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Review

T-cell signal transduction and the role of protein kinase C

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Abstract. The T lymphocyte has a vital part to play in maintaining the host response to bacterial and viral infection and also appears to play a key pathological role in autoimmune diseases such as rheumatoid arthritis. In this review, we summarize the signalling pathways which trigger antigen-driven T-cell proliferation and examine the evidence which suggests that protein kinase C (PKC) is fundamental to this process. Finally, we discuss the therapeutic potential that PKC inhibitors may have in the treatment of autoimmune disease.

Key words. T-cell; signal transduction; protein kinase C.

T-lymphocyte function in health and disease

T lymphocytes play a major role in the acquired immune response by which the host eliminates bacterial and viral infections and destroys tumourigenic cells. This response is orchestrated by $CD4^+$ T helper lymphocytes which promote intracellular killing by macrophages, antibody production by B lymphocytes and clonal expansion of cytotoxic T lymphocytes. Cytotoxic $CD8^+$ T lymphocytes recognize viral antigens expressed on the surface of virally infected cells and by destroying these cells play an important part in host defence against viral infection. In concert with natural killer (NK) cells, macrophages and B lymphocytes they also mediate the destruction of malignant cells. The strength of this system is its adaptability; T lymphocytes can rearrange the V, D and J elements of their T-cell receptor (TCR) genes to create many different clones each with a different TCR specificity. Only those clones of lymphocytes bearing TCRs with adequate affinity for a particular presented antigen will be triggered by that antigen to proliferate and develop into effector cells. This allows the host to tailor the immune response to the infective organism. Furthermore, after elimination of an infection, the antigen-specific clones remain expanded as 'memory' lymphocytes, providing a more rapid response to a second exposure to the same antigen. However, one major weakness of this system is an inability, on rare occasions, to distinguish between potential pathogens requiring an appropriate host immune response and antigens derived from the host which initiate an inappropriate, destructive and persistent immune response.

T-lymphocyte proliferation in autoimmune disease

Inappropriate proliferation of T lymphocytes in response to 'self' antigens is thought to underlie the aetiology of several autoimmune diseases, e.g. rheumatoid arthritis [1], multiple sclerosis [2] and psoriasis [3]. Rheumatoid arthritis is a chronic, autoimmune inflammatory disease that leads to destruction of hard tissue (especially cartilage) in joints. The aetiology of the disease is not known, but both genetic and environmental factors are thought to play important roles in the development of the disease. The most striking argu-

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ments in favour of T-cell involvement in the aetiology of this disease are the strong association of the disease with selected class II human leukocyte antigen (HLA) haplotypes (i.e. particular class II major histocompatibility complex (MHC) genes) [4, 5] and the fact that in experimental models of arthritis the disease can be transferred by isolated T-cell lines. All HLA haplotypes that correlate positively with rheumatoid arthritis share a common amino acid sequence in the polymorphic third hypervariable region of the HLA-DR β1 chain [5]. These observations accord with the idea that this shared epitope is involved in the binding and presentation of a rheumatoid arthritis autoantigen to the T-cell receptors of a population of $CD4^+$ 'rheumatogenic' T cells [6]. These activated T cells home to synovial tissue via specific interactions with 'addressin' molecules expressed on the surface of high endothelial venules in response to some, as yet undefined, stimulus. However, the search for these activated 'rheumatogenic' T cells in patients with disease and attempts to identify particular subpopulations of activated T cells in the inflammed joints of rheumatoid arthritis patients has met with mixed success. In fact, the proliferative response to recall antigens and to mitogens and secretion of cytokines following mitogen stimulation are reduced in peripheral blood [7] and synovial T cells [8] from patients with rheumatoid arthritis when compared with peripheral blood T cells from normal control subjects. Nevertheless, the disease may be induced and perpetuated by the activation of specific subpopulations of T cells which infiltrate the synovial membranes of inflamed joints, and the status of the peripheral T-cell population may not be relevant to this. In support of this, there is evidence for the focal synthesis of T-cell activation markers such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) in the synovial membrane from rheumatoid arthritis patients [9]. Furthermore, activated synovial T cells from patients with rheumatoid arthritis can induce the production of cartilage-degrading enzymes such as collagenase from dermal fibroblasts via mechanisms involving direct cell-cell contact [10]. However, the idea that the disease is driven by a particular subpopulation of T cells with restricted expression of TCR variable β gene segments which are localized to the inflamed synovium is still subject to debate [11, 12]. The lines of evidence which support T-cell involvement in the aetiology of multiple sclerosis and psoriasis are similar, i.e. strong association of these diseases with particular HLA haplotypes [13–15] and transfer of disease by T cells in animal models [16, 17]. Again, there is evidence for the presence of activated T cells at the lesional sites, but association of disease with restricted Vβ usage of lesional T cells has not been unequivocally demonstrated.

Naive and memory T cells; differentiation of T cells into effectors; importance of IL-2 in the proliferative response

Circulating $CD4^+$ T cells contain both naive T cells and cells previously primed with antigen, i.e. memory T cells, and these are distinguishable from one another on the basis of differential expression of the isoforms of the tyrosine phosphatase, CD45 [18]. Naive T cells are relatively recent emigrants from the thymus that have not yet encountered antigen. They are selectively recruited from the bloodstream into peripheral lymph nodes during lymphocyte recruitment [19]. When stimulated by specific antigen in the discrete microenvironment of lymphoid tissue, they produce IL-2 [20] but little or no IL-4 or IFN-γ. However, it is questionable whether the antigen-driven proliferative response of $CD4^+$ naive T cells is mediated through IL-2, since they produce little or no IL-2R chain [21]. Following stimulation with antigen and subsequent expansion, naive T cells differentiate into effector cells and are released into the circulation. These effector T cells express various homing/adhesion molecules lacking on naive T cells, and these homing molecules assist effector T cells to penetrate the walls of capillary blood vessels and target them to different tissues [6]. In the mouse, T cells stimulated to proliferate by specific antigen will differentiate into one of three stable differentiation states distinguishable by their cytokine secretion patterns [22]. Activated Th1 (T helper 1) cells produce IFN-γ, IL-2, TNFα, TNFβ and IL-12 and assist in the elimination of virally and bacterially infected cells through the induction of a cell-mediated immune response [23]. IL-2 and IFN-γ produced by Th1 T cells may assist the differentiation of $CD8^+$ T cells into cytotoxic T cells capable of inducing elimination of virally infected cells. Another important role of Th1 T cells is probably to elicit phagocyte-mediated defence against pathogens, and the cytokines they produce stimulate macrophages to phagocytose microbes. Th1-like responses seem to be associated with a number of autoimmune diseases, e.g. rheumatoid arthritis [24, 25], multiple sclerosis [26], psoriasis [27] and insulin-dependent diabetes mellitus (IDDM) [28]. Activated Th2 (T helper 2) cells produce IL-4, IL-5, IL-6 and IL-13 and mediate antiparasite responses by providing help to B cells to differentiate into antibody-producing plasma cells. Although many of the cytokines produced by Th2 T cells, e.g. IL-4 and IL-13, suppress macrophage function and have anti-inflammatory effects, Th2-dominant responses are involved in the pathogenesis of allergy and asthma [29, 30]. Th0 cells are characterized by the production of cytokines of both Th1 and Th2 types and may be obligatory precursors of Th1 and Th2 cells. The differentiation pathway taken by a naive T cell is deter-

mined by a large number of factors which may include antigen [31], the presence of certain cytokines released by antigen-presenting cells and other inflammatory cells at the time of antigen presentation [32, 33], the nature of the antigen-presenting cell and the genetic predisposition of the individual. In cell culture experiments, both Th1 and Th2 cell clones and lines maintain the ability to mount a proliferative response to specific antigen. For some Th1 cell lines, the proliferation is probably driven through the production of IL-2 and its high-affinity receptor. It is interesting to note that these Th1 T-cell lines produce lower levels of their own growth factor, IL-2, when stimulated with Ag/antigen-presenting cell (APC) but higher levels of effector cytokines such as IFN-γ when compared with the cytokine pattern derived from Ag/APC-stimulated naive T cells (high IL-2, low IFN-γ) [34]. However, for other terminally differentiated Th1 T-cell clones the proliferative response to Ag/APC is mediated primarily through IL-12, not IL-2 [35]. For Th2 cells, IL-4 probably plays a key role in driving the proliferative response to specific antigen. Clearly, there are important differences in the signal transduction pathways mediating antigen-driven proliferation of Th1 and Th2 cells. In the mouse, and particularly in humans, where the distinction between Th1 and Th2-like cells is less clear [36], it is evident that other Th-cell subsets with different but discrete patterns of cytokine production exist and that Th1- and Th2 phenotypes may merely represent the opposite poles of a spectrum of effector phenotypes [37].

As mentioned above, $CD8^+$ T lymphocytes play a role in the cytolytic destruction of target cells. Additionally, they may also participate in the regulation of the immune response (influence $CD4^+$ T-cell development; exert differential effects on Th1/Th2 $CD4^+$ T-cell differentiation). While there is evidence that $CD8^+$ T cells may commit to different effector phenotypes based on patterns of cytokine production, most $CD8^+$ T cells secrete a Th1-like cytokine pattern [22].

Following the resolution of infection and disappearance of antigen, most of the proliferating and effector T cells generated in the primary immune response are rapidly eliminated. However, a population of antigen-reactive T cells survive the response to become long-lived memory T cells [38], and these are capable of mounting a faster and more efficient response on reexposure to antigen (so-called recall response). Furthermore, antigen-driven proliferation of memory $CD4^+$ and $CD8^+$ T lymphocytes is probably dependent on IL-2 [20]. Whilst it is clear that naive $CD4^+$ T cells eventually develop into memory T cells and that memory T cells are 'antigen experienced', the exact lineage of memory T cells, i.e. whether they develop from a separate subpopulation of naive precursors or from effectors, is still subject to debate [38]. Recent data suggest that memory T cells may well reflect the Th1 or Th2 commitment of the original immune response for a limited period of time after the response has disappeared [38]. After that they seem to revert to an IL-2-secreting phenotype similar to that associated with naive T cells.

Thus, the production of interleukin 2 and its high-affinity receptor (IL-2R) is thought to be a key step in the antigen-driven proliferative response of many, but not all, T cells. Furthermore, it is becoming clear that the signal transduction processes which mediate antigen-driven T-cell proliferation differ between naive and memory T cells (for example requirement for costimulatory CD28 molecule), between these T cells and effector T cells, and between different subsets of effector T cells [39]. Although it is clear that activation and transcription of many cytokine, growth factor and other activation-associated genes are involved in antigen-driven T-cell proliferation and differentiation, antigen-induced activation of the IL-2 gene has been most extensively investigated as the model system for examining the mechanism of inducible gene transcription in activated T cells.

Signalling pathways involved in T-cell activation

The specificity of the T-cell antigen receptor signalling response is provided by the mechanism through which the foreign antigenic peptide is presented to and recognized by the T cell. The physiological ligand for the TCR consists of processed antigenic peptide bound to the polymorphic region of plasma membrane-associated MHC class I or II molecules which are present on the surface of antigen-presenting cells. The antigen specificity of T-cell responses is governed by the recognition properties of these clonally distributed, Ig-like TCRs. In general, each lymphocyte clone has a single antigen-binding specificity that is unique to that clone. In response to a given antigen, only those lymphocytes whose TCRs bind that antigen with high affinity will be activated. Activated T cells transcribe and translate a large number of genes, resulting in both the synthesis and release of haemapoietic cell effector molecules and the clonal expansion of that particular T-lymphocyte subpopulation. During T-cell development, signals transduced through the receptor are instrumental in positively selecting cells capable of recognizing foreign antigens and eliminating those which recognize self. Mature cytotoxic T cells respond to T-cell receptor activation by secreting cytotoxic mediators, whilst helper T cells react to activation by releasing cytokines directed to stimulate either cytotoxic T-cell or B-lymphocyte function.

T-cell activation encompasses the 'modulation of cell surface phenotype and the regulation of secretion of critical cytokines' [40] and usually refers to the immune activation of mature T cells in peripheral blood,

lymphatics or tissue. The best characterized of the many changes which occur on the T-cell surface following activation during an immune response are upregulated expression of CD69, transferrin receptors and the IL-2 receptor, and downregulation of the T-cell receptor. Additionally, synthesis of a number of important haemopoietic growth factors, e.g. IL-2, IL-3, IL-4, granulocyte-macrophage-colony stimulating factor (GM-CSF) is upregulated [40].

The interaction of the T cell with the APC: role of accessory molecules; agonists and partial agonists; cross-linking of TCRs and accessory molecules

There are many receptors on the T-cell surface which contribute to the interaction between antigenic peptide and T cell; however, specificity of this interaction is due to the selective binding of the antigen to a discrete TCR. The TCR/CD3 complex comprises the polymorphic TCR α and β subunits in a noncovalent association with the invariant chains of the TCR complex, the TCR ζ chains and the γ, δ and ε of the CD3 antigen complex [41]. CD4 and CD8 molecules contribute to MHC recognition in T helper and cytotoxic T lymphocytes, respectively. Other extracellular proteins are also considered to play a crucial role in T-cell activation. For example, interactions between CD28 and its ligands, B7-1 and B7-2, deliver an important accessory signal, and adhesion molecules such as CD2 and LFA1 also have a part to play.

There is evidence to suggest that the accessory molecules CD4 and CD8 play a key role in determining the nature of the T-cell response to antigens. T-cell activation is generally believed to occur when an adequate number of TCRs are aggregated at the adhesion receptor-generated membrane interface between the T cell and the antigen-presenting cell. In this model, antigens fall into two categories: those inducing full T-cell activation and those which are ignored by the T cell because T-cell affinity for the peptide-MHC complex is below the threshold required to trigger a response. Yet recent data suggest that the true picture is more complicated than this in that TCR engagement can induce different signals, depending on the precise structure of the peptide MHC complex to which it binds [42, 43]. For instance, it seems that some antigens can act as partial agonists in that they are able to induce certain cytokine responses from T cells (e.g. IL-4 release) without evoking the full repertoire of effector responses (IL-2 and IL-2 receptor synthesis). Furthermore, certain peptide MHC complexes are able to selectively block IL-2 production from particular murine Th1 T-cell clones without preventing secretion of IL-3 and IL-2Rα upregulation [44]. Several observations suggest that the accessory molecules CD4 and CD8 play a key role in determining the 'quality' of the T-cell response. Antigens which behave as full agonists (upregulate IL-2 and IL-3 release) can convert to partial agonists (IL-3 release, no IL-2 release) when the contribution of CD4 to the response is abrogated with anti-CD4 antibodies [45]. Furthermore, an MHC class II ligand unable to bind CD4 induces TCR signalling resembling that seen with partial agonists [45]. Additionally, it is possible to separate the effector activities of both $CD4^+$ and $CD8^+$ T cells into those which require (IL-2 secretion) and those which do not require (IL-3 secretion, cell killing) co-stimulatory signals [46]. These observations are most readily explained by a model in which the dissociation rate of the antigen-MHC complex from the TCR determines the signalling output from the T cell. Antigen-MHC complexes which bind with optimal kinetics of interaction will trigger the engagement of the accessory molecules, CD4 and CD8, and the full repertoire of T-cell responses [45]. Those complexes which do not bind with sufficient affinity will fail to induce the engagement of these accessory molecules, and a partial response will result. Thus, specific cross-linking of cell surface molecules involved in antigen-driven T-cell proliferation may be required in order to initiate the full proliferative response to Ag/APC. One theory suggests that CD4 stably binds MHC class II only after assuming an oligomeric state [46]. This results in the formation of aggregates critical for T-cell activation. The TCR regulates specific cross-linking and is itself dependent on lattice formation to trigger the proliferative response. Presumably, cross-linking of TCRs and accessory molecules such as CD4 involved in the initiation of the response allows the *trans*-activation of receptor-linked tyrosine-specific protein kinases involved in the very early signalling events associated with T-cell activation.

