0026-895X/94/061207-08$03.00/0

MOLECULAR PHARMACOLOGY, 45:1207–1214, 1994.

Differential Inhibition of Protein Kinase C Isozymes by UCN-01, a Staurosporine Analogue

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Received November 24, 1993; Accepted March 10, 1994

SUMMARY

UCN-01 (7-hydroxystaurosporine) has been demonstrated to be a potent inhibitor of tumor cell growth both in cell culture and with *in vivo* xenograft models. The ability of UCN-01 to inhibit the kinase activity of recombinant protein kinase C (PKC) isozymes α, β, γ, δ, ϵ, and ζ was characterized using an *in vitro* kinase assay. Two distinct groups of isozymes could be defined on the basis of relative potency of kinase inhibition. UCN-01 was 15–20-fold more potent for inhibition of the Ca^{2+}-dependent isozymes, compared with the Ca^{2+}-independent isozymes. In contrast, UCN-02 (the diastereomer of UCN-01) and staurosporine exhibited less ability to discriminate between Ca^{2+}-dependent and -independent isozymes. PKC-ζ was not inhibited by UCN-01, UCN-02, or staurosporine. IC_{50} values for UCN-01 inhibition of the Ca^{2+}-dependent PKC-α, -β, and -γ were 29, 34, and 30 nM, respectively, and for the Ca^{2+}-independent PKC-δ and -ϵ were 530 and 590 nM, respectively. IC_{50} values for staurosporine inhibition of the isozymes α, β, and γ were 58, 65, and 49 nM, respectively, and for the isozymes δ and ϵ were 325 and 160 nM, respectively. UCN-02 was significantly less potent for the inhibition of PKC-α, -β, -γ, -δ, and -ϵ (IC_{50} values of 530, 700, 385, 2800, and 1200 nM, respectively). An analysis of the inhibition by UCN-01 and staurosporine of the kinase activity of PKC-α and -δ indicated mixed inhibition kinetics. Increasing the ATP concentration resulted in decreased potency, as shown by increased IC_{50} values. In contrast, increasing the peptide substrate concentration resulted in increased potency, as shown by decreased IC_{50} values. Increasing concentrations of myelin basic protein as a PKC-α or -δ substrate also caused increased potency of inhibition by UCN-01. Because of the competitive nature of inhibition with respect to ATP and the uncompetitive nature with respect to substrate, the concentrations of these substrates can have dramatically different effects on the degree of inhibition observed. These data also suggest that UCN-01 may be an important tool for the dissection of PKC isozyme contributions to signal transduction pathways.

A central role for PKC has been demonstrated in signal transduction and in the process of tumor promotion and development (1–4). More recently this has been further defined in molecular terms as a role for PKC in the activation of the mitogen-activated protein kinase cascade, linking extracellular mitogenic stimuli, through PKC, with increased cell growth (5, 6). UCN-01 was developed as an inhibitor of PKC (7) and has been demonstrated to inhibit tumor cell growth *in vitro* and *in vivo* (8, 9). However, the exact isozymes that are inhibited by UCN-01 have not been defined, and the nature of inhibition produced by UCN-01 is unknown.

A number of different molecular forms of PKC have been identified, allowing the PKC family to be divided into subgroups comprising phospholipid-dependent species that are activated by phorbol esters and diacylglycerol and are either Ca^{2+} dependent (PKC-α, -β_I, -β_{II}, and -γ) or Ca^{2+} independent (PKC-δ, -ϵ, -η, and -θ), as well as the atypical PKC isozymes (PKC-ζ and -λ) that lack a portion of the C1 region from the regulatory domain and are independent of Ca^{2+}, phorbol ester, and diacylglycerol activation (10–13). Specific roles are being defined for individual PKC isozymes in diverse biological responses. PKC-α and -β have been demonstrated to be important for neural development in *Xenopus* embryos (14), PKC-α and -δ mediate phorbol ester-induced differentiation of myeloid cells (15), and differential stimulation of PKC-α and -β_{II} strongly influences the pathway of erythroleukemia cell proliferation and differentiation (16). Specific induction of PKC isozyme expression has been found in response to oncogene-induced transformation of rat fibroblasts (17), and multiple drug resistance in breast cancer cell lines has been associated with increased expression of PKC-α and increased phosphorylation of P-glycoprotein (18). The development and characterization of inhibitors selective for the different PKC isozymes would be extremely useful for the pharmacological study of signal transduction pathways and for potential therapeutic use.

Staurosporine is the most potent inhibitor of PKC described

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ABBREVIATIONS: PKC, protein kinase C; MBP, myelin basic protein.

to date and provides a structural basis for the development of more selective inhibitors that are capable of discriminating between different protein kinases. Characterization of inhibitors that exhibit PKC isozyme selectivity is an important goal. Recent studies with a different class of staurosporine analogues, the bisindolylmaleimides, have shown that some degree of selectivity for inhibition of PKC isozymes is possible (19–21). UCN-01 (7-hydroxystaurosporine) has potency similar to that of staurosporine for the inhibition of PKC but appears to be relatively more specific for PKC than for other kinases (8). In contrast to staurosporine, UCN-01 shows promise as a therapeutic agent because *in vivo* antitumor activity has been observed (8, 9).

