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Development of Affinity Labeling Agents Based on Nonsteroidal Anti-inflammatory Drugs: Labeling of the Nonsteroidal Anti-inflammatory Drug Binding Site of 3α-Hydroxysteroid Dehydrogenase[†,‡]

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ABSTRACT: Nonsteroidal anti-inflammatory drugs (NSAIDs) exert their effect by inhibiting the target enzyme cyclooxygenase (prostaglandin H_2 synthase); however, little is known about the peptides comprising its NSAID binding site. Hydroxyprostaglandin dehydrogenases also bind NSAIDs, but their NSAID binding sites have not been well characterized. Using existing synthetic strategies, we have incorporated the bromoacetoxy affinity labeling moiety around the perimeter of two potent NSAIDs, indomethacin and mefenamate, a *N*-phenylanthranilate. The compounds synthesized were 1-(4-(bromoacetamido)benzyl)-5-methoxy-2-methylindole-3-acetic acid (**1**), 3-(2-(2-bromoacetoxy)ethyl)-1-(4-chlorobenzyl)-5-methoxy-2-methylindole (**2**), 4-(bromoacetamido)-*N*-(2,3-dimethylphenyl)anthranilic acid (**3**), *N*-(3-(bromoacetamido)phenyl)-anthranilic acid (**4**), and *N*-(4-(bromoacetamido)phenyl)anthranilic acid (**5**). To access whether these compounds have general utility in labeling NSAID binding sites, the compounds were evaluated as affinity labeling agents for 3α-hydroxysteroid dehydrogenase (3α-HSD) from rat liver cytosol. This enzyme displays 9-, 11-, and 15-hydroxyprostaglandin dehydrogenase activity, is inhibited potently by NSAIDs, and is homologous to bovine lung prostaglandin F synthase. Compounds **1–5** were shown to affinity label the NSAID binding site of 3α-HSD. They inactivated 3α-HSD through an E·I complex in a time- and concentration-dependent manner with $t_{1/2}$ values ranging from seconds to hours. Ligands that compete for the active site of 3α-HSD (NAD^+ and indomethacin) afforded protection against inactivation, and the inactivators could demonstrate competitive kinetics against 3α-hydroxysteroid substrates by forming an E·NAD^+·I complex. Further, when compounds **1–3** were radiolabeled with [^{14}C]bromoacetate, inactivation of 3α-HSD was accompanied by a stoichiometric incorporation of inactivator, indicating the labeling of discrete amino acids at the enzyme active site. This analysis suggests that these NSAID analogues may have general utility in affinity labeling the drug binding site of NSAID target enzymes.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of arthritis, rheumatism, and other inflammatory disorders. The presumed target enzyme for these drugs is cyclooxygenase (prostaglandin H_2 synthase, EC 1.14.99.1) (Vane, 1971, 1974; Flower, 1974). This enzyme is responsible for the initial step in the biosynthesis of the primary prostaglandins, one class of inflammatory mediators. Cyclooxygenase has been purified to homogeneity from ram and bovine seminal vesicles (Miyamoto et al., 1976; van Der Ouderra et al., 1977), and its cDNA has been cloned and the protein sequence has been deduced (DeWitt & Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988). Despite the wealth of information about the protein, little is known about the peptides that comprise the NSAID binding site. Aspirin is known to acetylate Ser_{530}, 70 amino acids from the C-terminus (Roth et al., 1983); however, site-directed mutagenesis

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[‡] Preliminary accounts of this study were presented at the FASEB Societies Meeting in Las Vegas, NV, in May 1988, and in Washington, D.C. in April 1990, and have been published [(1988) *FASEB J. 2*, A1109, and (1990) *FASEB J. 4*, A1123, respectively].
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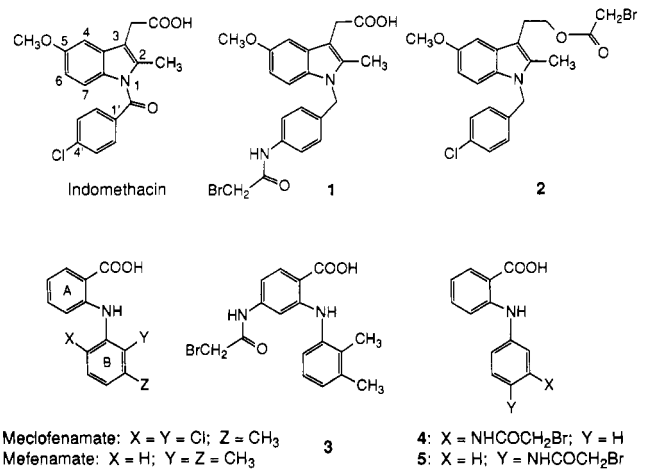
suggests that this site is distinct from the major NSAID binding site (DeWitt et al., 1990). The existing models of the NSAID binding site of this enzyme come from studies in which the crystal structure of indomethacin has been superimposed onto the flexible structure of arachidonic acid (Sankawa et al., 1973; Gund & Shen, 1977; Appleton & Brown, 1979). Another model has proposed that the binding site for NSAIDs and arachidonic acid do not overlap (Kulmacz, 1989). At this time, a good topological model does not exist for the NSAID binding site of cyclooxygenase. Without such a model, the rational design of superior NSAIDs is severely impeded.

