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Cytosolic Phospholipase C Activity: II. Relationship to Concanavalin A-Induced Phosphatidylinositol-Turnover in Splenocytes

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Abstract We have described in the first paper the coupling between cytosolic Giα and cytosolic PLC activity in a cell free preparation. In order to establish the functional significance of the cytosolic Giα coupled soluble PLC, we examined the effects of DEX, NaF, and trifluoperizine (TFP) on concanavalin A (Con A)-induced PI-turnover in intact splenocytes and, in parallel, on soluble PLC activity in cytosol preparations. Cytosolic PLC activity was measured with [^{3}H]PI and [^{3}H]PIP_2 as substrates. 1) The Con A-induced increase (2–4 fold) in PI-turnover in intact splenocytes was paralleled by an 1.2–5-fold increase in soluble PLC activity in vitro. Con A administration also increased cytosolic Giα immunoreactivity 3–6-fold as expected if cytosolic Giα was coupled to soluble PLC activation. 2) DEX (10^{-7} M), administered 6 h prior to Con A administration, inhibited the Con A-induced increase in PI-turnover in intact splenocytes. This was paralleled by DEX inhibition of the Con A-induced increase in soluble PLC activity measured in vitro and cytosolic Giα immunoreactivity. 3) We have demonstrated in the first paper that NaF and TFP inhibited soluble PLC activity. Here we show that NaF and TFP inhibited the Con A-induced increase in PI-turnover extending the similarities between soluble PLC activity and Con A-stimulated PLC activity in intact splenocytes. 4) In order to examine whether or not the Con A-induced PLC was similar to PLCγ, we measured PI-turnover induced by Con A or $NaVO_3$ in combination with DEX and PMA. Whereas the Con A-induced PI-turnover was significantly inhibited (40–60%) by DEX, the $NaVO_3$-induced PI-turnover was not affected by DEX. The Con A-induced PI-turnover was not affected by PMA (50 nM), but the $NaVO_3$-induced PI-turnover was increased over 2-fold by PMA (50 nM), suggesting that the Con A-induced PLC in intact splenocytes is different from $NaVO_3$-induced PLC. Based on these results a model for the sequential activation of substrate-specific PLCs in splenocyte by mitogen is presented.

Key words: G proteins, sodium metavanadate, phorbol esters, dexamethasone, NaF, trifluoperizine

Phosphatidylinositol-specific phospholipase C is found in the membrane and cytosol of several tissues [Rhee et al., 1989; Hoffman and Majerus, 1990]. Inspite of the fact that the substrates for PLC and the receptor systems that have been shown to stimulate PLC are membrane-associated; PLC is cytosolic in most cells [Rhee et al., 1989]. Very little is known about the role cytosolic PLC plays in cellular signal transduction even in cells with over 90% of the PLC activity in the cytosol.

All the PLC isozymes perform the same reaction, but the mechanism and factors required by each isotype subfamily seem to be different [Rhee and Choi, 1992]. For example the stimulation of PLC β requires the G_q/G_{11} family of G-proteins [Gutowski et al., 1991; Smrcka et al., 1991; Taylor, 1991], while the activation of PLC γ subfamily on the other hand does not appear to require G-proteins, but may involve tyrosine phosphorylation and dephosphorylation on the enzyme itself [Nishibe et al., 1990; Wahl et al., 1992]. The mechanism(s) and factors required for the activation of PLC α, and PLC δ are not known at present. The picture is further complicated by the recent identification of a soluble PLC activity that appears to be regulated by

Abbreviations used: Con A, concanavalin A; DAG, diacylglycerol; DEX,dexamethasone; G-protein, any GTP-binding protein that resembles a family of homologous proteins consisting G_s, G_i, G_o, and G_t; $G_{i\alpha}$, α subunit of the G-protein(s) that mediate the inhibition of adenylate cyclase; PLC, phosphatidylinositol-specific phospholipase C; PI, phosphatidylinositol, PIP_2, phosphatidylinositol 4,5,bisphosphate; PMA, phorbol 12-myristate 13-acatate; TFP, trifluoperizine.

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G-protein βγ-subunits [Camps et al., 1992; Blank et al., 1992; Boyer et al., 1992].

The stimulation of lymphocytes by mitogenic lectins like concanavalin A has been used extensively to study the signaling pathways involved in T-cell activation [Gelfand et al., 1987; Milner, 1977; Sasaki and Hasegawa-Sasaki, 1985; Kimball et al., 1988]. Although the mechanism(s) by which Con A acts via its receptors to stimulate lymphocytes is not known, several reports have indicated that the early events in the activation process include the rise in intracellular calcium and an increase in PI-turnover [reviewed by Altman et al., 1990].

