $[Ca^{2+}]$, the second resuspension was in the simplified medium without $CaCl_2$ and containing 0.25 mM-EGTA. The total Ca^{2+} in this medium measured with a Perkin-Elmer 380 atomic-absorption spectrophotometer was 5 μ M. The free $[Ca^{2+}]$ was calculated to be 8 nM by using a computer program developed by Dr. A. N. Corps from that of Fabiato & Fabiato [14]. Thymocytes from BALB/c mice were prepared and loaded with quin2 as described previously [15].

Fluorescence measurements and calibration of the fluorescence signal

The fluorescence measurements and calibrations were carried out as described previously [15], with the following exceptions. F_{max} , the fluorescence signal of quin2 saturated with Ca^{2+} , was obtained by the addition of 0.04% Triton X-100. This value was the same as that obtained with 50 nM-ionomycin. $[Ca^{2+}]_i$ values were calculated from fluorescence-intensity measurements integrated over 20 s intervals. As the presence of any extracellular quin2 leads to an over-estimate of $[Ca^{2+}]_i$, the amount of extracellular quin2 was estimated from the immediate decrease in fluorescence on the addition of 0.1 mM- Mn^{2+} (which completely quenches quin2 fluorescence [15]), and all calculated $[Ca^{2+}]_i$ values were corrected accordingly. The intracellular quin2 concentration was estimated by counting a sample of cells for ^3H radioactivity and using a cell volume of 639 fl for S49 cells (measured by ^3H_2O -space determination) and 105 fl for thymocytes [15].

ATP assay

S49 cells were loaded with a range of quin2 concentrations by using the method described for fluorescence measurements, and incubated for 15 min before assay of ATP as described by Pozzan *et al.* [16]. Control cells were treated identically, but without addition of quin2.

Cyclic AMP assay

Cyclic AMP was assayed as described in Moore *et al.* [17]. Care was taken to ensure that the conditions for the assay were as close as possible to those for the fluorescence measurements. All experiments were carried out at 37 °C, and the S49 cells and thymocytes were loaded with quin2 as described above. The ^3H label of quin2 did not interfere with the cyclic AMP assay. Neither Con A (Miles Yeda) nor quin2 had a detectable effect on basal $[cAMP]_i$ or on the elevation of $[cAMP]_i$ stimulated by isoprenaline or prostaglandin E_1 (PGE_1) (Sigma). Measurements of cyclic AMP and $[Ca^{2+}]_i$ after treatment with isoprenaline (in simplified medium) or PGE_1 (in ethanol) were made under the same conditions and at the same time after completion of quin2 loading. This procedure permits direct comparison between the $[Ca^{2+}]_i$ and $[cAMP]_i$ assays, and takes into account the desensitization phenomenon which occurs with time after the isolation of thymocytes, resulting in lesser stimulation of $[cAMP]_i$ by PGE_1 and isoprenaline [3,18].

S49-cell growth assay

Con A was added directly to cultures of S49 cells, and their growth was assayed by measuring cell density. For determinations of quin2 effects, cells were loaded with the indicator as described above before transfer to culture medium: control cells were treated identically but without quin2 loading.

Inositol incorporation into PtdIns

S49 cells were resuspended in simplified medium containing 4 μ Ci of *myo*- $[2-^3H]$ inositol (16.9 Ci/mmol; Amersham International)/ml and incubated in 0.5 ml portions at 37 °C: the incorporation was terminated by addition of 1.88 ml of chloroform/methanol (1:2, v/v), 0.62 ml of water and 0.62 ml of chloroform. The upper phase was removed and the lower phase transferred to glass tubes containing 3 ml of chloroform/methanol (2:1, v/v). The upper phase was removed, and the lower phase was washed with 3×2 ml of a 1:1 (v/v) mixture of chloroform/1 M-KCl and 10 mM-inositol, dried and assayed for ^3H by liquid-scintillation counting.

Labelling S49 cells with $[^3H]$ inositol to assay phosphoinositides and phosphoinositide breakdown

S49 cells were washed by centrifugation (5 min, 65 g) and resuspended at 7×10^5 cells/ml in Basal Medium Eagle diploid (modified) (inositol-free; Flow Laboratories), supplemented with 20 mM- $NaHCO_3$, 10% horse serum and 1.5–2.0 μ Ci of *myo*- $[2-^3H]$ inositol/ml (treated with Dowex resin (formate form) to remove radiolytic decomposition products that interfere with the $[^3H]$ -inositol phosphates assay [19]) and incubated for 16 h in a humidified atmosphere of CO_2 /air (1:19). This procedure labelled the phosphoinositides to steady state, as judged by the amount of ^3H incorporated. The cells were washed twice by centrifugation (5 min, 65 g) into

simplified medium (supplemented with 5 mM-LiCl in the phosphoinositide breakdown experiments to enhance InsP accumulation [20,21]) at 6×10^6 cells/ml. In most phosphoinositide-breakdown experiments, the cells were loaded with quin2 before the final resuspension. No effect of quin2 loading on the accumulation of any of the inositol phosphates was detected.