TCR/CD3 signal transduction; activation of ZAP-70; activation of CD4/8-associated $p56^{lck}$

Antigen binding to the TCR generates a signal which is transduced across the membrane and into the cytoplasm via the intracellular tails of the CD3γ, δ and ε chains and the TCR ζ molecule (fig. 1). The CD3 γ, δ and ε chains each contain a common immunoglobulin receptor family tyrosine-based activation motif (ITAM), and the ζ chain contains three such ITAMs in its cytoplasmic domain. Each ITAM contains the motif $EX_2YX_2L/IX_7YX_2L/I$ and, when phosphorylated on one or both of its tyrosine residues, provides binding sites for a number of proteins involved in early TCR signal transduction, including two protein tyrosine kinases (zeta-associated protein ZAP-70 and $p59^{fyn}$) which bind via their SH2 regions [47]. ZAP-70 requires a doubly phosphorylated ITAM for binding via its tandem SH2 domains and binds preferentially to one of the three TCR ζ chain ITAMs. The CD4/8

molecules also contain *trans*-membrane and intracellular domains to which a third tyrosine kinase ($p56^{lck}$) binds [48]. So the immediate consequence of antigen recognition is the activation of a protein tyrosine kinase signalling cascade and nonselective inhibitors of tyrosine-specific protein kinases (TSPKs) block T-cell activation probably by acting at this stage [49]. The exact sequence of events has not been completely resolved but probably involves TCR cross-linking and commences with the activation of the src kinases by the dephosphorylation of their negative regulatory sites, probably by CD45, a transmembrane molecule with an intracellular tyrosine phosphatase region. The src kinases then phosphorylate the ITAMs, resulting in the recruitment of ZAP kinase, which is subsequently phosphorylated and activated by $p56^{lck}$ [50, 51]. In agreement with this hypothesis, partial agonists induce formation of the p21 form of phosphorylated ζ but do not induce formation of the p23 form of phosphorylated ζ, nor do they induce phosphorylation of the CD3ε ITAM. However, full agonists induce formation of both phosphorylated forms of ζ, p21ζ and p23ζ, as well as CD3ε. More significantly, complete agonists, unlike partial agonists, induce tyrosine phosphorylation and activation of ZAP-70 [52]. Furthermore, in situations where the TCR is stimulated using a wild-type peptide MHC ligand and CD4 availability is reduced by addition of anti-CD4 antibodies, the phosphorylation pattern changes from that produced by a full agonist to that produced by a partial agonist, and ZAP-70 phosphorylation and activation does not occur [45]. These observations fit nicely with the idea that only complete agonists are able to productively associate $p56^{lck}$-bearing CD4 molecules with the TCR. Arguably, $p56^{lck}$ can then phosphorylate additional ITAMs (on ζ to create p23ζ, and perhaps on the tails of other CD3 components), and this creates binding sites for the SH2 domains of ZAP-70, resulting in a productive association of ZAP-70 with the TCR complex. This allows $p56^{lck}$ to phosphorylate and activate ZAP-70, and this is a key event for initiating downstream signals required to produce the full repertoire of activated T-cell responses.

The role of ZAP-70; activation of Vav and p21 Ras

Downstream of these early activation events, the signalling pathways diverge, and many of these pathways are incompletely elucidated. It has been clearly estab-

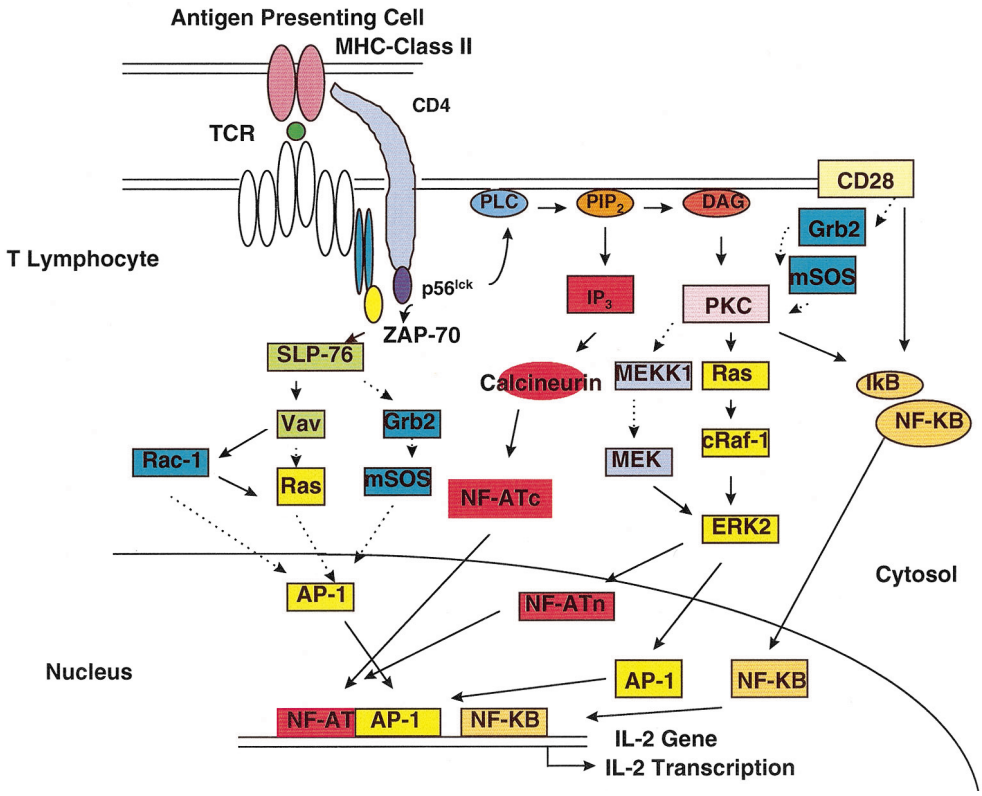

Figure 1. Antigen-driven T-cell signal transduction pathways.

lished that ZAP-70 plays an essential role in full T-cell activation. The evidence for this is that $CD4^+$ T cells taken from severe combined immunodeficiency disease (SCID) patients with defective ZAP-70 function are incapable of T-cell signalling [53, 54], that ZAP-70 point mutants devoid of kinase activity act as dominant-negative mutants and suppress TCR-mediated activation of IL-2 gene activation [55], and finally gain-of-function mutations of ZAP-70 enhance T-lymphocyte responsiveness [56]. The downstream signalling pathways and physiological substrates for ZAP kinase are only slowly being identified. However, one strong candidate is the SH2 domain containing leukocyte protein, SLP-76. Studies have shown that both SLP-76 and the protoongogene product Vav undergo tyrosine phosphorylation in response to T-cell ligation, interact with each other and augment transcription factor activity [57]. Phosphorylation of SLP-76 is diminished in T cells that express a catalytically inactive ZAP-70, and the enzyme will phosphorylate in vitro peptides modelled around the putative Vav-binding sites on SLP-76 [58]. Vav is a 95-kDa protooncogene product expressed specifically in haematopoetic cells and participates in a number of signalling processes activated by cell surface receptors. It contains a number of functional domains, including SH2 and SH3 domains, and a guanine nucleotide exchange factor domain. The identity of the tyrosine-specific protein kinase which activates and phosphorylates Vav following antigen-driven TCR activation is unknown. A number of candidates have been implicated in Vav phosphorylation and activation, and these include probably not ZAP-70 itself [59], but the IFN-receptor-associated Tyk-2 kinase [60] and tyrosine-specific protein kinases in the Tec family [61]. The downstream consequences of the SLP-76-Vav phosphorylation and activation are unclear, but activated Vav may play a role in the activation of Ras-like guanosine 5′-triphosphate (GTP)-binding proteins, a critical step for downstream signalling. The evidence for this comes from claims that, in some studies (but not others), Vav will bind Ras and that Ras activity can be increased by tyrosine-phosphorylated Vav [62, 63]. There is also evidence that tyrosine-phosphorylated, but not nonphosphorylated, Vav will catalyse the GTP/guanosine 5′-diphosphate (GDP) exchange of the related Rho-like GTP/GDP proteins, e.g. Rac-1 [64]. An interesting recent suggestion is that CD45 may participate in these downstream signalling processes by serving as a docking site and assembly point for components of the Ras signalling pathway [65]. The evidence to support this comes from the finding that the components of the signalling pathways immediately upstream of Ras (ras GAP, Grb-2, mSos and Vav) bind to phosphotyrosine residues on CD45 SH2 domains, whereas other SH2-containing proteins involved in T-cell activation (phospholipase Cγ1 (PLCγ1), p85 phosphoinostide 3-kinase (PI3-K)) do not bind to this domain.

Other routes to p21ras activation

While SLP-76-Vav interaction contributes to antigen-driven T-cell activation in most T cells, this association is not required for TCRζ-CD3 ligation-induced IL-2 production in the T-cell hybridoma DC27.10 [66]. It is clear that other pathways are also involved in the activation of p21 ras in antigen-stimulated T cells, and one such pathway involves the accessory molecule, CD28. The APC-associated molecules B7.1 (CD80) and B7.2 (CD86) ligate CD28 on T cells and thus provide a costimulatory signal required for optimal lymphokine production in response to TCR stimulation [67–69]. CD28 contains a (p)YMNM activation motif in its cytoplasmic tail. When tyrosine phosphorylated (probably by $p56^{lck}$) [70], this motif provides a docking site for the SH2 domains of at least two important downstream signalling molecules. One of these is the p85 subunit of PI3-K. The other is Grb-2 (growth factor receptor-binding protein), an adaptor molecule which binds mSos, the gene product of *Drosophila son-of-sevenless* [71]. mSos is a guanine nucleotide exchange factor which activates p21ras. Thus a pathway exists coupling the activation motif in the cytoplasmic tail of CD28 via Grb-2 and mSos to activation of p21ras. Additionally, a third pathway which involves SLP-76-Grb-2-mSos may also modulate of p21ras activity in stimulated T cells. Thus, at least three pathways may operate to trigger p21ras activity in antigen/APC-stimulated T cells: two of these involve activation of SLP-76 (SLP-76-Vav-p21ras and SLP-76-Grb-2-mSos); the third involves activation of the accessory molecule CD28 (CD28 YMNM phosphorylation-Grb-2-mSos-p21ras). The relative importance of each of these pathways in activating p21ras in antigen-driven T-cell activation has not been determined.

Members of the Ras superfamily of proteins function as GDP/GTP-regulated switches that cycle between active GTP-complexed and inactive GDP-complexed states [72]. Guanine-nucleotide exchange factors such as mSos and Vav stimulate formation of the GTP-bound state, whereas GTPase activating proteins (GAPs) catalyse the formation of the GDP-bound state. In T cells, activation of p21ras is coupled to the activation of at least two downstream pathways: the mitogen activated protein (MAP)-kinase cascade and activation of the related GTPase Rac-1. The exact mechanism by which the signals from these pathways are integrated with the Ca^{2+} signal to switch on cytokine transcription factor activity has not been completely elucidated.

Activation of the Ca^{2+} signal

The other key pathway triggered by engagement of the TCR by antigen/MHC is probably the first tyrosine specific protein kinase (TSPK)-controlled pathway to be reasonably well understood and involves the activation of PLCγ1 by tyrosine phosphorylation by either $p56^{lck}$ or $p59^{fyn}$ [73, 74]. Activation via the TCR also induces the formation of a complex between PLCγ1 and a tyrosine phosphoprotein, p36, which may act to recruit the enzyme to the membrane, exposing PLCγ1 to its substrate inositol phospholipids [75]. PLCγ1 then hydrolyses inositol phospholipids to generate two important intracellular messengers, inositol polyphosphates, which promote Ca^{2+} release from intracellular stores such as the endoplasmic reticulum, and diacylglycerol, which is the physiological activator of many isoforms of protein kinase C [76].

Ca^{2+} signal triggered responses: activation of calcineurin and nuclear localisation of NF-AT

The increase in intracellular Ca^{2+} (the so-called Ca^{2+} signal) following antigen/MHC-driven T-cell stimulation triggers a multitude of different pathways which modulate the activation process. Many of these downstream pathways have not been elucidated. However, one important consequence of the increase in intracellular Ca^{2+} following TCR-dependent T-cell activation is the activation of the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin. The cellular target for activated calcineurin is the preexisting form of NF-AT (NF-AT1; nuclear factor of activated T cells; see below) [77], one of several transcription factors required for expression of IL-2. The phosphorylated form of NF-AT, which is found in resting T cells, is restricted to the cytoplasm, and its ability to bind DNA and to associate with activator protein-1 (AP-1) (see below) is reduced when compared with its dephosphor-ylated form. The NF-AT molecule contains a regulatory domain which is distinct from its DNA-binding domain and which, in its phosphorylated form, prevents nuclear import of NF-AT. Deletion of this site in NF-ATx1 leads to nuclear localization of NF-ATx1 independent of the Ca^{2+} signal [78]. Calcineurin associates directly with this domain. Furthermore, this domain, when phosphor-ylated, is a substrate for calcineurin in isolated enzyme experiments. Thus, the association of this domain with calcineurin and, presumably, its dephosphorylation, results in nuclear localization of NF-AT and increased NF-AT transcription factor activity. In agreement with this, NF-AT1 is dephosphorylated, translocated from the cytoplasm to the nucleus and shows an increase in DNA-binding ability within minutes of TCR stimulation of T cells [79]. However, after several hours of TCR stimulation, the majority of NF-AT1 molecules in the T cell revert to their original phosphorylated form, reappear in the cytoplasm and show reduced affinity for DNA. The immunosuppressive drugs, cyclosporin A and FK506, when complexed with their corresponding immunophilins, cyclophilin and FKBP12, probably suppress cytokine gene transcription through inhibition of calcineurin [80, 81].

Signal transduction downstream of p21ras: the MAP-kinase cascade; integration of the p21ras and Ca^{2+} signals to activate NF-AT/AP-1 transcription factor activity

It is generally accepted that the triggering of the Ca^{2+} signal and the induction of p21ras activation are key events in TCR-driven T-cell activation. Furthermore, these pathways seem to operate synergistically to induce expression of IL-2 and its high-affinity receptor. Regulation of IL-2 production by the TCR requires the coordinate action of multiple transcription factors, which include NF-AT, AP-1, NF-κB and Oct-1 [82]. These transcription factors when activated bind to the promoter region upstream of the IL-2 gene to switch on transcription of the gene. One of the major targets for the Ras-activated and Ca^{2+} signal-activated pathways is the nuclear factor of activated T cells (NF-AT), a key transcription factor regulating IL-2 gene transcription [83]. Multiple NF-AT subunit isoforms exist, and some of these are found in the cytoplasm of quiescent T cells (NF-ATp, NF-ATc, NF-AT3, NF-AT4) and translocate to the nucleus in response to Ca^{2+}/calcineurin signals in TCR activated T cells [84]. There they combine with newly induced molecules of another transcription factor, AP-1, to form a functional transcription unit. Interaction of NF-AT with AP-1 is essential for NF-AT transcriptional activity. For NF-ATc, two clearly separable functional domains have been identified: the N-terminal domain (residues 1–415) controls the subcellular localization in response to the Ca^{2+} signal; the C-terminal domain (residues 416–591) is sufficient for DNA binding to the distal antigen-receptor response element of the IL-2 gene enhancer [85].