We have described in the first paper the association of cytosolic Giα with soluble PLC activation. The aims of the present report are first to establish the degree to which PLC activity induced by Con A in intact splenocytes involves the coupling of cytosolic Giα with PLC, and second to attempt to identify the Con A-stimulated PLC in terms of whether it differs from $NaVO_3$-induced PLC [PLCγ, Secrist et al., 1993]. Finally, a model is presented for the possible significance in intact splenocytes of PLC hydrolysis of two substrates PI and PIP_2.

MATERIALS AND METHODS

Antisera

Goat anti-rabbit ^{125}I labeled IgG was purchased from ICN radiochemicals (Costa Mesa, CA), [^3H] myo-inositol was purchased from NEN (Boston, MA). Antisera AS/7 was purchased from NEN Dupont. Dexamethasone was purchased from Sigma (St. Louis, MO). Sodium metavanadate was purchased from Aldrich (Milwaukee, WI).

Cell Culture

Spleens from male rats were removed and separated by mashing them through a wiremesh screen. The dispersed cells were briefly passed over glasswool to remove large particulate tissue and then centrifuged at 1,600 rpm for 10 min and resuspended in growth medium (RPMI 1640 complete media, supplemented with fetal bovine serum at a final concentration of 10%, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin and 0.2% mercaptoethanol); 5 ml of the resuspended cells, 3–5 million cells/ml, were placed in 25 cm^3 flasks. In the time course experiment, Con A (5 μg/mL) was added for the indicated times and all the groups were incubated for 48 h. The cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For the pertussis toxin experiments the toxin was administered in vivo for 48 h as described above, the spleens were then removed and cultured for 24 h with or without Con A. Cells were harvested by centrifugation at 1,600 rpm for 10 min, and washed two times with phosphate buffered saline and once with homogenization buffer (containing 50 mM Tris buffer pH 7.2, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 3 mM benzamidine, 1 mM leupeptin, 1 μg/ml pepsitenin, 1 μg/ml antipain, 1 μg/ml aprotinin, 10% (wt/vol) sucrose, and 1 μg/ml of soybean trypsin inhibitor) at 1,600 rpm for 10 min. The cells were frozen at −70°C until use.

Prelabelling Cells With [^3H] Myo-Inositol

Splenocytes isolated as described above were resuspended in Basal Eagle Medium, supplemented with fetal bovine serum at a final concentration of 10%, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin and 0.2% mercaptoethanol. [^3H] myo-inositol (2 μCi/mL) was added and the cells were grown for 40 h as described in cell culture above. The cells were harvested and washed once with RPMI 1640 and then resuspended in RPMI 1640 (supplemented with fetal bovine serum at a final concentration of 10%, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, 0.2% mercaptoethanol, and 10 mM $LiCl_2$) at a concentration of 7–10 million cells/mL; 1 mL of the resuspended cells was used in triplicates for each determination.

Isolating Inositol Phosphates From Prelabeled Cells

The prelabelled cells were harvested by centrifugation and 1 mL of a mixture containing methanol: chroloform: HCl(1:1:0.1) and 300 μL of 10 mM EDTA were added to lyse the cells. The cells were vortexed and centrifuged to separate the aqueuos and organic phases. The aqueous phase (700 μL) was extracted and applied to a Bio-Rad AG-1-X8 resin (formate form) column. The column was washed four times with 5 mM myo-inositol/0.1 M formic acid. The total inositol phosphates (IP-IP_3) were eluted with 6 mL of 1 M ammonium formate/0.1 M formic acid. The amount of inositol phosphates produced were determined by counting in a liquid scintillation counter.

Electrophoresis and Western Blotting

Electrophoresis and Western blots were performed according to standard procedure as previously described [Akompong et al., 1993].

PLC Activity

PLC activity was measured as described in the Akompong et al., 1994, this issue.

RESULTS

Time Course of Con A Stimulation of Proliferation, Induction of PLC Activity, Cytosolic Giα Levels, and PI-Turnover

Our first goal was to establish the role of PLC activity coupled to Giα in PI-turnover occurring in intact splenocytes. The way we chose to do this was to determine whether Con A stimulation of proliferation of splenocytes paralleled induction of PLC activity and cytosolic Giα levels. Activation of lymphocytes by Con A is a continuous process lasting for several hours. The biochemical events start seconds after the lectin is administered and culminate in blast transformation, which can be monitored by measuring uptake of [^{3}H]thymidine into DNA. A single dose of Con A (5 μg/ml) given to splenocytes in culture (see Materials and Methods for details) resulted in significant uptake of [^{3}H]thymidine (added at 36 h) after 12 h, but maximal incorporation of [^{3}H]thymidine occurred at 48 h (Fig. 1A). Soluble PLC activity (defined as the ability of soluble PLC to hydrolyze exogenous substrates) was measured with [^{3}H]PI and [^{3}H]PIP_2 as substrates.