Assay of phosphoinositides

S49-cell incubations (0.5 ml) were terminated by addition of 1.88 ml of chloroform/methanol (1:2, v/v) and the phosphoinositides were extracted under acidic conditions, deacylated with a methylamine reagent, and the glycerophosphorylinositides were separated by anion-exchange chromatography, as described by Berridge [22]. This procedure converted > 97% of extracted [^3H]lipid into ^3H -labelled water-soluble compounds and > 94% into ^3H -labelled compounds that were eluted in the predicted positions for deacylated PtdIns, PtdInsP and PtdInsP₂. No measurement was made of the positions of the phosphates on the inositol headgroup.

The above assay was compared with an extraction procedure using trichloroacetic acid and acidified chloroform/methanol. Samples extracted by either method were deacylated and products separated as described above. A similar amount of ^3H -labelled chloroform-soluble material was extracted by either procedure, but with the trichloroacetic acid method 22% of the ^3H label was recovered in the fraction eluted by water, which, in the method of Berridge [22], is used to remove a small amount of free inositol (about 3% of the total label in our experiments). This 22% of ^3H label was unidentified, and, because of this anomalous behaviour of the water-soluble deacylation products after trichloroacetic acid extraction, the method of Berridge [22] was used. This gave reproducible results, although the proportion of the phosphoinositides extracted is not defined. We note that problems with using trichloroacetic acid for termination have been found in phosphoinositide extraction from yeast [23] and in the assay of methylated phospholipid from lymphocytes [24]. For experiments with thymocytes, the cells were labelled with [^3H]inositol as described previously [25], and the phosphoinositides were assayed as described above.

Assay of phosphoinositide breakdown

S49-cell incubations (0.5 ml) were terminated with 1.88 ml of chloroform/methanol (1:2, v/v) and the inositol phosphates were assayed as described previously [19,22]. Loading S49 cells with [^3H]quin2 did not interfere with this assay. Direct quantitative comparisons between the phosphoinositides and inositol phosphates were not possible, as there are small differences in their efficiency of recovery and because the extent of inhibition by Li⁺ of inositol-1-phosphatase is not known.

RESULTS AND DISCUSSION

[Ca²⁺]_i measurements in S49 cells

S49 cells loaded with quin2 under the standard conditions described in the Materials and methods section had an average intracellular [quin2] of 0.94 ± 0.04 mM (S.E.M., $n = 47$) and a calculated [Ca²⁺]_i of 88 ± 3 nM ($n = 47$), which is similar to the [Ca²⁺]_i for mouse thymocytes of 97 ± 4 nM ($n = 13$) loaded with

0.85 ± 0.04 mM-quin2. S49 cells showed an increase in apparent [Ca²⁺]_i with increasing intracellular [quin2] from 0.1 to 1 mM, but an approximately constant [Ca²⁺]_i at higher intracellular [quin2] (results not shown), similar to the profile reported previously for thymocytes [15]. A plausible explanation for this effect is that the cells contain significant amounts of metal ions (Mⁿ⁺, e.g. Zn²⁺) that quench fluorescence and have a higher affinity for the indicator than has Ca²⁺ [15]. Quenching of quin2 fluorescence by Mⁿ⁺ ions to give very low apparent [Ca²⁺]_i values (< 40 nM) has been reported for Ehrlich ascites-tumour cells [26], and the ¹⁹F-n.m.r. Ca²⁺ indicator, 5FBAPTA, revealed a significant amount of Zn²⁺ in these cells [27]. 5FBAPTA measurements, which are unaffected by the presence of Zn²⁺, showed [Ca²⁺]_i to be approx. 100 nM in the Ehrlich ascites-tumour cells [27], and taken with the present data for S49 cells this suggests that an altered [Ca²⁺]_i is not a general characteristic of transformed cells.

Con A-induced [Ca²⁺]_i responses

For most experiments S49 cells were loaded with approx. 1.0 mM-quin2, which had no effect on the cellular ATP concentration of 6.3 mM, but which blocked cell growth for approx. 17 h before the normal growth rate was resumed (Fig. 1). All of the [Ca²⁺]_i responses to Con A and other agents described below were therefore examined at a lower intracellular [quin2] of approx. 0.3 mM, which had no effect on cell growth. Similar [Ca²⁺]_i responses over 15 min were obtained at both intracellular [quin2] values, but the higher of these was used routinely to obtain an improved signal-to-noise ratio.

