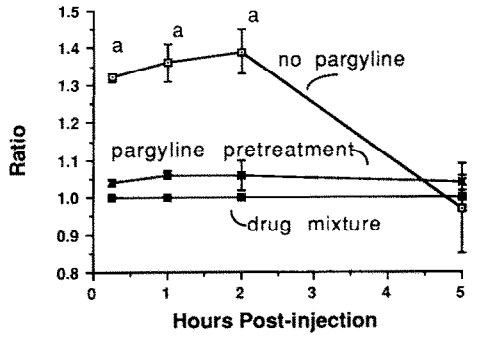

Fig. 2. Ratio of [$^2H_4$]phenelzine to phenelzine levels in rat brain at various times after injection of 50 mg/kg each of phenelzine and [$^2H_4$]phenelzine. Key: (a) $P < 0.05$ comparing the ratios of the drugs at a particular time period in rats with or without pargyline (50 mg/kg, 16 hr prior to rats being killed).

PLZ indirectly. Stereospecific deuterium substitution of various arylalkyl amines has been found to reduce their enzymatic deamination by MAO [7–10]. Intraperitoneally administered PLZ is subjected to extensive catabolism by the liver, primarily by MAO, before it is circulated throughout the body [16]. [$^2H_4$]PLZ appears to be protected from such catabolism on its first pass through the liver; thus, more [$^2H_4$]PLZ is available to enter the brain. This suggestion is supported by the present finding that pretreatment with an MAO inhibitor abolished the effect of deuteration. Similarly, at later time periods, the amount of [$^2H_4$]PLZ in the brain was no longer greater than the amount of PLZ, because of continuing metabolism of the drugs by the liver and other organs.

In summary, previous studies have shown that deuterium substitution in the $\alpha,\alpha,\beta,\beta$-positions of PLZ increases the behavioural and neurochemical potency of this drug, presumably through an attenuation of the metabolic inactivation of [$^2H_4$]PLZ by MAO. In this paper, we show that the concentration of [$^2H_4$]PLZ transported into the brain was significantly greater than that of the non-deuterated drug.

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Putative binding site(s) of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) on protein kinase C

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Protein phosphorylation is a major mechanism controlling intracellular events in mammalian tissues, as related to external physiological stimuli [1, 2]. Protein kinase C, an enzyme activated by calcium ion, phosphatidylserine and diacylglycerol, phosphorylates various protein substrates and relays transmembrane signalling in diverse Ca^{2+}-dependent cellular responses [3, 4]. As the manipulation of protein kinase by synthetic compounds should have profound effects on various cell functions, we developed selective inhibitors of protein kinase C, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) [5]. Studies using H-7 suggested that protein kinase C may play an important regulatory role in the function of various tissues and cells, including human platelets [6] and acinar cells of the pancreas [7]. However, the precise binding site of H-7 on protein kinase C had to be determined. As H-7 inhibits phosphotransferase activities, competitively with ATP [5], it is desirable to use reagents that covalently modify the

catalytic regions when attempting to identify the H-7 binding site on protein kinase C. We used 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) in the present study. The reactive sulfonyl fluoride of FSBA locates in a position analogous to the γ-phosphate of ATP [8] and so has been used as an affinity label for a number of kinases, synthetase, and dehydrogenases [9]. DTNB, a sulfhydryl-directed reagent was reported to inhibit protein phosphotransferase activity of the catalytic site, thereby suggesting that a cysteine residue is situated at or near the active site [10, 11]. We analysed the competitive protection of H-7 to these affinity labeling agents and found evidence for the interaction of the isoquinolinesulfonamide with the catalytic site of protein kinase C. Thus, information of drug binding site(s) on the protein kinase was gained.

Materials and methods

Protein kinase C was purified from rabbit brain as described [12]. Histone III-S, ATP, 5,5′-dithiobis (2-nitrobenzoic acid) and 5′-*p*-fluorosulfonylbenzoyladenosine were obtained from Sigma Chemical Co. (St. Louis, MO). Phosphatidylserine (pig liver) was purchased from Serdary Research Laboratories, Inc.

Protein kinase C was assayed by measuring the incorporation of ^{32}P into histone III-S from [γ-^{32}P]ATP, as described in Ref. 5.