Other target enzymes may also exist for NSAIDs. Indomethacin is known to inhibit 15-hydroxyprostaglandin dehydrogenase (Hansen, 1974), 9-hydroxyprostaglandin dehydrogenase, and 13-ketoprostaglandin reductase (Pace-Asciak & Cole, 1975) at low concentrations in vitro. However, the amino acids involved in drug binding and the location of the NSAID binding sites within these enzymes have not been determined.

Affinity labeling offers one approach by which NSAID binding sites may be mapped. However, affinity labeling analogues based on NSAIDs have not been developed. This paper describes the synthesis of (bromoacetoxy)- and (bromoacetamido)-NSAID analogues (Scheme I) which have the potential to affinity label NSAID binding sites. The compounds synthesized are based on the two potent classes of NSAIDs, the *N*-benzoylindoleacetic acids represented by indomethacin and the *N*-phenylanthranilic acids represented by mefenamate.

In accesssing the general utility of these agents to label NSAID binding sites, the newly synthesized compounds have been evaluated as affinity labeling agents for 3α-hydroxysteroid dehydrogenase (3α-HSD,[1] EC 1.1.1.50). This rat liver cytosolic enzyme is a versatile oxidoreductase (Penning et al., 1984) which can utilize 3α-hydroxysteroids as well as 9-, 11-, and 15-hydroxyprostaglandins (Penning & Sharp, 1987) as substrates. It satisfies many of the criteria expected of a NSAID target enzyme. It is inhibited by NSAIDs in rank order of their pharmacological potency and at concentrations relevant to therapeutic doses. It can discriminate between active and inactive drug isomers (Penning & Talalay, 1983; Penning, 1985). The indomethacin-sensitive 3α-HSD is widely distributed and of high specific activity in rat tissues, especially those which actively metabolize prostaglandins (i.e., spleen, lung, heart, and seminal vesicle) (Smithgall & Penning, 1985). In addition, a cDNA clone for 3α-HSD has been isolated and sequenced, and a high degree of sequence homology exists between 3α-HSD and bovine lung prostaglandin F synthase (Watanabe et al., 1988; Pawlowski et al., 1991).

Scheme I: Affinity Labeling Analogues Based on NSAIDs



In this paper we provide evidence that the (bromoacetamido)-NSAID analogues can affinity label the NSAID binding site of 3α-HSD in a stoichiometric manner. These compounds should have general utility in affinity labeling other NSAID binding sites including those of cyclooxygenase and 15-hydroxyprostaglandin dehydrogenase (Penning & Askonas, 1990). The ability of these compounds to affinity label cyclooxygenase has been described in a preliminary account (Askonas et al., 1990). These compounds represent the first affinity labeling agents, other than acetyl salicylate, that can be used to obtain structural information for the binding of NSAIDs to their target enzymes.

Experimental Procedures

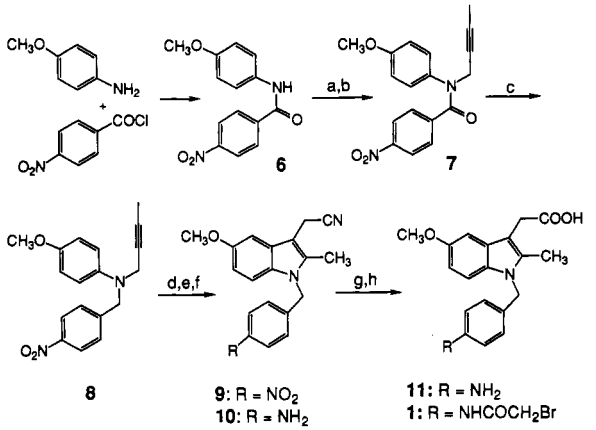
Materials. Pyridine nucleotides were purchased from Pharmacia-LKB Biotechnology (Piscataway, NJ) and Boehringer Mannheim (Indianapolis, IN). Steroids were purchased from Steraloids (Wilton, NH). Indomethacin was purchased from Sigma (St. Louis, MO). [1-^{14}C]Bromoacetic acid (51 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). 10% Pd/C (50% wet with water) was purchased from Kodak Laboratory Chemicals (Rochester, NY). Bromoacetic anhydride was purchased from Pfaltz & Bauer (Waterbury, CT). All other synthetic reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were of the highest commercial quality available. All solvents were of EM Science Omnisolve Grade and were obtained from Bodman Chemicals (Aston, PA).