Con A (5 µg/ml) caused an increase in fluorescence from quin2-loaded S49 cells (Fig. 2a), but had no effect on autofluorescence in unloaded cells. The response corresponded to an increase in [Ca²⁺]_i from 88 ± 3 nM in unstimulated cells to 133 ± 5 nM (S.E.M., $n = 41$) after the addition of Con A (Fig. 2b), a mean increase of 50%. No release of quin2 into the medium was detected, which would cause an increase in quin2 fluorescence and hence in the apparent [Ca²⁺]_i. The [Ca²⁺]_i response in S49 cells started after a delay of about 1 min, and the response was maximal in 7–8 min. In thymocytes, loaded with quin2 as described above, Con A (1 µg/ml) stimulated a [Ca²⁺]_i increase from 97 ± 4 nM with a delay of approx. 15 s to reach a maximal [Ca²⁺]_i of 173 ± 7 nM in 2–3 min.

In medium containing free Ca²⁺ buffered at 8 nM with EGTA, there was a steady decrease in the fluorescence signal from quin2-loaded S49 cells attributable to a decrease in [Ca²⁺]_i as the total cell Ca²⁺ declined through net efflux (Fig. 2a). In this low-extracellular-[Ca²⁺] medium there was an increase in [Ca²⁺]_i in response to Con A (5 µg/ml), although the response was only 20–40% of that in normal medium (Fig. 2b). This suggests that there is a component of the [Ca²⁺]_i response in S49 cells attributable to intracellular release, similar to that observed in quiescent thymocytes [1]. However, the cells differed in that NaN₃ caused a transient increase in [Ca²⁺]_i in quiescent thymocytes in low-Ca²⁺ medium, owing to intracellular Ca²⁺ release [1], whereas there was no comparable effect of NaN₃ on S49 cells.

The [Ca²⁺]_i response to Con A in S49 cells was also examined in the presence of 10% horse serum, which is present in the normal growth medium, to determine whether, in the presence of the growth factors in serum,

Fig. 1. Effect of quin2 on S49 cell growth and intracellular ATP concentrations

The growth of S49 cells treated as follows was assayed by cell number: ●, cells loaded with 0.28 mM-quin2; ▲, cells loaded with 1.11 mM-quin2; ○, cells treated as for quin2 loading, but with quin2 acetoxymethyl ester omitted; △, cells in normal exponential growth. Insert: effect of intracellular [quin2] on intracellular [ATP] ($[ATP]_i$); the latter was assayed 15 min after completion of the quin2 loading procedure. The results are expressed relative (%) to untreated cells ($[ATP]_i = 6.3$ mM). Most points are means of at least two separate determinations.

Con A is able to activate the receptors through which the $[Ca^{2+}]_i$ response is generated. Fig. 3 shows that $[Ca^{2+}]_i$ increases of up to at least 50% of the maximal response observed in the absence of serum were obtained under these conditions, albeit at much higher Con A concentrations. It should be noted, however, that glycoproteins in serum bind substantial amounts of Con A, lowering the free concentration in the medium. The data imply that the Con A receptors coupled to the $[Ca^{2+}]_i$ response are not substantially activated by the growth factors in serum. The data also show that some inhibition of growth was observed at Con A concentrations that did not cause a detectable increase in $[Ca^{2+}]_i$ (Fig. 3), suggesting that inhibition of growth by Con A is not directly correlated with activation of the receptors that cause the $[Ca^{2+}]_i$ response.

Effect of cyclic AMP on $[Ca^{2+}]_i$

Cyclic AMP is known to antagonize $[Ca^{2+}]_i$ increases in many cell types, including thymocytes [28,29]. Its effect was studied quantitatively to determine whether a similar $[Ca^{2+}]_i$ -regulatory system operates in the normal and transformed cells under investigation. Both isoprenaline and PGE_1 increased the intracellular concentration of cyclic AMP ($[cAMP]_i$) in S49 cells. The $[cAMP]_i$ response to these agents was rapid and coincided with a reversal of the $[Ca^{2+}]_i$ response to Con A (Figs. 4a and 4b). Partial reversal of the $[Ca^{2+}]_i$ response by both agents was detectable at a concentration of 1 nM, and the maximal effect of up to 90% reversal of the $[Ca^{2+}]_i$ increase was obtained at concentrations above 100 nM.