It seems that multiple p21ras effector pathways regulate NF-AT/AP-1 transcription factor activity in TCR-activated T cells. One pathway is mediated by the interaction of p21Ras GTP with the serine protein kinase p74Raf-1. The Ras/Raf interaction is essential to recruit p74Raf-1 to the plasma membrane, where it can be tyrosine phosphorylated and activated [86, 87]. Activation of Raf-1 switches on a Ras-dependent serine protein kinase cascade consisting of Raf-1, MEK-1 (MAP kinase kinase-1) and ERK-2 (a MAP kinase). This pathway is necessary, yet by itself not sufficient for induction of NF-AT/AP-1 transcription factor activity in T cells. A dominant-negative MEK-1 can inhibit NF-AT/AP-1 transcription factor activity, but expression of a constitu-

tively active MEK-1 mutant, which can activate ERK-2 in T cells, cannot substitute for constitutively active p21Ras and synergize with the Ca^{2+} signal to induce NF-AT/AP-1 transcription factor activity [88]. Another parallel pathway implicated in NF-AT/AP-1 activation involves ras activation of the ras-related GTPase Rac-1. The evidence for the involvement of this pathway in ras-activation of NF-AT/AP-1 transcription factor activity comes from a demonstration that a dominant-negative mutant of Rac-1 did not suppress p21ras-induced activation of ERK-2 but did inhibit TCR/p21ras-induced NF-AT/AP-1 transcription factor activity [88]. However, even a combination of constitutively active MEK-1 and Rac-1 could not substitute for activated p21ras and synergize with the Ca^{2+} signal to induce NF-AT/AP-1 transcription factor activity [88]. Therefore, at least one more p21ras effector pathway must be required to induce NF-AT/AP-1 transcription factor activity.

The precise mechanism by which activation of these pathways contributes to increased NF-AT/AP-1 transcription factor activity has yet to be established. The transcriptional activity of the NF-AT-binding site is mediated through the binding of a complex comprising a member of the NF-AT family of DNA-binding proteins and the AP-1 family of DNA-binding proteins. AP-1 proteins are dimers comprised of members of the fos and jun families of proteins, but the nature of the AP-1 component in the NF-AT/AP-1 complex in T cells has not been unequivocally established. However, it seems that, in T cells, Rac-1 activation is necessary and sufficient for AP-1 induction and that Rac-1 can entirely mediate the effects of Ras on AP-1 activation [88]. Thus, the role of Rac-1 in AP-1 activation could explain the Rac-1 requirement for induction of NF-AT/AP-1 transcription factor activity. One potential pathway through which Rac-1 might increase transcription of fos and jun family members which form AP-1 is through its ability to regulate a kinase cascade phosphorylating Elk-1 [88]. In support of this, expression of Rac-1 and ras in T cells will induce phosphorylation of Elk-1. Furthermore, Elk-1 is a substrate for a number of kinases in the SAPK and JUNK families [89, 90]. Elk-1 is one of a family of proteins that can form a ternary complex with the transcriptional activator serum response factor (SRF), and Elk-1/SRF complexes are necessary and sufficient mediators of c-fos serum response element induction. Additionally, Rac-1 may also regulate c-jun transcription (regulated by MAP-kinase pathways) and c-jun phosphorylation (possibly through control of JNK-1 activity) [91]. Thus, Rac-1 regulation of one or more unknown MAP-kinase cascades may play a role in regulating c-fos and c-jun gene expression and phosphorylation and thus AP-1 transcription factor activity.

CD28 costimulatory signalling pathways; triggering of NF-κ B transcription factor activity

Another important transcription factor involved in up-regulation of transcription of the genes for IL-2 and its high-affinity receptor is NF-κB. When activated, NF-κB binds specific DNA sequences in the promoter sequences upstream of several cytokines involved in host defence against disease and activates transcription of these genes. The transcription factor operates as heterodimeric complexes composed of members of the Rel/N NF-κB family of polypeptides. The members of this family include c-Rel, RelA/p50 and RelB/p65, and a number of regulatory proteins with inhibitory function, called I-κB.

The signal which initiates NF-κB activation following antigen-specific T-cell activation is probably provided by the costimulatory molecule CD28 [92]. Indeed, c-rel knockouts exhibit profound defects in IL-2 secretion from their T cells and in the T-cell proliferative response to CD28 and TCR costimulation [93]. The mechanism by which CD28 ligation induces NF-κB activation is not well understood but probably involves the activation of one or more tyrosine-specific protein kinases and the subsequent activation of phospholipase A2 and Δ5-lipoxygenase [94]. The products of the action of these enzymes generate reactive oxygen species (ROS) which activate NF-κB, and in primary T lymphocytes, ligation of CD28 results in the rapid formation of ROS. In their inactive state, NF-κB dimeric complexes are sequestered in the cytoplasm associated with an inhibitor, I-κB, which inhibits both the nuclear translocation and DNA binding of NF-κB. The signals provided by CD28, including ROS, induce a rapid degradation of I-κB molecules [95]. The precise mechanism for this has not been elucidated but appears to involve a signal (ROS?)-induced site-specific phosphor-ylation by an as yet incompletely characterized serine/threonine protein kinase termed CHUK [96], which appears to target I-κB for covalent addition of multiple copies of the ubiquitin polypeptide. This modification subsequently allows proteolytic degradation of the ubiquitinated I-κB by the 26S multicatalytic proteinase (proteosome) complex [97, 98]. The consequence of this is translocation of NF-κB dimers to the nucleus, their binding to specific elements in the promoters of genes involved in T-cell proliferation, e.g. IL-2 and IL-2R, and participation in activation of transcription of these genes.

Integration of transcription factor activity in controlling the expression of IL-2

The IL-2 transcriptional apparatus integrates multiple signals to determine expression of the IL-2 gene by using many diverse transcription factors (e.g. NF-AT, NF-κB,

Oct-1) [99] that are activated or inhibited by different signalling pathways. Blocking the activity of just one or two of these transcription factors is sufficient to completely inhibit IL-2 expression. The explanation behind this is that none of these transcription factors will interact stably with its target site in the IL-2 enhancer unless all the factors are present.

IL-2 and its receptor

IL-2 plays a vital role in the growth and clonal expansion of many types of T cells and in the differentiation of thymocytes, peripheral T and B lymphocytes and other cells of haemopoietic origin. The IL-2 required for antigen-driven T-lymphocyte proliferation may be derived from the stimulated T-lymphocyte itself or from other cells involved in the immune response. Thus the key factor in determining the T cell's ability to mount a proliferative response to IL-2 will be expression of the high-affinity IL-R on the surface of the antigen-activated T cell. The IL-2R comprises three subunits (IL-2Rα, IL-2Rβ and IL-2Rγ), which are encoded by different genes (reviewed in refs 100, 101). Il-2Rγ is constitutively expressed on various populations of haematopoietic cells, whilst expression of both IL-2Rα and IL-2Rβ is restricted to lymphocytes and monocytes/macrophages. Three classes of IL-2 receptor proteins exist: high-affinity receptors ($K_d \sim 10^{11}$ M) are heterotrimers containing α, β and γ subunits; intermediate-affinity IL-2Rs ($K_d \sim 10^9$ M) contain β and γ subunits, and low-affinity subunits contain only the α chain. Although both intermediate- and high-affinity IL-2Rs are capable of transducing mitogenic signals in response to IL-2, the high-affinity receptor (and, therefore, IL-2Rα) is probably required to bind the relatively low concentrations of IL-2 produced physiologically.

Transcriptional control of IL-2Rα expression in response to Ag/APC and IL-2

T lymphocytes regulate their responsiveness to IL-2 through the transcriptional control of one of the components of the high-affinity IL-2R, namely the IL-2Rα gene [102]. Of course, specific Ag/APC will induce expression of both IL-2 and its high-affinity receptor; however IL-2 itself can also induce transcription of IL-2Rα gene [103]. The IL-2RI promoter contains multiple regulatory sequence elements that bind several transcription factors, some of which are similar to those which control IL-2 gene transcription in response to Ag/APC. IL-2Rα transcription in response to Ag/APC is, at least in part, controlled by two regulatory regions in its promoter, termed PRRI and PRRII [104]. PRRI is an inducible proximal enhancer that contains DNA-binding sites for SRF and NF-κB. Thus, NF-κB proteins play an important regulatory role in the transcription of both IL-2 and IL-2Rα genes in response to Ag/APC. PRRII is a T-cell-specific enhancer element which binds the T-cell-specific ETS protein, ETS-related transcription factor (ELF) [105] and certain high-mobility group (HMG) proteins. The interactions between the proteins which bind to PRRI and PRRII probably result in the formation of a complex that regulates the transcriptional activity of the IL-2Rα promoter upon stimulation with Ag/APC. Interestingly, neither of these elements regulate IL-2Rα transcription induced by IL-2 [104]. This is mediated through PRRIII [104], a complex response element that lies upstream of PRRI and PRRII. PRRIII is composed of DNA-binding sites for the IL-2-inducible STAT proteins (see below), STAT 5A and STAT 5B, for the lymphoid/myeloid specific Ets family protein, Elf-1, for HMG-I(Y) and a gamma-amino-n-butyrate (GATA)-like protein. Binding of all these factors appears to be required for optimal transcription of the IL-2Rα gene in response to IL-2.

The proliferative response to IL-2

Activation of the heterodimeric IL-2R by high-affinity binding of IL-2 is thought to activate three major signal transduction pathways. These are: the activation of Shc and consequent activation of Ras (see above) [106]; activation of PI3-K, which activates S6 kinase through Ras independent MAP kinase pathways [107], and the activation of a JAK/STAT pathway [108]. Activation of the IL-2R induces the binding of Janus kinases (JAKs) 1 and 3 to the intracellular domains of IL-2Rα and IL-2Rγ [107]. The primary substrates for the JAK kinases are the transcription factors of the family of signal inducers and activators of transcription (STATs). Activation of the IL-2R induces the phosphorylation, subsequent dimerization, nuclear translocation and transcriptional activation of STAT3 and STAT5 [107].

CD95 receptor costimulatory signalling pathways: activation-induced cell death

The relative numbers of cells in the immune system are regulated by the balance between cell proliferation and programmed cell death, or apoptosis. CD95 (Fas or APO-1) plays a key role in the control of apoptosis in mature lymphocytes. The mechanism of cell death is largely unknown, but evidence is accumulating for the involvement of several proteases. A gene, ced-3, has been identified in the nematode *Caenorhabditis elegans* which is obligatory for apotosis [109]. The

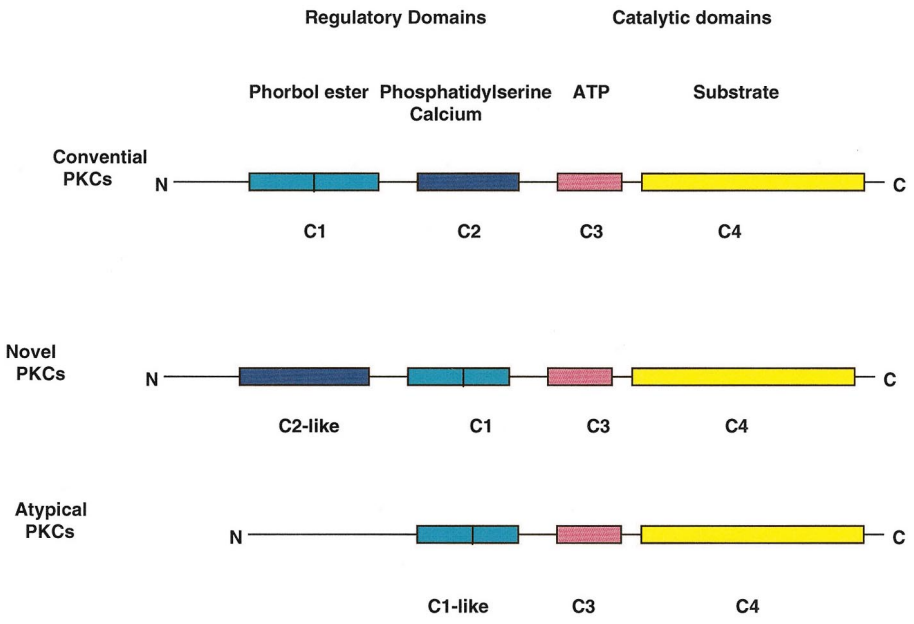

Figure 2. The primary structure of conventional, novel and atypical PKCs.

CED-3 protein shows homology to the interleukin-1β-converting enzyme (ICE) family, all of which are cysteine proteases [110]. CD95 appears to activate a molecule, FLICE, which then undergoes a conformation change activating its ICE/CED-3-like protease domain and triggering cell death [111]. Recent studies [112] suggest that ICE-like proteases regulate the release of ceramide, a bioactive lipid intracellular messenger which has been shown to play a positive role in both T-cell activation and apoptosis. Ceramide is generated through the action of spingomyelinases, implying these molecules are downstream targets of ICE/CED-3-like proteases.

Protein kinase C

PKC domain structure; the PKC isoenzyme family; activation requirements

PKC exists as a family of serine/threonine-specific isoenzymes with at least 12 members identified to date. All PKC isoenzymes comprise an N-terminal regulatory region (20–40 kDa) and a C-terminal catalytic domain ($\sim$45 kDa). These two domains are linked by a flexible hinge region that may be proteolysed when the enzyme becomes membrane-bound. The catalytic domain contains binding sites for the nucleotide phosphate donor, adenosine 5′-triphosphate (ATP), and the protein phosphate acceptor. The regulatory domain contains binding sites for the various activators of the different PKC isoenzymes. These isoenzymes have been classified into families according to their cofactor requirements. The conventional PKCs (cPKCs) PKC-α, -β_{I}, -β_{II}, -γ are activated by Ca^{2+} and diacylglycerol (DAG). Novel PKCs (nPKCs) PKC-δ, -ε, -η, -θ and -μ are distinguishable from the cPKCs in that they lack a functional C2 domain which allows them to function in a Ca^{2+}-independent manner. Furthermore, the individual members of this class are optimally activated by combinations of lipids which are different from those which activate cPKCs. For example, nPKCδ is optimally activated by phosphatidylinositol, nPKCε is activated by *cis*-unsaturated lipids, nPKCθ is activated by phosphatidylserine and nPKCη is activated by cholesterol sulphate. Both cPKCs and nPKCs are activated by phorbol esters. The atypical PKCs (aPKCs) PKC-ι, -ζ and -λ do not require DAG or phorbol ester for activation (fig. 2). aPKCs have only one cysteine-rich motif, and phorbol ester binding has not been detected. PKC-ζ does not respond to Ca^{2+}, 1,2-diacylglycerol or phorbol esters; however, the enzyme does require phosphatidylserine and is activated by *cis*-unsaturated fatty acids [113].

Maturation of PKC

Following ribosomal synthesis, phosphorylation of PKC is a posttranslational modification which appears

to be required to prime the enzyme ready for activation. In this state, the enzyme still exists in an inactive conformation (see below) prior to activation by second messengers. It is not clear to what extent phosphorylation can acutely regulate the activity of different PKC isoenzymes.

The details of this posttranslational modification mechanism have only been explored for a few PKC isoenzymes. In the case of PKC-β_{II}, translocation and activation of the enzyme have been shown to be preceded by a series of three phosphorylation events [114]. The enzyme is first synthesized as an inactive precursor which is associated with the detergent-insoluble fraction of cells. This is then phosphorylated on its activation loop at threonine 500 by a putative PKC kinase. This phosphorylation is believed to cause residues to realign such that the enzyme becomes catalytically competent, the primary consequence of which is autophosphorylation of threonine 641. A phosphate on this position replaces the requirement for negative charge on the activation loop. A second subsequent autophosphorylation at the carboxyl terminus, on serine 660, appears to regulate the enzyme's subcellular localization, releasing mature PKC into the cytosol. Finally, phosphatases dephosphorylate the activation loop, so that about half the PKC in the cytosol is not phosphorylated on this position. This may also be a point of regulation of enzyme activity. Although the activation of the other isoenzyme subgroups is less well understood, serine/threonine residues are conserved at these three positions across all the other family members apart from a glutamate at position 660 in PKC-ζ and -ι. It is therefore likely that a similar posttranslational priming mechanism may exist for all PKC isoenzymes.

