Signalling through CD28 T-cell activation pathway involves an inositol phospholipid-specific phospholipase C activity

Jacques NUNES,* Sandrine KLASEN,* Marie-Dominique FRANCO,* Carol LIPCEY,* Claude MAWAS,* Marcello BAGNASCO† and Daniel OLIVE*‡

*Unité de Cancérologie et Thérapeutique Expérimentales U 119 INSERM, 27 Bd Leï Roure, 13009 Marseille, France, and †DI.M.I. Università di Genova, Viale Benedetto XV, n.6 16132 Genova, Italy

Stimulation of the human T-cell line, Jurkat, by a monoclonal antibody (mAb) directed against the CD28 molecule leads to sustained increases in intracellular levels of Ca^{2+} ($[Ca^{2+}]_i$); the initial rise in Ca^{2+} comes from internal stores, followed by Ca^{2+} entry into the cells. The CD28 molecule also appears to activate polyphosphoinositide (InsPL)-specific phospholipase C (PLC) activity in Jurkat cells, as demonstrated by PtdInsP_2 breakdown, InsP_3 and 1,2-diacylglycerol generation and PtdIns resynthesis. We also observed that interleukin-2 (IL2) production induced via CD28 triggering was sensitive to a selective protein kinase C inhibitor. Of the four other anti-CD28 mAbs (CD28.2, CD28.4, CD28.5, CD28.6) tested, only one (CD28.5) was unable to generate any InsPL-specific PLC or IL2 secretion. However, the cross-linking of cell-bound CD28.5 with anti-mouse Ig antibodies led to an increase in $[Ca^{2+}]_i$. CD28-molecule clustering in itself appears to be a sufficient signal for induction of PLC activity.

INTRODUCTION

T lymphocytes recognize antigen in association with major histocompatibility antigens on the surface of antigen-presenting cells, and, as a consequence of this interaction, can be activated to perform their effector functions [1]. The T-cell receptor for antigen (TcR) comprises an α–β heterodimer, associated non-covalently with at least five different subunits of the CD3 antigen [2].

Aggregation of surface determinants by monoclonal antibodies (mAbs) directed against TcR/CD3 components mimics the effects of the TcR-complex and antigen–major histocompatibility complex interaction. It is accepted that CD3/TcR-mediated T-cell activation triggers hydrolysis of surface membrane PtdIns(1,4)P_2 to Ins(1,4,5)P_3 and diacylglycerol (DAG), which respectively promote an increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and activate protein kinase C (PKC) [3,4]. The polyphosphoinositide (InsPL)-specific phospholipase C isoform (PLC γ1) was identified and demonstrated to be activated in T lymphocytes [5,6] after stimulation by anti-CD3 mAb.

A number of surface molecules distinct from those involved in antigen recognition are able to mediate T-cell activation. These molecules are involved in the T-cell adhesion; for example, the CD2 molecule binds two ubiquitous proteins, namely CD58/LFA-3 and CD59 [7,8]. In T-cell lines, agonistic mAbs against the CD2 molecule (anti-CD2 mAbs) can induce a PtdIns pathway [9] with translocation of PKC from cytosol to plasma membrane [10]. In addition, PLC γ1 is activated by this alternative T-cell activation pathway [11]. Also, the CD2 molecule could be associated with a transducer element of the CD3–TcR complex [12]. An alternative activation pathway is mediated by the CD28 molecule, a 90 kDa homodimer [13]. This molecule has been described as being involved in the cell interactions between T cells and B cells via its ligand, B7/BB-1 [14]. Anti-CD28 mAbs can induce T-cell proliferation and interleukin-2 (IL2) release, synergizing with phorbol esters [13,15] and anti-CD3 or -CD2 mAbs in T-cell activation [15–18].

It is controversial whether signal transduction via CD28 involves PtdIns metabolism. Previously it has been shown that CD28 stimulation is associated with extracellular Ca^{2+} entry [19] or InsP_3 accumulation, with $[Ca^{2+}]_i$ increase from internal stores [16]. However, neither DAG generation nor PKC translocation has been demonstrated in these previous studies. Moreover, the CD28 stimulation is resistant to cyclosporin A, which can inhibit the Ca^{2+}-dependent pathway [20]. The CD28 pathway synergizes with pathways which are known to activate PtdIns metabolism, such as CD3 or CD2, and augments T-cell expression of several lymphokines and lymphokine-receptor genes by specifically stabilizing their transcripts [21,22]. So whether the CD28 T-cell activation pathway is associated with induction of PLC activity remains an open question. We have used a clone of the human leukaemic T cell Jurkat, JH6.2, which has been selected to produce high amounts of IL2 by the CD28 pathway. These cells, expressing high levels of the CD28 molecule on the cell surface, are a model to study molecular events in T-cell activation.

We have tested different parameters of PLC activity in Jurkat cells after stimulation by a soluble anti-CD28 mAb (248), namely PtdInsP_2 breakdown, InsP_3 and DAG accumulation, PtdIns turnover and $[Ca^{2+}]_i$ increase.

This PLC activity was analysed by using several anti-CD28 mAbs, which recognize different regions of the extracellular domain of the CD28 molecule.

MATERIALS AND METHODS

Materials

Different mouse mAbs were used directed against the CD28 molecule [23] (Table 1).

Abbreviations used: CD, cluster of differentiation; TcR, T-cell receptor; PKC, protein kinase C; DAG, 1,2-diacylglycerol; mAb, monoclonal antibody; InsPL, polyphosphoinositide; $[Ca^{2+}]_i$, intracellular concentration of free Ca^{2+}; PLC, phospholipase C; IL2, interleukin-2; PHA, phytohaemagglutinin; PMA, phorbol myristate acetate; GAM Ig, goat anti-(mouse Ig) antiserum; AMG, 1-alkyl-2-methylglycerol.

‡ To whom correspondence should be addressed.