Protein kinase C was chemically modified with FSBA and DTNB, as follows: protein kinase C was incubated with 1 mM FSBA or 0.05 mM DTNB at 30° in 50 mM HEPES–NaOH (pH 7.5), 2 mM EGTA containing 10% (v/v) dimethylsulfoxide. The reaction was halted at the indicated times by removing aliquots of 20 μl from the reaction mixture and diluting them into 800 μl of an above ice-cold buffer. A sample (20 μl) of this diluted enzyme was immediately assayed. The presence of diluted reagents, after cessation of the reaction, caused less than 3% inactivation in the course of the manipulations involved.

Results and discussion

1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7) is a novel, potent inhibitor of protein kinase C. The related inhibition is freely reversible and of the competitive type, with respect to ATP [5]. To elucidate the H-7 binding site(s) on the protein kinase, we examined the competition of H-7 with affinity labeling reagents at or near the ATP binding site of the enzyme. 5′-*p*-Fluorosulfonylbenzoyladenosine (FSBA) is an effective reagent for covalent labeling of the active center in the catalytic subunit of cAMP-dependent protein kinase II from bovine heart [13, 14].

Purified protein kinase C was inactivated time-dependently upon incubation with FSBA (1 mM) at 30° in 50 mM HEPES–NaOH (pH 7.5), 2 mM EGTA. In contrast, the enzyme incubated under similar conditions in the absence of the reagent lost no significant amount of activity. To establish whether inactivation of this enzyme proceeded through a reversible binding of the reagent, prior to modification, we calculate the apparent first order rates of kinase inactivation, as a function of FSBA concentration. A plot of rate constant versus reagent concentration yielded a hyperbolic curve and tended toward a maximum with increase in the concentrations of FSBA (Fig. 1). When the K_{obsd}^{-1} was plotted against $[FSBA]^{-1}$ (Fig. 1, insert) a straight line was obtained with a positive intercept on the abscissa. The data reveal a K_2 at pH 7.5 of 0.041 min^{-1} and a K_1 for FSBA of 0.22 mM. The negative K_i value for Mg-ATP protection against inactivation was approximately 0.5 mM (data not shown). This result is 10-fold higher than the K_m obtained when Mg-ATP was used as a substrate and similar to that for cyclic AMP dependent protein kinase [13]. A plot of the log E/E_0 vs time is shown in Fig. 2A. After 80 min of incubation with 1 mM FSBA, over 75% of the original activity of the enzyme was lost. Mg-ATP afforded a significant protection against this loss of activity. H-7, a competitive inhibitor with respect to Mg-ATP [5] also protected from inactivation by this reagent (Fig. 2A).

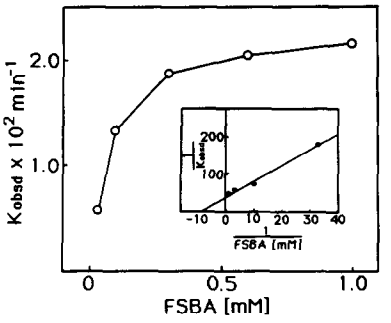

Fig. 1. Pseudo-first order rate constants of inactivation on the FSBA, as plotted against time. The inset is a double reciprocal plot of the data. The values for K_{obsd} were replotted according to Segel [15] to determine the K_1 and K_2.

When the enzyme was incubated at 30° in the presence of 0.05 mM DTNB, inhibition of the phosphotransferase activity exceeded 90% after 20 min, as indicated in Fig. 2B. Mg-ATP in a concentration of 1 mM completely protected against this inhibition and H-7 (1 mM) alone was without effect.

In an attempt to determine the H-7 related subsite, we compared the effect of ATP and its analogues known to bind to this site on the modification of the enzyme by FSBA and DTNB (Table 1). Ten mM Mg^{2+} and 1 mM ATP significantly protected from the inactivation by FSBA and DTNB and ADP and AMP were less effective.

Kinetical analyses revealed that FSBA and H-7 compete for the same site on the kinase molecule. Here we found that H-7 afforded full protection against modification by FSBA but not by DTNB. FSBA covalently modifies the NH_2 group of Lys-72 of the cAMP-dependent protein kinase, concomitant with the loss of catalytic activity, whereas DTNB alkylates SH groups of two cysteins (Cys-199 and Cys-343) of the catalytic subunit of cAMP-dependent protein kinase and the loss of phosphotransferase activity [16]. The catalytic domain shares substantial homology with other serine/threonin, and tyrosine kinases [17].