Preparation and Assay of Enzyme. 3α-HSD was purified to homogeneity as previously described (Penning et al., 1984). The enzyme was stored in 20 mM potassium phosphate buffer, pH 7.0, containing 30% glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol at −80 °C. Standard assay conditions were 75 μM androsterone, 2.3 mM NAD^+, 100 mM potassium phosphate, pH 7.0, and 4% acetonitrile (v/v) in a 1.0-mL system at 25 °C. The reaction was started by the addition of enzyme, and the rate of formation of NADH was monitored at 340 nm over 5 min. A Gilford 260 UV/vis spectrophotometer was used for all measurements, using a molar extinction coefficient of 6270 M^{-1} cm^{-1} for NADH.

Preincubation Experiments. Enzyme which was used in studies with affinity labeling compounds was dialyzed against (3 × 500 mL) 10 mM potassium phosphate, pH 7.0/1 mM

[1] Abbreviations and trivial names: 3α-HSD, 3α-hydroxysteroid dehydrogenase or 3α-hydroxysteroid-NAD(P)$^+$-oxidoreductase (EC 1.1.1.50); androsterone, 5α-androstan-3α-ol-17-one: cyclooxygenase, prostaglandin H_2 synthase (EC 1.14.99.1); NAD(P)(H), nicotinamide adenine dinucleotide (phosphate) (reduced); DMF, dimethylformamide; DMSO, dimethyl sulfoxide; TLC, thin layer chromatography; NSAIDs, nonsteroidal anti-inflammatory drugs; indomethacin, 1-(4-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid; benzylindomethacin, 1-(4-chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid; 4′-BrAc-benzylindomethacin 1-(4-(bromoacetamido)benzyl)-5-methoxy-2-methylindole-3-acetic acid; 4′-NO_2-benzylindomethacin nitrile, 1-(4-nitrobenzyl)-5-methoxy-2-methylindole-3-acetonitrile; 4′-NO_2-benzylindomethacin, 1-(4-nitrobenzyl)-5-methoxy-2-methylindole-3-acetic acid; 1-BrAc-benzylindomethacin ester, 3-(2-(2-bromoacetoxy)ethyl)-1-(4-chlorobenzyl)-5-methoxy-2-methylindole; indomethacin methyl ester, 1-(4-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid methyl ester; 4-BrAc-mefenamate, 4-(bromoacetamido)-*N*-(2,3-dimethylphenyl)-anthranilic acid; 4-NO_2-mefenamate, 4-nitro-*N*-(2,3-dimethylphenyl)-anthranilic acid; *m*-BrAc-fenamate, *N*-(3-(bromoacetamido)phenyl)-anthranilic acid; *p*-BrAc-fenamate, *N*-(4-(bromoacetamido)phenyl)-anthranilic acid; *m*-NO_2-fenamate, *N*-(3-nitrophenyl)anthranilic acid; *p*-NO_2-fenamate, *N*-(4-nitrophenyl)anthranilic acid; *m*-NH_2-fenamate, *N*-(3-aminophenyl)anthranilic acid; *p*-NH_2-fenamate, *N*-(4-aminophenyl)anthranilic acid.

Scheme II: Synthesis of 1-(4-(Bromoacetamido)benzyl)-5-methoxy-2-methylindole-3-acetic Acid (1)[a]



[a] Reagents used: (a) NaH; (b) but-2-yne bromide; (c) $LiAlH_4/AlCl_3$; (d) mCPBA/DMF; (e) KCN; (f) $NH_4COOH/Pd/C$; (g) KOH/EtOH; (h) $(BrCH_2CO)_2O$.

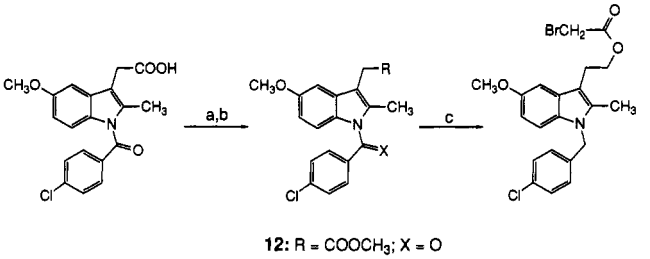
EDTA. Preincubation experiments were conducted at 25 °C in 100 μL of 10 mM potassium phosphate, pH 7.0/1 mM EDTA/5% acetonitrile or DMSO, containing 10 μM 3α-HSD and varying concentrations of the inactivators and/or protecting agents. Time courses were started by the addition of inactivator, and aliquots were removed over time and diluted at least 100-fold into a standard assay without androsterone. The assay was then initiated by the addition of androsterone, and the amount of enzyme activity remaining was determined.

