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Wealth of opportunity – the C1 domain as a target for drug development

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Abstract

The diacylglycerol-responsive C1 domains of protein kinase C and of the related classes of signaling proteins represent highly attractive targets for drug development. The signaling functions that are regulated by C1 domains are central to cellular control, thereby impacting many pathological conditions. Our understanding of the diacylglycerol signaling pathways provides great confidence in the utility of intervention in these pathways for treatment of cancer and other conditions. Multiple compounds directed at these signaling proteins, including compounds directed at the C1 domains, are currently in clinical trials, providing strong validation for these targets. Extensive understanding of the structure and function of C1 domains, coupled with detailed insights into the molecular details of ligand – C1 domain interactions, provides a solid basis for rational and semi-rational drug design. Finally, the complexity of the factors contributing to ligand – C1 domain interactions affords abundant opportunities for manipulation of selectivity; indeed, substantially selective compounds have already been identified.

Keywords

protein kinase C; phorbol ester; RasGRP; bryostatin; C1 domain

Background

Sn-1,2-diacylglycerol (DAG) represents one of the central second messengers in the cell, generated by the hydrolysis of phosphatidylinositol 4,5-bisphosphate subsequent to the activation of phospholipase C by either G-protein coupled receptors or receptor tyrosine kinases [1–3]. An additional source of DAG is from phosphatidic acid, liberated by the hydrolysis of phosphatidylcholine by phospholipase D. The primary mechanism of action of DAG is through its interaction with a specialized recognition motif, the so-called C1 domain [4–6] (Fig. (1)). C1 domains are zinc finger structures of approximately 50 amino acids in length. They can be subclassified into two families, those that are responsive to DAG and those that are not. The C1 domains of protein kinase C (PKC) provide the paradigm for the DAG-responsive C1 domains; the C1 domains of the atypical PKCs (zeta and iota) provide the paradigm for the so-called “atypical” C1 domains, which do not respond to DAG.

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The classic and novel PKC isoforms represent the first recognized and most extensively studied family of effectors for DAG [7, 8]. The PKCs are ser/thr specific protein kinases. Structurally, they consist of an N-terminal inhibitory regulatory domain and a C-terminal catalytic domain (Fig. (2)). The regulatory domain functions by positioning a pseudosubstrate sequence in the catalytic site of the enzyme, blocking its access to substrates. The regulatory domain contains two C1 domains (C1a and C1b, respectively) and, in the classical PKCs, a C2 domain. Interaction of DAG with the C1 domains and of Ca^{2+} with the C2 domain stabilizes association of these domains at the membrane. The resultant conformational change of the enzyme extracts the pseudosubstrate from the catalytic site, thereby activating the enzyme. Concomitantly, the membrane association of the enzyme affects its proximity to substrates, contributing to activity and specificity. Finally, the conformational change enhances access of the hinge region between the regulatory and catalytic domains of PKC to calpain and caspase, leading to its cleavage [9]. Depending on circumstances, this cleavage can provide either for down regulation of PKC through its breakdown or for novel activities, reflecting liberation of an active catalytic domain with different localization as well as of a regulatory domain, which may have functions of its own.

Following the identification of the PKCs, six other families of proteins with homologous, DAG-responsive C1 domains have been identified with different effector domains (Fig. (2)) [10, 11]. The protein kinase D family possesses a distinct kinase domain more closely resembling the calcium-calmodulin dependent protein kinase [12, 13]. The PKDs are involved in Golgi function, proliferation, metastasis, and apoptosis. The chimaerins act as inhibitors (GAPs, GTPase activating proteins) for Rac and are candidate tumor suppressors [14]. The RasGRP family members function as activators (GEFs, GTP exchange factors) for Ras [15], are oncogenic in T-cell lymphoma [16], contribute to tumor progression in skin carcinogenesis [17], and are involved in angiogenesis [18]. The Unc-13 family members promote vesicle priming [19, 20]. The DAG kinases phosphorylate DAG, terminating DAG signaling [21]. Finally, MRCK acts as a downstream effector of cdc42 involved in filopodia formation, contributing to tumor invasion [22, 23]. The existence of these other families of effectors for DAG enhances the opportunities for dissecting out subpathways of response downstream from DAG, provided that ligands selective for these distinct effector families can be designed.

Potential of C1 domains as drug targets

PKC has emerged as a clear, validated therapeutic target for cancer (see Chapter by Newton in this volume, as well as [24 – 28]. Among the many examples, PKC beta is overexpressed in diffuse large B-cell lymphoma and associated with poor prognosis for response to chemotherapy [29, 30]. Clinical trials are evaluating the treatment of diffuse large B-cell lymphoma [31] with enzastaurin, a PKC beta specific kinase inhibitor [32]. PKC iota functions as an oncogene in human non-small cell lung cancer [33]. Aurothioglucose, a compound clinically used in the treatment of rheumatoid arthritis, has been found to act as a PKC iota inhibitor and as an inhibitor of non-small cell lung cancer growth [34] and is now entering clinical trials.