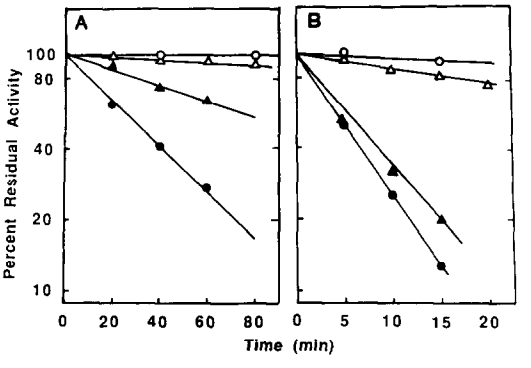

Fig. 2. Time course of inactivation by FSBA and DTNB in the presence of Mg-ATP or H-7. Protein kinase C was incubated with 1 mM FSBA or 0.05 mM DTNB in 50 mM HEPES–NaOH, pH 7.5, 2 mM EGTA and 10% DMSO at 30°, as described under Materials and Methods. A: control (○), FSBA alone (●), FSBA + 1 mM ATP + 10 mM $MgCl_2$ (△), FSBA + 1 mM H-7 (▲). B: control (○), DTNB alone (●), DTNB + 1 mM ATP + 10 mM $MgCl_2$ (△), DTNB + 1 mM H-7 (▲).

Table 1. Effect of nucleotides or H-7 on the inactivation of protein kinase C by FSBA or DTNB

Addition to assay mixture	% Residual activity	
	1 mM FSBA	0.05 mM DTNB
Control	100 ± 3.5	96.3 ± 6.4
None	47.7 ± 6.0	22.3 ± 4.1
ATP (1 mM)	90.0 ± 11.2	71.3 ± 6.1
ADP (1 mM)	59.3 ± 10.5	71.3 ± 3.2
AMP (1 mM)	56.7 ± 3.5	52.6 ± 5.0
H-7 (1 mM)	71.2 ± 6.0	36.6 ± 5.6

Protein kinase C was incubated with 1 mM FSBA or 0.05 mM DTNB at 30° in 50 mM HEPES–NaOH, pH 7.5, 2 mM EGTA, as described under Materials and Methods. Residual activity at 30 min (FSBA) or 10 min (DTNB) is given.

By analogy with the cAMP-dependent protein kinase, one can assume that Lys-371 and Cys-502 of rabbit protein kinase C (α type) would lie at or near the ATP binding site [17]. When considering the pattern of protection against FSBA or DTNB, the region binding to H-7 may be Lys-371 and not Cys-502. The selectivity of this compound should facilitate studies on the molecular mechanism of phosphotransferase reactions of protein kinase C.

In summary, protein kinase C purified from rabbit brain is irreversibly inactivated by 5′-*p*-fluorosulfonylbenzoyl-adenosine (FSBA) and 5,5-dithiobis (2-nitro-benzoic acids) (DTNB), compounds postulated to modify residues at or near the active site of the catalytic region, in various protein kinases. Inactivation by FSBA followed pseudo-first order kinetics. The pseudo-first order rate constant for inactivation showed saturation kinetics, thereby indicating a reversible binding of the reagent to the enzyme, prior to inactivation. We examined the effects of Mg-ATP and 1-(isoquinolinylsulfonyl)-2-methylpiperazine (H-7), the latter a selective inhibitor for protein kinase C on the inactivation of the enzyme by these reagents. As Mg-ATP and H-7 protected the enzyme against inactivation by FSBA, H-7 probably binds at or near the ATP-binding site on the protein kinase C molecule. However, H-7 did not protect the enzyme from DTNB and Mg-ATP afforded full protection from DTNB. These data suggest that H-7 may interact selectively with the region labelled by FSBA and does not interfere with the domain labelled by DTNB in the ATP binding site of protein kinase C.

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In vivo role of the microsomal ethanol-oxidizing system in ethanol metabolism by deermice lacking alcohol dehydrogenase*

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Two pathways of alcohol dehydrogenase (ADH) independent ethanol metabolism have been reported: the microsomal ethanol-oxidizing system (MEOS) and catalase. The respective role of each pathway in ethanol metabolism is still actively debated [1–7]. To examine the contribution of catalase, many investigators have used 3-amino-1,2,4-triazole (AT), widely reported to be a good catalase inhibitor [8, 9]. AT was found to have little effect on *in vivo* ethanol metabolism in either rats [10–15] or ADH-deficient deermice [1, 3]. This suggested a major role for MEOS rather than catalase in non-ADH ethanol metabolism. By contrast, a recent paper [4] reported that, 6 hr after AT administration in deermice, AT inhibited

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