Con A stimulated splenocytes showed an increase in soluble PLC activity over unstimulated controls as early as 12 h. The biggest difference in activity between stimulated and unstimulated cells for both PI and PIP_2 as substrate occurred at 48 h (Fig. 1B). With PI as substrate the increase in soluble PLC activity was about 3-fold. With PIP_2 as substrate the increase in soluble PLC activity of stimulated cells after 48 h was about 1.5-fold over control.

Soluble PLC from different cell types has been shown to require G-proteins for activation, [Deckmyn et al., 1986; Camps et al., 1990]. We have previously reported [Akompong et al., 1993] that soluble PLC activity and cytosolic Giα immunoreactivity were co-regulated by glucocorticoids. We have presented evidence in another study (see Akompong et al., 1994, this issue) that suggests cytosolic Giα may be associated

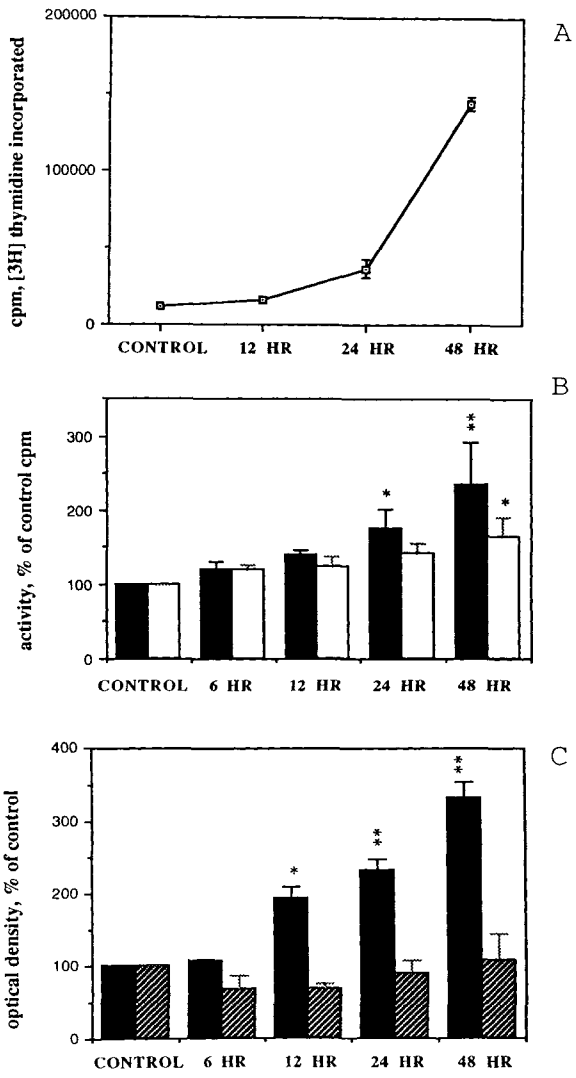

Fig. 1. Time course of Con A administration on splenocyte proliferation, soluble PLC activity and Giα immunoreactivity in cultured spleen cells. Spleen cells were isolated from four rats and cultured as described in Materials and Methods. Con A (5 μg/mL) was added for the indicated times; all the cells were harvested after 48 h incubation. **A:** ^{3}H-thymidine incorporation in splenocytes was used as an index of proliferation. The cells were pulsed 12 h (36–48 h) with ^{3}H-thymidine (5 μCi/mL). The points represent the means ±SEM (n = 3). **B:** PLC activity was measured with [^{3}H]PI (solid bars) and [^{3}H]PIP_2 (open bars) as substrates; 25 μg of splenocyte cytosol proteins were used at pH 6.8 and calcium (10^{-6} M). The bars represent the means ±SEM (n = 4). **C:** Giα immunoreactivity from cytosol (solid bars) and membrane (hatched bars) were measured by western blots. The optical densities from the autoradiograms were expressed as percent of control. The bars represent the means ±SEM (n = 3). Statistics were done by one way analysis of variance with the Tukey test for multiple comparisons between means. *significantly different from control $P < 0.05$ and **$P < 0.01$.

with the activation of soluble PLC. We therefore measured Giα immunoreactivity in this study, to confirm our hypothesis that cytosolic Giα is involved in the activation of soluble PLC.

Cytosolic Giα immunoreactivity increased about 2-fold after 12 h of Con A administration, and the levels increased to 4-fold at 48 h which is consistent with our previous results (Fig. 1C). It is important to note that under these conditions the membrane Giα immunoreactivity from Con A-stimulated splenocytes tended to decrease, but the decrease was not statistically significant at any time point (Fig. 1C).