Table 1 Anti-CD28 mAbs used in this study

Name	Ig subclass	Laboratory origin
248	Murine IgM	A. Moretta
CD28.2	Murine IgG1	D. Olive
CD28.4	Murine IgM	D. Olive
CD28.5	Murine IgG1	D. Olive
CD28.6	Murine IgG2a	D. Olive

Binding of purified anti-CD28 mAbs to the CD28 molecule was analysed on the Jurkat cell surface: ED_{50} was determined as 10 ng/ml for CD28.2 and 80 ng/ml for CD28.5 or CD28.6 (results not shown). In this study, they were used at saturating concentration (10–50 μg/ml).

Other mAbs used were 6A11.2 (IgM), 10D11.5 (CD8, IgG1) and 289 (CD3, IgG2a), obtained from Professor Alessandro Moretta (Genova, Italy).

Purified phytohaemagglutinin (PHA) and phorbol myristate acetate (PMA) were respectively supplied by IBF (Villeneuve-la-Garenne, France) and Sigma (St. Louis, MO, U.S.A.). GF 109203X has been shown to inhibit PKC activities [24] and was obtained from Dominique Toullec and Jorge Kirilovski (Glaxo France, Les Ulis, France).

Cells

The subclone JH6.2 of the leukaemic differentiated human T-cell line Jurkat was obtained by limiting dilution, selected for high $CD28^+$ surface expression and high IL2 secretion induced by the soluble anti-CD28 mAb 248 with either PMA or anti-CD2 mAbs. These Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum.

Antibody-binding determination

For indirect immunofluorescence, 2×10^5 Jurkat cells were incubated for 45 min at 4 °C with different concentrations of mAbs. The cells were then washed and incubated with a saturating concentration of fluorescein-labelled goat anti-(mouse Ig) antibodies (Jackson Laboratories, West Grove, PA, U.S.A.). The samples (5000 cells) were analysed by flow cytometry using a FACSSCAN instrument (Beckton Dickinson, Mountain View, VA, U.S.A.).

Green fluorescence emission was measured at 525 nm. Mean Fluorescence Intensity (MFI) is expressed as

$$MFI = \sum_{i=1}^{255} n \times m / \sum_{i=1}^{255} m$$

where n = channel number (n = 1–255) and m = number of cells in channel.

Intracellular Ca^{2+} mobilization studies

Jurkat cells were loaded with Indo-1 AM as previously described [10,25]. Briefly, 10^7 cells/ml were loaded with 5 μM Indo-1 in RPMI 1640 supplemented with 2% fetal-calf serum/25 mM Hepes for 45 min at 37 °C. Stimulation was performed at 37 °C in loading medium or RPMI/0.5% BSA/25 mM Hepes, with 10 mM EGTA. Cell response was monitored with a cytofluorograph (ATC 3000; Odam Brucker, Wissenbourg, France). U.v. excitation (100 mW, 351–364 nm) was obtained from an argon ion laser (90/s uv; Coherent, Palo Alto, CA, U.S.A.). Violet fluorescence emission of Indo-1 was measured at 380–410 nm (Dichroic Filter 395:25 nm: Omega Optical, Brattleboro, MN, U.S.A.). Blue fluorescence emission of Indo-1 was collected with a BP 520 nm (Corion, Holliston, MA, U.S.A.) instead of the BP 500 nm filter initially used by Rabinovitch et al. [25], in order to stop the 488 nm laser beam. This combination allows good detection of $[Ca^{2+}]_i$ variations (results not shown).

Cells were transferred to a 37 °C thermostatically controlled sample cup and analysed at rates of 1000 cells/s. Ratio histograms of 3000 cells were stored every 15 s or 30 s using an 'ATC 3000' kinetic mode. Relative Fluorescence Ratio Intensity (RFRI) was expressed as

$$RFRI = \sum_{i=1}^{255} n \times m / \sum_{i=1}^{255} m$$

where n = channel number (n = 1–255) and m = number of cells in channel.

Ins*P* phosphate determination

[^{3}H]Ins*P*s were measured by a slight modification of previous methodology [26]. Briefly, Jurkat cells were incubated at 10^7 cells/ml in inositol-free RPMI medium containing 0.1% fetal-calf serum with 20 μCi/ml *myo*-[2-^{3}H]inositol (New England Nuclear, Boston, MA, U.S.A.), and incubated for 4 h at 37 °C in 5% CO_2/air. The cells were washed three times in inositol-free RPMI medium and once in buffer A (125 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.5 mM glucose, 0.1% BSA, 25 mM Hepes, pH 7.4), and resuspended at 10^7 cells/ml. Samples (100 μl) of this cellular suspension were then transferred to microfuge tubes and the appropriate additions were made.

The Ins*P*s were extracted by the acidic procedure of Bligh and Dyer [27]. The [^{3}H]Ins*P*s obtained from the aqueous phase were separated by anion-exchange chromatography using Dowex AG1X8 (formate form) (Bio-Rad, Richmond, CA, U.S.A.) and quantified by scintillation counting in Emulsifier Safe (Packard, Downers Grove, IL, U.S.A.).

Measurement of DAG

DAGs were labelled, separated and quantified by the methods previously described [26]. Briefly, 2×10^6 cells/ml were incubated with 10 μCi/ml [^{3}H]arachidonic acid (Amersham France) for 18 h at 37 °C in 5% CO_2/air. The cells were washed three times in buffer A, stimulated at 2×10^6 cells/ml with the appropriate mAbs, and then the reaction was terminated by addition of 0.75 ml of chloroform/methanol/HCl (100:200:1, by vol.). Phospholipids obtained from the organic phase were applied with appropriate standards to t.l.c. on silica gel 60 (Whatman, Maidstone, Kent, U.K.). Neutral lipids and [^{3}H]diacylglycerol were separated by using hexane/diethyl ether/formic acid (80:20:1, by vol.) as solvent.