Fig. 2. Con A-induced increase in $[Ca^{2+}]_i$ in S49 cells

(a) Typical fluorescence signals from quin2-loaded S49 cells 10–15 min after resuspension (see the Materials and methods section) either in standard simplified medium, which contains 1 mM- Ca^{2+} (upper trace), or in simplified medium in which the free Ca^{2+} concentration was buffered to 8 nM with EGTA (lower trace). Con A (5 μ g/ml) was added as indicated. (b) $[Ca^{2+}]_i$ calculated from the integrated fluorescence data shown in (a) as described in the Materials and methods section: ●, 1 mM- Ca^{2+} ; ▲, 8 nM- Ca^{2+} .

The relationship between the reversal of the $[Ca^{2+}]_i$ response and $[cAMP]_i$ is shown in Fig. 4(d). Prior addition of the $[cAMP]_i$ -elevating agents or of the membrane-permeant cyclic AMP analogue 8-bromo cyclic AMP (0.1–10 mM) before Con A decreased the $[Ca^{2+}]_i$ response to the same extent as in the reversal experiments, but did not affect significantly the time

Fig. 3. Effect of Con A on $[Ca^{2+}]_i$ and growth of S49 cells in the presence of 10% horse serum

The $[Ca^{2+}]_i$ increase was measured after the addition of a range of concentrations of Con A to quin2-loaded S49 cells preincubated for 10 min in the absence (○) or presence (●) of 10% horse serum. The results in the presence of serum, added 10 min before Con A, are from three determinations (means $\pm$ S.D.) and those in the absence of serum are the means of two determinations. S49 cell growth (▲) was assayed by the increase in cell number above the starting number 48 h after the addition of Con A and is expressed relative (%) to control cells without added Con A (data are from three separate experiments).

course of the $[Ca^{2+}]_i$ response to Con A. In thymocytes, the effect on the Con A-induced $[Ca^{2+}]_i$ response of elevating $[cAMP]_i$ to 2–10 μM with isoprenaline (Fig. 4c) or PGE_1 was similar to that in S49 cells, except that the antagonism was less pronounced. Thus, concentrations of agonist that elevated $[cAMP]_i$ by up to 20-fold reversed the $[Ca^{2+}]_i$ response to Con A by < 50% (Figs. 4c and 4d). Nevertheless, the $[Ca^{2+}]_i$ response in both S49 cells and thymocytes is inhibited over the same $[cAMP]_i$ range, which is consistent with a similar mechanism of inhibition in the two cell types.

None of the $[cAMP]_i$ -elevating agents affected the basal $[Ca^{2+}]_i$ in either S49 cells or thymocytes. Furthermore, in contrast with the marked antagonistic action of 1 μM -isoprenaline on the $[Ca^{2+}]_i$ response to Con A in both cell types, this concentration of isoprenaline did not

reverse the $[Ca^{2+}]_i$ increase of 60% caused by the Ca^{2+} ionophore ionomycin. These results suggest that, in the absence of Con A, cyclic AMP does not affect the Ca^{2+} fluxes across the plasma membrane at either resting or elevated $[Ca^{2+}]_i$. Evidence that β -adrenergic stimulation has no effect on Ca^{2+} efflux from S49 cells is consistent with this conclusion [30]. Taking these data together, it is unlikely that cyclic AMP exerts its antagonistic effect in S49 cells by modulating the Ca^{2+} -transport proteins in the plasma membrane that regulate $[Ca^{2+}]_i$. It remains possible, however, that the effect of cyclic AMP on these fluxes is only exerted after they have been modulated by Con A.

An alternative explanation of the effects of cyclic AMP on $[Ca^{2+}]_i$ responses to Con A is that cyclic AMP inhibits the activating pathway before the $[Ca^{2+}]_i$ increase, either by direct effects on the Con A receptor which initiates the response, or by inhibiting subsequent intermediate steps in the pathway. If cyclic AMP inhibited the primary signal from the Con A receptor, it would be expected to antagonize all of the early responses in thymocytes or S49 cells. This holds for all of the responses to Con A that have been examined in thymocytes [3,10], except for the increase in amount of $PtdInsP_2$, which is enhanced by cyclic AMP [25]. It is therefore concluded that cyclic AMP is unlikely to inhibit the primary signal from the Con A receptor.