Effect of DEX on Cytosolic PLC Activity, Giα Immunoreactivity, and PI-Turnover in Con A-Treated Splenocytes

Con A stimulation not only increases proliferation of splenocyte but also PI-turnover. To further establish the relationship between Con A-induced increase in soluble PLC and Con A-induced increase in PI-turnover in intact splenocytes we measured the PI-turnover due to Con A over time. As shown in Figure 2, Con A administration produced an increase in PI-turnover for up to 4 h. We used this to find out whether an agent, dexamethasone, that inhibits basal soluble PLC activity [Akompong et al., 1993] also inhibits the Con A-induced PI-turnover, as well as Con A-induced increase in soluble PLC activity and cytosolic Giα levels. To this end we treated five groups of splenocytes with DEX as follows: i) control (no addition), ii) Con A (5 μg/mL), iii) DEX (10^{-7} M) and, iv) Con A (5 μg/mL) and DEX (10^{-7} M) given at the same time; v) DEX (10^{-7} M) given 6 h before Con A (5 μg/mL) administration.

Pretreatment of splenocytes with DEX (10^{-7} M), 6 h prior to adding Con A (5 μg/mL), inhibited Con A-induced increase in PI-turnover, but concurrent administration of DEX (10^{-7} M) with Con A (5 μg/mL) had no significant effect on Con A-induced increase in PI-turnover (Fig. 2). These results are similar to DEX effects on basal soluble PLC activity.

In a parallel experiment we examined the effects of DEX on Con A induced increase in soluble PLC activity and cytosolic Giα levels. The results shown in Table I indicate that DEX (10^{-7} M) given alone inhibited serum induced incorporation of ^{3}H-thymidine into splenocytes by about 90% compared to control. DEX (10^{-7} M) did not inhibit the Con A-stimulated ^{3}H-thymidine incorporation when DEX and Con A were given together. On the other hand when DEX (10^{-7} M) was given 6 h prior to Con A administration, the ^{3}H-thymidine incorporation was inhibited by about 50% compared to Con A alone (Table I).

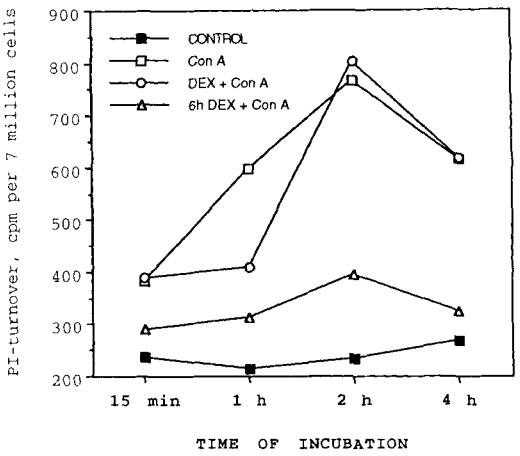

Fig. 2. *Time-dependent inhibition of Con A-induced PI-turnover by DEX.* Splenocytes were isolated and labeled with [^{3}H] inositol as described in Materials and Methods. One group of cells were treated with DEX (10^{-7} M) for 6 h before adding Con A (5 μg/mL); the other groups were *ii) control, iii) Con A* (5 μg/mL) and iv) Con A (5 μg/mL) + DEX (10^{-7} M) given concurrently. The cells were incubated for the indicated times. The results are expressed as cpm per 7×10^{-6} cells.

The administration of DEX (10^{-7} M) alone decreased soluble PLC activity to about 20% of control when PI was used as substrate and to 40% of control activity when PIP_2 was used as substrate. Con A (5 μg/mL) administered alone for 48 h induced an increase in soluble PLC activity by 3–5-fold over control with PI as substrate and 1.5–3-fold over control with PIP_2 as substrate (Table I). Simultaneous treatment with DEX (10^{-7} M) and Con A increased soluble PLC activity by 1.5-fold over control for both substrates. When DEX (10^{-7} M) was given 6 h prior to Con A addition the Con A-induced increase in soluble PLC activity was completely blocked, and the resulting PLC activity was lower than that observed for the control. It is interesting to note that in all instances the effect, whether it is inhibition or stimulation of soluble PLC activity, was greater with PI as substrate than with PIP_2 as substrate (Table I).