All resolved lipids were revealed by iodine staining, compared with standard lipids, and each lipid spot was scraped into a scintillation vial and counted for radioactivity in a β-counter.

Measurement of phosphoinositides

PtdIns and PtdInsP_2 were labelled, separated and quantified by the methods previously described [26]. Briefly, 10^7 cells were incubated with 20 μCi/ml [^{32}P]phosphoric acid (PB4; 20 mCi/

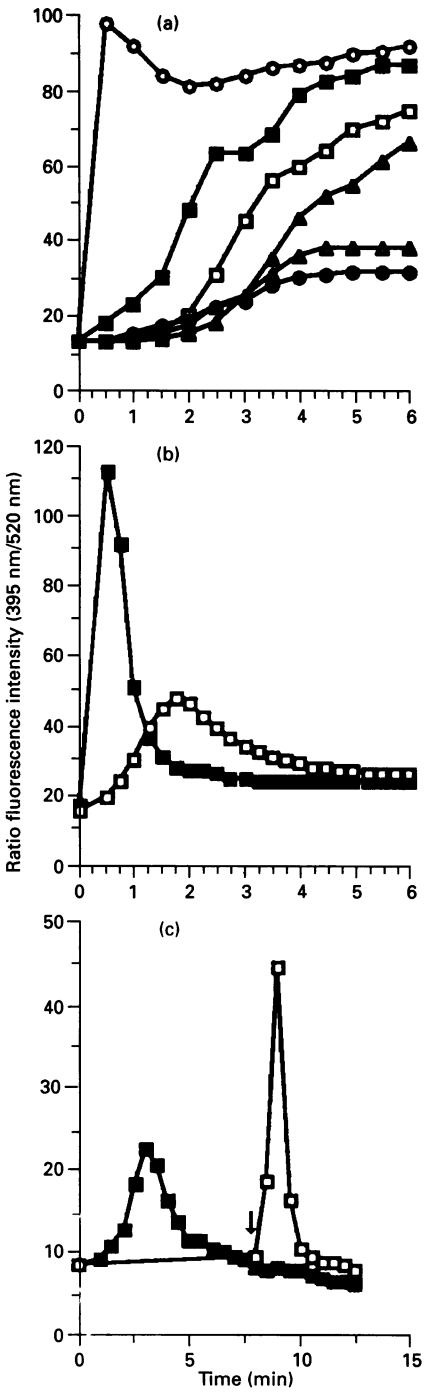

Figure 1 $[Ca^{2+}]_i$ mobilization induced by anti-CD28 mAb 248

Jurkat cells were labelled with Indo-1 AM, stimulated by mAbs and analysed by flow cytometry. Results are expressed as variations in violet-to-blue ratio of fluorescence intensity (arbitrary units) as a function of time. (**a**) Dose–response curves of mAb-248-induced $[Ca^{2+}]_i$ accumulation. Experiments performed in the presence of extracellular Ca^{2+}. Different ascitic dilutions were used: 1:100 (■), 1:200 (□), 1:400 (▲), 1:800 (△) and 1:1600 (●). $[Ca^{2+}]_i$ mobilization seen with an anti-CD3 mAb, 289, at 10 μg/ml (○) is also shown. The experiment shown is one representative of three determinations. (**b**) Time course of mAb-248-induced $[Ca^{2+}]_i$ increase from internal stores. Experiments performed in the absence of extracellular Ca^{2+} (10 mM EGTA). The mAbs were used at saturating concentration: mAb 289 at 10 μg/ml (■) and mAb 248 at 1:100 ascitic dilution (□). The experiment depicted is one representative of five determinations. (**c**) Effect of sequential stimulation of Jurkat cells with anti-CD28 mAb (248 at 1:100 ascitic dilution) and anti-CD3 mAb (289 at 10 μg/ml) on $[Ca^{2+}]_i$ mobilization. In Ca^{2+}-deprived medium, cells were sequentially stimulated with mAb 248 (■) or mAb 6A11.2 (negative control) (□), and then with anti-CD3 mAb at a time indicated by the arrow (8 min). The experiment shown is one representative of three experiments.

mmol; Amersham France) for 30 min (PtdIns determination) and 120 min (PtdInsP_2 determination) at 37 °C in 5% CO_2/air. The cells were washed three times in a phosphate-free buffer (125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5.5 mM glucose, 0.5% BSA, 25 mM Hepes, pH 7.4), stimulated at 10^6 cells/100 μl with the appropriate mAbs, and then the reaction was terminated by addition of 0.75 ml of chloroform/methanol/HCl (100:200:1, by vol.). Phospholipids obtained from the organic phase were applied with appropriate standards to t.l.c. on silica gel 60 (Whatman). ^{32}P-labelled lipids were separated by placing the plates in equilibrated tanks containing chloroform/methanol/33% ammonia/water (250:190:13:42, by vol.). All resolved lipids were revealed by iodine staining, compared with standard lipids, and each lipid spot was scraped into a scintillation vial and counted for radioactivity in a β-counter.

IL2 production

Jurkat cells were washed several times in culture medium, then resuspended at a concentration of 0.5×10^6/ml. Duplicate cultures (200 μl) in 96-well flat-bottomed plates were set up in the presence of saturating concentrations of anti-CD28 mAbs (50 μg/ml for purified antibodies and 1:100 dilution for fluid ascites) in the presence or absence of PMA (15 ng/ml). After 24 h, supernatants were collected and stored at −80 °C for IL2 assay. IL2 concentrations were measured by e.l.i.s.a., using a commercially available kit (Immunotech S.A., Marseille, France) according to the manufacturer's instructions.