In this study, we have defined the ability of UCN-01 to inhibit individual recombinant isozymes of PKC-α, -β, -γ, -δ, -ϵ, and -ζ. UCN-02 (the diastereomer of UCN-01) and staurosporine were included for comparison. Our results demonstrate that UCN-01 displays a markedly better capacity to discriminate between PKC isozymes than does UCN-02 or staurosporine. These results will allow better definition of PKC isozyme function in studies using UCN-01 and will aid in the clarification of the antiproliferative mechanism of UCN-01.

Experimental Procedures

Materials. UCN-01 (NSC 638850) and UCN-02 (NSC 642739) were supplied by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan) and staurosporine was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Phosphatidylserine, phosphatidylcholine, MBP, and benzamidine were obtained from Sigma Chemical Co. (St. Louis, MO). PKC-α pseudosubstrate peptide [α-peptide, [Ser^{25}]PKC-α(19–31)] was purchased from GIBCO (Gaithersburg, MD). Recombinant PKC isozymes were expressed in a baculovirus expression system, as described previously, from full length cDNA clones for bovine PKC-α and mouse PKC-β_I, -γ, -δ, -ϵ, and -ζ (22). Purification of the individual isozymes was done as described previously (22).

PKC activity assay. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the α-peptide substrate or MBP, as described previously (23). Phospholipid vesicles (20% phosphatidylserine/80% phosphatidylcholine) were prepared by sonication (24), and phorbol-12-myristate-13-acetate was used as a PKC activator at a final concentration of 1 μM. Incubation was at 30° for 8 min. The kinase assay was linear with time over this period and with amount of protein over the concentrations of PKC isozymes used in these assays. Unless otherwise stated, the α-peptide concentration was 10 μM and the ATP concentration was 25 μM. Protein concentration was determined using a Bio-Rad protein assay kit, with bovine serum albumin as the standard.

Calculation of kinetic parameters. Initial estimates of K_m, V_{max}, and K_i were determined from double-reciprocal plots and secondary plots (25). These estimates were then used to directly fit the rate data to equations derived for competitive, noncompetitive, uncompetitive, or mixed inhibition (25) using the NonlinearFit module of the Mathematica software system, version 2.1 (26), running on a Convex C3830 computer.

Results

UCN-01 showed better discrimination between the Ca^{2+}-dependent (PKC-α, -β, and -γ) and Ca^{2+}-independent (PKC-δ and -ϵ) isozymes than did staurosporine or UCN-02 for the inhibition of kinase activity (Fig. 1; Table 1). These data showed a 15–20-fold difference in relative potency of UCN-01

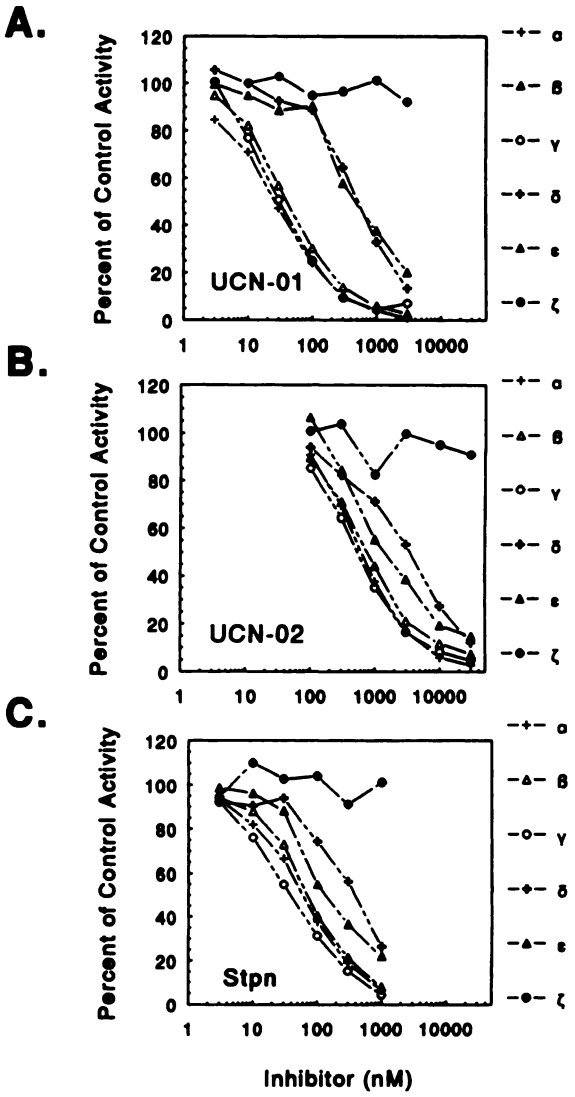

Fig. 1. Inhibition of PKC isozymes α, β, γ, δ, ϵ, and ζ. A, UCN-01; B, UCN-02; C, staurosporine. Substrate concentrations were 10 μM α-peptide and 25 μM ATP. Data are typical of results obtained in two or three experiments.

between the two groups. In contrast, staurosporine and UCN-02 showed only a 2–6-fold difference in relative potency for the inhibition of Ca^{2+}-dependent and -independent isozymes. In absolute terms, UCN-02 was approximately 10-fold less potent than either UCN-01 or staurosporine (Table 1). PKC-ζ was not inhibited by UCN-01, UCN-02, or staurosporine at any of the concentrations employed (up to 30 μM). This result indicates clearly that PKC-ζ kinase activity may be separated from that of the other isozymes tested in these experiments by these drugs.