Analysis of Kinetic Data. Enzyme kinetic data from reversible inhibition studies were fit to the appropriate rate equation with the computer programs described by Cleland (1977, 1979). Patterns and kinetic constants shown in the Results section are those judged to be the best fit according to the criteria of Cleland (1979). Enzyme kinetic data from irreversible inhibition studies were analyzed by semilogarithmic plots and transformed by the method of Kitz and Wilson (1962).

Synthesis. The compounds synthesized are described for the first time and were obtained by adapting existing synthetic strategies. Complete synthetic details and spectroscopic data are available as supplementary material. Brief descriptions of the syntheses are presented below. Satisfactory ^1H NMR, IR, and MS or elemental analysis were obtained for all new compounds. High-field ^1H NMR data were obtained on either a Bruker WH-360 spectrometer operating at 360.04 MHz equipped with an ASPECT 2000 computer or a Bruker AM-500 spectrometer operating at 500.13 MHz equipped with an ASPECT 3000 computer.

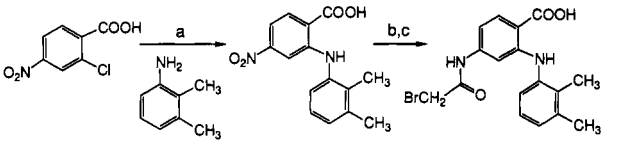
Synthesis of 1-(4-(Bromoacetamido)benzyl)-5-methoxy-2-methylindole-3-acetic Acid (1). The indomethacin analogue **1** was synthesized as illustrated in Scheme II in 6.2% overall yield (Makisumi & Takada, 1976). Anisidine and *p*-nitrobenzoyl chloride were reacted to form the benzanilide **6**, which on treatment with sodium hydride, followed by the addition of but-2-ynyl bromide (Ashworth et al., 1957) formed the *N*-but-2-ynyl-substituted benzanilide **7**. Reduction of the anilide **7** with a 5-fold excess of 1:1 lithium aluminum hydride/aluminum chloride afforded the benzyl intermediate **8**. N-Oxidation of **8** with *m*-chloroperbenzoic acid, accompanied by consecutive sigmatropic rearrangements, ketalization, and finally nucleophilic attack by potassium cyanide resulted in the intermediate indole-3-acetonitrile **9**. The nitro moiety was reduced under phase-transfer conditions to the amine **10** (Ram & Ehrenkaufer, 1984), and subsequent base hydrolysis of the nitrile resulted in the indole-3-acetic acid **11**. Finally, the amine was reacted with bromoacetic anhydride in acetone to afford **1**: ^1H NMR of **1** ($CDCl_3$, 360 MHz) δ 2.29 (3 H, s, indole-2-C*H*$_3$), 3.42 (2 H, s, indole-3-C*H*$_2$COOH), 3.74 (3 H, s, indole-5-OC*H*$_3$), 3.99 (2 H, s, COC*H*$_2$Br), 5.29 (2 H, s, N-C*H*$_2$-benzyl), 6.65 (1 H, dd, indole-6-*H*), 6.81 (1 H, br s, benzyl-N*H*CO), 6.96 (1 H, d, indole-7-*H*), 7.06 (1 H, d, indole-4-*H*), 7.21 (2 H, d, benzyl-*H*), 7.47 (2 H, d, benzyl-*H*), 9.50 (1 H, br s, COO*H*).

Scheme III: Synthesis of 3-(2-(2-Bromoacetoxy)ethyl)-1-(4-chlorobenzyl)-5-methoxy-2-methylindole (2)[a]



[a] Reagents used: (a) CH_2N_2; (b) $LiAlH_4/AlCl_3$; (c) $BrCH_2COOH$.

Scheme IV: Synthesis of 4-(Bromoacetamido)-*N*-(2,3-dimethylphenyl)anthranilic Acid (3)[a]



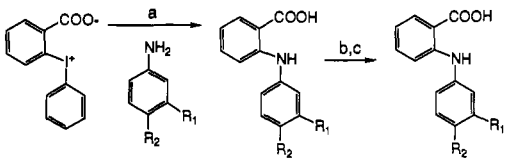
[a] Reagents used: (a) CuCl; (b) $SnCl_2/EtOH$; (c) $(BrCH_2CO)_2O$.