RESULTS

Ca^{2+} mobilization induced by anti-CD28 mAb 248

We evaluated the ability of anti-CD28 mAb 248 to induce this event in a dose-dependent manner, using Indo-1-labelled cells and cytofluorimetry. As shown as Figure 1(a), mAb 248 elicited a dose-dependent increase of $[Ca^{2+}]_i$ in Jurkat cells. Maximal Ca^{2+} response was obtained at a 1:100 ascitic dilution corresponding to the saturating concentration of mAb 248 on Jurkat cells (as determined by indirect immunofluorescence staining; results not shown). We then investigated whether mAb 248 was able to mobilize Ca^{2+} from internal stores by repeating the experiments in Ca^{2+}-deprived medium [16]. The results are depicted in Figure 1(b); mAb 248 induced a transient $[Ca^{2+}]_i$ increase (peak value 43 arbitrary units at 2 min). This increase was comparable with the $[Ca^{2+}]_i$ increase induced by anti-CD3 mAb 289, in terms of area under the peak, but the mAb-289-induced response was more rapid and gave a higher peak value (112 arbitrary units). One may conclude that CD28 stimulation results in Ca^{2+} mobilization from internal stores, possibly due to InsP_3 generation [3]. That the pools of intracellular Ca^{2+} mobilized via CD3 or CD28 are the same is demonstrated by the fact that Jurkat cells, once they have released intracellular Ca^{2+} after CD28 stimulation, are unable to release further Ca^{2+} when stimulated with anti-CD3 mAb (Figure 1c).

CD28 is associated with a PLC activity in Jurkat cells

On the basis of the above results, one could hypothesize that the CD28 molecule is functionally linked to PLC activity. To test this hypothesis further, we measured the PtdInsP_2 breakdown induced by mAb 248.

^{32}P-labelled Jurkat cells were stimulated, and phospholipids were analysed at different times for PtdInsP_2 content. The polyclonal mitogen PHA was used as positive control. mAb 248 induced rapid PtdInsP_2 breakdown: 60% decrease at 5 min,

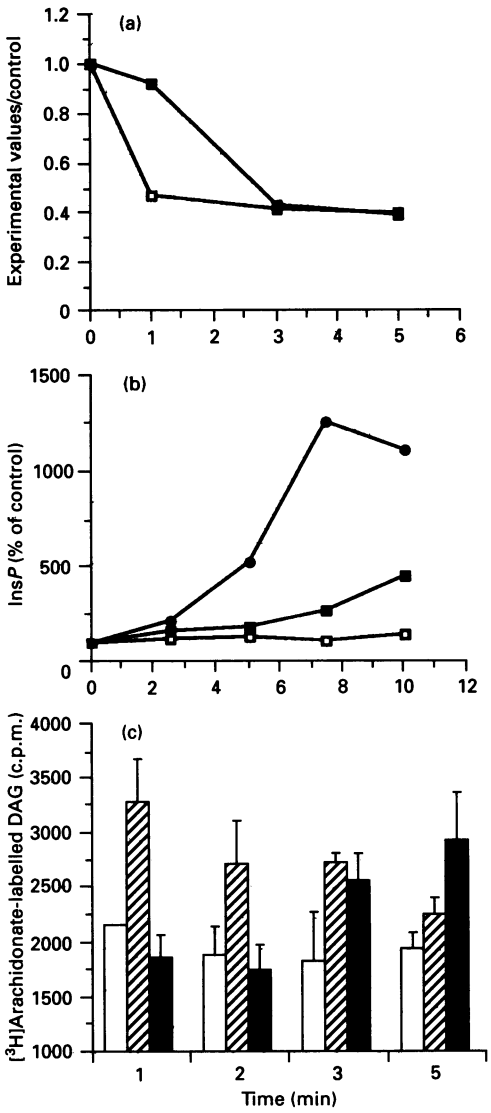

Figure 2 Studies of PLC activity induced by mAb 248 in Jurkat cells: analysis of different parameters

(**a**) PtdInsP_2 breakdown induced by mAb 248 at 1:100 ascitic dilution (■) and control PHA at 10 μg/ml (□) on ^{32}P-labelled Jurkat cells. These results are representative of one of three separate experiments. The results are expressed as experimental values versus negative control. Values remained constant during the course of the experiment (mean ± S.E.M. 717 ± 26 c.p.m.). (**b**) Production of Ins*P*s induced by mAb 248 at 1:100 ascitic dilution. Jurkat cells were labelled with *myo*-[2-^{3}H]inositol; InsP_1 (□), InsP_2 (■) and InsP_3 + InsP_4 (●) were measured at the indicated time points by anion-exchange chromatography. The results are expressed as percentages of basal values. Basal levels corresponded to 484 c.p.m. for InsP_1, 88 for InsP_2 and 63 for (InsP_3 + InsP_4). The values shown are representative of two determinations. (**c**) DAG accumulation in [^{3}H]arachidonate-labelled Jurkat cells after stimulation with mAb 248 at 1:100 ascitic dilution (■), mAb 289 at 10 μg/ml (▨) or in unstimulated Jurkat cells (□). The results are expressed as c.p.m. (means ± S.E.M.) and the values shown are representative of three experiments.

which is comparable with that by PHA. The kinetics of the phenomenon correlated with the increase in $[Ca^{2+}]_i$ (Figure 2a).

We then evaluated the ability of mAb 248 to raise the levels of the two products of PtdInsP_2 breakdown, i.e. the second messengers InsP_3 and DAG. As shown in Figure 2(b), mAb 248 induced a remarkable increase in InsP_3 + InsP_4. This increase was apparent as early as 2.5 min, reached a peak at 7.5 min and

Table 2 Dose–response effect of the PKC inhibitor GF109203X on IL2 secretion

The Jurkat cells were stimulated by anti-CD28 mAb (248) at 1:200 ascitic dilution in the absence or presence of PMA at 15 ng/ml, or anti-CD3 mAb (289) at 10 μg/ml with PMA. PKC inhibitor GF109203X was added to cells 5 min before the mAb. IL2 concentration was measured in 24 h culture supernatants by e.l.i.s.a. The IL2 concentration is expressed in pg/ml.