The inactive conformation

All PKC isoenzymes contain an amino acid sequence in their N-terminal domain (e.g. between residues 19 and 36 for PKC-α) which resembles the substrate phosphorylation site [115]. This 'pseudosubstrate' sequence in the regulatory domain binds to the peptide-binding site on PKC and is responsible for maintaining the enzyme in an inactive conformation in the absence of allosteric activators. Thus, in the absence of second messengers such as DAG, cPKCs are present in the cytosol in an inactive conformation with the pseudosubstrate bound to the active site and the hinge region masked from proteinase attack.

Activation and translocation mechanisms for cPKC isoenzymes

Although it is becoming clear that different physiological agonists are involved in the activation of these three PKC isoenzyme families, only the activation of the members of the cPKC family has been thoroughly studied, and this is believed to be initiated by the binding of Ca^{2+} to the second conserved (C2) region of these isoenzymes. This allows a low-affinity binding to the membrane, which is likely to be mediated through C2 domain interactions with the membrane. For nPKCs it may be that this lipid-binding surface in the C2 domain is already set in the membrane-accessible conformation, hence their Ca^{2+}-independent status. This low-affinity binding does not by itself result in enzyme activation, since the pseudosubstrate remains bound in the active site of the enzyme, but it does render the hinge region susceptible to proteolysis. An additional interaction occurring through the C1 domain is required to establish high-affinity binding and enzyme activation. This occurs through binding to the C1 domain of diacylglycerols, which are generated in the plasma membrane and serve as hydrophobic anchors which recruit cPKCs to sites on the plasma membrane. They cause a dramatic increase in the affinity of cPKCs for the plasma membrane, and this results in a translocation of cPKCs from the cytoplasm to the cell membrane. This effect is reversible, directly proportional to the amount of DAG present in the membrane, requires phosphatidylserine and is likely to be mediated through C1 domain interactions. The change in affinity is believed to be occasioned by a change in surface hydrophobicity brought about by the capping of a hydrophilic groove on the surface of the enzyme by the binding of DAG and does not involve any conformational change by the protein. DAG may also act to stabilize the active conformation of the enzyme [116]. Although the DAG binds exclusively to the C1 domain, high-affinity productive binding of cPKCs to the membrane requires the interactions at both the C2 and C1 domains mentioned above. This high-affinity interaction results in a conformational change which removes the pseudosubstrate region from the active site cleft, allowing substrate binding and catalysis. Phorbol esters appear to work by the same mechanism and simply differ from DAG in that they are over two orders of magnitude more potent than DAG. Thus, activation of cPKCs is accompanied by translocation from one cell compartment (cytoplasm) to another (plasma membrane). While it is not clear to what extent the mechanism for the activation of cPKCs can serve as a model for the activation of other isoenzyme families, it seems that both nPKCs and aPKCs are also translocated during physiological activation.

Downregulation of PKC activity in cells

Prolonged treatment of cells with phorbol esters leads to the disappearance of PKC activity from both the cytosol and membrane fractions of the cell. This phe-

nomenon is referred to as PKC downregulation and has been used to probe the role of PKC in a variety of signalling pathways. Downregulation results from an increased rate of PKC degradation rather than a decreased rate of synthesis of the enzyme [117]. Generally, all PKC isoenzymes which are activated by phorbolesters can be downregulated by prolonged treatment with these agents, although there are some exceptions. The explanation for these exceptions may be that the particular isoenzyme is not recognized by the proteinase system which forms the downregulatory apparatus in the cell or that biosynthesis of that particular PKC isoenzyme outruns its degradation in the particular cell line chosen.

The mechanisms by which PKC isoenzymes are downregulated is unclear. As noted above, the conformational changes which accompany PKC activation expose the hinge region in the PKC molecule to proteolysis. One idea is that a specific cleavage in this region may allow the transient generation of a catalytic domain (PK-M) which functions independently of the regulatory domain. Indeed this species is seen transiently in cells responding to certain agonists. Although the enzyme responsible for this cleavage has not been identified, several cysteinyl proteinases would figure as candidates. Subsequently, this species would be destroyed as a consequence of general proteolysis. The consequences of the transient generation of a soluble catalytic domain are that it may gain access to target proteins, e.g. certain cytosolic proteins, not available to the activated holoenzyme in the plasma membrane. Although this may serve a signalling function, it is difficult to conceive how the activity of the catalytic domain could be controlled in this scenario. An alternative hypothesis which probably makes more sense is that the membrane-associated PKC phosphorylates a component of the cell's endocytotic machinery [117]. This stimulates an endocytotic process in which the membrane-attached PKC molecule remains attached to a membrane vesicle and is transported to general degradative departments within the cell (e.g. lysosomes, proteosomes) where destruction occurred. In this scenario, the catalytic domain is not even transiently generated as a free species.

Inhibitors of PKC

There are three areas on the PKC molecule which are obvious targets for rational drug design – the DAG-binding domain, the protein substrate-binding region and the ATP-binding domain. The regulatory domain of PKC would appear to offer the best target for the design of a selective inhibitor of PKC in that this region is unique to certain PKC isoenzymes and is not present in other serine/threonine kinases. However, it should be noted that compounds which belong to this category may well be inactive against members of the aPKC subgroup which lack the phorbol ester-binding site. The perylenequinone calphostin C, originally isolated from *Cladosporium cladosporioides* and showing at least 1000-fold greater potency against PKC than against any other kinase [118], is an example of one such inhibitor. It would seem that the ATP-binding site offers the most difficult target in the PKC molecule against which to design potent and selective inhibitors. There are two reasons for this. First, it is not clear how selectivity over other protein kinases can be achieved, since this the ATP-binding domain is conserved across the whole spectrum of protein kinases. Second, these agents have to work in the presence of very high concentrations of ATP in cells, typically 100-fold greater than the K_m of PKC for ATP. Nevertheless, it is in this area that the greatest progress has been made in the design of selective PKC inhibitors. Despite this progress, three nonselective ATP-competitive compounds have frequently been used in isolated enzyme and cellular studies as inhibitors of PKC; the indolocarbazoles staurosporine and K252a, and the isoquinoline sulphonamide H7. Staurosporine and K252a are indeed potent PKC inhibitors and will inhibit the enzyme in cellular systems, but these compounds are far from specific for PKC. Staurosporine has been shown to act as a broad specificity inhibitor of serine/threonine kinases and to block both receptor and src-family tyrosine-specific protein kinase activity [119, 120]. K252a exhibits a similar profile and has also shown some selectivity for the Ca^{2+}/calmodulin-dependent phosphorylase kinase [121]. H7 is a broad specificity serine/threonine kinase inhibitor and is only weakly active against PKC in vitro [122]. The compound binds to the enzyme in competition with ATP, and in intracellular systems where the concentration of ATP is in the millimolar range (rather than the micromolar concentrations routinely used in isolated enzyme assays) the compound is a very poor inhibitor of phorbol ester-induced cellular responses. The effects of H7 in whole-cell systems may be mediated through the inhibition of an unknown serine/threonine kinase [123].

Although staurosporine acts as a broad spectrum inhibitor of both tyrosine-specific and serine/threonine-kinases, it is undeniably a potent inhibitor of PKC and has provided a useful starting point for the design of more selective inhibitors of the enzyme. These agents include the indolocarbazole CGP 41251 [124] and bis-indolylmaleimides Ro 31-8425 and Ro 32-0432 [125]. Whilst these compounds show a high degree of selectivity for PKC over many other protein kinases, several of

the bis-indolylmaleimides have recently been shown to potently inhibit S6 kinase and MAPKAP1 kinase [126], whilst the indolocarbazoles also tend to be potent inhibitors of Ca^{2+}/calmodulin protein kinases [124]. Nevertheless, agents such as these have been used as tools to explore the role of PKC in T-cell activation.

The role of PKC in T-lymphocyte function

Evidence from the use of phorbol esters

The ability of phorbol esters to bind to the DAG-binding site and to activate PKC in vitro, in cellular systems and in animal models, has provided researchers with convenient and apparently highly selective tools to probe the role of this enzyme in T-lymphocyte activation and proliferation. High concentrations of phorbol ester alone will induce resting T cells to proliferate; however, lower concentrations require an additional calcium signal to be mitogenic [127]. Phorbol ester treatment also induces the phosphorylation of several proteins in T cells and modulates the expression of several cell surface proteins, e.g. CD4 and CD3 downregulated, CD2 upregulated. As expected, both the nonselective protein kinase inhibitor staurosporine and a number of selective bis-indolylmaleimide PKC inhibitors block phorbol ester-induced T-cell proliferation and CD3 and CD4 downregulation [128]. Activation of PKC by phorbol esters antagonizes apoptosis induced by TNF-α, factor activating exoenzyme S (FAS) ligand and ionizing radiation, suggesting that a PKC-dependent pathway counteracts ceramide-mediated apoptosis [129].

At concentrations that induce IL-2 production and T-cell proliferation, phorbol esters and mezerein, another potent but nonphysiological activator of PKC, produce a sustained translocation of PKC more than 1 h following the initial stimulation [130]. In contrast, a single exposure to diacylglycerol or one of its analogues results in only a transient translocation of PKC in T cells. This peaks at around 15 min and has disappeared 1 h after the initial stimulation of the T lymphocytes. This is sufficient for induction of functionally active IL-2R expression but does not stimulate synthesis of IL-2. Thus, a single exposure to diacylglycerol, either alone or in combination with Ca^{2+} ionophore, does not produce a proliferative response from the T cell [130]. One explanation for this is that both phorbol esters and mezerein are much more stable in intracellular systems than the physiological activator DAG, and the short-lived pulse of DAG generated in the plasma membrane of T cells following single exposure may be insufficient to produce the sustained translocation of PKC required for a proliferative response. In agreement with this, experiments involving multiple additions of the diacylglycerols have confirmed that a sustained translocation of PKC does occur and that repeated exposure to DAG can induce the proliferation of resting T cells [131]. Another interesting idea is that DAG will optimally activate only cPKCs, whereas phorbol esters activate both cPKCs and nPKCs and that nPKC translocation is required for the proliferative response. Some support was given to this idea by experiments in which addition of unsaturated fatty acids, which will activate certain nPKC isoenzymes, to a combination of DAG and Ca^{2+} ionophore was shown to induce a sustained translocation of PKC, IL-2 production and T cell proliferation [132].

While several studies have shown that cPKCs PKC-α and -β translocate rapidly to the cell membrane of T lymphocytes following 10 min treatment with phorbol ester and that the redistribution was maintained for some hours after phorbol ester stimulation, the picture is more confused with nPKC isotypes [133]. In the human T-cell line K-4, PKC δ exists in the perinuclear envelope and has a polar cytoplasmic distribution in resting cells but, after 5-min exposure of these cells to phorbol 12-myristate 13-acetate (PMA), forms aggregates in the cytoplasm [134]. PKC-ε has a diffuse distribution in the cytoplasm of resting K-4 cells and does not appear to translocate to the membrane after 1-h exposure to PMA. However, in studies carried out in Jurkat T cells, PKC-ε, but not PKC-δ, appears to translocate to the membrane fraction after exposure to phorbol ester [135]. In other studies in Jurkat T cells and in Th D10 cells, another nPKC isoform, PKC-θ, was shown to respond rapidly to phorbol esters by translocating to the cellular membrane [136, 137]. However, the atypical PKC-ζ which is not activated by phorbol esters does not appear to translocate in response to these agents [117]. The pattern that emerges from these studies is, in the main, very much the expected one in that those PKC isoenzymes which are activated in isolated enzyme experiments by phorbol esters, in general, translocate when T cells are stimulated by these agents. It is interesting to note that in some scenarios PKC translocation appears to occur to sites in the cell other than the plasma membrane.

The observation that phorbol esters were able to induce sustained translocation of PKC in T lymphocytes, and that this appeared to correlate with their ability to induce IL-2R and IL-2 expression and T-lymphocyte proliferation, inevitably led to the assumption that PKC had a pivotal role to play in T-cell activation. However, doubts have now been cast on the validity of these observations as line of evidence for PKC involvement in antigen-driven T-lymphocyte proliferation [138]. One question which arises is whether phorbol esters are able to modulate the activity of other proteins involved in the T-cell signalling pathways triggering the proliferative response and thus their proliferative effect on T cells may not be mediated through activation of PKC. Recent discoveries have shown that members of the PKC isoenzyme family

are not the only receptors for phorbol esters. Neuronal chimaerin is a protein found in the brain which binds phorbol esters with high affinity, stereospecificity and requirement for phospholipid [139, 140], enabling it to regulate $p21^{rac}$-GTPase activity. The possible existence of other phorbol ester-binding proteins which mediate signal transduction events involved in T-cell proliferation cannot now be ignored. A more serious concern which arises is the extent to which phorbol esters mimic the pattern and duration of translocation and activation of various PKC isotypes which occur during stimulation of a T cell with specific Ag/APC. Certainly, phorbol esters are also much more stable in intracellular systems than the physiological activator of cPKCs, DAG, which is rapidly metabolized. The presence of a persistent agonist may lead to the activation of PKC isoenzymes not normally activated under physiological conditions or to a continuous rather than transient activation of a particular isoenzyme. This may result in the phosphorylation of substrates which are not normally physiological targets for PKC and to the initiation of cellular functions which are not usually mediated through PKC activation. Some support for this is provided by studies carried out in a range of other cell types. For instance, in human neutrophils, phorbol esters will stimulate neutrophil adhesion to endothelial cells, and this process is inhibited by potent and selective bis-indolylmaleimide PKC inhibitors. However, the same process stimulated by a more physiological agonist, C5a, is not blocked by the PKC inhibitor [141]. As early as 1984, Nishizuka pointed out that the activation of PKC by phorbol esters was not subject to the feedback control by cyclic nucleotides normally observed after physiological stimulation of PKC in many cell types [142]. Conversely, aPKC isoforms such as PKC-ζ are not activated in isolated enzyme systems by phorbol esters, nor translocated in cell systems treated with phorbol esters, and apparently play no role in phorbol ester-driven T-lymphocyte proliferation. Yet these isoforms may well be activated and translocate in response to Ag/APC.

Evidence from the use of antibodies to TCR components and accessory molecules

Antibodies to the α and β chains of the T-cell receptor and to the CD3 component of the TCR/CD3 complex will induce IL-2 production, expression of high-affinity IL-2Rα and proliferation of many, but not all, T lymphocytes. For example, cloned Th D10 cells can be stimulated to proliferate with specific Ag/APC and release cytokines in response to anti-TCR/CD3 but fail to proliferate in response to these antibodies [137]. These antibodies act as a surrogate for the antigen/MHC complex by inducing the cross-linking of TCRs which is required for T-cell activation (see above). Therefore, those antibodies which are able to induce cross-linking of TCRs most effectively are the strongest T-cell activators. Thus, in the case of T-cell activation induced by anti-CD3 or anti-TCR $\alpha\beta$ antibodies, Fab fragments themselves cannot trigger activation, bivalent intact IgGs are weak stimulators, pentameric IgM anti-TCR mAbs are stronger activators and anti-TCR monoclonal antibodies (mAbs) coupled to solid supports (which give infinite cross-linking ability) are the best activators of cytokine production and the proliferative response. Additionally, the ability of anti-TCR antibodies to induce TCR association with CD4 also correlates with their ability to activate the T cell.