In a wide variety of cells $[Ca^{2+}]_i$ is increased via $InsP_3$ [31], and possibly $InsP_4$ [32], released by receptor-linked $PtdInsP_2$ hydrolysis. Hence, an explanation for the inhibitory effect of cyclic AMP on the $[Ca^{2+}]_i$ response is that cyclic AMP inhibits $PtdInsP_2$ phosphodiesterase action. Consistent with this hypothesis, cyclic AMP inhibits $PtdInsP_2$ hydrolysis in response to Con A in thymocytes, as assayed by $InsP_3$ accumulation [25], and preliminary results indicate a similar effect in S49 cells. Since cyclic AMP has no detectable effect on basal $InsP_3$ concentrations in either type of cell, the postulated inhibitory action of cyclic AMP would only affect the activated phosphodiesterase, which would account for the net increase in $PtdInsP_2$ in Con A-stimulated thymocytes treated with cyclic AMP [25]. This hypothesis is consistent with a role for $PtdInsP_2$ hydrolysis in generating the $[Ca^{2+}]_i$ response to Con A in S49 cells.

Con A-induced phosphoinositide responses

The incorporation of $[^3H]$ inositol into the phosphoinositides was faster in growing S49 cells than in quiescent

Table 1. Phosphoinositides in thymocytes and S49 cells

The phosphoinositides $PtdIns$, $PtdInsP$ and $PtdInsP_2$ in S49 cells and thymocytes were assayed as described in the Materials and methods section. The results are means $\pm$ S.E.M. for n individual determinations.

Cell type	Treatment	$\frac{PtdInsP}{PtdIns}$	(%)	$\frac{PtdInsP_2}{PtdIns}$	(%)	$\frac{PtdInsP + PtdInsP_2}{PtdIns}$	(%)
S49	None	0.71 ± 0.02	(16)	2.46 ± 0.05	(16)	3.17 ± 0.06	(16)
Thymocyte	None	0.50 ± 0.02	(43)	1.63 ± 0.04	(43)	2.13 ± 0.06	(43)
Thymocyte	Con A (1 $\mu g/ml$) (20 min)	0.71 ± 0.03	(9)	1.90 ± 0.08	(9)	2.61 ± 0.07	(9)
Thymocyte	5–10 nM-TPA + 30–100 nM-A23187 (20 min)	0.99 ± 0.07	(4)	2.38 ± 0.06	(4)	3.38 ± 0.07	(4)

Fig. 4. Effect of cyclic AMP on Con A-induced increases in $[Ca^{2+}]_i$ in S49 cells and thymocytes

(a), (b) and (c): Con A [$5 \mu\text{g/ml}$ in (a) and (b), $1 \mu\text{g/ml}$ in (c)] was added to suspensions of quin2-loaded S49 cells (a and b) or thymocytes (c) as indicated. When the $[Ca^{2+}]_i$ increase was complete, $1 \mu\text{M}$ -isoprenaline or -PGE_1 was added ($\bullet$). The time course of the $[cAMP]_i$ increase caused by $1 \mu\text{M}$ -isoprenaline or PGE_1 ($\circ$) was determined under identical conditions, as described in the Materials and methods section. (d): Con A was added as in (a), (b) and (c) to S49 cells ($\bullet$, $\blacktriangle$) or thymocytes ($\circ$, $\triangle$). When the resulting $[Ca^{2+}]_i$ increase was complete (8 min for S49 cells, 3 min for thymocytes) a range of concentrations (0.5 nM – $10 \mu\text{M}$) of isoprenaline ($\bullet$, $\circ$) and PGE_1 ($\blacktriangle$, $\triangle$) was added. The reversal in $[Ca^{2+}]_i$ was complete after 2–3 min in both cell types. The $[cAMP]_i$ at 2 min after each of these additions of isoprenaline or PGE_1 was determined under the same conditions. Each point is the mean of at least two separate determinations. [In these experiments a small reversal owing to the addition of ethanol (0.1% final concn.), the solvent for PGE_1 , was sometimes observed.]

thymocytes, reaching half the steady-state value in 4.5 h compared with 9 h (results not shown). In S49 cells stimulated by Con A ($5 \mu\text{g/ml}$) the rate of incorporation of $[^3\text{H}]$ inositol into PtdIns increased by $24 \pm 8\%$ (S.D., $n = 5$), which is comparable with the effect of Con A ($1 \mu\text{g/ml}$) on thymocytes [15].