Expt. no.	GF109203X (nM)	IL2 secretion (pg/ml) mAb 248	mAb 248 + PMA	mAb 289 + PMA
1	0	1100	15800	5200
	1	850	12800	4200
	3	780	12800	400
	10	800	12800	3500
	30	580	12400	1000
	100	320	8000	60
	300	60	250	< 10
	1000	< 10	< 10	< 10
2	0	790	17600	4800
	5	555	15800	4160
	500	< 10	600	< 10

tended to decline at 10 min after stimulation. Conversely, as expected, a lower increase of InsP_1 was detected at these early time points, presumably due to the absence of LiCl from the stimulation medium. In Figure 2(c) is reported the kinetics of DAG generation in [^{3}H]arachidonate-labelled Jurkat cells after CD28 stimulation by mAb 248. As clearly shown, CD28 stimulation induced a significant DAG increase after 5 min (more than 150% of control levels in four separate experiments). At 10 min, accumulation of [^{3}H]arachidonate-labelled DAG was no longer detectable (results not shown).

CD28 stimulation is inhibited by the PKC inhibitor GF109203X

The role of PKC in CD28-mediated activation was investigated by using a PKC inhibitor. The Jurkat cells were stimulated with saturating concentrations of mAb 248 in combination or not with PMA for 24 h in presence of the synthetic aminoalkyl bisindolylmaleimide GF109203X (Table 2). mAb 248 alone induced IL2 secretion, which increased in the presence of PMA (mean of two experiments, 945 versus 16700 pg/ml). PMA at 15 ng/ml could not induce detectable IL2 secretion (results not shown). GF109203X inhibited the CD28-induced IL2 secretion, and the ED_{50} was evaluated in Expt. 1: 30 nM for mAb 248 stimulation and 100 nM for mAb 248 plus PMA. After CD3 stimulation (anti-CD3 mAb + PMA), the ED_{50} was evaluated as being between 10 and 30 nM. The anti-CD3 mAb alone could not induce IL2 secretion in Jurkat cells (results not shown).

Anti-CD28 mAbs directed against distinct regions of the molecule induce PLC activity to different extents

Five anti-CD28 mAbs have been compared for IL2 secretion and PLC activation in Jurkat cells. As shown in Figure 3(a), four out of the five anti-CD28 mAbs could induce IL2 secretion at different levels (248, CD28.6, CD28.4, CD28.2: 8475, 2962, 910 and 250 pg/ml respectively). In contrast, CD28.5 did not induce any IL2 secretion (< 10 pg/ml) detectable by IL2 e.l.i.s.a. mAb 248 could only induce IL2 secretion without addition of PMA (375 pg/ml).

We then evaluated the ability of each anti-CD28 mAb to mobilize intracellular Ca^{2+} from internal stores at saturating

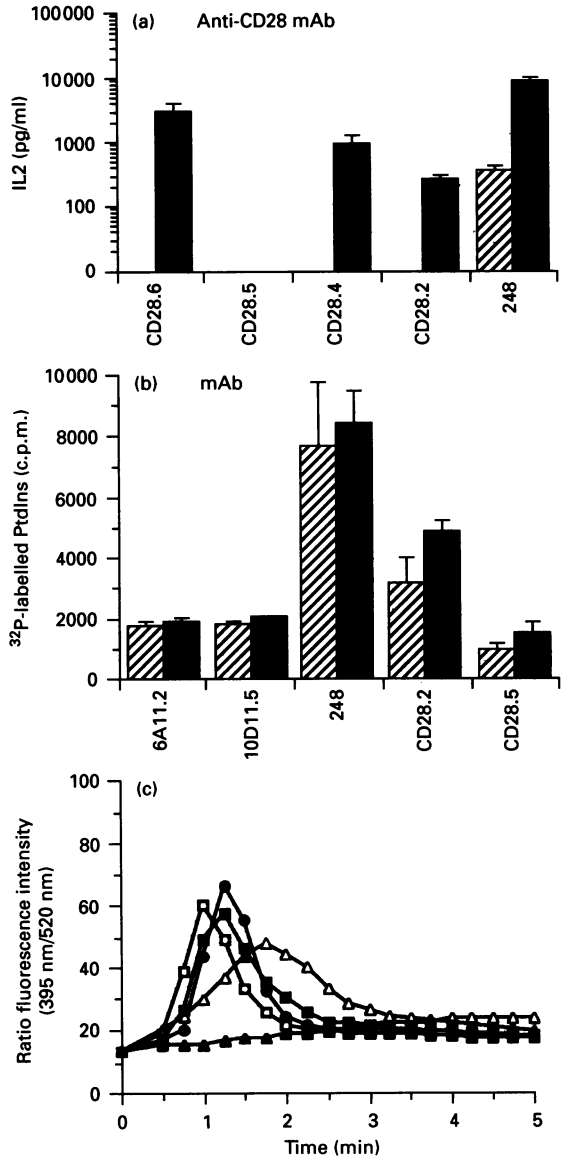

Figure 3 Activation of Jurkat cells by different anti-CD28 mAbs

(**a**) Effect of anti-CD28 mAbs on IL2 secretion in Jurkat cells, in the absence (▨) or presence (■) of PMA at 15 ng/ml. The mAbs were used at 50 μg/ml for IgG anti-CD28 mAbs (CD28.2, CD28.5 and CD28.6) and at 1:100 ascitic dilution for IgM anti-CD28 mAbs (CD28.4 and 248). IL2 concentration was measured in 24 h culture supernatants by e.l.i.s.a. and was expressed as pg/ml. The data shown are means ± S.E.M. of four separate experiments. (**b**) Effect of the different anti-CD28 mAbs, control IgM (6A11.2 mAb) and IgG1 (10D11.5 mAb) on PtdIns resynthesis in ^{32}P-labelled Jurkat cells after incubation for 30 min (▨) or 60 min (■). IgM mAbs were used at 1:100 ascitic dilution, 10D11.5 and CD28.2 at 10 μg/ml, and CD28.5 at 50 μg/ml. The values shown are mean values ± S.E.M. from two separate experiments. The results are expressed as c.p.m. (**c**) Intracellular Ca^{2+} mobilization induced by the different anti-CD28 mAbs in the absence of extracellular Ca^{2+} (10 mM EGTA). The mAbs were used at saturating concentrations: mAbs 248 (●) and CD28.4 (□) at 1:100 ascitic dilution, CD28.2 at 10 μg/ml (■), CD28.5 (▲) and CD28.6 (△) at 50 μg/ml on the Indo-1-labelled Jurkat cells. The results are expressed as variations in violet-to-blue ratio fluorescence intensity as a function of time. The experiment shown is representative of four separate experiments.