A number of studies established that triggering of peripheral T cells and T-cell lines by anti-CD3 mAbs and by anti-TCR mAbs induced rapid synthesis of inositol triphosphate and a rise in intracellular Ca^{2+}, presumably derived from phospholipase C-induced phosphoinositide breakdown [143, 144]. It was assumed that this would also generate DAG and thus activate PKC. However, there are few studies which measure production of diacylglycerols and unsaturated fatty acids (activators of nPKCs) directly and attempt to correlate this with translocation of PKC.

In contrast to PMA, which produces a sustained translocation of PKC, early studies (late 1980s) with antibodies to the TCR established that they induced a biphasic redistribution of PKC from cytosol to membrane fraction in T lymphocytes (PBMC) [145]. An initial translocation which peaked at 15 min following antibody stimulation diminished and was followed by a slower and more sustained translocation (up to 2 h after stimulation). In one study with Jurkat T cells, those antibodies which were effective in triggering a proliferative response (i.e. Sepharose-linked anti-TCR/CD3 Abs) induced prolonged translocation of PKC (>2 h), whereas soluble antibodies which were incapable of triggering the proliferative response induced only a transient (10 min) redistribution [146]. In these studies no attempt was made to distinguish between the behaviour of different PKC isoenzymes in response to the mitogenic signal; PKC activity was simply measured as Ca^{2+}, phospholipid-dependent phosphorylation of histone in different subcellular fractions. Thus, it is likely that these studies focused on redistribution of cPKCs. Subsequent studies do not appear to agree completely over which isoenzymes redistribute. Futop et al. [147] showed that in T lymphocytes taken from young subjects, stimulation with anti-CD3 for 60 min caused redistribution of PKCs-α, -β, -δ and -ε. However, a subsequent study using immunofluorescent microscopy to visualize isoenzyme location in the T cell claimed that activation of PBMC with anti-CD3 had no effect on PKC-δ redistribution [148]. This study also claimed

no effect of anti-CD3 on redistribution of PKC-η and PKC-θ. This is in agreement with studies carried out in Th D10 cells in which capping with specific monoclonal antibodies to the TCR or accessory molecules (e.g. CD4, CD28) failed to induce translocation of PKC-θ from the cytoplasm to the plasma membrane in the vicinity of the cap [149].

Thus, antibodies against the TCR and accessory molecules can, under certain circumstances, drive IL-2R production, IL-2 production and the proliferation of certain T-cell lines. However, engagement of the APC by the T cell involves many interactions between receptors on the T cell and the APC, aside from the interaction between Ag/MHC and the TCR/CD3 complex itself. These interactions initiate multiple signals that can be provided only by correctly engaged APC. Although the cross-linking of the TCR with antibody can induce a proliferative response, it is unlikely to exactly mimic all these interactions and may trigger signalling pathways which differ from those used by the physiological agonist.

Experimental evidence from antigen-driven T-cell proliferation studies

Evidence from inhibitor studies in vitro: selective PKC inhibitors block Ag/APC driven T-cell proliferation. A range of selective PKC inhibitors has been shown to inhibit antigen-driven T-cell proliferation. For instance, bis-indolylmaleimide inhibitors of PKC such as Ro 31-8425 and Ro 32-0432 inhibit the allogeneic mixed lymphocyte reaction [120] and T-cell proliferation in response to specific antigen. In these experiments, a human T-cell clone, HA27, specific to influenza peptide 307–319, was exposed to antigen-pulsed autologous presenting cells. Ro 32-0432 inhibited HA27 proliferation with an inhibitory concentration of 50 percent (IC_{50}) of 150 nM. Another selective PKC inhibitor, the indolocarbazole CGP 41251, inhibited antigen-induced proliferation of human peripheral blood lymphocytes and murine Th1 and Th2 clones equally well. Although these agents show a high degree of selectivity for PKC over a range of other protein kinases, the possibility that these effects are mediated through inhibition of another protein kinase cannot be completely dismissed. For instance, it is known that Ro 31-8425 and Ro 32-0432 are also potent inhibitors of MAPKAP kinase 1 and S6 kinase and these enzymes may well play a role in antigen-driven T-cell proliferation [126].

Additionally, there is some disagreement over which stages in the proliferative process these agents target. The bis-indolylmaleimides Ro 31-8425 and Ro 32-0432, at concentrations up to 10 μM, had no effect on the IL-2-induced proliferation of peripheral blood T cells which had been induced to express high-affinity IL-2Rs by 24-h exposure to phorbol ester. These studies would suggest that PKC appears to be required for the generation of IL-2 and/or IL-2R required for T-cell proliferation but does not play a role in subsequent IL-2-driven T-cell proliferation [120]. This is supported by studies which show that IL-2 driven T-cell proliferation can occur in PKC-depleted cell lines and in PKC-negative mutant cell lines [149]. However, the indolocarbazole CGP 41251 inhibits IL-2-dependent growth of a murine Th1 cell line and the IL-2- and IL-4-induced growth of a murine Th2 cell line [150]. The reasons for these differences are unclear but may relate to the different protein kinase or PKC isoenzyme selectivity profiles of the agents studied. Alternatively, IL-2-driven signalling pathways may vary in the different T-cell lines that were used in these studies.

Evidence from inhibitor studies in vivo: selective PKC inhibitors block T-cell-mediated inflammatory responses in vivo. The ability of these agents to inhibit a phorbol ester-induced inflammatory response has been used to demonstrate that these compounds are sufficiently bioavailable when dosed orally to inhibit PKC-mediated events in vivo. For instance, the injection of phorbol ester into rat paws induces an acute oedema which subsides after 6 to 8 h and which disappears after 24 h. Treatment with Ro 32-0432 at a single oral dose of 20 mg kg^{-1}, 1 h before injection of phorbol ester produced a significant decrease in the extent of paw swelling [120].

In vivo these compounds have also been shown to inhibit a range of T-cell-mediated inflammatory events. Injection of adjuvant (*Mycobacterium tuberculosis* in liquid paraffin) into the hind paw of a rat induces two phases of inflammation in the paws of the rat. The primary swelling phase (days 0–4) occurs only in the injected hind paw and represents an acute inflammatory response. The secondary inflammatory response (days 7–14) is a systemic immune response which afflicts both hind paws. Additionally, lesions appear on the nose, ears, forepaws and tail of afflicted animals. Ro 32-0432, dosed orally every day, had no effect on the primary, presumably neutrophil-mediated inflammatory response, but did inhibit the secondary phase [120]. This inhibition of the secondary inflammatory response in the injected paw by Ro 32-0432 was dose-related, as was inhibition of noninjected hind paw swelling (ED_{50} 11 mg kg^{-1}), inhibition of lesion score (ED_{50} 15 mg kg^{-1}) and restoration of degrees of joint mobility in the noninjected hind paw [effective dose at 50% inhibition (ED_{50}) 18 mg kg^{-1} per os (p.o.)]. At the highest dose tested, 50 and 100 mg kg^{-1} p.o., Ro 32-0432 completely inhibited the secondary hind paw swelling, lesion score and loss of joint mobility. Ro 32-0432 inhibited the body weight loss induced by arthritis at doses of 20 to 100 mg kg^{-1} p.o. (ED_{50} 17 mg kg^{-1}). Plasma levels of

acute phase proteins such as haptoglobin, a systemic marker of inflammation, were restored towards normal by treatment with Ro 32-0432 (ED_{50} 32 mg kg^{-1} p.o.), although complete inhibition was not seen at doses of 50 and 100 mg kg^{-1} p.o. Finally, the hind leg popiteal lymph node increases in size in animals with adjuvant arthritis. Dosing of arthritic animals with Ro 32-0432 restored lymph node weight towards normal, compared with the untreated arthritic controls (ED_{50} 11 mg kg^{-1} p.o.). This is consistent with inhibition of T-lymphocyte proliferation by Ro 32-0432 and may explain the selective effects of Ro 32-0432 upon the secondary, T-cell-mediated, phase of the disease. Ro 32-0432 also significantly inhibited the ongoing secondary phase of inflammation in the established adjuvant arthritis model. The compound reduced paw swelling at doses of 50 and 100 mg kg^{-1} p.o. (ED_{50} 42 mg kg^{-1}).

A simple model of the host-vs.-graft response has been developed by injecting mitomycin C-treated mouse spleen cells into the hind paw of rats [151]. The immune reaction of the rat to the mouse cells can be followed by measuring the weight change in the draining popliteal lymph node over a period of 4 days; a three- to fourfold increase in popliteal lymph node weight is observed by day 3. Treatment of the rats with a nonsteroidal anti-inflammatory drug, indomethacin, had no inhibitory effect, whilst immunomodulatory drugs such as cyclosporin A or dexamethasone induced a dose-related inhibition of weight change. Ro 32-0432, at doses of 50 and 100 mg kg^{-1} p.o., significantly reduced the weight increase by 45 and 61%, respectively [120]. The lowest dose, 20 mg kg^{-1}, had no significant effect on lymph node weight increase.

Experimental allergic encephalomyelitis (EAE) is a T-cell-dependent autoimmune disease of the central nervous system (CNS) characterized by paralysis predominantly affecting hind limbs [152]. Histopathologically, the disease is characterized by mononuclear cell infiltration within the CNS parenchyma and a variable degree of demyelination. Studies using cell-specific mAbs showed that the cellular infiltrate consists of T cells and macrophages [153, 154]. The mechanisms by which the autoreactive T cells induce the clinical symptoms of the disease are not well understood, but many of the features of EAE are similar to those of the human disease multiple sclerosis. EAE was induced in female AHH/R rats by the injection of a guinea pig spinal cord homogenate/Freund's complete adjuvant emulsion into both hind paws (N. Lad et al., unpublished observations). Control animals attained maximum paralysis on day 14 with a subsequent recovery from symptoms. Animals dosed with 30 mg kg^{-1} Ro 32-0432 p.o. exhibited a delay in the development of paralysis, reaching maximum paralysis on day 20, followed by recovery. Clinical symptoms were graded on a scale of 1 to 4 (0 = normal, 4 = tetraplegia) on day 14. One hundred percent of control animals scored 2.5 to 3, i.e. full hind limb paralysis, whilst 90% of the group dosed with Ro 32-0432 showed a score of 1 or less. This study suggested a possible role for PKC in the immunoinflammatory component of the EAE model as assessed by clinical symptoms.

Collectively, studies with selective inhibitors indicate a key role for PKC in antigen-driven T-cell proliferation. One important consequence of this is that inhibitors of PKC are able to prevent the inappropriate activation of T lymphocytes that occurs in adjuvant-induced arthritis and in EAE. This may point to a therapeutic potential for selective PKC inhibitors in the treatment of autoimmune diseases. Although the types of 50 inhibitors used in these studies generally show selectivity for cPKCs over nPKCs and are probably poor inhibitors of aPKCs, complete isoenzyme selectivity profiles have not been determined for these agents. In truth, it is the PKC isoenzyme selectivity demonstrated by these agents in antigen-activated T cells, rather than that measured against the isolated isoenzymes, that will determine the selectivity profile in vivo. Therefore, it has not, as yet, been possible to use agents such as these to determine which PKC isoenzymes are involved in the proliferative response.

Immunofluorescence microscopy studies in intact cells; selective activation and translocation of PKC-θ. Immunofluorescence microscopy has been utilized to visualize, at the single-cell level, the PKC isoenzymes responsible for T-cell activation following the interaction of the T-cell clone Th D10 with an antigen-presenting cell, the B-cell lymphoma CH12.LX [137]. The T cell and APC remained in contact long after the period of initial stimulation, and cytokine production was localized to the contact region. Depletion of all PKC from the T lymphocyte prevented signalling through the TCR, and the role of each PKC isoenzyme was investigated using anti-PKC isoenzyme selective antibodies. Only PKC-θ was translocated to the site of cell contact. Unlike the other PKC isoenzymes, PKC-θ has a limited tissue distribution and is preferentially expressed in haemopoietic cells, particularly T cells, and skeletal muscle. The translocation was rapid (less than 10 min) and persistent in that 55 min after cell mixing PKC-θ was observed at the contact point in 88% of the cell conjugates. In vitro assays of immunoprecipitates from these specific antigen-activated T cells showed PKC-θ to be enzymatically active. This redistribution of PKC-θ in response to specific antigen/APC was also observed in a number of T-cell clones and in primary lymph node T cells from TCR transgenic mice. Conversely, partial activation of Th D10 cells, for example with anti-TCR/CD3 mAbs or with APCs which do not present specific antigen, failed to induce PKC-θ activation and translocation and also failed to induce T-cell proliferation.

Evidence from PKC depletion studies

Phorbol ester-induced PKC depletion. Chronic treatment of T-cell clones with phorbol esters leads to a complete downregulation of cPKCs and nPKCs, resulting in loss in PKC activity and a concomitant loss in the proliferative response to T-cell receptor ligands such as antigen or lectins [117]. Treatment of T lymphocytes with a variety of agonists, such as phorbol esters and mezerein, for 24 h leads to the downregulation of PKC and the concomitant suppression of IL-2 production and mitogenesis [155]. However, these cells retain the ability to proliferate to IL-2, suggesting that this response is PKC-independent. Unfortunately, these data are not conclusive. In cases where PKC depletion leads to the loss of a particular response, it may be that phorbol ester treatment induces changes in other molecules, independent from the downregulation of PKC, and it is these changes which have abrogated that particular response. In cases where PKC depletion does not alter the ability of a cell to mount a particular response, it may be that this response can be triggered by the minimal activity of a particular PKC isoenzyme that is not completely downregulated by phorbol ester. Alternatively, the response may involve PKC isoenzymes that are refractory to phorbol ester downregulation, e.g. PKC-ζ.

Depletion of PKC with isoenzyme-specific antibodies. There have been a limited number of studies in which isoenzyme-specific antibodies have been introduced in T cells, usually by electroporation. When anti-PKC-α antibodies were introduced in to Jurkat T cells, anti-TCR/CD3-induced IL-2 gene expression was completely suppressed, suggesting that this may be the major isoenyme involved in regulating IL-2 receptor expression in stimulated human lymphocytes [156]. Electroporation of PKC-β_1, but not -α or -γ, also into Jurkat T cells, led to an increase in the rate of Ca^{2+} influx following following T-cell stimulation via the TCR [157]. This suggests the isoenzyme may have a role to play in the downregulation of increases in Ca^{2+} influx associated with T-cell activation.

Depletion of PKC with antisense oligonucleotides. Isoenzyme-selective antisense oligonucleotides have now been derived for most PKC isoenzymes. These agents have been used to implicate certain PKC isoenzymes in signalling pathways that are likely to be triggered during antigen-driven T-cell proliferation. For example, experiments with antisense to PKC-ζ suggest that this isoenzyme may be involved in NF-κB activation and in the IL-2-induced activation of PI-3 kinase [158]. However, antisense oligonucleotides have not been used to systematically assess the effect of depleting each PKC isoenzyme from T cells on T-cell function i.e. cytokine release and proliferation in response to Ag/APC.

Evidence from studies with PKC isoenzyme transgenics

There is some evidence from PKC-α and PKC-β transgenics that overexpression of these isoenzymes can influence T-cell development and activation [159]. Two major difficulties arise in interpreting the results from these types of experiments. One is the difficulty of distinguishing between the effects of overexpressing a particular PKC isoenzyme on thymocyte development and maturation and direct effects on proliferation of the mature T-cell population in response to antigen. This difficulty arises because gene expression in the transgenic animal may skew thymocyte development in such a way as to affect the proliferative response of the mature T-cell population. A second concern which arises is to what extent overexpression of a particular PKC isoform subverts the true physiological situation by triggering signalling pathways that do not normally occur.