Synthesis of 3-(2-(2-Bromoacetoxy)ethyl)-1-(4-chlorobenzyl)-5-methoxy-2-methylindole (2). The bromoacetate ester of benzylindomethacin (**2**) was synthesized from indomethacin in three steps in 25% overall yield (Scheme III). Indomethacin was reacted with diazomethane to form the methyl ester **12**, which was subsequently reduced to the alcohol **13** with 1:1 lithium aluminum hydride/aluminum chloride. The alcohol **13** was then esterified with bromoacetic acid in the presence of dicyclohexylcarbodiimide and catalytic pyridine to yield **2**: ^1H NMR ($CDCl_3$, 500 MHz) δ 2.29 (3 H, s, indole-2-C*H*$_3$), 3.08 (2 H, t, CH_2CH_2O-), 3.80 (2 H, s, COC*H*$_2$Br), 3.87 (3 H, s, indole-5-OC*H*$_3$), 4.35 (2 H, t, indole-3-C*H*$_2$CH$_2$), 5.22 (2 H, s, N-C*H*$_2$-benzyl), 6.78 (1 H, dd, indole-6-*H*), 6.85 (1 H, d, indole-7-*H*), 7.01 (1 H, d, indole-4-*H*), 7.04 (2 H, d, benzyl-*H*), 7.22 (2 H, d, benzyl-*H*).

Synthesis of 4-(Bromoacetamido)-N-(2,3-dimethylphenyl)anthranilic Acid (3). Compound **3** was synthesized starting from 2-chloro-4-nitrobenzoic acid and 2,3-dimethylaniline (Scheme IV) (Acheson, 1973). The intermediate nitro acid **14** was reduced to the amine with stannous chloride in ethanol, and upon reaction of the amine with bromoacetic anhydride in DMF, the bromoacetamide **3** was formed in 3.8% overall yield: ^1H NMR (acetone-d_6, 360 MHz) δ 2.15 (3 H, s, aryl-C*H*$_3$), 2.29 (3 H, s, aryl-C*H*$_3$), 3.97 (2 H, s, COC*H*$_2$Br), 6.56 (1 H, d, anthranil-5-*H*), 6.82 (1 H, s, anthranil-3-*H*), 6.92 (1 H, d, anthranil-6-*H*), 7.03–7.15 (3 H, m, *N*-phenyl-*H*), 9.22 (1 H, s, COO*H*).

Synthesis of N-(3- and N-(4-(Bromoacetamido)phenyl)-anthranilic Acid (4 and 5). Using Ullmann-type chemistry (Scheme V), diphenyliodonium-2-carboxylate (Scherrer & Beatty, 1980) was coupled with either *m*- or *p*-nitroaniline in the presence of cupric acetate, resulting in the substituted anthranilates **15** and **16**. The nitro groups were then reduced

Scheme V: Synthesis of *N*-(3- and *N*-(4-(Bromoacetamido)phenyl)-anthranilic Acid (**4** and **5**)[a]



15 $R_1 = NO_2$; $R_2 = H$ **17** $R_1 = NH_2$; $R_2 = H$
16 $R_1 = H$; $R_2 = NO_2$ **18** $R_1 = H$; $R_2 = NH_2$
4 $R_1 = NHCOCH_2Br$; $R_2 = H$
5 $R_1 = H$; $R_2 = NHCOCH_2Br$

[a] Reagents used: (a) $Cu(OAc)_2$; (b) NH_2NH_2; (c) $(BrCH_2CO)_2O$.

with hydrazine to the amines **17** and **18**, and upon addition of bromoacetic anhydride, the bromoacetamides **4** and **5** were formed in 16.5% and 17.2% overall yields, respectively. ^1H NMR of **4** (acetone-d_6, 360 MHz) δ 2.79 (1 H, br s, N*H*), 4.03 (2 H, s, $COCH_2Br$), 6.82 (1 H, split t, anthranil-4-*H*), 7.01 (1 H, m, aryl-*H*), 7.31–7.43 (4 H, m, aryl-*H*), 7.72 (1 H, s, *N*-phenyl-2-*H*), 8.03 (1 H, dd, anthranil-3-*H*), 9.45 (1 H, s, COO*H*). ^1H NMR of **5** (acetone-d_6, 360 MHz) δ 4.01 (2 H, s, $COCH_2Br$), 6.74 (1 H, t, anthranil-5-*H*), 7.15 (1 H, d, anthranil-6-*H*), 7.20 (2 H, d, *N*-phenyl-*H*), 7.37 (1 H, split t, anthranil-4-*H*), 7.66 (2 H, d, *N*-phenyl-*H*), 7.99 (1 H, dd, anthranil-3-*H*), 9.20 (1 H, s, COO*H*).