The staurosporine analogue K252a has been suggested to inhibit protein kinases in a manner that is competitive with respect to ATP (27). To determine whether UCN-01 had a similar mechanism of action, we performed the kinase assays in the presence of either varied ATP concentrations or varied peptide [[Ser^{25}]PKC-α(19–31)] substrate concentrations, while the concentration of the other substrate was held constant. For these experiments, PKC-α and PKC-δ were selected as representatives of the PKC isozyme subgroups that were differen-

TABLE 1

IC_{50} values for the inhibition of PKC isozymes by UCN-01, UCN-02, and staurosporine

Values in parentheses show the relative potency differences, expressed as PKC-x IC_{50}/PKC-α IC_{50} for each of the inhibitors. Experiments were done with 10 μM α-peptide and 25 μM ATP. Data are expressed as mean ± standard error unless noted otherwise.

	IC_{50}					
	α	β	γ	δ	ϵ	ζ
	nM					
UCN-01	29 ± 5	34 ± 3 (1)	30 (1)[a]	590 ± 90 (21)	530 ± 38 (19)	NI[b]
UCN-02	530 ± 80	700 ± 50 (1)	390 ± 90 (1)	2830 ± 620 (5)	1230 ± 90 (2)	NI
Staurosporine	58[a]	65 (1)[a]	49 (1)[a]	330 (6)[a]	160 (3)[a]	NI

[a] Data are from two experiments done in duplicate.
[b] NI, isozyme was not inhibited over the concentration range of inhibitor employed in the study.

tially inhibited by UCN-01. Increasing the peptide substrate concentration from 0.5 to 10 μM in the presence of inhibitor resulted in an increased relative potency for UCN-01, UCN-02, and staurosporine. A typical result is shown in Fig. 2A for the inhibition of PKC-α by UCN-01 in the presence of varied α-peptide substrate concentrations. In contrast, when the concentration of ATP was increased from 25 to 500 μM, a decreased relative potency was observed, with a shift to the right in the percentage inhibition curve of PKC-α kinase activity, suggesting competitive inhibition (Fig. 2B). Lineweaver-Burk plots (Fig. 2, *insets*) indicated the uncompetitive and mixed nature of inhibition with respect to α-peptide and ATP, respectively. These results were also obtained for inhibition of PKC-δ by UCN-01. Consistent with these results for UCN-01, both staurosporine and UCN-02 exhibited the mixed-competitive type of inhibition with respect to ATP and uncompetitive inhibition with respect to peptide substrate when tested against either PKC-α or PKC-δ (data not shown).

The increased potency of inhibition observed with increasing concentrations of the α-peptide substrate prompted us to consider whether this effect could be achieved with a physiological substrate. MBP, a protein important for central nervous system myelination (28), is a substrate for PKC (29–31). The data shown in Fig. 3 indicate an uncompetitive inhibition for both PKC-α and -δ, with increased potency as the concentration of the physiological substrate MBP is increased. Table 2 shows the IC_{50} values for UCN-01, UCN-02, and staurosporine for inhibition of PKC-α or PKC-δ with MBP as a substrate. The relative sensitivity to inhibition produced by these agents with MBP as a substrate mirrors the data obtained with the α-peptide substrate (Table 1).