As shown in Figure 3 the Giα immunoreactivity was decreased in the DEX (10^{-7} M) treated cells compared to controls. Con A (5 μg/mL) administered alone induced an increase in Giα immunoreactivity 4–6-fold compared to control cells. Simultaneous treatment with DEX (10^{-7}

TABLE I. Effects of Con A (5 μg/mL) and DEX (10^{-7} M), on Splenocyte Proliferation, and Soluble PLC Activity†

Treatment	PI	PIP_2	^{3}[H]-thymidine
Control	100*	100*	100***
CON A (5 μg/mL)	348 ± 84	223 ± 41	408 ± 185
DEX (10^{-7} M)	21 ± 4***	41 ± 7***	11 ± 1***
DEX + CON A	165 ± 22	154 ± 26	426 ± 126
6 h DEX + CON A	65 ± 3**	72 ± 3**	212 ± 80*

†^{3}H-thymidine incorporation into splenocytes was used as an index of proliferation. The cells were pulsed 6 h (42–48 h) with ^{3}H-thymidine (5 μCi/mL). PLC activity was measured with [^{3}H]PI and [^{3}H]PIP_2 as substrates; 25 μg of splenocyte cytosol proteins were used at pH 6.8 and calcium (10^{-6} M). Spleen cells were isolated from four rats and cultured as described in Materials and Methods. Con A (5 μg/mL) and DEX (10^{-7} M) were added as indicated in the table; all the cells were harvested after 48 h incubation. The results are expressed as % of control cpm. The values represent means ±SEM (n = 4). Statistics were done by one way analysis of variance on the log transformed data with the Tukey test for multiple comparisons between means. *Significantly different from Con A treated cells $P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

M) and Con A (5 μg/mL) resulted in a 1.5–2-fold increase in Giα immunoreactivity compared to control. Pretreatment with DEX (10^{-7} M) for 6 h prior to Con A (5 μg/mL) addition resulted in a reduction in Giα immunoreactivity similar to that produced by DEX (10^{-7} M) alone (Fig. 3).

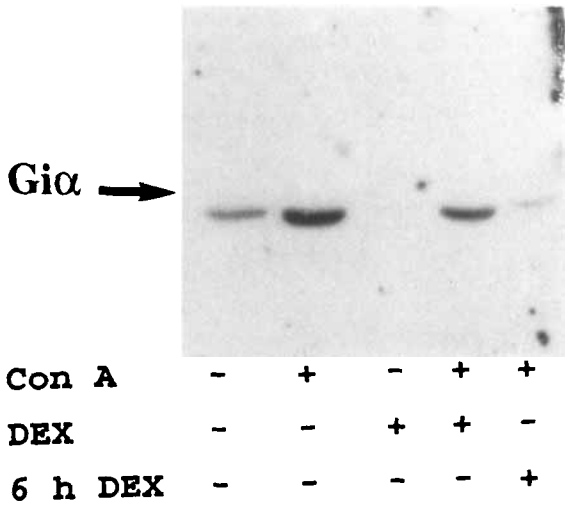

Fig. 3. Autoradiogram showing the effects of Con A (5 μg/mL) and DEX (10^{-7} M) on Giα immunoreactivity from splenocytes. Sixty micrograms of soluble proteins from splenocytes were used to generate the autoradiograms as described in Materials and Methods. Autoradiogram showing the effects of Con A (5 μg/mL), DEX (10^{-7} M), and DEX (10^{-7} M) administered with Con A at the indicated times on cytosolic Giα immunoreactivity.

Effects of NaF (5 mM) and TFP (100 μM) on Concanavalin A-Stimulated PI-Turnover in Splenocytes

We have shown in Akompong et al. [1994], this issue, that NaF and TFP, two serine/threonine phosphatase inhibitors, inhibited soluble PLC activity which suggested that serine/threonine phosphatases may be involved in the activation process. If serine phosphatases are important to the Con A activation of PLC turnover in intact cells, then NaF and TFP should block this process. In order to examine whether or not serine/threonine protein phosphorylation/dephosphorylation may be a means of regulating Con A-induced PLC activity in an intact splenocyte, we prelabeled splenocytes with [^{3}H] myo-inositol (2 μCi/mL) for 40 h. The cells were harvested as described in Materials and Methods. Phosphatidylinositol turnover was initiated by adding Con A (5 μg/mL) and/or NaF (5 mM), and TFP (100 μM) to splenocytes for 1 or 2 h at 37°C.

As can be seen from Table II, NaF (5 mM) and TFP (100 μM) blocked the Con A-mediated PI-turnover at 1 and 2 h after Con A addition. NaF (5 mM) did not have any effect on the basal PI-turnover. TFP (100 μM), on the other hand, inhibited the basal PI hydrolysis as well as inhibiting the Con A stimulation. These results are consistent with the inhibitory effects of NaF and TFP on soluble PLC activity.