Synthesis of [^{14}C]-*1-(4-(Bromoacetamido)benzyl)-5-methoxy-2-methylindole-3-acetic Acid* (*1*). This synthesis was conducted in the manner described above by using 5 mg of the amine **11** and 4.73 mg of bromo[1-^{14}C]acetate diluted to a specific activity of 12.6 mCi/mmol. Bromo[1-^{14}C]acetic anhydride was synthesized by the method of Wilcheck and Givol (1977). Compound **1** was purified to constant specific radioactivity by TLC. The final specific activity of [^{14}C]-labeled **1** was determined from a solution whose concentration was measured spectrophotometrically (λ_{max} = 227 nm; ϵ = 28 800) and was found to be 13.7 mCi/mmol.

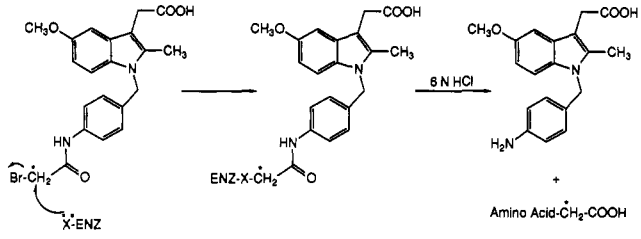
Synthesis of [^{14}C]-*3-(2-(2-Bromoacetoxy)ethyl)-1-(4-chlorobenzyl)-5-methoxy-2-methylindole* (*2*). This synthesis was conducted in the manner described above by using 4.63 mg of the alcohol **13** and 2.92 mg of bromo[1-^{14}C]acetate diluted to a specific activity of 13.9 mCi/mmol. [^{14}C]-labeled **2** was recrystallized from ethanol to a constant specific radioactivity of 7.64 mCi/mmol.

Synthesis of [^{14}C]-*4-(Bromoacetamido)-N-(2,3-dimethylphenyl)anthranilic Acid* (*3*). This synthesis was conducted in the manner described above by using 5 mg of the amine and 5.97 mg of bromo[1-^{14}C]acetate diluted to a specific activity of 10.3 mCi/mmol. Bromo[1-^{14}C]acetic anhydride was synthesized by the method of Wilcheck and Givol (1977). [^{14}C]-labeled **3** was purified to constant specific radioactivity by repetitive TLC (final specific activity is 10.2 mCi/mmol).

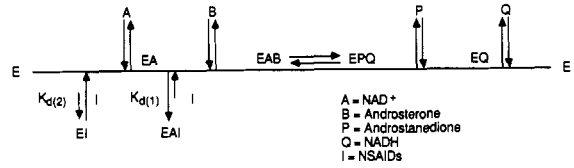
Results

Design of (Bromoacetyl)- and (Bromoacetamido)-NSAID Analogues. In designing affinity labeling analogues based on NSAIDs, we chose to develop flexible synthetic routes that would permit the incorporation of the bromoacetoxy moiety around the perimeter of a parent NSAID. In this manner we would be able to identify points of contact between a NSAID and amino acid residues at the drug binding sites of target enzymes. The bromoacetoxy group was chosen because it is well characterized as an affinity labeling group and can readily carboxymethylate reactive amino acids. In addition, if the resultant covalent linkage is stable to acid hydrolysis, the identity of the carboxymethylated amino acid can be ascertained by amino acid analysis, provided the carboxymethyl group is radiolabeled (Scheme VI). This method has been employed successfully by others to map the active sites of hydroxysteroid dehydrogenases with (bromoacetoxy)steroids (Sweet et al., 1972; Stricker et al., 1975; Penning et al., 1987, 1991).

Scheme VI: Affinity Labeling with (Bromoacetamido)- or (Bromoacetoxy)-NSAID Analogues (Asterisk Indicates ^{14}C)



Scheme VII: Kinetic Mechanism of 3α-HSD in the Presence of NSAIDs[a]



[a] An EQI complex also forms in the reverse direction. This is not shown in the present scheme since it is not pertinent to the current data analysis.

In the indomethacin series, we successfully replaced the *p*-chlorobenzoyl group of indomethacin with the *p*-(bromoacetamido)benzyl moiety in **1** and have substituted the bromoacetate ester **2** for the acid moiety (Scheme I). By selecting appropriately substituted starting materials, our synthetic route would also permit the incorporation of the bromoacetyl group at the 2, 5, and 6 positions of the indole and at all the positions of the *N*-benzyl ring. For the *N*-phenylanthranilate nucleus, synthetic routes were devised to substitute positions on both the A and B rings. By the prudent selection of appropriately substituted 2-chlorobenzoates and anilines, all the ring positions of the *N*-phenylanthranilate nucleus could ultimately be substituted with bromoacetate groups.