Transgenic mice carrying rabbit PKC-α complementary DNA (cDNA) under control of the human CD2 regulatory element will overexpress PKC-α protein some hours after stimulation with anti-CD3 mAb [160]. The newly synthesized protein is translocated to the membrane and appears to enhance T-cell responses in that thymocytes from these transgenic mice produce IL-2 and proliferate in response to anti-CD3 mAb, which is usually an incomplete agonist for normal thymocytes. Transgenic mice have been also produced in which PKC expression can be temporally regulated, and this may partially address the first of the concerns described above [161]. The lck proximal promoter is active predominantly in thymocytes, and expression of PKC-β from this promoter has suggested a role for this isoenzyme in the positive and negative selection of T lymphocytes in the thymus, in that the normal distribution of thymocyte subsets is altered in transgenic animals. The lck distal promoter is active in mature T cells, and mice carrying PKC-β driven by this promoter develop a lymphoproliferative disease in which all lymphoid organs become greatly enlarged. Arguably, these data suggest that PKC-β may have a role to play in both thymocyte maturation and in the activation of mature T cells.

Evidence from mechanistic studies

The individual roles of particular protein kinases in transmitting signals along defined pathways have in many cases been identified. Additionally, the functions of the signal transduction cascades in which these kinases are active are at least partially understood. For example, the components of the ras/raf/MAPK and the stress-activated protein kinase pathways were identified subsequent to the first identification of many PKC

isoenzymes. Yet, the mapping of these protein kinases in the different cascades in which they operate is well advanced, and the precise role of many of these enzymes in cellular signalling is far better understood than that of PKC. Why then has dissecting the role of PKC in signal transduction processes proved particularly difficult? There may be a number of reasons for this. First, many of the tools (phorbol esters, inhibitors) used to investigate the individual functions of this family do not distinguish between the different PKC isoenzymes. This can prove important when different isoenzymes may perform disparate or even opposing functions within the same signal transduction pathway. One such example is the effect of the bis-indolylmaleimide PKC inhibitor, Ro 31-8425, on opsonized zymosan-induced-respiratory burst in the neutrophil [162]. Low concentrations of the inhibitor potentiate the response, whereas higher concentrations of the same compound inhibit the respiratory burst. This possibly implies distinct roles for two different isoenzymes at different points on the same pathway. Conversely, certain PKC family members, i.e. aPKCs, are insensitive to phorbol ester and DAG activation and are poorly inhibited by many of the ATP-competitive PKC inhibitors and completely insensitive to inhibitors which bind at the DAG/phorbol ester-binding site. Thus, insensitivity of a process to phorbol ester stimulation or to inhibition by a range of PKC inhibitors does not imply a lack of aPKC involvement in the process. Second, PKC activation invariably leads to PKC downregulation. It can, therefore, be difficult to separate events which involve PKC upregulation from those which are driven by downregulation of an isoenzyme.

However, there is some understanding of the role that PKC plays in the signal transduction processes following T-cell stimulation. Inhibitors of PKC, such as Ro 31-8425, have been shown to inhibit both antigen and anti-CD3-driven T-cell activation [162]. These compounds do not affect anti-CD3-induced rises in $[Ca^{2+}]_i$ in T lymphocytes, nor do they affect phospholipase C activity. However, Ro 31-8425 and Ro 32-0432 do inhibit the allogeneic mixed lymphocyte reaction [120] and the T-cell response to specific antigen. For example, a human T-cell clone, HA27, specific to influenza peptide 307–319, was exposed to antigen-pulsed autologous presenting cells. Ro 32-0432 inhibited HA27 proliferation with an IC_{50} of 150 nM. However, Ro 31-8425 and Ro 32-0432, at concentrations up to 10 μM, had no effect on IL-2-induced T-cell proliferation of peripheral blood T cells which had been induced to express high-affinity IL-2Rs by 24-h exposure to phorbol ester. Thus, at a functional level, PKC appears to be required for the generation of both the cytokine and cytokine receptor required for T-cell proliferation but does not play a role in subsequent IL-2-driven T-cell proliferation [120]. Nevertheless, the PKC isoenzymes which are involved in this process have not been identified.

T-cell-activation signals converge and cooperate to induce the synthesis of a variety of transcription factors, such as NF-κB, NF-AT, Oct-1 and AP-1, which together regulate the induction of IL-2 and IL-2R expression. Ro 31-8425 has been used to try to unravel the mechanisms leading to IL-2 gene transcription in Jurkat T cells using reporter gene constructs which are dependent upon either NF-κB, AP-1 or NF-AT activities for expression [163, 164]. Using transient expression in Jurkat T cells activated by phytohaemagglutinin (PHA)/phorbol dibutyrate (PDBu), Williams et al. demonstrated that NF-κB-induced reporter gene activity is inhibited at concentrations of Ro 31-8425 required for inhibition of the IL-2 promoter. This suggests that a PKC-mediated event is required for NF-κB activation and IL-2 gene expression in the activation of T cells. Another PKC inhibitor, calphostin C, has also been shown to block NF-κB activation and subsequent nuclear localization [165]. Furthermore, a bis-indolylmaleimide PKC inhibitor, GF109203X, abrogated phorbol ester-induced IκB-α phosphorylation/degradation irrespective of the activation of Ca^{2+}-dependant pathways but not the phosphorylation and degradation of IκB-α induced by TNF-α, a PKC-independent stimulus [166]. Collectively, these observations suggest that a PKC-mediated event is required for NF-κB activation and IL-2 expression in activated T cells. Interestingly, Ro 31-8425 potentiated NF-AT-induced reporter gene activity at the IC_{50} required for IL-2 promoter inhibition, suggesting that the presence of NF-AT alone is insufficient for IL-2 gene activation and that PKC may act to downregulate some early event in NF-AT activation.

One PKC isoenzyme that has been implicated in antigen-driven T-cell proliferation and which has been studied in some detail is PKC-θ. This nPKC has a limited tissue distribution profile, in that it is preferentially expressed in T lymphocytes and is also found in skeletal muscle. The translocation of PKC-θ to the T cell plasma membrane and its consequent activation which occur when T cells are activated by antigen-presenting cells has been described above. The role of PKC-θ in activation of the IL-2 promoter was identified from experiments in which the response of the defined enhancer elements within the IL-2 promoter to PKC overexpression was measured [167]. EL4 thyoma cells were transiently transfected with IL-2 promoter-derived enhancer/promoter reporter gene constructs – synthetic multimers of either the Oct-1 or NF-AT elements, an NF-κB-chloramphenicol acetyl transferase (CAT) construct or $^{-73/+60}$Col-CAT, a CAT reporter gene under the control of a collagenase promoter that contains a

single AP-1 phorbol ester response element. Overexpression of both PKC-α and PKC-θ caused a significant increase in phorbol ester-induced transcriptional activation of IL2-CAT and NF-AT-CAT but not of Oct-1-CAT or NF-κB-CAT. However, overexpression of PKC-θ but not PKC-α, -ε or -ζ led to an enhanced stimulation of $^{-73/+60}$Col-CAT in EL4 cells. This suggests that AP-1 activation is controlled by PKC-θ. In agreement with this, expression of a constitutively active PKC-θ mutant induced phorbol ester-independent transcriptional AP-1 activation, whereas expression of a 'kinase-dead' mutant of the isoenzyme abrogated phorbol ester-mediated AP1 transcriptional control in EL4 cells. Cotransfection of a dominant-negative Ras mutant with PKC-θ at a ratio of 1:1 completely inhibited phorbol ester-dependent PKC-θ wild-type and PKC-θ constitutively active mutant-induced phorbol ester-independent AP-1 CAT signals, placing PKC-θ either upstream of or parallel to Ras in the signalling cascade.

PKC-θ has also been shown to coprecipitate with the 14-3-3-tau protein from Jurkat T cells [168]. Transient overexpression of this protein suppressed stimulation of the IL-2 promoter mediated by ionomycin and/or phorbol ester-treated cells but not PKC-independent, Ca^{2+}-dependent activation of an IL-4 promoter. Overexpression of 14-3-3-tau also inhibited phorbol ester-induced translocation of PKC-θ to the cell membrane in Jurkat cells, whilst a membrane-targeted form of the protein increased localization of PKC-θ in the particulate fraction of unstimulated cells. Whilst the significance of this interaction between PKC-θ and 14-3-3-tau protein is not understood, the interaction appears to be specific, since attempts to demonstrate an association between this protein and other PKC isoenzymes have so far proved unsuccessful.

Conclusion

Autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and psoriasis are all believed to be T-cell mediated, and this has fuelled the search for therapeutic agents which will modulate T-lymphocyte activation and thus alleviate these conditions. Evidence from studies in animals with selective inhibitors of protein kinase C suggests that these agents may be useful in the treatment of autoimmune diseases. However, despite the increasing availability of PKC inhibitors and activators, the precise role that PKC plays in antigen-driven T-cell proliferation is only just starting to emerge. The evidence accumulated to date suggests an essential role for the PKC isoenzyme family in T-cell activation and subsequent downstream signalling. PKC-θ, in particular, appears to be involved in early activation events, whereas many of the other isoenzymes may be important further downstream in the signal transduction pathway.

1 Breedveld F. C. and Verweij C. L. (1997) T cells in rheumatoid arthritis. Br. J. Rheumatol. **36:** 617–621

2 Hohlfield R., Londei M., Massacesi L. and Salvetti M. (1995) T-cell autoimmunity in multiple sclerosis. Immunol. Today **16:** 259–261

3 Valdimarrson H., Baker B. S., Jonsdottir I., Powles A. and Fry L. (1995) Psoriasis: a T-cell mediated autoimmune disease induced by streptococcal superantigens? Immunol. Today **16:** 145–149

4 Sasny P. N. (1987) HLA and the role of T cell in the predisposition to disease. Rheum. Dis. Clin. North Am. **13:** 1–6

5 Zanelli E., Gonzalez-Gay M. A. and David C. S. (1995) Could HLA-DRB1 be the protective locus in rheumatiod arthritis? Immunol. Today **16:** 274–278

6 Meeusen E. N. T., Premier R. R. and Brandon M. R. (1996) Tissue-specific migration of lymphocytes: a key role for Th1 and Th2 cells? Immunol. Today **17:** 421–424

7 Carruthers D. M., Naylor W. G., Allen M. E., Kitas G. D., Bacon P. A. and Young S. P. (1996) Characterisation of altered calcium signalling in T lymphocytes from patients with rheumatoid arthritis. Clin. Exp. Immunol. **105:** 291–296

8 Van Den Broek M., Boeije L., Swaak T., Smeenk R. and Aarden L. (1995) Functional analysis of synovial fluid and peripheral blood T cells from patients with rheumatoid arthritis. Rheumatol. Int. **15:** 137–143

9 De Keyser F., Elewaut D., Vermeersch J., DeWever N., Cuvelier C. and Veys E. M. (1995) The role of T cells in rheumatoid arthritis. Clin. Rheumatol. **14** (Suppl. 2)**:** 5–9

10 Miltenburg A. M. M., Lacraz S., Welgus H. G. and Dayer J-M. (1995) Immobilised anti-CD3 antibody activates T cell clones to induce the production of interstitial collagenase, but not tissue inhibitor of metalloproteinases, in monocytic THP-1 cells and dermal fibroblasts. J. Immunol. **154:** 2655–2667

11 Ramanujam T., Luchi M., Schumacher H. R., Zwillich S., Chang C. P., Callegari P. E. et al. (1995) Detection of T cell receptors in early rheumatoid arthritis synovial tissue. Pathobiology **63:** 100–108

12 Fischer D.-C., Opalka B., Hoffmann A., Mayr W. and Haubeck H.-D. (1996) Limited heterogeneity of rearranged T cell receptor V-alpha and V-beta transcripts in synovial fluid T cells in early stages of rheumatoid arthritis. Arthritis Rheum. **39:** 454–462

13 Multiple Sclerosis Genetics Group. (1996) A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. Nat. Genet. **13:** 469–471

14 Ikaheimo I., Tiilikainen A., Karvonen J. and Silvennoinen-Kassinen S. (1996) HLA risk haplotype Cw6,DR7,DQA 1*0201 and HLA-Cw6 with reference to the clinical picture of *Psoriasis vulgaris*. Arch. Dermatol. Res. **288:** 363–365

15 Trembath R. C., Clough R. L., Rosbotham J. L., Jones A. B., Camp R. D. R., Frodsham A. et al. (1997) Identification of a major susceptibility locus on chromosome 6p and evidence for further disease loci by a two-Stage genome-wide search in psoriasis. Hum. Mol. Genet. **6:** 813–820

16 Berger T., Weerth S., Kojima K., Linington C., Wekerle H. and Lassmann H. (1997) Experimental autoimmune encephalomyelitis: the antigen specificity of T lymphocytes determines the topography of lesions in the central and peripheral nervous system. Lab. Invest. **76:** 355–364

17 Wrone-Smith T. and Nickoloff B. J. (1996) Dermal injection of immunocytes induces psoriasis. J. Clin. Invest. **98:** 1878–1887

18 Hamann D., Baars P. A., Hooibrink B. and Van Lier R. A. W. (1996) Heterogeneity of the Human $CD4^+$ T-cell population: two distinct $CD4^+$ T-cell subsets characterised by co-expression of CD45RA and CD45R0 isoforms. Blood **88:** 3513–3521

19 Hwang S. T., Singer M. M., Giblin P. A., Yednock T. A., Bacon K. B. and Simon S. I. (1996) GlyCAM-1, a physiological ligand for L-selectin, activates beta-2 integrins on naive peripheral lymphocytes. J. Exp. Med. **184:** 1343–1348

20 Sad S. and Mossmann T. R. (1995) Interleukin 4, in the absence of antigen stimulation induces an anergy-like state in differentiated $CD8^+$ TC1 cells: loss of IL-2 synthesis and autonomous proliferation but retention of cytotoxicity and synthesis of other cytokines. J. Exp. Med. **182:** 1505–1515

21 Kanegane H. and Tosato G. (1996) Activation of naive and memory T cells by interleukin-15. Blood **88:** 230–235

22 Mossman T. R. and Sad S. (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today **17:** 138–146

23 DiPiro J. T. (1997) Cytokine networks with infection: mycobacterial infections, leishmaniasis, human immunodeficiency virus infection and sepsis. Pharmacotherapy **17:** 205–223

24 Schultze-Koops H., Lipsky P. E., Kavanaugh A. F. and Davis L. S. (1995) Th1- or Th0-like cytokine mRNA in peripheral circulation of patients with rheumatoid arthritis: modulation by treatment with anti-ICAM-1 correlates with clinical benefit. J. Immunol. **155:** 5029–5037

25 Dolhain R. J. E. M., Van der Heiden A. M., TerHaar N. T., Breedvald F. C. and Mittenburg A. M. M. (1996) Shift toward T-lymphocyte with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis. Arthritis Rheum. **39:** 1961–1969

26 Balashov K. E., Smith D. R., Khoung S. J., Hafler D. A. and Weiner H. L. (1997) Increased interleukin-12 production in progressive multiple sclerosis: induction by activated $CD4^+$ T cells via CD40 ligand. Proc. Natl. Acad. Sci. USA **94:** 599–603

27 DeJong R., Bezemer A. C., Zomerdijk T. P. L., Van der Poun-Kraan T., Ottenhof T. H. M. and Nibbering P. H. (1996) Selective stimulation of T helper 2 cytokine responses by the anti-psoriasis agent monomethylfumarate. Eur. J. Immunol. **26:** 2067–2074

28 Liblau R. S., Singer S. M. and McDevitt H. O. (1995) Th1 and Th2 $CD4^+$ T cells in the pathogenesis of organ-specific autoimmune disease. Immunol. Today **16:** 34–38

29 Umetsu D. T. and DeKruyff R. H. (1997) Th1 and Th2 $CD4^+$ in the pathogenesis of allergic diseases. Proc. Soc. Exp. Biol. Med. **215:** 11–20