The Effects of DEX and PMA on Con A- or $NaVO_3$-Induced PI-Turnover

The Con A-induced PI-turnover in T-lymphocytes is qualitatively different from PLC activation in other cells. Whereas in most cells the rise in DAG and IP_3 elicited by agonists returns to baseline in the order of seconds to minutes, the mitogen stimulated PI-turnover in lymphocytes lasts for at least 2–4 h for successful lymphocyte transformation [Altman et al., 1990; Goldsmith and Weiss, 1988]. Vanadate in different oxidation states has been shown to induce PI-turnover [Zhang et al., 1991; Secrist et al., 1993]. In the human Jurkat, lymphoma cell line, Secrist et al. [1993] have suggested that the pervanadate induced PLC is PLCγ. We compared the effects of DEX (10^{-7} M) and PMA (50 nM) on

TABLE II. Effects of NaF (5 mM), and TFP (100 μM) on Con A-Induced PI Turnover in Splenocytes†

Time after Con A addition	PI Turnover: Total inositol phosphates produced (cpm) 1 hr	2 hr
Control	144 ± 8*	ND
Con A (5 μg/mL)	318 ± 39	445 ± 44
NaF (5 mM)	158 (n = 2)	ND
TFP (100 μM)	103 (n = 2)	ND
Con A + NaF	166 ± 18*	186 ± 12**
Con A + TFP	100 ± 7**	92 ± 10**

†Spleen cells from three rats were grown in Basal Eagle Medium (BEM) with [^{3}H] myo-inositol (2 μCi/mL) for 40 h. The cells were harvested and resuspended in RPMI 1640/10 mM $LiCl_2$ (see Materials and Methods for details). The resuspended cells were incubated with No addition, Con A (5 μg/mL), and/or NaF (5 mM), and TFP (100 μM) for 1 or 2 h. The values represent Means ± SEM (n = 3). Statistics were by one way analysis of variance with the Tukey test for multiple comparisons between means. *Significantly different from Con A only, $P < 0.05$ and **$P < 0.01$.

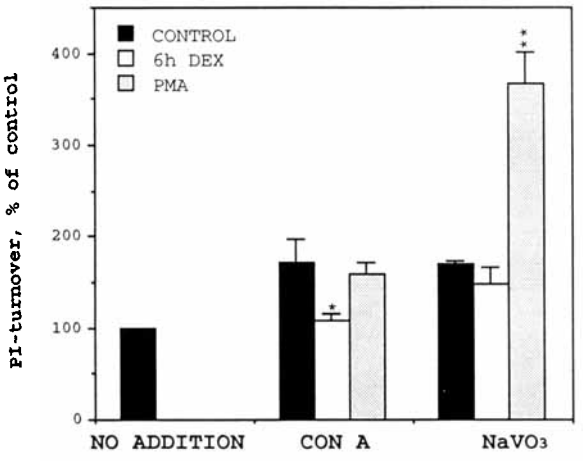

Fig. 4. Effects of DEX and PMA on Con A or metavanadate induced PI-turnover. Splenocytes were isolated from three rats and incubated with [^{3}H]inositol (2 μCi/mL) for 40 h as described in Materials and Methods. The cells were incubated with i) no addition, ii) Con A (5 μg/mL), iii) $NaVO_3$ (5 mM), iv) 6 h DEX + Con A (5 μg/mL), v) Con A (5 μg/mL) + PMA (50 nM), vi) 6 h DEX + $NaVO_3$ (5 mM), and vii) $NaVO_3$ (5 mM) and PMA (50 nM). The values represent Means ± SEM ($n = 3$). Statistics were by one way analysis of variance with the Tukey test for multiple comparison between means. *Significantly different from Con A alone, $P < 0.05$ and **Significantly different from $NaVO_3$ alone, $P < 0.01$.

Con A- and $NaVO_3$-induced PI-turnovers to establish whether or not Con A-stimulated PLC was PLCγ. As shown in Figures 2 and 4, prior treatment of splenocytes with DEX inhibited Con A-induced PI-turnover over time; but DEX had no significant effect on $NaVO_3$-induced PI-turnover (Fig. 4).

The protein kinase C activating phorbol esters like PMA are believed to inhibit PI-turnover in several cell types via a negative feedback mechanism. In splenocytes, PMA (50 nM) had no effect on the Con A-induced PI-turnover (Fig. 4). The $NaVO_3$-induced PI-turnover, on the other hand, was increased about two fold by PMA (50 nM) (Fig. 4). These results suggest that the Con A-induced PLC is different from the $NaVO_3$-induced PLC that is identified as PLCγ.

DISCUSSION

Association of Cytosolic Giα With Con A-Inducible Soluble PLC Activity

Our previous finding that glucocorticoid administration in vivo and in vitro inhibit cytosolic PLC activity in spleen cells [Akompong et al., 1993] led us to further study the mechanism of activation, and the role the cytosolic PLC may play in spleen cell activation. In Akompong et al. [1994], this issue, we described the coupling of cytosolic Giα to soluble PLC activation in spleen cells. The goals of this report are 1) to establish the relationship between the cytosolic Giα associated soluble PLC activity and the PLC stimulated by Con A in intact splenocytes, 2) to compare and contrast the characteristics of the Con A-stimulated PLC to $NaVO_3$-stimulated PLC [PLCγ, Secrist et al., 1993], and 3) to provide a model for a sequential substrate hydrolysis during Con A-stimulated PI-turnover.