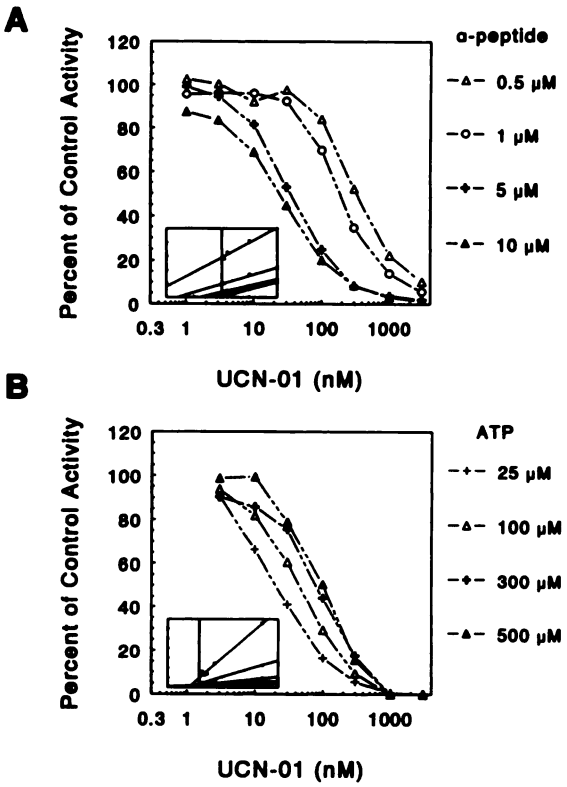

Fig. 2. Concentration dependence of UCN-01 inhibition of PKC-α. A, Effect of α-peptide substrate concentrations of 0.5, 1, 5, and 10 μM, with 25 μM ATP. B, Effect of ATP concentrations of 25, 100, 300, and 500 μM, with 10 μM α-peptide. *Insets*, double-reciprocal plots of the data. Data are typical of results obtained in two or three experiments.

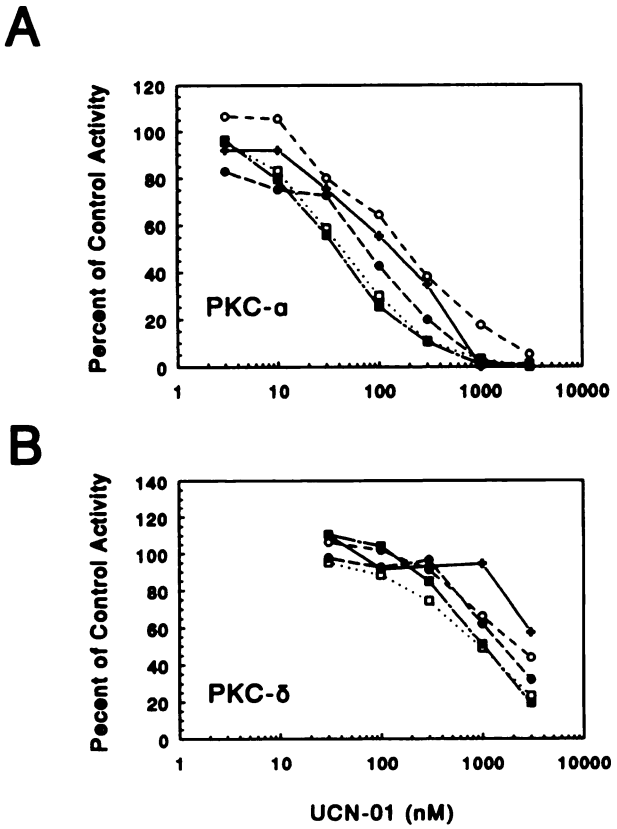

Fig. 3. Concentration dependence of UCN-01 inhibition of PKC with MBP as substrate. A, Effect of MBP substrate concentration on PKC-α inhibition; B, effect of MBP substrate concentration on PKC-δ inhibition, with 0.01 (+), 0.03 (○), 0.1 (●), 0.3 (□), or 1 (■) mg/ml MBP and 25 μM ATP. Data are typical of results obtained in two experiments.

TABLE 2

IC_{50} values for the inhibition of PKC isozymes by UCN-01, UCN-02, and staurosporine, with MBP as substrate

Experiments were done with 1 mg/ml MBP and 25 μM ATP. Data are from two experiments done in duplicate.

	IC_{50}	
	α	δ
	μM	
UCN-01	33, 40	710, 1000
UCN-02	670, 950	4900, 6000
Staurosporine	53, 73	660, 680

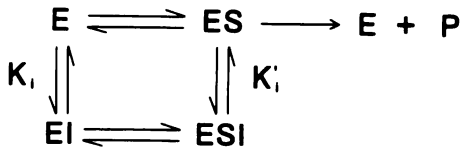

Fig. 4. Model for the inhibition of PKC by staurosporine and analogues, allowing the inhibitor to bind either to free enzyme or to enzyme-substrate complex, as defined by the inhibition constants K_i and K_i'. *E*, enzyme; *S*, substrate; *I*, inhibitor; *P*, product.

The results shown in Figs. 2 and 3 suggest that the inhibitor has increased affinity for PKC when peptide substrate is bound but has less capability to bind the enzyme in the presence of ATP. Based on these observations, a model can be proposed (illustrated by the schematic diagram in Fig. 4) in which the inhibitor can bind either to the enzyme free of that particular substrate or to the enzyme-substrate complex. This model as proposed is simplified from the reality of a two-substrate/two-product system, to reduce the complexity and to facilitate analysis of the system. Under conditions where one substrate concentration is varied while the other is held constant, we can then determine the relative K_i and K_i' values describing the formation of enzyme-inhibitor complex or enzyme-substrate-inhibitor complex with respect to either ATP or peptide substrate.