30 Erb K. J. and Le Gros G. (1996) The role of Th2 type $CD4^+$ T cells and Th2 type $CD8^+$ T cells in asthma. Immunol. Cell Biol. **74:** 206–208

31 Hosken N. A., Shibuya K., Heath A. W., Murphy K. M. and O'Garra A. (1995) The effect of antigen dose on $CD4^{+4}$ T helper cell phenotype development on a T cell receptor-α-β-transgenic model. J. Exp. Med. **182:** 1579–1584

32 Hilkens C. M. V., Snijders A., Snijdement F. G. M., Wieranga E. A. and Kapsenberg M. L. (1996) Modulation of T-cell cytokine secretion by accessory cell-derived products. Eur. Respir. J. **9** (Suppl. 2)**:** 905–945

33 Sornasse T., Laranas P., Davis K. A., DeVries J. E. and Yssel H. (1996) Differentiation and stability of T helper 1 and 2 cells derived from naive human neonatal $CD4^+$ T cells, analysed at the single cell level. J. Exp. Med. **184:** 413–483

34 Swain S. L., Weinberg A. D. and English M. (1990) $CD4^+$ T cell subsets: Lymphokine secretion of memory cells and of effector cells that develop from precursors in vitro J. Immunol. **144:** 1788

35 Maruo S., Toyo-oka K., Oh-hora M., Tai X.-G., Iwata H., Takenaka H. et al. (1996) IL-12 produced by antigen-presenting cells induces IL-2 independent proliferation of T-helper cell clones. J. Immunol. **140:** 4211–4216

36 Umetsu D. T., Jabara H. H., DeKruyff R. H., Abbas A. K., Abrams J. S. and Geta R. S. (1988) Functional heterogeneity among human inducer T cell clones. J. Immunol. **140:** 4211–4216

37 Kelso A. (1995) Th1 and Th2 subsets: paradigms lost? Immunol. Today **16:** 374–379

38 Bradley L. M., Croft M. and Swain S. L. (1993) T cell memory: new perspectives. Immunol. Today **14:** 197–199

39 Chiocchia D. A., Baldwin M. and Smith K. A. (1995) Memory T cells are hypersensitive and hyper-responsive compared with naive T cells. Ninth International Congress of Immunology meeting, San Francisco, CA, abstract 162

40 Cantrell D. A., Pastor M. I., Reif K. and Woodrow M. (1995) T cell activation in 'T cell receptors', pp. 151–163, Bell J. I., Owen M. J. and Simpson E. (eds), Oxford University Press, Oxford

41 Weiss A. (1993) T cell antigen receptor signalling transduction: a tale of tails and cytoplasmic protein kinases. Cell **73:** 209–212

42 Kersh G. J. and Allen P. M. (1996) Structural basis of T cell recognition of altered peptide ligands. A single T cell receptor can productively recognise a large continuum of related ligands. J. Exp. Med. **184:** 1525–1530

43 Sloan-Lancaster J., Steinberg T. H. and Allen P. M. (1996) Selective activation of the calcium signalling pathway by altered peptide ligands. J. Exp. Med. **184:** 1525–1530

44 Racioppi L., Ronchese F., Matis L. A. and Germain R. A. (1993) Peptide-major histocompatability complex class II complexes with mixed agonist/antagonist properties provide evidence for ligand-related differences in T cell receptor-dependent intracellular signalling. J. Exp. Med. **177:** 1047–1060

45 Madrenas J., Chau L. A., Smith J., Bluestone J. A. and Germain R. N. (1997) The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist-partial agonist properties of peptide-MHC molecule ligands. J. Exp. Med. **185:** 219–229

46 Otten G. R. and Germain R. N. (1991) Split anergy in a $CD8^+$ T cell: receptor-dependent cytolysis in the absence of interleukin-2 production. Science **251:** 1228–1231

47 Osman N., Turner H., Lucas S., Reif K. and Cantrell D. A. (1996) The protein interactions of the immunoglobulin receptor family tyrosine-based activation motifs present in the T cell receptor zeta sub-units and the CD3 gamma, delta and epsilon chains. Eur. J. Immunol. **26:** 1063–1068

48 Weiss A. and Littman D. R. (1994) Signal transduction by lymphocyte antigen receptor. Cell **76:** 263–274

49 Klausner R. D. and Samelson L. E. (1991) T cell antigen receptor activation pathways: the tyrosine kinase connection. Cell **64:** 875–878

50 Strauss D. B., Chan A. C., Patai B. and Weiss A. (1996) SH2 domain function is essential for the role of lck tyrosine kinase in T cell receptor signal transduction J. Biol. Chem. **271:** 9976–9981

51 Weil R., Cloutier J.-F., Fournei M. and Veilette A. (1995) Regulation of ZAP-70 by Src family tyrosine kinases in an antigen-specific T cell line. J. Biol. Chem. **270:** 2791–2799

52 Madrenas J., Wange R. L., Wang J. L., Isakov N., Samelson L. E. and Germain R. N. (1995) ζ Phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. Science **267:** 515–518

53 Arpaia E., Shahar M., Dadi H., Cohen A. and Roifman C. M. (1994) Defective T cell receptor signalling and $CD8^+$ thymic selection in humans lacking ZAP-70 kinase. Cell **76:** 947–958

54 Elder M. E., Lin D., Clever J., Chan A.-C., Hope T. J., Weiss A. et al. (1994) Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell Tyrosine kinase. Science **264:** 1596–1599

55 Qian D., Mollenauer M. N. and Weiss A. (1996) Dominant negative receptor signalling. J. Exp. Med. **183:** 611–620

56 Zhao Q. and Weiss A. (1996) Enhancement of lymphocyte responsiveness by a gain-of-function mutation of ZAP-70. Mol. Cell Biol. **16:** 6765–6774

57 Wu. J., Motto D. G., Koretsky G. A. and Weiss A. (1996) A complex between p95-vav and SLP-76 is induced in TCR-mediated IL-2 gene induction. FASEB J. **10:** A1027

58 Wardenburg J. B., Fu C., Jackman J. K., Flotow H., Wilkinson S. E., Williams D. H. et al. (1996) Phosphorylation of SLP-76 by ZAP-70 protein tyrosine kinase as required for T-cell receptor function. J. Biol. Chem. **271:** 19641–19644
59 Huby R. D. J., Carlisle G. W. and Ley S. C. (1995) Interactions between the protein-tyrosine kinase ZAP-70, the proto-oncoprotein Vav and tubulin in Jurkat T cells. J. Biol. Chem. **270:** 30241–30244
60 Uddin S., Sweet M., Colamonici O. R., Krolewski J. J. and Platanids L. C. (1997) The Vav proto-oncogene product (p95–vav) Interacts with the Tyk-2 protein tyrosine kinase. FEBS Lett. **403:** 31–34
61 Machicle M., Mano H. and Todokoro K. (1995) Interleukin 3 and Erythropoietin induce association of Vav with Tec kinase through Tec homology domain. Oncogene **11:** 619–625
62 Gulbins E., Schlottmann K., Brenner B., Linderkamp D., Long F. and Coggeshall K. M. (1995) Molecular analysis of Ras activation by tyrosine phosphorylated Vav. Biochem. Biophys. Res. Commun. **217:** 876–885
63 Boonefoy-Barard N., Munshi A., Yron I., Wu S., Collins T. L., Deckert M. et al. (1997) Vav: function and regulation in haemopoetic cell signalling. Stem Cells (Dayton) **14:** 250–268
64 Crespo P., Schwebel K. E., Ostrom A. A., Crutkind J. S. and Bustelo X. R. (1997) Phospho-tyrosine-dependent activation of Rac-1 GDP-GTP exchange by the Vav proto-oncogene Product. Nature **385:** 169–172
65 Lee J. M., Fournel M., Veilette A. and Branton P. E. (1996) Association of CD45 with Lck and components of the Ras signalling pathway in pervanadate-treated mouse T-Cell lines. Oncogene **12:** 253–263
66 Raab M., DaSilva A. J., Findell P. R. and Rudd C. E. (1997) Regulation of Vav-SLP-76 binding by ZAP-70 and its relevance to TCR-ζ-CD3 ligation of interleukin-2. Immunity **6:** 155–164
67 Stein P. H., Fraser J. D. and Weiss A. (1994) The cytoplasmic domain of CD3 is both necessary and sufficient for co-stimulation of interleukin-2 secretion and association with phosphatidylinositol-3-kinase. Mol. Cell. Biol. **14:** 3392–3402
68 Mindino A. and Jenkins M. K. (1994) Surface proteins involved in T cell costimulation. J. Leukoc. Biol. **55:** 805–815
69 Kariv I., Reddy M., Eichman C., Lyn S., Truneh A. and Sweet R. (1995) Deteminations of residues on CD28 involved in interaction with its counter receptors CD80(B7.1) and CD86(B7.2)-B70. Ninth International Congress of Immunology, San Francisco CA, abstract 285
70 Raab M., Cai Y.-C., Bunnell S. C., Heyeck S. D., Berg L. J. and Rudd C. E. (1995) $p56^{lck}$ and $p59^{fyn}$ regulate CD28 binding to phosphatidylinositol 3-kinase, growth factor receptor-bound protein Grb-2 and T-cell specific protein tyrosine kinase ITK: implications for T cell co-stimulation Proc. Natl. Acad. Sci. USA **92:** 8891–8895
71 Schneider H., Cai Y.-C., Prasal K. V. S., Shoelson S. E. and Rudd C. E. (1995) T cell antigen CD28 binds to the Grb-2-SOS complex regulator of p21-ras. Eur. J. Immunol. **25:** 1044–1050
72 Macara I. G., Lounsbury K. M., Richards S. A., McKiernan C. and Bar-Sagi D. (1996) The Ras superfamily of GTPases. FASEB J. **10:** 625–630
73 Chu K. and Littman D. R. (1994) Requirement for kinase activity of CD4 associated $p56^{lck}$ in antibody-triggered T cell signal transduction J. Biol. Chem. **269:** 24095–24101
74 Hall C. G., Sancho J. and Terhorst C. (1993) Redistribution of T cell receptor ζ-mediated calcium mobilization in non-lymphoid cells. Science **261:** 915–918
75 Sieh M., Batzer A., Schlessinger J. and Weiss A. (1994) Grb2 and phospholipase C-γ1 associate with a 36 to 38 kilodalton phosphotyrosine protein after T cell receptor stimulation. Mol. Cell. Biol. **14:** 4435–4442
76 Burns D. J., Bloomenthal J., Lee M. H. and Bell R. M. (1990) Expression of the α, βII and γ protein kinase C isozymes in the baculovirus-insect cell expression system. Purification and characterisation of the individual isoforms. J. Biol. Chem. **265:** 12044–12051
77 Loh C., Shaw K. T.-Y., Carew J., Viola J. P. B., Luo C., Perrino B. A. et al. (1996) Calcineurin binds the transcriptional factor NF-AT1 and reversibly regulates its activity. J. Biol. Chem. **271:** 10884–10891
78 Masuda E. S., Liu J., Imamura R., Imai S.-I., Arai K.-I. and Arai N. (1997) Translocation by a calcineurin-regulated inhibitory domain. Mol. Cell. Biol. **17:** 2066–2075
79 Loh C., Carew J. A., Kim J., Hogan P. G. and Raq A. (1996) T-cell receptor stimulation elicits an early phase of activation and a later phase of deactivation of the transcription factor NF-AT1. Mol. Cell. Biol. **16:** 3945–3954
80 Park J., Yaseen N. R., Hogan P. G., Rao A. and Sharma S. (1995) Phosphorylation of the transcription factor NF-AT-p inhibits its DNA binding activity in cyclosporin A-treated human B and T cells. J. Biol. Chem. **270:** 20653–20659
81 Griffith J. P., Kim J. L., Kim E. E., Sintchak M. D., Thomson J. A., Fitzgibbon M. J. et al. (1995) X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP-12-FK506 complex. Cell **82:** 507–522
82 Granelli-Piperno A. and Nolan P. (1991) Nuclear transcription factors that bind to elements of the IL-2 promoter induction requirements in primary human T cells. J. Immunol. **147:** 2734–2739
83 Lyakh L., Ghuship P. and Rice N. R. (1997) Expression of NF-AT-family proteins in normal human T cells. Mol. Cell. Biol. **17:** 2475–2484
84 Sun L. J., Peterson B. R. and Verdine G. L. (1997) Dual role of the nuclear factor of activated T cells insert region in DNA recognition and cooperative contacts to activator protein 1. Proc. Natl. Acad. Sci. USA **94:** 4919–4924
85 Wolfe S. A., Zhou P., Dotsch V., Chen L., You A., Ho S. N. et al. (1997) Unusual Rel-like architecture in the DNA-binding domain of the transcription factor NF-ATc. Nature **385:** 172–176
86 Marshall M. (1995) Interactions between Ras and Raf: key regulatory proteins in cellular transformation. Mol. Reprod. Dev. **42:** 493–499
87 Marais R., Light Y., Paterson H. F., Mason C. S. and Marshall C. J. (1997) Differential regulation of Raf-1, A-Raf and B-Raf by oncogenic Ras and tyrosine kinases. J. Biol. Chem. **272:** 4378–4383
88 Genot E., Cleverley S., Henning S. and Cantrell D. (1996) Multiple p21ras effector pathways regulate nuclear factor of activated T cells. EMBO J. **15:** 3923–3933
89 Marais R., Wynne J. and Treismann R. (1993) The SRF accessory protein, Elk-1, contains a growth-factor-regulated transcriptional activation domain. Cell **73:** 381–393
90 Whitmarsh A. J., Shore P., Sharrocks A. and Davis R. J. (1995) Integration of MAP kinase signal transduction pathways at the serum response element. Science **269:** 403–407
91 Derijard B., Hibi M., Wu I.-H., Barrett T., Su B., Deng T. et al. (1994) JNK-1: a protein kinase stimulated by UV light and H-Ras that bind and phosphorylates the c-jun activation domain. Cell **76:** 1025–1037
92 Shapiro V. S., Truitt K. E., Imhoden J. B. and Weiss A. (1997) CD28 mediates transcriptional upregulation of the interleukin-2 promoter through a composite element containing the CD28 RE and NF-IL-2B AP-1 sites. Mol. Cell. Biol. **17:** 4051–4058
93 Kongen F., Grumant R.-J., Strasses A., Metcalf D., Li R., Tarlington D. et al. (1995) Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity and interleukin-2 expression. Genes Dev. **9:** 1965–1977
94 Los M., Schenk H., Hexel K., Boeverke P. A., Droege W. and Schulze-Osthoff K. (1995) IL-2 gene expression and NK-κB activation through CD28 requires reactive oxygen production by 5-lipoxygenase. EMBO J. **14:** 3731–340
95 Harhaj E. W., Maggirwar S. B., Good L. and Sin S.-C. (1996) CD28 mediates a potent co-stimulatory signal for a rapid degradation of I-κ-B-beta which is associated with