Relationship Between Cytosolic Giα-Associated Soluble PLC Activity and Con A-Stimulated PI-Turnover (PLC)

In order to establish the functional significance of Giα-associated soluble PLC activity, we compared the effects of different agents on soluble PLC activity and Con A-stimulated PI-turnover. First, the time dependent stimulation of PI-turnover was paralleled by a time dependent increase in soluble PLC activity and cytosolic Giα immunoreactivity. There was a time lag, however, between the Con A-induced rise in PI-turnover and the increase of soluble PLC activity. The delay in observing the increase in soluble PLC activity may be due to sensitivity of our assay system to measure very small changes. Second, pretreatment of splenocytes with DEX

for 6 h inhibited Con A-induced soluble PLC activity and cytosolic Giα immunoreactivity below control levels. This treatment also inhibited the Con A-stimulated increase in PI-turnover. However, when DEX and Con A were administered concurrently, DEX failed to significantly inhibit both soluble PLC activity and PI-turnover, further suggesting that the two PLC activities may be similar. Third, we have shown in Akompong et al. [1994] that NaF and TFP, two serine/threonine phosphatase inhibitors inhibit soluble PLC activity. Con A-stimulated increase in PI-turnover in intact splenocytes was also inhibited by NaF and TFP. This suggests that the activation of soluble PLC in vitro and the stimulation of PLC in intact splenocytes by Con A may occur by a similar mechanism and may require the participation of serine/threonine protein phosphatases. Taken together the data strongly suggest that the PLC that is stimulated by Con A to produce an increase in PI-turnover is similar to the cytosolic Giα associated soluble PLC.

The Nature of Con A-Stimulated PLC

The Con A-induced PI-turnover in T-lymphocytes is different from the PI-turnover in other cells. Whereas in most cells the PI-turnover is curtailed in seconds to minutes after its initiation, the Con A-induced PI-turnover lasts for hours [Goldsmith and Weiss, 1988]. We found that the Con A-induced PI-turnover may be sustained for over 30 h (data not shown). One explanation of how the Con A-induced PI-turnover lasts so long is that the components of the PLC system are replenished over time probably by an increase in the synthesis of the proteins involved in the activation process. This idea is consistent with the long term increase in soluble PLC activity and Giα immunoreactivity induced by Con A that was found in this study.

In order to elucidate the nature of this PLC we compared some of its characteristics to the $NaVO_3$-induced PLC that have been suggested to be PLCγ in the human lymphoma cell line, Jurkat, [Secrist et al., 1993]. Because PLCβ (the other PLC whose mechanism of activation is fairly well understood) is activated via a pertussis toxin independent pathway we did not consider it in these studies. Whereas DEX inhibited the Con A-stimulated PI-turnover, it had no effect on the $NaVO_3$-stimulated PI-turnover. PMA, on the other hand, had no effect on the Con A-stimulated PI-turnover, but enhanced the $NaVO_3$-stimulated PI-turnover by about 2-fold. The mechanism by which PMA acts to enhance $NaVO_3$-induced PI-turnover may involve PMA's ability to increase protein tyrosine phosphorylation when administered together with vanadate [Ferrell and Martin, 1988, and data not shown]. These results clearly suggest that the Con A-stimulated PLC in splenocytes is different from the $NaVO_3$-stimulated PLC (that has been identified as PLCγ).

Relationship Between PLC Substrates

Both PI and PIP_2 are hydrolyze to contribute to the increase in phosphoinositide metabolism during the activation of intact lymphocytes by mitogen [Gelfand et al., 1987; Hui and Harmony, 1980]. We have shown in Akompong et al. [1994], this issue, that there was a difference in PLC activity, depending on which substrate (PI or PIP_2) was used to measure the activity. When PI was used as substrate there was no activity above background in the absence of added calcium. PIP_2, on the other hand, had a basal activity in the absence of calcium that was significantly above background. Pertussis toxin treatment of splenocytes resulted in a significant reduction in soluble PLC activity only when PI was the substrate, and this decrease was associated with a significant decrease in cytosolic Giα levels. The results presented in this paper show that the Con A-induced increase in soluble PLC activity correlated better with the increase in cytosolic Giα levels (3–5-fold increase) when PI was the substrate (3–5-fold increase) than when PIP_2 was the substrate (1.2–3-fold increase). There was a better correlation between DEX reduction in cytosolic Giα and soluble PLC activity when PI was the substrate rather than PIP_2. These results suggest that there are at least two different forms of PLC activity in splenocyte cytosol. One of these activities is responsible for the hydrolysis of PI that seems to be better coupled to cytosolic Giα, while another activity is responsible for the hydrolysis of PIP_2 that may not be entirely dependent on cytosolic Giα for activation.