Complementing the more conventional approach of inhibiting PKC enzymatic activity is the strategy of targeting its C1 domains. Considerable confidence in this latter approach is provided by the fact that several inhibitors directed at the C1 domain of PKC are already in or approaching clinical trials. All of this first generation of compounds targeting the C1 domain are natural products, isolated from traditional medicines or identified from natural products screening. PEP005 (ingenol 3-angelate), initially isolated from *Euphorbia antiquorum* [35], is in clinical trials for actinic keratosis and for non-melanotic skin cancer

[36]. Bryostatin 1, identified through the highly productive marine natural products screening program of Dr. G.R. Pettit, is in multiple cancer clinical trials [37]. Prostratin (12-deoxyphorbol 13-acetate), initially isolated from *Euphorbia prostrata* and subsequently from *Homalanthus accuminata*, is a candidate for combination with HAART treatment to address viral latency in HIV-AIDS [38, 39]. Phorbol 12-myristate 13-acetate (PMA), the paradigmatic phorbol ester from *Croton tiglium*, is being evaluated as a differentiation agent in cancer [40].

A major difficulty in the design of catalytic site inhibitors of PKC has been the high conservation of the ATP binding site among the more than 500 kinases in the human genome [41]. As yet, selective inhibitors for PKC have only been developed for PKC beta (and these indeed are only semi-selective, in that they also inhibit some kinases other than the PKCs)[42]. An advantage of targeting the C1 domains of PKC is the much smaller number of C1 domains that may mediate off-target effects. Nonetheless, a potential concern is the high conservation of the C1 domain structure. Years ago, we were able to show similar phorbol ester binding in organisms as diverse as nematodes, flies, and mice [43]. On the other hand, an important concept is that ligand recognition by PKC is highly dependent on the cellular context in which it is found. Elements contributing to this effect of cellular context include the lipid composition of the membranes, since phospholipids form the half-site for ligand recognition along with the C1 domain, and intracellular calcium levels, modulating the membrane association of the classical but not the novel PKC isoforms. In *in vitro* binding assays, Lorenzo and co-workers [44] demonstrated, for example, that the relative affinities of phorbol 12,13-dibutyrate for PKC alpha versus a RasGRP1 construct depended markedly on the phospholipid composition. Similarly, we demonstrated dramatic differences in selectivity of PMA and bryostatin 1 for the PKC isoforms alpha and delta in mouse 3T3 cells [45] and in mouse keratinocytes [46].

Is it possible to achieve selectivity among subpathways of biological response with C1 domain ligands? The clear answer from the early analysis of the biological activities of phorbol derivatives was that selectivity was achievable. Whereas PMA was potent both as a tumor promoter in the mouse skin two-stage carcinogenesis model and as a skin irritant, some phorbol derivatives were solely irritant, leading Hecker to conclude that “the inflammatory and cocarcinogenic activities (*of phorbol esters*) were different and independent parameters” [47]. Similarly, Slaga and colleagues reported that mezerein, an irritant daphnane derivative, functioned as a promoter on mouse skin only if preceded by one or more applications of PMA and thus defined a sub-stage of tumor promotion [48], and Hennings et al. [49] described a marked difference in the efficiency of mezerein as compared to PMA for promotion of squamous cell carcinomas relative to papillomas. Most dramatic of all, we demonstrated that prostratin not only failed to induce hyperplasia, in contrast to PMA [50], but was a potent inhibitor of the induction by PMA of skin hyperplasia, ornithine decarboxylase, and edema [51, 52] as well as of tumor promotion [53]. Such results unambiguously establish that selectivity of responses to DAG analogs is attainable. Remaining questions, which will provide the basis for rational drug design, are the extent the selectivity, the ability to target desired subpathways selectively, and the mechanisms underlying selectivity.

Overall strategies for biological specificity

Choice of target

The most straightforward mechanism for achieving different biological responses to different C1 domain ligands is selectivity within the 7 families of effectors for C1 domain ligands and/or selectivity among members within a single family of effectors. Not only do the individual PKC isoforms show marked differences in biological functions, but a striking

observation is that the function of one PKC isoform may be antagonistic of another [54]. For example, in many systems PKC epsilon promotes cell proliferation, inhibits apoptosis, and is transforming [55]. Correspondingly, in mouse skin PKC epsilon overexpression enhances tumor formation following UV irradiation [56]. In contrast, PKC delta typically inhibits cell proliferation, induces cell differentiation, and promotes apoptosis [57, 58], although the specific effect depends on the cell system and the apoptotic stimulus [59]. Likewise, in the mouse skin two-stage carcinogenesis protocol, overexpression of PKC delta suppresses tumor promotion [60]. For the colon, the elegant studies of Fields and coworkers document that PKC beta II expression is associated with cancer formation [61] whereas PKC alpha acts as a tumor suppressor [62]. It therefore follows that a strategy complementary to the design of a selective inhibitor for one PKC isoform would be the design of a selective activator for an antagonistic isoform. The same concept is valid for different families of effectors for C1 domain ligands with antagonistic functions. DAG kinases, by converting DAG to phosphatidic acid, abrogate physiological responses through the DAG pathways, such as stimulation of PKC. Similarly, the chimaerins, which act to inhibit Rac, are predicted to function as tumor suppressors [14].