At steady state, the ratio of polyphosphoinositides to PtdIns was $3.17 \pm 0.06\%$ (S.E.M.) for S49 cells, compared with a ratio of $2.13 \pm 0.06\%$ for unstimulated thymocytes, increasing to $2.61 \pm 0.07\%$ and $3.38 \pm 0.07\%$ when the thymocytes were stimulated with mitogenic concentrations of Con A or TPA + A23187 respectively. Similar patterns of responses were observed in the individual polyphosphoinositides (see Table 1). Thus, when compared in the proliferating state, the proportion of polyphosphoinositides to PtdIns was similar in the normal and transformed cells. These ratios were measured by the same methods in both types of cell, and are therefore directly comparable in spite of the technical uncertainties in the absolute values (see the Materials and methods section). The membrane concentrations of

phosphorylated phosphoinositides in the two cell types may also be very similar, as the proportion of PtdIns relative to total phospholipid has been found to be similar in normal fibroblasts and their transformed counterparts [4,33].

Con A stimulated a slow decrease in PtdIns P_2 of 17% over 30 min in S49 cells, with little change in the amounts of PtdIns P or PtdIns (Fig. 5). This may be contrasted with the responses reported previously for mouse thymocytes [25], in which, after an initial decrease in the amount of PtdIns P_2 , there was a net increase in the phosphorylation of the phosphoinositides in response to Con A (Fig. 5). The two types of cell also differed in their responses to 10 nM-TPA or 100 nM-A23187, which both stimulate net phosphorylation of the phosphoinositides in mouse thymocytes [25], but have no significant effect on either polyphosphoinositide ($< 8\%$) in S49 cells after 30 min. This comparison of the effects of three mitogens on S49 cells and thymocytes suggests that the relatively high proportion of polyphosphoinositides in S49 cells is related to their rapid rate of growth.

Fig. 5. Comparison of the effect of Con A on the polyphosphoinositides in S49 cells and thymocytes

Con A ($10 \mu\text{g/ml}$) was added to [^3H]inositol-labelled S49 cells, and the amounts of PtdInsP ($\blacktriangle$) and PtdInsP₂ ($\bullet$) were assayed as described in the Materials and methods section. The results are expressed relative (%) to untreated control (mean $\pm$ s.d.) and are from at least four determinations in two separate experiments. In control untreated cells the mean amount of [^3H]PtdIns was 105480 d.p.m./ 10^6 cells, of [^3H]PtdInsP was 740 d.p.m./ 10^6 cells, and of [^3H]PtdInsP₂ was 2560 d.p.m./ 10^6 cells. The data for PtdInsP ($\triangle$) and PtdInsP₂ ($\circ$) for thymocytes are from Taylor *et al.* [25]. The amount of [^3H]PtdIns was constant throughout the time course shown in both S49 cells and thymocytes. The decrease of 17% in PtdInsP₂ over 30 min was significant by Student's *t* test ($P < 0.01$).

Con A-induced inositol phosphate responses

Inositol phosphates were assayed to determine whether the increased amounts of the polyphosphoinositides in S49 cells, compared with quiescent thymocytes, were associated with similarly elevated amounts of inositol phosphates in S49 cells. The inositol phosphates ratios were calculated on the assumption that the specific radioactivities of each of the inositol phosphates and phosphoinositides were the same. This is very probable, as the time course of labelling of each phosphoinositide with [^3H]inositol to steady state was similar, which is consistent with other data, which show that the monoester phosphates of PtdInsP₂ and PtdInsP turn over much more rapidly than other parts of the lipids [34]. The ratio of total inositol phosphates to the phosphoinositides in S49 cells labelled with [^3H]inositol to steady state was $0.93 \pm 0.08\%$ (S.E.M., $n = 6$), compared with $0.90 \pm 0.05\%$ ($n = 12$) for quiescent thymocytes [25]. The ratios for the separated inositol phosphates were 0.75%, 0.08% and 0.11% for InsP, InsP₂ and InsP₃ respectively in S49 cells, similar to the ratios obtained for quiescent thymocytes [25]. Additionally, when thymocytes were stimulated with a mitogenic combination of A23187 plus

Fig. 6. Time courses of the stimulation of phosphoinositide hydrolysis and the increase in $[\text{Ca}^{2+}]_i$ by Con A in S49 cells

S49 cells were labelled with [^3H]inositol and loaded with quin2 as described in the Materials and methods section, treated with 5 or $10 \mu\text{g}$ of Con A/ml and assayed simultaneously for inositol phosphates and $[\text{Ca}^{2+}]_i$. (a) $\bullet$, [^3H]InsP₃; $\circ$, $[\text{Ca}^{2+}]_i$; (b) [^3H]inositol phosphates. Points with error bars are means $\pm$ S.E.M. of at least three determinations. The results are expressed relative (%) to control values in cells not treated with Con A ([^3H]InsP₃, 100 d.p.m./ 10^6 cells, equivalent to 90 c.p.m. per sample; [^3H]InsPs, 950 d.p.m./ 10^6 cells, equivalent to 850 c.p.m. per sample).