The significance of the substrate specific activities for PI and PIP_2 is that there could be sequential hydrolysis of phosphatidylinositols during the long lasting Con A-stimulated increase in PI-turnover in splenocyte; the alternative hydrolysis of PI in preference to PIP_2 may serve as an important regulatory mechanism to

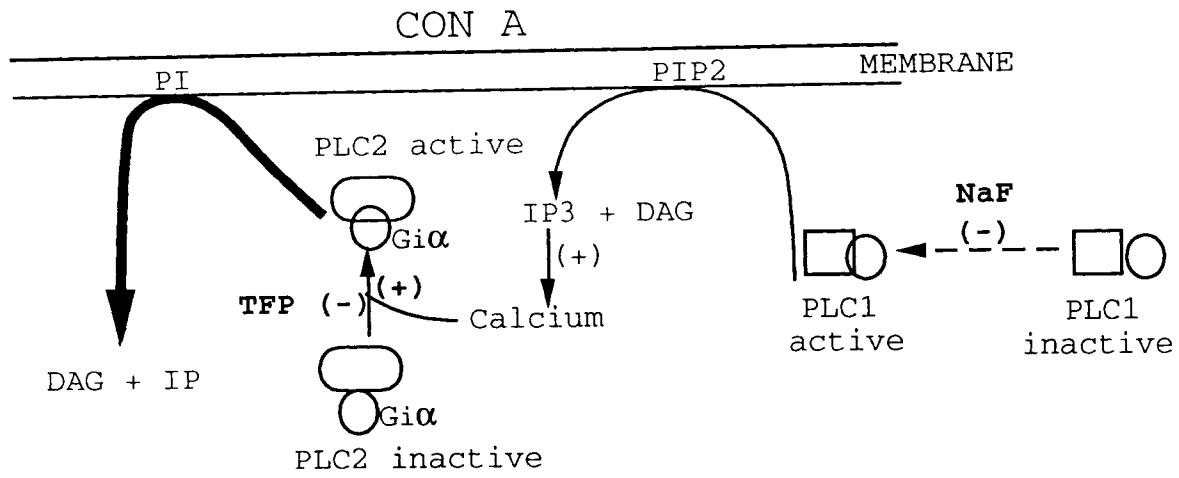

Fig. 5. Model for sequential activation of substrate specific PLCs by Con A in splenocytes. The model suggests that PLC 1 (PIP_2 specific PLC) is the first to be activated by Con A but has a limited contribution to the overall phosphoinositol turnover. The release of calcium by IP_3 initiates the activation of the cytosolic Giα coupled PLC (PLC 2) which utilizes PI to sustain the long lasting phosphoinositol turnover (see text for other details).

preserve the less abundant PIP_2 when PI-turnover is sustained over several hours. Figure 5 is a schematic representation of how the two substrate dependent PLC activities may act in concert to sustain a long term increase in mitogen induced PI-turnover. The initial signal by the mitogen activates the putative PIP_2 dependent PLC which is less dependent on Ca^{2+} to generate DAG and IP_3. The IP_3 then mobilizes Ca^{2+} from internal stores, initiating the activation of the PI-dependent PLC that is dependent on Ca^{2+} and cytosolic Giα for activity. Since the elevated Ca^{2+} levels are sustained [mainly by influx of extracellular Ca^{2+}; Kimball et al., 1988; Gardner, 1989] for several hours there will be no need to further hydrolyze PIP_2, which is 10× less abundant than PI. Hui and Harmony [1980] examined mitogen stimulated PI-turnover in lymphocytes by following the incorporation of ^{32}P into phosphatidylinositides and found that only the radioactivity associated with PI (but not PIP or PIP_2), was coupled to Ca^{2+} accumulation, suggesting that PI hydrolysis was the essential component of phosphatidylinositol metabolism in mitogen activated lymphocytes. This is consistent with our scheme which suggests that in the long term PI may be the preferred substrate used to maintain the mitogen stimulated PI-turnover in splenocytes.

In conclusion we have addressed some of the ways that cytosilic PLC may be activated to modulate signal transduction. We have identified cytosolic Giα as one of the components involved in the activation of soluble PLC in splenocytes. Our data also suggest that the cytosolic Giα coupled PLC may mediate the Con A-induced increase in PI-turnover in splenocytes. Finally, a synergistic relationship between PLC hydrolysis of PI and PIP_2 in splenocytes is proposed.

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