Of course, a complicating factor in the rational design of selective ligands is the complicated direct and indirect cross-talk among targets. Thus, among C1 domain DAG effectors, PKC phosphorylates PKD [13] and RasGRP, leading to their activation [63 – 66]. The role of the C1 domains of PKD and RasGRP is apparently to drive localization rather than activation *per se*. Nonetheless, this localization role may be critical for its biological function. Similarly, PKC phosphorylates some of the DAG kinase isoforms. Depending on the isoform, this phosphorylation may be activating or inhibitory [21]. A second level of cross-talk is more indirect, where a pathway induced by one target feeds upstream of itself or another target. For example, EGF receptor activation stimulates c-Src and PLC- γ , activating RasGRP3, which activates Rap2B and in turn PLC- ϵ . PLC- ϵ , through its formation of DAG, can maintain activation of RasGRP3 and PKC [67]. A third level of complication is the association of C1 domain family members. For example, DGK ξ associates with RasGRP1 [68] and PKC alpha [69], inhibiting their action. DGK α associates with RasGRP1 [70] and DGK ι associates with RasGRP3 [71], leading to inhibition in both cases. A practical consequence of these interlocking pathways is that the most realistic approach to drug design is semi-rational, whereby biological responses provide validation of the underlying design concepts.

Difference in kinetics of action

An important hypothesis emerging from the experience with natural products is that the lipophilicity of ligands may be a critical variable. Prominent among the differences in biological response of mouse skin to the non-promoting 12-deoxyphorbol esters as compared to the typical phorbol esters was that short chain substituted 12-deoxyphorbol esters induced transient inflammation whereas the typical phorbol esters induced inflammation of much longer duration [47]. Attention was focused on the nature of the side chains, since homologous 12-deoxyphorbol derivatives differing only in the substitution of a long aliphatic side chain for the short, branched chains on the non-promoting derivatives led to potent tumor promoters [72]. Likewise, PEP005 (ingenol 3-angelate), currently in clinical trials, is conspicuous for having only a short ester side chain, whereas once again derivatives such as ingenol 3-hexadecanoate are potent tumor promoters [73]. Although rates of penetration of phorbol esters through skin have not been studied in detail, it is clear in cellular systems that lipophilicity determines their rates of cellular penetration, as evidenced by direct measurements using a series of fluorescent phorbol ester derivatives of varying lipophilicities [74]. Lipophilicity has likewise been shown to influence the structure activity relations of phorbol esters for inducing cellular response [75].

Differences in cellular localization

Constructs fusing GFP (green fluorescent protein) to C1 domains and to intact PKC isoforms or other C1 domain containing effectors have provided a convenient tool to measure the kinetics of translocation in response to ligands as well as the subcellular location of that translocation [76, 77]. Not only may different PKC isoforms translocate to different cellular fractions but, for PKC delta, the pattern of localization depends on the specific ligand [78]. Of particular potential significance was the comparison between a pair of homologues, one of which was tumor promoting (12-deoxyphorbol 13-tetradecanoate) and the other an inhibitor of tumor promotion (12-deoxyphorbol 13-phenylacetate). The tumor promoting derivative caused PKC delta to translocate first to the plasma membrane, followed subsequently by redistribution to internal membranes and to the nuclear membrane. In contrast, the inhibitor of tumor promotion caused the immediate association with internal membranes and the nuclear membrane (Fig. (3)). The paradigmatic tumor promoter PMA induced a similar pattern of translocation as did the tumor promoting 12-deoxyphorbol ester. In subsequent studies, we showed that ligand hydrophobicity was a clear determinant of the pattern and kinetics of PKC delta translocation [79]. Because the localization of PKC will dictate those substrates to which it will have access, these different patterns of localization should fit with different patterns of response.

The above studies evaluated the kinetics of localization of individual, over-expressed GFP-tagged PKC isoforms. An elegant complementary approach was that of Gallegos et al. [80]. These authors developed a selective PKC substrate that generated a FRET signal upon phosphorylation and that they could direct to specific subcellular compartments by fusing to the appropriate targeting sequences. Using these localizable reporters of PKC activity, they found that PDBu stimulated total PKC activity at different cellular locations with different time courses. The plasma membrane showed the fastest response; the mitochondria showed the slowest response. Furthermore, the pattern of behavior depended on the specific activating stimulus. Thus, PKC activation in response to purinergic receptor stimulation by UTP, as compared to PKC activation by PDBu, showed marked differences in the kinetics as well as in the time dependent ratios of stimulation at the different membrane compartments.