Initial analysis, by double-reciprocal plots, of rate data generated over a range of either peptide or ATP concentrations and inhibitor concentrations suggested uncompetitive inhibition with respect to peptide and mixed inhibition with respect to ATP. The final fit to the model was obtained by simultaneously fitting the complete data set for each experiment over all the inhibitor and substrate concentration ranges. An equation describing the model illustrated in Fig. 4, derived under equilibrium conditions (25), is

$$v = V_{max}/[(K_m/s) \cdot (1 + i/K_i) + (1 + i/K_i')] \quad (1)$$

where s and i are the substrate and inhibitor concentrations, respectively, gave the best data fit. This model resulted in a smaller error sum of squares than the next best model, which was competitive inhibition with respect to ATP and uncompetitive inhibition with respect to α-peptide substrate. The derived constants are shown in Table 3. It is apparent that the inhibitor has greater affinity for the enzyme-α-peptide complex ($K'_{apep} < K_i$). With respect to ATP, the inhibitor shows greater preference for the form of the enzyme without complexed ATP, either free enzyme or enzyme-α-peptide complex ($K_i < K'_{ATP}$). Representative fitted data curves for PKC-α are shown in Fig. 5, using the model described by eq. 1. Fig. 5, A-C, shows the data and curve fits at fixed ATP concentration over a range of α-peptide substrate concentrations with the inhibitors UCN-01, UCN-02, and staurosporine. Fig. 5, D-F, shows the data and curve fits for inhibition of PKC-α by UCN-01, UCN-02, and staurosporine as a function of ATP concentration at fixed α-peptide concentration.

TABLE 3

Derived parameters V_{max}, K_m, K_i, and K_i' for the PKC isozymes α and δ

Parameters are the averaged values from the indicated number of experiments.

Isozyme	No. of experiments	Inhibitor	Peptide substrate concentration dependence[a]			
			V_{max}	K_m	K_{iapep}	K'_{iapep}
			pmol/min	μM	nM	nM
PKC-α	2	UCN-01	81	6.1	352	26
	2	UCN-02	84	6.3	13,400	410
	2	Staurosporine	85	6.6	515	46
PKC-δ	2	UCN-01	41	3.1	4,950	580
	2	UCN-02	41	2.9	121,500	3,400
	2	Staurosporine	41	3.0	2,250	305
Isozyme	No. of experiments	Inhibitor	ATP concentration dependence[b]			
			V_{max}	K_m	K_{iATP}	K'_{iATP}
			pmol/min	μM	nM	nM
PKC-α	5	UCN-01	54	26.0	11	260
	1	UCN-02	37	15	610	700
	5	Staurosporine	47	23	36	230
PKC-δ	4	UCN-01	38	10	146	3,210
	1	UCN-02	18	6.9	125	2,210
	3	Staurosporine	43	13	113	14,500

[a] 25 μM ATP.
[b] 10 μM α-peptide.

This simplified mixed-inhibition model predicts that as s goes to 0 the IC_{50} for inhibition of enzyme activity tends toward K_i and as s goes to ∞ the IC_{50} tends toward K_i'. Therefore, for $K_i > K_i'$, as s goes to ∞ we should observe a shift to the left for the percentage inhibition curves. Conversely, for $K_i' > K_i$, as s goes to ∞ the percentage inhibition curves move to the right. The former behavior was observed in the experimental data shown in Fig. 2A for increasing peptide substrate concentration; the latter behavior was observed for increasing ATP concentration (Fig. 2B).

When a reaction proceeds with two substrates, an inhibitor can bind either free enzyme or enzyme complexed with one or two substrates. Assuming that the inhibitor has preference for a particular enzyme state, under equilibrium conditions the potency of inhibition can be predicted to depend on the order of enzyme binding to ATP and peptide substrate. Assuming an order of reaction of

$$E + A \rightleftarrows E \cdot A + B \rightleftarrows E \cdot A \cdot B \rightarrow E + P + Q \quad (2)$$

the equation derived is

$$v = (V_{max}/(1 + K_b/b + K_a \cdot K_b/a \cdot b + i/K_{ib} + i \cdot K_b/b \cdot K_{ia} + i \cdot K_a \cdot K_b/K_i \cdot a \cdot b) \quad (3)$$

where K_a and K_b are the equilibrium constants for a and b binding to E and EA, respectively, a is the concentration of substrate binding first, b is the concentration of substrate binding second, i is the inhibitor concentration, K_i is the inhibition constant for inhibitor binding to E, K_{ia} is the inhibition constant for inhibitor binding to EA, and K_{ib} is the inhibition constant for inhibitor binding to EAB. From this model, using the substrate concentrations employed in Fig. 2 and the derived parameter values shown in Table 3 for PKC-