Evaluation of (Bromoacetyl)- and (Bromoacetamido)-NSAID Analogues as Affinity Labeling Agents: Influence of the Kinetic Mechanism of the Target Enzyme. To determine whether the (bromoacetoxy)- and (bromoacetamido)-NSAID analogues could act as affinity labeling agents for NSAID binding sites, we chose to evaluate these compounds as site-specific agents for 3α-HSD. As already described, 3α-HSD can function as a hydroxyprostaglandin dehydrogenase, bears sequence homology with prostaglandin F synthase, and has a well-defined NSAID binding site. In evaluating these compounds, it is important to understand the kinetic mechanism of 3α-HSD and the identity of the complexes through which NSAIDs inhibit the enzyme. We have shown that 3α-HSD catalyzes an ordered kinetic mechanism in which pyridine nucleotide binds first and leaves last (Scheme VII) (Askonas et al., 1991). Dead-end inhibition studies with NSAIDs reveal that only one kinetically significant complex forms, a tight binding ternary complex of E·NAD$^+$·NSAID ($K_{d(1)}$ for indomethacin = 0.8 μM). In addition, equilibrium binding studies have revealed that in the absence of NAD$^+$ NSAIDs can bind reversibly with free enzyme to form a low-affinity binary E·NSAID complex ($K_{d(2)}$ for indomethacin = 20.0 μM). In the evaluation of the (bromoacetyl)-NSAID analogues, it is possible to distinguish between these two complexes. Inhibition constants for an E·NAD$^+$·NSAID

Table I: Kinetic Constants for the Formation of Reversible E·NAD⁺·NSAID Analogue Complexes[a,b]

compound (trivial name)	IC_{50} (μM)	K_i (μM) ± SD[c]
1 4′-BrAc-benzylindomethacin	52	7.85 ± 1.06
2 1-BrAc-benzylindomethacin ester	NI	
3 4-BrAc-mefenamate	200	ND
4 *m*-BrAc-fenamate	3.75	0.96 ± 0.11
5 *p*-BrAc-fenamate	5.7	1.72 ± 0.24
9 4′-NO_2-benzylindomethacin nitrile	NI	
12 indomethacin methyl ester	NI	
14 4-NO_2-mefenamate	4.4	2.3 ± 0.42
15 *m*-NO_2-fenamate	6.1	2.6 ± 0.40
16 *p*-NO_2-fenamate	28	7.9 ± 1.1
17 *m*-NH_2-fenamate	20	ND
18 *p*-NH_2-fenamate	7.8	ND

[a] Assays were performed as described in the text. [b] NI, no inhibition; ND, not determined. [c] All compounds display competitive kinetics against androsterone and uncompetitive kinetics against NAD^+ as determined by fitting the data to the programs described by Cleland (1977) for dead-end inhibitors.

analogue ternary complex were obtained under initial velocity conditions in the presence of saturating concentrations of NAD^+, while the inhibition constants for an E·NSAID analogue binary complex were calculated from inactivation experiments with free enzyme.

Reversible Inhibition: Formation of E·NAD⁺·NSAID Analogue Complexes. Under initial velocity conditions in which the concentration of one substrate is varied and the other substrate is held constant, the (bromoacetamido)-NSAID analogues **1** and **3–5** yield the same kinetic profile observed with other NSAIDs; i.e., they act as competitive inhibitors against varied androsterone and as uncompetitive inhibitors against varied NAD^+. In these experiments, the (bromoacetamido)-NSAID analogues **1** and **3–5** presumably bind to the E·NAD^+ binary complex in a rapid reversible fashion but are prevented from alkylating the enzyme by the presence of competing substrate. The results of these studies are summarized in Table I. The (bromoacetamido)-NSAID analogues **1** and **3–5** inhibit 3α-HSD reversibly and with micromolar affinity, presumably by the formation of E·NAD^+·NSAID analogue complexes.

In analyzing the binding constants for analogues **1–5** it is important to emphasize that substitution of the *p*-chlorobenzoyl ring of indomethacin with a *p*-chlorobenzyl ring has little effect on the binding affinity of the drug for the E·NAD^+ complex [compare a K_i = 0.8 μM for indomethacin (Penning & Talalay, 1983) with a K_i = 0.3 for benzylindomethacin]. However, replacement of the *p*-chlorobenzyl ring with a *p*-(bromoacetamido)benzyl ring to yield compound **1** reduces the binding affinity 20-fold to yield a K_i = 7.8 μM. By contrast, the 1-BrAc-benzylindomethacin ester **2** does not exert an inhibitory effect under initial velocity conditions, implying that a free acid is essential for binding the NSAID analogue to the E·NAD^+ complex.

In the *N*-phenylanthranilate series, substitution of the B ring with a bromoacetamido group is allowed, since K_i values of the meta and para compounds **4** and **5** are 0.96 and 1.72 μM respectively, and are similar to those observed with meclofenamic acid (Penning & Talalay, 1983). By contrast, substitution of the A ring with a bromoacetamido group is not well tolerated, and the corresponding binding constant increases at least 100-fold, which is reflected by a significant increase in IC_{50} value.