Using fluorescent phorbol derivatives, it was possible to compare in the same cell the kinetics of uptake of the ligand and the kinetics and pattern of localization of the C1 domain containing effector [74]. More hydrophilic ligands equilibrated quickly within the cell; more hydrophobic ligands equilibrated slowly, over a time of up to several hours. For PKC alpha, localization was always to the plasma membrane, independent of whether ligand was predominantly in the plasma membrane or in internal membranes. The conclusion therefore is that factors other than ligand localization play a determinative role in the subcellular localization preference of PKC alpha. In contrast, PKC delta at all times showed colocalization with ligand. *A critical strategic concept which emerges is that design of C1 targeted ligands with unique patterns of localization should be able to generate unique patterns of response, selectively affecting a subset of C1 domain containing target proteins.* Although the GFP constructs permitted visualization of localization to subcellular compartments, it is also plausible that it may be possible to design ligands directed to subcompartments, such as lipid rafts within the plasma membrane. Once again, such localization should permit access to the restricted set of substrates present in that subcompartment.

Antagonism through inappropriate localization

Several conceptually promising strategies for the design of C1 domain directed ligands are in the early stages of development. Highly hydrophilic ligands afford the potential, at least

theoretically, to bind to the C1 domain but, by introducing a hydrophilic cap where the typical ligands complete a hydrophobic surface, prevent stabilization of the C1 domain at the membrane; they would thereby block the action of the C1 domain containing effector by preventing its proper localization in proximity to the requisite substrates. An early test of this approach was through the use of the unsubstituted parent alcohols of the phorbol and ingenol esters, viz. phorbol and ingenol themselves. Neither compound was very active. Ingenol had weak activity by itself as a ligand, binding with a K_i of 30 μM and inducing several responses typical of the phorbol esters at concentrations between 30 μM and 1 mM [81]. Phorbol indeed inhibited PKC, with an EC_{50} of 10 mM, but the inhibition appeared independent of its binding activity [82]. Conceptually, both ligands were flawed in that they lack the ester group of the typical ligands. Although the ester carbonyl is not identified as interacting with the C1 domain in the crystallographic structure of the binary ligand – C1 domain complex [83], it is clear that the ester carbonyl represents a pharmacophoric element, interacting with the phospholipid headgroups in the physiological ternary binding complex [84, 85]. A second approach was to use phorbol esters substituted with polar functional groups on their side chains. Bertolini et al. [86] described that derivatives functionalized with free carboxylic acid groups on both side chains inhibited PKC activity at very high (mM) concentrations. Less intensely polar ligands were still able to induce lipid association. Yamatsugu et al. [87] used phorbol derivatives substituted with a perfluorinated alkyl group and a polyether hydrophilic chain on a terephthaloyl aromatic ring in the C12 position ester. These compounds proved to have significant potency as PKC α inhibitors (approximately 200 nM) and to have specificity for PKC α relative to PKC δ . Ironically, however, the elements other than the polyether hydrophilic chain appeared to dominate the behavior of the compound, so that it partitioned well into the membrane. The authors concluded that the compounds may have a different mechanism of action from what they had intended; they suggested that the binding directs the ligand to the C1 domain, where the polyether chain, which is known to potently bind metals, extracts the zinc atoms from the C1 domain structure and thereby disrupts its structure. *This would thus be an example of a distinct general strategy of antagonism through specific tethering of an inactivating functionality.*

The approach underlying the design of hydrophilic ligands, to prevent translocation of PKC to the membranes, is a specialized example of a broader strategy, to maintain PKC in the wrong location within the cell, thereby preventing its access to the necessary substrates. Abundant examples are cited in the preceding section that localization to different membranes, which may achieve a similar result, can be attained by ligands upon variation of the degree of hydrophobicity of their side chains. Although those examples are directed at differences in localization among membranes, approaches in which ligands are directed to subdomains within the membranes, such as lipid rafts, or to signaling complexes bound through protein-protein associations, would fall within this same concept. A RasGRP selective agent might provide an example of this latter strategy. Since RasGRP requires phosphorylation to have exchange activity on Ras, a ligand which bound to RasGRP but not to PKC would be expected to translocate an inactive RasGRP to the membrane, bringing along its associated DAG kinase and thereby inhibiting DAG signaling.