TPA, there was no effect on the inositol phosphates [3]. There is therefore no evidence that elevated amounts of inositol phosphates are obligatory for cell growth in either the transformed cells or normal thymocytes. Additionally, the amounts of inositol phosphates in the two cell types are similar when their Con A receptors are not activated.

The effect of Con A ($5\text{--}10 \mu\text{g/ml}$) on the $[\text{Ca}^{2+}]_i$ in S49 cells was compared with the accumulation of InsP₃, which is a direct indication of PtdInsP₂ hydrolysis (Fig. 6a). The earliest time at which the InsP₃ increase was statistically significant was 10 min (Student's *t* test, $P < 0.01$), when the $[\text{Ca}^{2+}]_i$ increase was already maximal, although the total inositol phosphates increase was

significant at 5 min (Student's *t* test, $P < 0.01$). The data show that Con A stimulates early increases in both PtdIns P_2 hydrolysis and $[Ca^{2+}]_i$ in S49 cells and that the $[Ca^{2+}]_i$ response is sustained during the continuous accumulation of Ins P_3 , but they do not define whether Ins P_3 release precedes the $[Ca^{2+}]_i$ response in S49 cells. It may be noted that a recent sub-second kinetic study indicated that the hormonally induced increase in $[Ca^{2+}]_i$ in GH4 cells preceded any detectable increase in Ins P_3 [35]. Fig. 6(b) also shows the initial increase in the amount of total inositol phosphates, which is much smaller (10-fold at 15 min) than in thymocytes [25]. This precluded analysis of the Ins P_3 isomers or of whether PtdIns P_2 was the only phosphoinositide initially hydrolysed. However, the accumulation of Ins P_3 and the concomitant decrease in PtdIns P_2 , with no change in the amounts of PtdIns P or PtdIns, described above (see Fig. 5) is consistent with a small activation of PtdIns P_2 phosphodiesterase by the Con A receptors. Furthermore, the decrease in PtdIns P_2 at 15 min (339 d.p.m./ 10^6 cells) was comparable with the corresponding increase in total inositol phosphates (227 d.p.m./ 10^6 cells), suggesting that all of the inositol phosphates were derived from PtdIns P_2 . The data for S49 cells therefore differ significantly from those for thymocytes stimulated by Con A, where there is substantial and rapid resynthesis of PtdIns P_2 to account for the amounts of inositol phosphates released [25]. The simplest explanation of the data is that exponential-phase S49 cells grown in serum are already stimulated to synthesize PtdIns P_2 at the maximal net rate of which the cells are capable, but that serum does not stimulate PtdIns P_2 breakdown or accumulation of inositol phosphates. Maximal stimulation of PtdIns P_2 synthesis in the exponential-phase cells would account for the high polyphosphoinositides/PtdIns ratio and the inability of TPA, A23187 or Con A to stimulate a net increase in PtdIns P_2 or PtdIns P in S49 cells. The ability of Con A to stimulate a $[Ca^{2+}]_i$ increase in these cells, even in the presence of serum, suggests that PtdIns P_2 breakdown occurs under these conditions, and this response is not stimulated significantly by serum. More complicated explanations of the data cannot be rigorously excluded, but it would appear that transduction of the primary signal to the secondary phosphoinositide and $[Ca^{2+}]_i$ responses is very similar in S49 cells and thymocytes.

Conclusions

It is probable that the Con A-induced $[Ca^{2+}]_i$ increase in S49 cells is produced by a similar mechanism to the persistent $[Ca^{2+}]_i$ response in thymocytes [1]. Both responses show extracellular Ca^{2+} -dependence (the present paper; [1]) and are inhibited by cyclic AMP over the same intracellular concentration range of 2–10 μ M. The persistent $[Ca^{2+}]_i$ response correlates closely with the release of inositol phosphates, but a causal relationship has not been established. Such a relationship would imply a role for PtdIns P_2 or inositol phosphates (e.g. Ins P_3 [36] or Ins P_4 [32,37]) in regulating $[Ca^{2+}]_i$ through Ca^{2+} fluxes across the plasma membrane. It is likely that the elevated contents of polyphosphoinositides in S49 cells are characteristic of their continuous growth. The observation that S49 cells and thymocytes respond similarly to the mitogen Con A underlines the need for more extensive comparisons of responses between normal and transformed cells. It is highly probable that

such comparisons will be essential in understanding the role of autocrine stimulation in transformed cell growth.

We thank John Moore for many discussions and critical reading of the manuscript, Gerry Smith for synthesizing the [^{3} H]quin2 and Tony Corps for developing the calcium computer program. This work was supported by an MRC Studentship to M.V.T. and an SERC Special Project grant to J.C.M.