mAb concentrations. The results are depicted in Figure 3(c): mAbs CD28.2, CD28.4, CD28.6 and 248 induced a rapid (75–105 s), strong (peak value between 68 and 48 arbitrary units) and a transient $[Ca^{2+}]_i$ increase. In contrast, intracellular Ca^{2+} mobilization was not detected after mAb CD28.5 stimulation. As

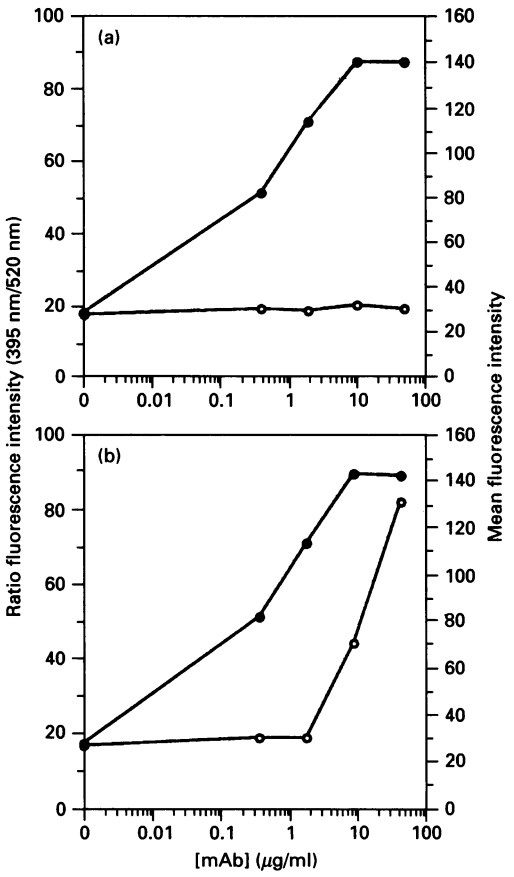

Figure 4 Dose–response in $[Ca^{2+}]_i$ increase induced by anti-CD28 IgG mAbs of low affinity for the CD28 molecule

CD28.5 (**a**) and CD28.6 (**b**) were used at different concentrations (0.4, 2, 10, 50 μg/ml). For each concentration, two events were analysed: site occupation at cell surface, represented by mean fluorescence intensity (●), and $[Ca^{2+}]_i$ rise, by ratio fluorescence intensity (○).

shown in Figure 4, CD28.5 and CD28.6 displayed similar binding characteristics, although only the latter was able to induce a $[Ca^{2+}]_i$ increase.

PLC activation results in PtdInsP_2 breakdown, followed by PtdIns resynthesis. In this light, we measured ^{32}P-labelled PtdIns contents in Jurkat cells, 30 min and 60 min after stimulation by different anti-CD28 mAbs. As shown in Figure 3(b), mAbs 248 and CD28.2, but not CD28.5, increased [^{32}P]PtdIns levels at 30 or 60 min. The results for CD28.4 are similar to the results for CD28.2 (results not shown).

There is a fair correlation between the ability of each individual mAb to mobilize intracellular Ca^{2+} and to induce PtdIns resynthesis: mAbs 248 and CD28.2 induced both $[Ca^{2+}]_i$ increase and PtdIns resynthesis. Conversely, mAb CD28.5, which was unable to mobilize $[Ca^{2+}]_i$, was indistinguishable from irrelevant control mAbs in inducing PtdIns resynthesis.

The anti-CD28 mAbs 248, CD28.2 and CD28.6 induce long-term $[Ca^{2+}]_i$ increase in Jurkat cells

We next evaluated the duration of the $[Ca^{2+}]_i$ increase induced by activating anti-CD28 mAbs, CD28.2, CD28.6 and 248 (Figure 5). In these experiments, the three anti-CD28 mAbs (CD28.2, CD28.6 and 248) tested gave almost similar results. The Ca^{2+} response was stable until at least 1 h after stimulation, then

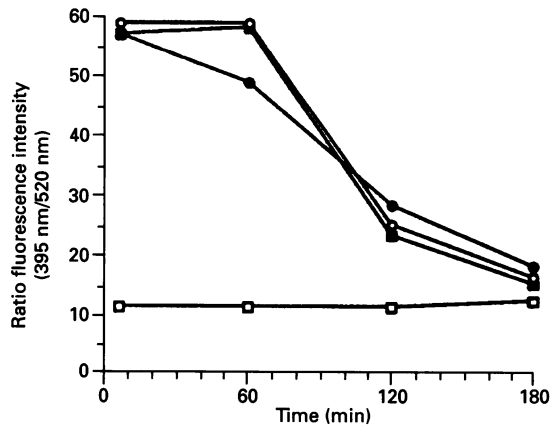

Figure 5 $[Ca^{2+}]_i$ accumulation induced by different anti-CD28 mAbs at long term

The Indo-1-labelled Jurkat cells were incubated without mAb (□) or with anti-CD28 mAbs at saturating concentration [248 at 1:100 ascitic dilution (■), CD28.2 at 10 μg/ml (○) and CD28.6 at 50 μg/ml (●)]. $[Ca^{2+}]_i$ rise was evaluated at 5, 60, 120 and 180 min. Data shown are representative of one of three separate experiments.