Enhanced specificity with bidentate ligands

A long-standing concept in drug design is that combination of two interacting binding moieties in a single ligand should produce a ligand with a dissociation constant equal to the product of the dissociation constants of the independent binding moieties ($K_{\text{comb}} = K_a \times K_b$). Thus, two weakly binding ligands with μM affinity should generate a bidentate ligand with femtomolar affinity, leaving very strong affinity even if there were some interference between the two binding moieties [88]. In the case of the C1 domain containing DAG

effectors, only the PKCs and PKDs have twin C1 domains. The chimaerins, the RasGRP, Unc13, and MRCK family members possess only a single C1 domain and the DAG kinases have only a single C1 domain involved in binding. Furthermore, the spacing of the C1 domains varies among the PKC and PKD isoforms. In the classic PKCs (alpha, beta, gamma) 15 residues are interposed between the two C1 domains; in the novel PKCs (delta, epsilon, theta, and eta) the number increases to 22–23; and for the PKDs there are 67–76 residues between the two C1 domains. Finally, as discussed in additional detail below, the structure activity relations of the two C1 domains differ. The strong prediction therefore is that a dimeric ligand with a linker of appropriate length could be selective for the PKCs and PKDs and, within this group, be selective for only those few members with the corresponding spacing between C1 domains. Further selectivity could be engineered with a non-symmetric dimer optimized for the respective structure-activity requirements of the two C1 domains.

Several groups have designed bidentate ligands of variable linker length and assayed their activity on PKC [89]. For example, Sridhar et al. [90] designed a series of benzolactam dimers with linkers of 4–20 carbons. Spacers of 10–20 carbons gave similar, optimal results in the low nM range but without selectivity among PKC isoforms. Giorgione et al. [91] used a similar approach with dimeric phorbol esters. Careful controls were used to assess the potential engagement of both C1 domains contributing to enhanced binding. The activity on the isolated C1b domain of PKC beta II was compared with activity on the intact PKC beta II. Likewise, binding to intact PKC delta was compared with binding to PKC delta mutants with either the C1a or C1b domains mutated to be inactive. Again, potent ligands were obtained but only a quite modest (3–4 fold) enhancement of activity was found that might be attributed to the dimeric nature of the ligand. A plausible explanation for the modest success so far is that the linkers used function both as spacers and as hydrophobic anchors, penetrating into the lipid bilayer. The results so far suggest that these two roles are in conflict for the specific linkers used.

Specificity through controlled depth of penetration

The C1 domains are highly conserved in the structure of the ligand binding cleft. There is less conservation of residues on the exterior of the domain, influencing their interaction with the phospholipid bilayer (Fig. (4)). Reflecting this diversity, different C1 domains are able to insert to variable degrees into the bilayer, as revealed by the elegant studies of Cho and co-workers [92 – 94]. In initial studies to exploit these differences, Malolanarashimhan et al. [95] designed a series of ligands displaying oligo(p-phenyleneethynylene) acyl domains of variable lengths. Among other properties, such compounds if capped with a hydrophilic terminus could span the plasma membrane bilayer and present a DAG-lactone or other C1 domain binding moiety at variable depths or heights relative to the bilayer surface. Although these initial studies were directed simply at exploring the properties of this general class of structures, the compounds showed complex, variable patterns of biological activity, supporting this general strategy.

Altered pattern of interaction at the C1 domain

Based on the X-ray crystallographic determination of the structure of the complex between phorbol 13-acetate and the C1b domain of PKC delta [83], the interaction of other classes of ligands with the C1 domain has been assessed by molecular modeling. The modeling indicates that structurally distinct classes of ligands – phorbol esters, ingenol esters, daphnane esters, and indolactams – use a combination of identical and unique interactions to drive their binding [96]. In each case, the hydrophobic side chains project out from the C1 domain, where they can interact with the phospholipid, but their specific orientations differ. Bryostatins contrast with the other ligands. This compound, which induces rather different

biology, appears to insert into the binding cleft with a major portion of the structure overlaying the C1 domain [97]. It thus presents a complicated interface to the lipid bilayer, although it is not known to what degree this feature of the bryostatins is responsible for their unusual behavior.

The extensive studies analyzing DAG-lactones (Fig. (5)) as ligands for C1 domains will be discussed in detail below. However, it should be noted here that modeling has suggested that both DAGs and DAG-lactones can insert into the binding cleft of the C1 domain in either of two orientations, termed *sn*-1 and *sn*-2, in which the binding interactions with the C1 domain are with the C3 primary hydroxyl group and either the *sn*-1 or *sn*-2 carbonyl respectively [85, 98, 99](Fig. (6)). The preference between orientations is driven in part by the nature of the two side chains, where preference is given to the orientation which directs the more hydrophobic side chain away from the C1 domain and into the lipid bilayer. A further element is the nature of the template, with the *sn*-1 orientation is preferred for the DAGs and the *sn*-2 orientation is preferred for the DAG-lactones.