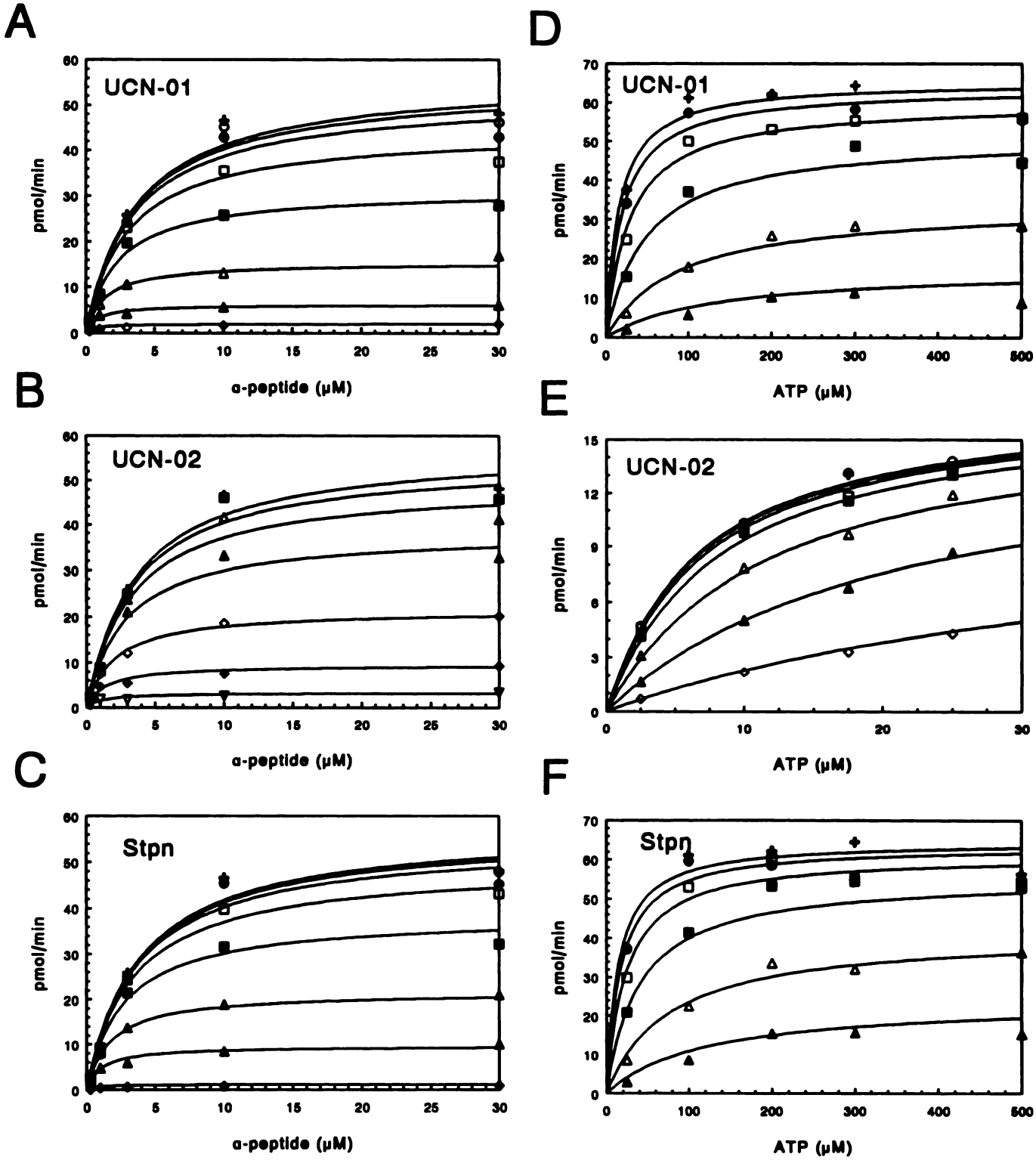

Fig. 5. Fit of derived curves to the PKC-α rate data. Data are shown with varied α-peptide substrate concentrations and the ATP concentration (25 μM) held constant (A, UCN-01; B, UCN-02; C, staurosporine) or with varied ATP concentrations and the α-peptide substrate concentration (10 μM) held constant (D, UCN-01; E, UCN-02; F, staurosporine). Inhibitor concentrations are 0 (+), 1 (○), 3 (●), 10 (□), 30 (■), 100 (△), 300 (▲), 1000 (◇), 3000 (◆), and 10,000 (▽) nM. Data are typical of results obtained in two to five experiments.

α, we determined whether our data could allow conclusions about the order of substrate binding. Simulation of a two-substrate reaction with inhibition by UCN-01 can be done in two ways. Assuming that ATP binds to the enzyme first, values from Table 3 for PKC-α were applied as follows (units are μM): K_a, 26; K_b, 6.1; K_i, 0.011; K_{ia}, 0.306; K_{ib}, 0.026 [in this simulation, K_{iapep} and K'_{ATP} (Table 3) describe the same step and the average value for K_{ia} was used]; this produced the results shown in Fig. 6, A and B. If ATP is considered to bind the enzyme first, then the model predicts little difference in the apparent