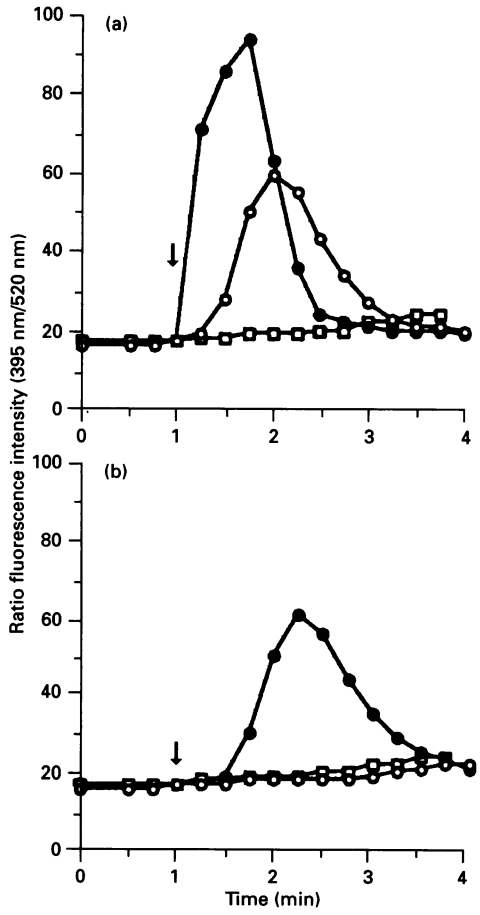

Figure 6 Effect of anti-CD28 mAbs cross-linking on intracellular Ca^{2+} mobilization

The Indo-1-labelled Jurkat cells were stimulated by CD28.2 at 10 μg/ml (**a**) or CD28.5 at 50 μg/ml (**b**) in the absence (○) or presence (●) of GAM Ig; 40 μl of the latter was added after 1 min of incubation with anti-CD28 mAb (indicated by the arrow). In controls, the cells were incubated with the antiserum alone (□).

decreased, but was still detectable at 2 h (25, 28 and 23 arbitrary units respectively) and reached baseline levels at 3 h.

Cross-linking of non-activating anti-CD28 mAb CD28.5 results in $[Ca^{2+}]_i$ increase in Jurkat cells

We performed these experiments in Ca^{2+}-deprived medium, with two IgG anti-CD28 mAbs, CD28.2 and CD28.5. mAbs were added to the cells for 1 min at 37 °C before addition of goat anti-(mouse Ig) antiserum (GAM Ig). CD28.2 alone induced a $[Ca^{2+}]_i$ increase, and addition of GAM Ig induced both a stronger (peak value 58 versus 93 arbitrary units, when GAM is added at 1 min) and more rapid response (Figure 6a). CD28.5 alone at saturating concentration did not induce a $[Ca^{2+}]_i$ increase (Figure 6b); however, addition of GAM Ig induced a $[Ca^{2+}]_i$ increase, starting 45 s after GAM Ig addition, with a peak at 145 s.

DISCUSSION

A role for PtdIns PLC in CD28 signalling has been proposed on the basis of several findings. Ledbetter et al. [28] showed that CD28 triggering induced $[Ca^{2+}]_i$ increase and InsP_3 generation in peripheral blood T lymphocytes upon cross-linking. In Jurkat cells, it was shown that an anti-CD28 mAb (9.3) induced InsP_3 generation and $[Ca^{2+}]_i$ increase [16]. However, in peripheral-blood T cells CD28-induced Ins*P*s generation was increased by PMA treatment, whereas that induced by CD3 was inhibited [28]; moreover, no PKC translocation was demonstrated in Jurkat cells after CD28 stimulation, in contrast with CD3 [16]. Finally, we were unable to detect a significant increase in Ins*P*s induced by anti-CD28 mAbs using the JA3 Jurkat clone [6].

In the present study, we have tested the role of PtdIns PLC activation after CD28 triggering, using a $CD28^+$ Jurkat T-cell clone and a panel of anti-CD28 mAbs of various isotypes identifying different CD28 sub-regions [23].

We demonstrate that the transduction mechanism of CD28 signalling involves a PtdIns pathway in Jurkat cells, similar to that with CD3/TcR or CD2.

This conclusion is based on the following experimental evidence: (1) rapid breakdown of PtdInsP_2; (2) InsP_3 accumulation (involved in Ca^{2+} release) and DAG generation (implicated in PKC activation); (3) Ca^{2+} release from intracellular stores followed by Ca^{2+} entry; (4) and finally resynthesis of PtdIns.

These biochemical events fulfil the requirements for demonstration of PLC activity. Previous studies failed to demonstrate the ability of an anti-CD28 mAb (9.3) to mobilize intracellular Ca^{2+} in the absence of extracellular Ca^{2+} in Jurkat cells [19]. Discrepancy with the present results could be mainly due to the different methodologies (spectrofluorimetry or cytofluorimetry) and/or the Jurkat cell clones tested (clone JA3 in the previous, clone JH6.2 in the present study), since JH6.2 studied in this paper was selected as a high responder to stimulation by anti-CD28 mAbs.

mAb 248 led to an accumulation of InsP_3 in *myo*-[^{3}H]inositol-labelled Jurkat cells, reaching a peak between 5 and 10 min. Nonetheless, InsP_3 increase could occur before 2.5 min. In fact, Weiss et al. [16] tested the InsP_3 increase induced by an anti-CD28 mAb (9.3) at early stages (between 1 and 3 min), showing that mAb 9.3 could induce InsP_3 increase at 1 min. Long-term InsP_3 generation can also be induced by anti-CD3 mAbs and be sustained up to 20 min in CD3-stimulated Jurkat cells [29]. This phenomenon could provide an explanation for Ca^{2+} entry via the plasma-membrane Ins(1,4,5)P_3 receptor [30]. We also demonstrated that the pool of $[Ca^{2+}]_i$ mobilized by CD28 appeared superimposable on that mobilized by CD3/TcR activation.