accelerated activation of various NF-κ-B Rel heterodimers. Mol. Cell. Biol. **16:** 6736–6743
96 Regnier C. H., Song H. Y., Gao X., Goecklel D. V., Cao Z. and Rothe M. (1997) Identification and characterisation of an I-κ-B kinase. Cell **90:** 373–383
97 Traenckner E. B. and Bauerle P. A. (1995) Appearance of apparently ubiquitin-conjugated I-κ-B-alpha during its phosphorylation-induced degradation in intact cells. J. Cell. Sci. (Suppl. 19)**:** 79–84
98 Schow S. and Joly A. (1996) Inhibition of NF-κ-B activation by the proteosome inhibitor *N*-acetyl-leucinyl-leucinyl-naleucinal. Mol. Biol. **7** (Suppl.)**:** 180A
99 Rottenberg E. V. and Ward B. (1996) A dynamic assembly of diverse transcription factors integrates activation and cell-type information for interleukin-2 gene regulation. Proc. Natl. Acad. Sci. USA **93:** 9358–9365
100 Theze J., Alzani P. M. and Berloglio J. (1996) Interleukin 2 and its receptors: recent advances and new immunological functions. Immunol. Today **17:** 481–486
101 Theze J. (1994) Cytokine receptors: a combinative family of molecules. Eur. Cytokine Netw. **5:** 353–368
102 Algarte M., Lecine P., Costello R., Plet A., Olive D. and Imbert J. (1995) In vivo regulation of interleukin-2 receptor alpha gene transcription by the co-ordinated binding of constitutive and inducible factors in the human primary T cells. EMBO J. **14:** 5060–5072
103 Ascherman D. P., Migene T.-S., Friedmann M. C. and Leonard W. J. (1997) Interleukin-2 (IL-2)-mediated induction of the IL-2 receptor alpha chain gene. J. Biol. Chem. **272:** 8704–8709
104 Lecine P., Algarte M., Rameil P., Beadling C., Bucher P., Mabholz M. et al. (1996) Elf-1 and Stat 5 bind to a critical element in a new enhancer of the human interleukin-2 receptor alpha gene. Mol. Cell. Biol. **16:** 6829–6840
105 John S., Marais R., Child R., Light Y. and Leonard W. J. (1996) Importance of low affinity Elf-1 sites in the regulation of lymphoid-specific inducible gene expression. J. Exp. Med. **183:** 743–760
106 Ravichandran K. S., Igras V., Shoelson S. E., Fesik S. W. and Burakoff S. J. (1996) Evidence for a role for the phosphotyrosine-binding domain of Shc in interleukin 2 signaling. Proc. Natl. Acad. Sci. USA **93:** 5275–5280
107 Abraham R. T. and Wiederrecht G. J. (1996) Immunopharmacology of rapamycin. Ann. Rev. Immunol. **14:** 483–510
108 Schindler C. (1995) Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Ann. Rev. Biochem. **64:** 621–651
109 Ellis H. M. and Horvitz H. R. (1986) Genetic control of programmed cell death in the nematode *Caenorhabditis elegans*. Cell **44:** 817–829
110 Yuan J., Shaham S. and Ledoux S. (1993) The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1-β converting enzyme. Cell **75:** 641–652
111 Muzio M., Chinnaiyan A. M., Kischkel F. C., O'Rourke K., Shevehenko A., Ni J. et al. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signalling complex. Cell **85:** 817–827
112 Pronk G. J., Ramer K., Amiri P. and Williams L. T. (1996) Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER. Science **271:** 808–810
113 Hug H. and Saare T. F. (1993) Protein kinase C isoenzymes: divergence in signal transduction? Biochem. J. **291:** 329–343
114 Keranen L. M., Dutil E. M. and Newton A. C. (1995) Protein kinase is regulated in vivo by three functionally distinct phosphorylations. Curr. Biol. **5:** 1394–1404
115 Newton A. C. (1997) Regulation of protein kinase C by cofactors. In: Protein Kinase C, pp. 25–44, Parker P. J. and Dekker L. V. (eds), Chapman and Hall, New York
116 Newton A. C. (1995) Protein kinase C: structure, function and regulation. J. Biol. Chem. **270:** 28495–28498
117 Pears C. J. and Goode N. T. (1997) PKC downregulation: signal or adaptation. In: Protein Kinase C, pp. 45–56, Parker P. J. and Dekker L. V. (eds), Chapman and Hall, New York
118 Kobayashi E., Nakano H., Morimoto M. and Tamaoki T. (1989) Calphostin C (UCN-1028C), a novel microbial compound is a highly potent and selective inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. **159:** 548–553
119 Ruegg U. T. and Burgess G. M. (1989) Staurosporine K-252 and UCN-**01:** potent but nonspecific inhibitors of protein kinases. Trends Biochem. Sci. **10:** 218–220
120 Birchall A. M., Bishop J., Bradshaw D., Cline A., Coffey J., Elliott L. H. et al. (1994) Ro 32-0432, a selective and orally active inhibitor of protein kinase C prevents T-cell activation. J. Pharmacol. Exp. Ther. **268:** 922–928
121 Elliott L. H., Wilkinson S. E., Sedgwick A. D., Hill C. H., Lawton G., Davis P. D. et al. (1990) K252a is a potent and selective inhibitor of phosphorylase kinase. Biochem. Biophys. Res. Commun. **171:** 148–154
122 Nixon J. S., Wilkinson S. E., Davis P. D., Sedgwick A. D., Wadsworth J. and Westmacott D. (1991) Modulation of cellular processes by H7, a non-selective inhibitor of protein kinases. Agents Actions **32:** 188–193
123 Nakajima K., Kusafuka T., Takeda T., Fujitani Y., Nakae, K. and Hirano T. (1993) Identification of a novel interleukin-6 response element containing an Ets-binding site and a CRE-like site in the junB promoter. Mol. Cell. Biol. **13:** 3027–3041
124 Meyer T., Regenass U., Fabbro D., Alteri E., Rosel J., Muller M. et al. (1989) A derivative of staurosporine shows selectivity for protein kinase C inhibition and in vitro anti-proliferative as well as in vivo anti-tumour activity. Int. J. Cancer **43:** 851–856
125 Bit R. A., Davis P. D., Elliott L. H., Harris W., Hill C. H., Keech E. et al. (1993) Inhibitors of protein kinase C 3. Potent and highly selective bisidolylmaleimides by conformational restriction. J. Med. Chem. **36:** 21–29
126 Alessi D. R. (1997) The protein kinase C inhibitors Ro 31-8220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1β (Rsk-2) and p70 S6 kinase. FEBS Lett. **402:** 121–123
127 Nobrega A. F., Maldonado M. S. and Dos Reis G. A. (1986) Analysis of isolated and combined effects of calcium ionophore and phorbol ester on T lymphocyte activation. Clin. Exp. Immunol. **65:** 559–569
128 Nixon J. S., Bishop J., Bradshaw D., Davis P. D., Hill C. H., Elliott L. H. et al. (1992) The design and biological properties of potent and selective inhibitors of PKC. Biochem. Soc. Trans. **20:** 419–425
129 Cuvillier O., Pirianov G., Kleuser B., Vanek P. G., Coso O. A., Gutkin J. S. et al. (1996) Suppression of ceramide-mediated programed cell death by sphingosine-1-phosphate. Nature **381:** 800–803
130 Mills G. B., May C., Hill M., Ebanks R., Roifman C., Mellors A. et al. (1989) Physiologic activation of protein kinase C limits IL-2 secretion. J. Immunol. **142:** 1995–2003
131 Berry N., Ase K., Kishimoto A. and Nishizuka Y. (1990) Activation of resting T-cells requires prolonged stimulation of protein kinase C. Proc. Natl. Acad. Sci. USA **87:** 2294–2298
132 Rossetti R. G., Seiler C. M., Laposata M. and Zurier R. B. (1995) Differential regulation of human T lymphocyte protein kinase C activity by unsaturated fatty acids. Clin. Immunol. Immunopathol. **76:** 220–224
133 Tsutsumi A., Kubo M., Fujii H., Freire-Moar J., Turck C. W. and Ransom J. T. (1993) Regulation of protein kinase C isoform proteins in phorbol ester stimulated Jurkat T lymphoma cells. J. Immunol. **150:** 1746–54
134 Keenan C., Kelleher D. and Long A. (1995) Regulation of non-classical protein kinase C isoenzymes in a human T cell line. Eur. J. Immunol. **25:** 13–17
135 Tsutsumi A., Kubo M., Fujii H., Freire-Moar J., Turck C. W. and Ransom J. T. (1993) Regulation of protein kinase

C isoform proteins in phorbol ester-stimulated Jurkat T lymphoma cells. J. Immunol. **150:** 1746–1754
136 Baier G., Baier-Bitterlich G., Meller N., Coggeshall K. M., Giampa L., Telford D. et al. (1994) Expression and biochemical characterization of human protein kinase C-θ. Eur. J. Biochem. **225:** 195–203
137 Monks C. R. F., Kupfer H., Tamir I. and Kuper A. (1997) Selective modulation of protein kinase-θ during T-cell activation. Nature **385:** 83–85
138 Wilkinson S. E. and Hallam T. J. (1994) Protein kinase C: is its pivotal role in cellular activation over-stated? Trends Pharmacol. Sci. **15:** 53
139 Ahmed S., Kozma R., Monfries C., Hall C., Lim H. H., Smith P. et al. (1990) Human brain n-chimaerin cDNA encodes a novel phorbol ester receptor. Biochem. J. **272:** 767–773
140 Ahmed S., Lee J., Best A., Monfries C. and Lim L. (1993) A novel functional target for tumor-promoting phorbol esters and lysophosphatidic acid. The p21rac-GTPase activating protein n-chimaerin. J. Biol. Chem. **268:** 10709–10712
141 Merritt J. E., Sullivan J. A., Tse J., Wilkinson S. E. and Nixon J. S. (1997) Different sensitivities of neutrophil responses to a selective protein kinase C inhibitor, Ro 31-8425: redundancy in signal transduction. Cell. Signal. **9:** 53–57
142 Nishizuka Y. 1984) The role of PKC in cell surface signal transduction and tumour promotion. Nature **308:** 693–698
143 Berney S. M. and Atkinson T. P. (1995) Phosphatidylinositol hydrolysis in freshly isolated human T lymphocytes. J. Immunol. Methods **186:** 71–77
144 Vassilopoulos D., Zurier R. B., Rossetti R. G. and Tsokos G. C. (1997) Gammalinolenic acid and dihomogammalinolenic acid suppress the CD3-mediated signal transduction pathway in human T cells. Clin. Immunol. Immunopathol. **83:** 237–244
145 Szamel M., Retiermann B., Krebs B., Kurrle R. and Resch K. (1989) Activation signals in human lymphocytes. J. Immunol. **143:** 2806–2813
146 Imboden J., Weyand C. and Govonzy J. (1987) Antigen recognition by a human T cell clone leads to increases in inositol triphosphate. J. Immunol. **138:** 1322–1324
147 Fulop T. Jr., LeBlanc C., Lacombe G. and Dupuis G. (1995) Cellular distribution of protein kinase C isozymes in CD-3 mediated stimulation of human T-lymphocytes with aging. FEBS Lett **375:** 69–74
148 Keenan C., Long A., Volkov Y. and Kelleher D. (1997) Protein kinase C isotypes θ, -δ, -η in human lymphocytes: Differential responses to signalling through the T-cell receptor and phorbol esters. Immunology **90:** 557–563
149 Valge V. E., Wong J. G., Datlof B. M., Sinskey A. J. and Rao A. (1988) Protein kinase C is required for responses to T cell receptor ligands but not to interleukin-2 in T cells. Cell **55:** 101–102
150 Alkan S. S., Ritschmann S., Grigg D. and Erb P. (1993) Effects of a new protein kinase C inhibitor CGP 41251, on T cell functions: inhibition of activation, growth and target cell killing. Cell. Immunol. **150:** 137–148
151 Bishop J., Greenham A. K., Lewis E. J. and Pole D. S. (1993) A Simple host-versus-graft model in the rat for the study of immunomodulatory drugs. Eur. J. Rheumatol. Inflamm. **13:** 17–20
152 Heber-Katz E. (1994) Is experimental allergic encephalomyelitis a model of multiple sclerosis? In: Autoimmunity and Physiological Diseases, pp. 353–366, Coutinho A. and Kazatchkine M. D. (eds), Wiley-Liss Inc., New York
153 Lassman H., Vass K., Brunner C. and Seitelburger F. (1986) Characterization of inflammatory infiltrates in experimental allergic encephalomyelitis. In: Progress in Neuropathology, vol. 6, pp. 33–36, Zimmerman H. M. (ed.), Raven Press, New York
154 Matsumoto Y. and Fujiwara M. (1987) Immunopathology of adoptively transferred experimental allergic encephalomyelitis (EAE) in Lewis rats. Part I. Immunohistochemical examination of developing lesions of EAE. J. Neurol. Sci. **77:** 35–47
155 Grove D. S. and Mastro A. M. (1991) Differential activation and inhibition of lymphocyte proliferation by phorbol esters, mezerein, teleocidin and okadaic acid. Cancer Res. **51:** 82–88
156 Szamel M., Ebel U., Uciechowski P., Kaever V. and Resch K. (1997) T cell antigen receptor dependent signalling in human lymphocytes: cholera toxin inhibits interleukin-receptor expression but not interleukin-2 synthesis by preventing activation of a protein kinase C isotype PKC-α. Biochim. Biophys. Acta **1356:** 237–248
157 Haverstick D. M., Dicus M., Resnick M. S., Sando J. J. and Gray L. S. (1997) A role for protein kinase C-β_I in the regulation of Ca^{2+} entry in Jurkat T cells. J. Biol. Chem. **272:** 15426–15433
158 Gomez J., Martinez C., Garcia A. and Rebolla A. (1996) Association of phosphatidylinositol 3 kinase to protein kinase C zeta during interleukin-2 stimulation. Eur. J. Immunol. **26:** 1781–1787
159 Iwata M., Ohoka Y., Kuwata T., Asada A., Zhao Y. and Mukai M. (1997) Regulation of thymocyte lineage commitment by the level of classical protein kinase C activity. J. Allergy Clin. Immunol. **99:** S111
160 Freire Moar J., Tsutumu A. and Baya P. (1992) Use of an antisense RNA experimental system to explore the functions of the PKC isoenzymes in T cell function. Ann. NY Acad. Sci. **660:** 288–290
161 Snyder J. T. II and Finn O. J. (1997) The role of protein kinase C-beta in T-lymphocyte activation and tolerance induction. J. Allergy Clin. Immunol. **99:** S307
162 Muid R. E., Dale M. M., Davis P. D., Elliott L. H., Hill C. H., Kumar M. K. H. et al. (1991) A novel conformationally restricted protein kinase C inhibitor Ro 31-8425, inhibits human neutrophil superoxide generation by soluble, particulate and post-recepter stimuli. FEBS Lett. **293:** 169–172
163 Williams D. H., Hallam T. J. and Murray E. J. (1993) The role of protein kinase C in TCR-induced interleukin 2 secretion in human Jurkat T-cells. Biochem. Soc. Trans. **21:** 383s
164 Williams D. H., Woodrow M., Cantrell D. A. and Murray E. J. (1995) Protein kinase C is not a downstream effector of p21ras in activated T cells. Eur. J. Immunol. **25:** 42–47
165 Lindholm P. F., Tamami M., Makowski J. and Brady J. N. (1996) Human T-cell lymphotropic virus type 1 Tax-1 activation of NF-κ-B: involvement of the protein kinase C pathway. J. Virol. **70:** 2525–2532
166 Steffan N. M., Bren G. D., Frantz B., Tocci M. J., O'Neill E. A. and Paya C. V. (1995) Regulation of Iκ-B-α phosphorylation by PKC- and Ca^{2+}-dependent signal transduction pathways. J. Immunol. **155:** 4685–4691
167 Baier-Bitterlich G., Uberall F., Bauer B., Fresser F., Wachter H. et al. (1996) Protein kinase C-θ isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. Mol. Cell. Biol. **16:** 1842–1850
168 Meller N., Liu Y.-C., Collins T. L., Bonnefoy-Berard N., Baier G., Isakov N. et al. (1996) Direct interaction between PKC-θ and 14-3-3-tau in T cells: 14-3-3 overexpression results in inhibition of PKC-θ translocation and function. Mol. Cell. Biol. **16:** 5782–5791