Factors influencing the function of the C1 domains

Although it would be convenient to analyze the behavior of the C1 domain itself and to transfer that knowledge to predict the behavior of the protein in which the C1 domain lies, it is clear that the context in which the C1 domain is found has important effects on its behavior. A straightforward example is provided by the extensive studies of Irie and coworkers [100]. Their approach has been to chemically synthesize the various C1 domains for characterization of their structure activity relations. An observation is that the inclusion of additional charged residues C-terminal to the C1 domain is able to enhance apparent ligand binding affinity by up to a factor of 7.5. The probable mechanism, of course, is that binding is typically assayed not for the binary complex of ligand and C1 domain but rather for the ternary complex of C1 domain, ligand, and anionic lipid. Enhanced positive charge on the C1 domain outside of the region needed for insertion into the bilayer may increase association with the anionic lipids, contributing to the apparent affinity of the ternary complex.

A second example is provided by comparison of affinities measured for the individual C1 domains and those measured for the intact proteins. As will be discussed below, the DAG-lactone 130C037 was found to have high affinity for the C1b domain of PKC delta but to have very low affinity for the C1a domain of PKC delta or either C1 domain of PKC alpha [101]. In contrast, there was only a modest (4-fold) difference in the affinities of these two intact PKC isoforms for the ligand.

Elegant mechanistic insight into the divergence between the behavior of the C1 domain and the intact protein has been provided by Canagarajah et al. [102]. These authors determined the crystallographic structure of β 2-chimaerin. This protein has a C1 domain which binds phorbol 12,13-dibutyrate with high affinity, whereas the intact protein only translocates in intact cells at much higher concentrations of phorbol ester. The X-ray structure revealed that the binding cleft of the C1 domain is buried by contacts with the N-terminus, the SH2 domain, the RacGAP domain and the linker between the SH2 and C1 domains. Mutation of residues contributing to these contacts sensitized the protein to translocation by phorbol ester. The implication is that physiological activation of β 2-chimaerin is cooperative, with DAG acting as a co-stimulus along with some other factor (phosphotyrosine interacting with the SH domain?) which promotes the unfolding.

Indirect evidence with PKC supports a similar situation. Oancea and Meyer [76] showed that the fragment of PKC gamma containing the twin C1 domains showed immediate translocation to the plasma membrane in response to phorbol ester. In contrast, the intact

enzyme showed a delay in response, suggesting that the enzyme first needed to undergo a conformational change, rendering the C1 domains accessible. Such a model is consistent with the prediction that the hydrophobic face of the C1 domains would not be expected to be exposed to solvent in the unstimulated enzyme but rather would interact with some complementary surface on the enzyme. Consistent with this model, Stahelin et al. [103] present evidence that the C1a domain and the C2 domains of PKC alpha interact through complementary charges on the two domains. Disruption of these interactions enhances membrane affinity and the kinetics of translocation.

The above examples are of a C1 domain which is responsive to ligand, where the activity is to some degree masked by other elements in the protein. Our initial understanding of MRCK implies a different pattern of behavior. MRCK was reported to respond to high levels of phorbol ester [104]. We have shown that the isolated C1 domains have an affinity for phorbol ester which is appreciably (50-fold) less than that to the C1b domain of PKC delta but approximates that of the C1b domain of PKC alpha [105]. This finding strongly suggests that other elements in the MRCK will be required, in combination with the C1 domain, in order to drive responsiveness to phorbol ester or, under physiological conditions, to DAG.

DAG-lactones as ligands for C1 domains

The phorbol esters, indolactams, polyacetates, macrocyclic lactones, and iridals are potent ligands for C1 domains, fixing on rigid templates the appropriate hydrophilic and hydrophobic groups in the proper three dimensional configuration for interaction with the C1 domains. Unfortunately, the complicated chemistry of these natural products has limited their utility for the detailed exploration of structure activity relations. Phorbol, for example, has 8 asymmetric centers in its structure and the bryostatins have 11 asymmetric centers. The endogenous ligand for C1 domains, *sn*-1,2-diacylglycerol, in contrast, has a much simplified structure with only a single asymmetric center, greatly facilitating chemistry. On the other hand, DAG displays rather weak affinity, reflecting in part the flexibility of the glycerol backbone, with the consequence that the immobilization of the glycerol upon binding is entropically unfavorable.

An approach adopted by the groups of Marquez and Blumberg was to explore whether DAG could afford a suitable template if the DAG were cyclized, rigidifying the structure so as to eliminate this entropic penalty. Among the various potential routes for cyclization, the DAG-lactone structure proved to be successful [106]. Further enhancement of activity, approaching that of the phorbol esters in *in vitro* binding assays, was attained through appropriate manipulation of the hydrophobic side chains [107], and an efficient stereospecific synthesis of the single chiral center was developed [108]. Using the DAG-lactone template, extensive exploration of ligand structure activity relations has been possible, as reviewed elsewhere [98]. A powerful strategy has been the combination of the purposeful structural modification of ligands together with their evaluation on C1 domains mutated to address postulated mechanisms of interaction, complemented by computer modeling to yield insight into the energetics of the various combinations.