However, this does not necessarily mean that the PLC activity induced by CD3/TcR complex and by the CD28 molecule is the same: the $[Ca^{2+}]_i$ increase associated with CD3 and CD28 signalling is differently regulated by phorbol esters in peripheral-blood T cells [27].

Moreover, there are some differences between the Ca^{2+} response induced by CD3 as opposed to CD28 molecules. Anti-CD3 mAb induced a rapid Ca^{2+} increase (peak level at 30 s), whereas the Ca^{2+} response to CD28 was slightly delayed (peak level at 60–120 s). This difference could be due to different surface molecule occupancy requirements, since CD3-induced $[Ca^{2+}]_i$ increase starts when less than 10% of membrane sites are saturated [31], as demonstrated by simultaneous monitoring of mAb binding and $[Ca^{2+}]_i$ increase. Conversely, in the case of CD28, we observed that full site saturation was required before intracellular Ca^{2+} mobilization took place (results not shown). This suggests that CD28 aggregation and a high level of interaction with its ligand B7/BB-1 may be important in cellular responses.

We observed both $[Ca^{2+}]_i$ increase and DAG generation after CD28 stimulation, which have been implicated in PKC activation [32]. To evaluate the importance of PKC in the CD28 activation pathway, we have tested the effect of a potent PKC inhibitor on CD28-mediated IL2 secretion.

The bisindolylmaleimide GF 109203X belongs to a new family of compounds structurally related to staurosporine [24]. GF 109203X blocks IL2 secretion induced by an anti-CD28 mAb: mAb 248 alone or in combination with PMA (Table 2) or with PHA (results not shown). This suggests that PKC activity is involved in CD28-mediated T-cell activation.

In another study, Van Lier et al. [33] used another PKC inhibitor, 1-alkyl-2-methylglycerol (AMG), and were unable to inhibit IL2 secretion after stimulation of Jurkat cells by a combination of soluble anti-CD3 and anti-CD28 mAbs. AMG recognizes a different PKC region, namely the agonist-binding site. This could explain the variable effects of the different PKC inhibitors.

The PKC activity used in CD28 signalling or CD3/TcR signalling, or their regulation, could be different. In fact Weiss et al. [16] demonstrated that CD28 activation of Jurkat cells was not associated with a PKC translocation, unlike that by CD3.

In the present study we used five anti-CD28 mAbs: one of them had been already characterized [18], and the others have been generated by fusing mouse spleen cells immunized against human CD28 cDNA-transfected murine T-cell hybridoma [23]. Interestingly, the anti-CD28 mAbs differed in their capacity to induce IL2 secretion in Jurkat cells. Differences in mAb isotypes cannot explain such findings, e.g. two IgM mAbs, 248 and CD28.4, differed in terms of IL2 secretion (8475 and 910 pg/ml respectively).

Using this panel of mAbs, we have raised the question of the relevance of PLC activity in CD28-mediated IL2 release. We observed that the soluble anti-CD28 mAb CD28.5 was unable to induce PtdInsP_2 hydrolysis (results not shown), $[Ca^{2+}]_i$ release or PtdIns resynthesis. The difference between mAb CD28.5 and other anti-CD28 mAbs in their ability directly to cause an increase in $[Ca^{2+}]_i$ could not be ascribed to differences in affinity, because another mAb, CD28.6, with the same affinity as mAb CD28.5, could induce a $[Ca^{2+}]_i$ increase. Alternatively, this may be due to differences in the extracellular region recognized by the anti-CD28 mAbs. Results on cross-inhibition of binding on Jurkat cells tend to support this view, since mAb CD28.5 could recognize a region different from those recognized by the other anti-CD28 mAbs [23].

Anti-CD28 mAbs, which trigger quantitatively different IL2 responses (from 250 pg/ml to 8475 pg/ml) in Jurkat cells, can induce grossly the same $[Ca^{2+}]_i$ increase in early events (60–120 s). Similar results have been described in Jurkat cells stimulated by the CD3/TcR pathway using three anti-CD3 mAbs [34].

Differences in degree of $[Ca^{2+}]_i$ increase at long term could explain differences in IL2 secretion in Jurkat cells and duration of the Ca^{2+} signal [35]. But the anti-CD28 mAbs, 248, CD28.2 and CD28.6, induce a similar Ca^{2+} response at long term (2–3 h). An increase in $[Ca^{2+}]_i$ after surface-molecule cross-linking can be detected in T cells [36]. mAb CD28.5, which cannot directly induce a $[Ca^{2+}]_i$ increase, causes a Ca^{2+} response after cross-linking. The role of receptor cross-linking in signal transduction has been demonstrated in various systems [37–39].

Receptor clustering, even when mediated via domains distinct from the hormone-binding site on the receptor molecule, appears to be a sufficient signal for cell-activation induction. mAb CD28.5 induces a $[Ca^{2+}]_i$ increase, but only after cross-linking with goat anti-(mouse IgG); this suggests that CD28 homodimer aggregation is sufficient to induce a $[Ca^{2+}]_i$ increase. In addition, Williams et al. [40] have recently shown that Sepharose-beads-linked, but not soluble, anti-CD28 mAb is sufficient to induce detectable IL2 secretion in Jurkat cells. Consequently, it will be of interest to investigate which are the regions recognized by the different anti-CD28 mAbs.

In conclusion, the present study demonstrated that PLC activity can be initiated by the CD28 molecule in Jurkat cells. However, the CD28 pathway synergizes with CD3/TcR or CD2 in T-cell activation. The latter are also associated with a PtdIns-PLC activation. In order to explain the synergies between these molecules, putative differences between the PtdIns-PLC activated by CD28 and the CD3/TcR or CD2 pathway should be demonstrated. Alternatively, another second-messenger system could be involved, such as protein tyrosine kinases which could play a role in the CD28 T-cell pathway [41,42].

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