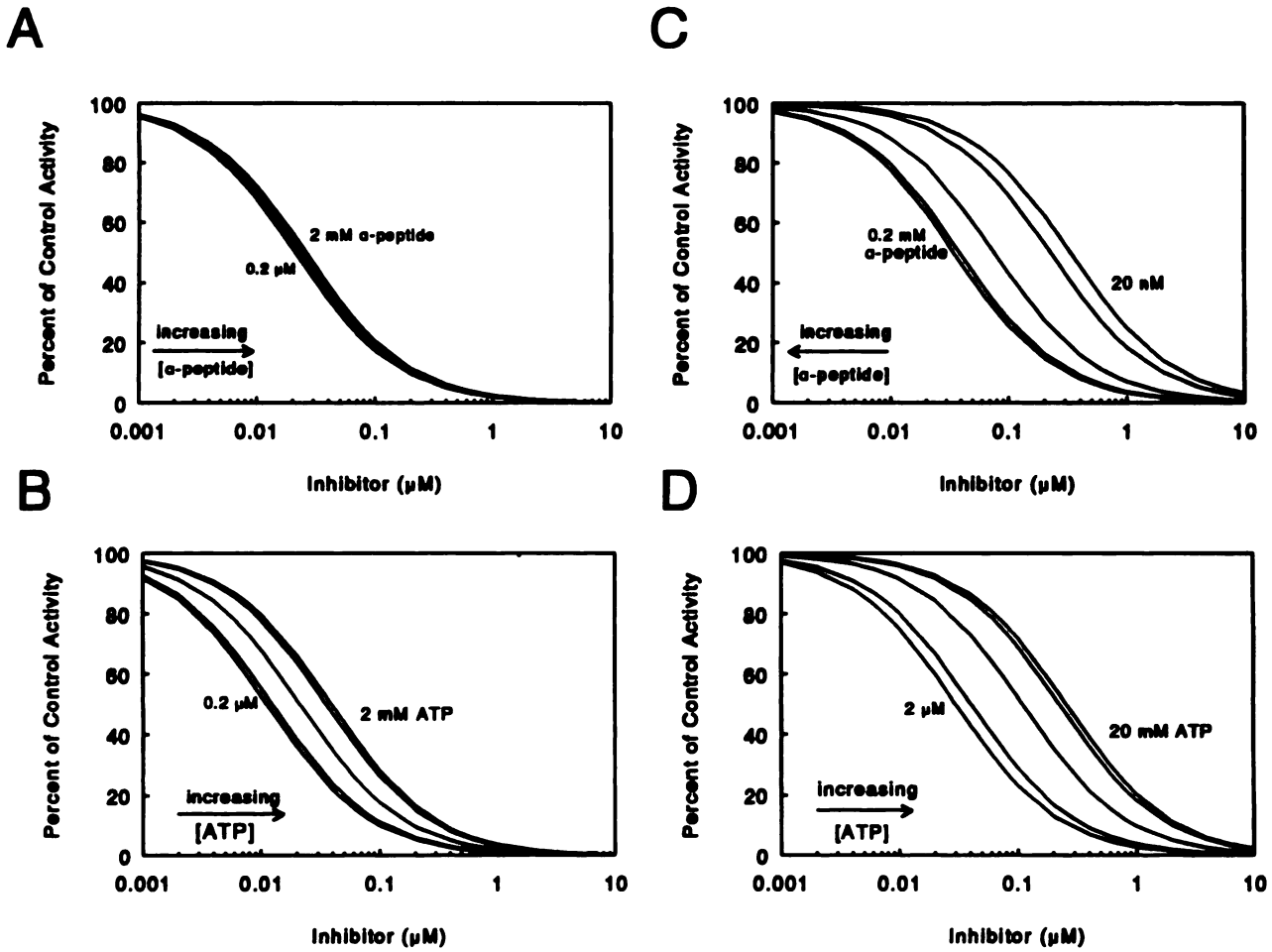

Fig. 6. Simulated data obtained using the parameters from Table 2 for PKC-α inhibition by UCN-01, with the two-substrate model. Data are plotted as percentage inhibition curves. A and B, Predicted inhibition in the presence of varied α-peptide (0.2, 2, and 20 μM or 0.2 and 2 mM, respectively) and ATP (0.2, 2, and 20 μM or 0.2 and 2 mM, respectively) concentrations, when ATP is assumed to bind to the enzyme first. C and D, Predicted inhibition in the presence of varied α-peptide (0.02, 0.2, 2, and 20 μM or 0.2 mM, respectively) and ATP (2 and 20 μM or 0.2, 2, and 20 mM, respectively) concentrations, when α-peptide is assumed to bind to the enzyme first.

efficacy of inhibition. This does not fit with the experimental data in Fig. 2.

When the α-peptide was treated as binding to the enzyme first, then the parameter values (as μM) from Table 3 for PKC-α were applied as follows: K_a, 6.1; K_b, 26; K_i, 0.352; K_{ia}, 0.0185; K_{ib}, 0.26 [in this simulation, K_{iATP} and $K'_{\alpha pep}$ (Table 3) describe the same step and the average value for K_{ia} was used]; this produced the results shown in Fig. 6, C and D. The latter results are consistent with the experimental data shown in Fig. 2 and contrast with the data generated when ATP is considered to bind first. When peptide concentration is increased over the range from 20 nM to 0.2 mM, the simulation predicts that IC_{50} values will reflect increased potency, with the corresponding IC_{50} values ranging from 330 nM to 34 nM, respectively. When ATP concentration is increased from 2 μM to 20 mM, an apparent decrease in potency is predicted (predicted IC_{50} values of 30 and 260 nM, respectively). These predictions are consistent with the experimental data shown in Fig. 2. These limits are more complex than for the simplified single-substrate system and are dependent not only on the concentration of ATP and α-peptide but also on a combination of K_a, K_b, K_i, K_{ia}, and K_{ib}. These data are consistent with the proposal of α-peptide substrate binding preferentially to the enzyme before ATP.