An illustration of this approach is provided by the dioxolanones [109]. The question was whether it would be possible to introduce additional interacting groups into the DAG-lactone structure. The dioxolanones represent derivatives in which the bridging methylene group of the DAG-lactone structure has been replaced with oxygen. The dioxolanones indeed bound to the C1b domain of PKC delta with high affinity, albeit without enhanced affinity compared to comparably substituted DAG-lactones. Modeling revealed that this oxygen group could form a new hydrogen bond with the conserved glutamine at position 27 in the C1b domain of PKC delta. Consistent with this interaction indeed being critical for the interaction of the dioxolanones, a mutated C1b domain in which this glutamine residue was

mutated to glutamate showed a modest decrease in binding affinity for phorbol ester but a profound loss of binding for the dioxolanone. Why was the binding affinity not enhanced if an additional point of interaction had been introduced? The modeling again provided insight. In order to exploit this new point of interaction, the dioxolanone had to shift its orientation for binding from the *sn*-2 orientation preferred by DAG-lactones to the disfavored *sn*-1 orientation, essentially canceling the potential gain in energy.

Complementing the design of specific ligands based on modeling insights, combinatorial chemistry is providing a powerful tool to efficiently probe the impact of variation in the hydrophobic domains on the biological effects of ligands [101, 110]. As discussed above, we already know that this structural aspect has been responsible for much of the diversity in biological outcome observed with the potent natural product ligands for C1 domains. From an initial library of 96 elements, we identified compound 130C037 as a highly selective ligand. It bound to the isolated C1b domain of PKC delta with 1.8 nM affinity, whereas its affinity for either the C1a domain of PKC delta or for either of the C1 domains of PKC alpha was of the order of μ M or weaker. In contrast, the affinity of PDBu for these four C1 domains was similar within a factor of 10. For intact proteins, 130C037 bound to the Ras activators RasGRP1 and RasGRP3 with high affinity (approximately 4 nM). It bound with 8-fold weaker affinity to PKC epsilon and with 80-fold weaker affinity to PKC alpha. Similar selectivity was observed in intact cells for membrane association or translocation, as well as for activation of ERK phosphorylation downstream of Ras. In ongoing studies on the initial two libraries, a clear finding was that library members showed a different pattern of selectivity in each of a variety of biological assays [111]. Although the actual mechanisms responsible for the wide diversity in specificities of response has not been demonstrated for these individual biological endpoints, the plausible model is that of differential localization, whether to different membranes within the cell or to different microdomains within the same cellular membrane, directed by the hydrophobic “chemical zip codes” on the library molecules. The power of the combinatorial approach is that it can efficiently generate a wide variety of structures to probe the multiplicity of interactions in the DAG signaling network to yield an integrated selective response.

Indolactams and benzolactams as C1 domain ligands

The second class of structures which have proven amenable to extensive manipulation are those of the indolactams and a modified template, the benzolactams (see [112 and 113] for reviews). Some of these compounds have again shown appreciable selectivity. On individual C1 domains, 5-prenyl-indolactam-V showed marked selectivity for the C1b domains of the novel PKCs compared to either of the C1 domains of the classical PKCs or the C1 domains of the chimaerins, RasGRPs, or Unc13 [100]. 8-octyl-benzolactam-V9 showed selectivity for the translocation of the novel PKCs compared to the classical PKCs [114]. Compounds of this class, like other PKC activators, have attracted interest as potential therapeutic agents in Alzheimer’s disease because of their ability to enhance alpha-secretase activity subsequent to PKC stimulation, leading to reduced levels of the amyloid precursor protein [115].

Summary

The consistent picture which emerges for the C1 domains as regulatory motifs is that they represent individual elements driving response in combination with other regulatory elements within the target protein. Thus, for the classical PKC isoforms, the active conformation at the membrane is stabilized by ionic interactions of the pseudosubstrate with the membrane, by stabilization of the C1 domains with the membrane-partitioned ligand, by further interactions of the C1 domain with the phospholipids, and by interactions of the C2 domain with the phospholipid headgroups via calcium bridges. Further contributions are

made by the state of phosphorylation of the PKC and, presumably, by the presence of binding proteins such as RACKS [116]. The multiplicity of elements being integrated into the response gives the cell the opportunity to control subsets of the 7 families and multiple family members with C1 domains.

A superimposed level of regulation is provided by multiple C1 domain containing proteins sequentially positioned on the same pathway. PKC phosphorylates and activates PKD, RasGRP, and some DAG kinases. Multiple points of activation in the same pathway predict positive cooperativity of response, generating a steep dose response curve. Conversely, DAG kinases abrogate the responses of all the C1 domain containing proteins.