REFERENCES

- Hesketh, T. R., Moore, J. P., Morris, J. D. H., Taylor, M. V., Rogers, J., Smith, G. A. & Metcalfe, J. C. (1985) *Nature* (London) **313**, 481–484
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Metcalfe, J. C., Hesketh, T. R., Smith, G. A., Morris, J. D. H., Corps, A. N. & Moore, J. P. (1985) *J. Cell Sci. Suppl.* **3**, 199–228
- Fleischman, L. F., Chahwala, S. B. & Cantley, L. (1986) *Science* **231**, 407–410
- Wolfman, A. & Macara, I. G. (1987) *Nature* (London) **325**, 359–361
- Wakelam, M. J. O., Davies, S. A., Houslay, M. D., McKay, I., Marshall, C. J. & Hall, A. (1986) *Nature* (London) **323**, 173–176
- Horibata, K. & Harris, A. W. (1970) *Exp. Cell Res.* **60**, 61–77
- Darfler, F. J., Murakami, H. & Insel, P. A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5993–5997
- Coffino, P., Gray, J. W. & Tomkins, G. M. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 878–882
- Ling, N. R. & Kay, J. E. (1975) *Lymphocyte Stimulation*, North-Holland/Elsevier, Amsterdam and New York
- Ralph, R. K. & Nakoinz, I. (1973) *J. Natl. Cancer Inst.* **51**, 883–890
- Sporn, M. B. & Todaro, G. J. (1980) *N. Engl. J. Med.* **303**, 878–880
- Johansson, A., Betsholtz, C., Heldin, C. H. & Westermark, B. (1985) *Nature* (London) **317**, 438–440
- Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- Hesketh, T. R., Smith, G. A., Moore, J. P., Taylor, M. V. & Metcalfe, J. C. (1983) *J. Biol. Chem.* **258**, 4876–4882
- Pozzan, T., Corps, A. N., Montecucco, C., Hesketh, T. R. & Metcalfe, J. C. (1980) *Biochim. Biophys. Acta* **602**, 558–566
- Moore, J. P., Smith, G. A., Hesketh, T. R. & Metcalfe, J. C. (1983) *Biochem. J.* **216**, 207–213
- Nordeen, S. K. & Young, D. A. (1978) *J. Biol. Chem.* **253**, 1234–1239
- Moore, J. P., Johannsson, A., Hesketh, T. R., Smith, G. A. & Metcalfe, J. C. (1984) *Biochem. J.* **221**, 675–684
- Hallcher, L. M. & Sherman, W. R. (1980) *J. Biol. Chem.* **255**, 10896–10901
- Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595
- Berridge, M. J. (1983) *Biochem. J.* **212**, 849–858
- Hanson, B. A. & Lester, R. L. (1980) *J. Lipid Res.* **21**, 309–315
- Moore, J. P., Smith, G. A., Hesketh, T. R. & Metcalfe, J. C. (1982) *J. Biol. Chem.* **257**, 8183–8189
- Taylor, M. V., Metcalfe, J. C., Hesketh, T. R., Smith, G. A. & Moore, J. P. (1984) *Nature* (London) **312**, 462–465
- Arslan, P., Di Virgilio, F., Beltrane, M., Tsien, R. Y. & Pozzan, T. (1985) *J. Biol. Chem.* **260**, 2719–2727
- Metcalfe, J. C., Hesketh, T. R. & Smith, G. A. (1985) *Cell Calcium* **6**, 183–195

28. Berridge, M. J. (1975) *Adv. Cyclic Nucleotide Res.* **6**, 1-98
29. Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *Nature (London)* **295**, 68-71
30. Erdos, J. J. & Maguire, M. E. (1983) *J. Physiol. (London)* **337**, 351-371
31. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315-321
32. Irvine, R. F. & Moore, R. M. (1986) *Biochem. J.* **240**, 917-920
33. Diringer, H. & Friis, R. R. (1977) *Cancer Res.* **37**, 2979-2984
34. Downes, C. P. & Michell, R. H. (1982) *Cell Calcium* **3**, 467-502
35. Tashjian, A. H., Heslop, J. P. & Berridge, M. J. (1987) *Biochem. J.* **243**, 305-308
36. Kuno, M. & Gardner, P. (1987) *Nature (London)* **326**, 301-304
37. Zilberman, Y., Howe, L. R., Moore, J. P., Hesketh, T. R. & Metcalfe, J. C. (1987) *EMBO J.* **6**, 957-962

Received 24 April 1987/21 August 1987; accepted 30 September 1987