Discussion

The data presented in this paper show that UCN-01 can selectively inhibit the PKC isozymes and has a greater ability than staurosporine to discriminate between the isozyme subclasses. PKC-α, -β, and -γ were inhibited similarly by UCN-01, with greater potency than PKC-δ and -ϵ, and PKC-ζ was not inhibited under the conditions used for our experiments. For the selected isozymes PKC-α and -δ, the inhibition produced by UCN-01 appeared more mixed-competitive in nature with respect to ATP and uncompetitive with respect to the peptide substrate or MBP. Inhibition constants were obtained by fitting experimental rate data to a simplified model that allows for binding of the inhibitor to the enzyme either with or without bound substrate (the substrate being varied in its concentration while the other substrate is held constant). Extension of the simplified model to accommodate a two-substrate reaction maintains consistency with the observed data and indicates a specific order of binding of substrates to the enzyme, with α-peptide predicted to bind first.

There are now several reports of PKC inhibitors that exhibit some degree of selectivity between the isozymes (19–21), based only on comparison of IC_{50} values. These studies include the more recently developed bisindolylmaleimides, which are more

selective for PKC than are the indole carbazole inhibitors (32–34). A number of these derivatives possess quite a range of differences in isozyme selectivity when PKC-α, -β, -γ, and -ϵ are compared (20). The ability of staurosporine to discriminate between isozymes appears to vary with the report and the isozymes tested (20, 21). It is remarkable that UCN-01, with slight modification from the parent compound staurosporine, is capable of discriminating between isozymes and has inhibition properties that are similar to those of some of the more potent, PKC-selective bisindolylmaleimides. When a physiological substrate such as MBP is used, UCN-01 maintains its ability to discriminate isozymes and a slight improvement is found with staurosporine (compare Tables 1 and 2). Other studies have demonstrated similar effects with different substrates, such as histone H1 (19) or protamine sulfate (21). As suggested by this and other work (19–21, 32, 34), the defined structures that are capable of discriminating isozyme activity should allow the definition of the binding determinants on the PKC isozymes and lead to an understanding of why selectivity can be achieved with an agent that targets the conserved ATP binding site. The development and characterization of these agents that possess relative differences in PKC selectivity, differential capability for the inhibition of the PKC isozymes, and, in some tested examples, different abilities to inhibit tumor cell growth *in vitro* and *in vivo* (9, 32, 35) will facilitate progress in delineating PKC isozyme functions in signal transduction and cellular growth.

An ordered sequence of substrate binding to and product release from PKC has been suggested (36, 37). The proposed order suggested that ATP binding preceded second substrate binding, but it included the concession that there may be some randomness in the PKC kinetic mechanism (37). After phosphate transfer, ADP is released first, followed by phosphorylated peptide/protein substrate. An investigation of the kinetics of staurosporine inhibition of PKC suggested that first PKC combines with ATP and histone (the substrate used in that study) and then staurosporine forms the inhibited enzyme complex (38). In that study, using the catalytic fragment of PKC-γ, a lack of competitive inhibition with respect to ATP was observed. This suggests that the inhibitor staurosporine may bind to a site overlapping the ATP binding site (38). The data we have obtained using recombinant, baculovirus-expressed, whole enzyme and the model we derived contrast with the findings of those previous studies. Our data support a model in which peptide substrate binds first to the enzyme, followed by ATP. The proposed order of substrate binding is consistent with the experimental data indicating the type of inhibition produced by UCN-01, where binding of peptide substrate increases the affinity of the enzyme for UCN-01, and with the mixed-competitive nature of inhibition by UCN-01 with respect to ATP. Under these conditions, where the phospho-acceptor substrate binds to the enzyme first, it is possible that different physiological PKC substrates may influence the degree of inhibition observed. Extension of enzymology determined *in vitro* to the whole-cell environment can be difficult, taking into account subcellular localization, enzyme and substrate concentrations, and substrate type. However, the *in vitro* data provide a framework to facilitate the design of future experiments that will allow the evaluation of particular kinases and their roles in intracellular signaling.

In summary, we have shown that UCN-01, which contains a slight structural change from the potent but nonselective protein kinase inhibitor staurosporine, is capable of discriminating between PKC isozymes for the inhibition of kinase activity. Greater potency was observed against the Ca^{2+}-dependent PKC isozymes than the Ca^{2+}-independent PKC isozymes. Additionally, there is a clear, diverse, concentration dependence of the observed IC_{50} values for these inhibitors with respect to either ATP or peptide substrates.

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