A third level of regulation is provided by C1 domain containing proteins lying within pathways which feed back upon themselves. A multiplicity of receptors coupled to phospholipase C β and phospholipase C γ generate DAG to activate PKC, which in turn sensitizes directly or indirectly a multiplicity of receptors coupled to phospholipase C β and phospholipase C γ , yielding positive feedback loops.

At the functional level, the increased hydrophobicity of the liganded C1 domain plays two, non-exclusive roles. In the case of the PKCs, the C1 domains both promote association with membranes and, together with the membrane, stabilize the activated conformation of the enzyme. In the case of PKD or RasGRP, the C1 domains are involved in localization of the protein, whereas the activation is a separate process driven by phosphorylation; this latter step is also C1 domain dependent, but driven by the C1 domains on PKC. Finally, one might distinguish between the role of the C1 domain to localize a functional activity of the protein itself, as exemplified by the guanine nucleotide exchange functionality of RasGRP, and the role of the C1 domain containing protein as an adapter, where it localizes an associated functional protein, for which RasGRP and DAG kinase provide an example.

A third potential role, which has received relatively little attention, is the involvement of C1 domains in protein-protein interactions, and the possible modulation of these interactions by ligand binding [6]. For example, Wang and Kazanietz [117] have described interaction between the C1 domain of chimaerin and Tmp21-I, and Yasuda et al. [118] have described interaction of the chimaerin C1 domain with DAG kinase gamma.

The initial view, driven by homology among C1 domains, was that characterization of a paradigmatic C1 domain would reveal the details of behavior of C1 domains generally. It has become increasingly apparent that this is an oversimplification, and that the specific behavior of each C1 domain in its receptor will require analysis.

The very good news is that these interlocking pathways of regulation and the multiple elements contributing to ligand response liberate C1 domain targeted therapeutics from the constraints otherwise imposed by a relatively conserved binding cleft. The target is not the binding cleft but rather the C1 domain in the complicated context of the rest of the C1 domain, of the specific receptor, and of the cellular environment in which that receptor is present. The corollary of this complexity, on the other hand, is that, as for much of biology, the complexity of the systems means that drug development for C1 targeted ligands will tend to be semi-rational rather than rational.

Again, the very good news is that the multiplicity of therapeutic targets potentially addressed by C1 domain directed agents provides a wealth of opportunities for matching the right pharmacological solution to the right biological problem. The not so good news is that this approach is orthogonal to the usual organizational structure in the pharmaceutical industry, which is organized around specific therapeutic conditions which drive drug development, not around specific pathways impacting multiple conditions. It is thus not

surprising that academic and government efforts have been in the forefront of identifying structural leads among C1 ligands. The current state of the field is very exciting.

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Abbreviations

DAG	<i>sn</i> -1,2-diacylglycerol
PKC	protein kinase C
PKD	protein kinase D
MRCK	myotonic dystrophy kinase-related cdc42 binding kinase
PMA	phorbol 12-myristate 13-acetate
PDBu	phorbol 12,13-dibutyrate
GFP	green fluorescent protein

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Figure 1. Structure of the complex between phorbol 13-acetate and the C1b domain of PKC delta. A) Interaction of phorbol 12,13-dibutyrate within the binding cleft of the C1b domain of PKC delta. B) Ternary complex between the C1b domain, phorbol ester, and the lipid bilayer (from [83]).

Figure 2.

Structure of the C1 domain containing proteins. PS, pseudosubstrate domain; TD, transmembrane domain; PH, plekstrin homology domain; SH2, Src homology 2 domain; Rac-GAP, Rac GTPase-activating protein domain; REM, Ras exchange motif; GEF, guanine nucleotide exchange factor; CC, coiled-coil domain; CH, citron homology domain; PBD, p21 GTPase binding domain

Figure 3.

Different ligands cause differential membrane translocation of PKC delta in living Chinese hamster ovary cells. Cells expressing GFP-PKC delta were imaged by confocal microscopy after treatment with 1 μ M ligand. 12-deoxyphorbol 13-tetradecanoate is a tumor promoting phorbol derivative whereas its more hydrophilic analog, 12-deoxyphorbol 13-phenylacetate, is an inhibitor of tumor promotion (from [78]).

Figure 4.

Diversity of surfaces among C1 domains. The C1 domains are all shown in the same relative orientation, with the binding cleft at the top front. The normalized consensus hydrophobicity scale is from [119].

Figure 5. Comparison of the structures of the phorbol esters, bryostatins, DAG, and DAG-lactone. Chiral centers are shown in violet. The DAG-lactone represents a conformationally constrained DAG analog (adapted from [107]).

Figure 6.
Alternative orientations of DAG-lactones in the binding cleft of the C1 domain (from [98]).