Additional information regarding the nature of the NSAID binding site of 3α-HSD could also be obtained by examining the inhibition constants obtained for some of the synthetic

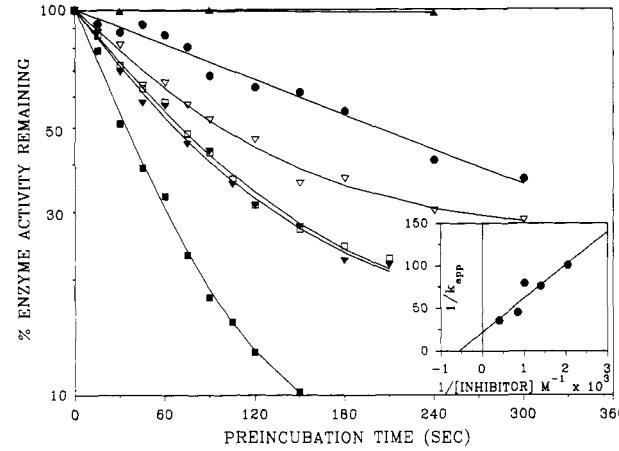


FIGURE 1: Time- and concentration-dependent inactivation of 3α-HSD by 4-BrAc-mefenamate (**3**). 3α-HSD (10 μM) was incubated with various concentrations of 4-BrAc-mefenamate (**3**) (0 μM (▲), 23.6 μM (●), 48.5 μM (▽), 70.9 μM (▼), 97.7 μM (□), and 118.2 μM (■)) at 25 °C in 100 μL of 10 mM potassium phosphate, 1 mM EDTA, pH 7.0, containing 5% DMSO. At the indicated times, 5 μL of the incubation mixture was withdrawn, and the remaining activity was determined by diluting into the standard assay, as described in the text. Semilogarithmic plots of the percent of 3α-HSD activity remaining versus time are shown. Rate constants for the inactivation were calculated by drawing tangents to the inactivation progress curves. These estimates of the pseudo-first-order rate constants were plotted by the method of Kitz and Wilson (1962) to yield the double-reciprocal plot for the inactivation of 3α-HSD (shown in the inset).

intermediates. In the benzylindomethacin series, no inhibition of 3α-HSD activity could be detected with either the methyl ester **12** or the nitrile **9** (Table I), corroborating that a free acid is essential for binding and that substitution of the carboxyl group with an alternative electronegative group still yields a compound that is without an inhibitory effect.

In the *N*-phenylanthranilate series, the B-ring synthetic intermediates **15–18** all bind with high affinity, regardless of the size, electronic nature, or position of the substituents. Substitution of the A ring of the fenamate nucleus, however, produced mixed results. The highly polar nitro substituent in compound **14** is a very good competitive inhibitor, but substitution through to the bulkier bromoacetamido group (**3**) yields a compound that binds poorly. This may suggest that there is less tolerance in terms of steric hindrance around the A ring than around the B ring of the fenamate nucleus. Alternatively, the electron-donating effect of the *p*-bromoacetamido moiety of **3** may sufficiently weaken the acid so as to destabilize its binding, while the *p*-nitro group in **14** is serving to increase the acid strength through its electron-withdrawing effect.

Irreversible Inhibition: Formation of E·NSAID Complexes. Under conditions in which free enzyme was preincubated with the (bromoacetamido)-NSAID analogues, compounds **1–5** were found to inactivate 3α-HSD in a time- and concentration-dependent manner. Semilogarithmic plots of enzyme activity remaining versus time show that $t_{1/2}$ values can vary from a few seconds to several hours, suggesting that amino acids of varying reactivity are being labeled. For each compound, the progress curves for inactivation appear to follow pseudo-first-order kinetics. To obtain estimates of the pseudo-first-order rate constants (k_{app} values), tangents were drawn to the progress curves. These data were then analyzed by the method of Kitz and Wilson (1962) in which $1/k_{app}$ is plotted against 1/[I]. This plot usually gives a measure of $1/k_{+2}$, the limiting rate constant for inactivation, and $1/K_{i(2)}$ the dissociation constant for the E·I complex. Typical inactivation data obtained with compound **3** are shown in Figure 1, and the

Table II: Kinetic Constants for Irreversible Inhibition[a]

compound (trivial name)	K_i[b] (μM)	$k_{+2} \times 10^{-3}$ (s^{-1})	$t_{1/2}$[c] (s)
1 4′-BrAc-benzylindomethacin[d]	>673	>40.9	<17
2 1-BrAc-benzylindomethacin ester[d]	>552	<0.096	>7200
3 4-BrAc-mefenamate	210	50.2	13.8
4 *m*-BrAc-fenamate	140	94.9	7.3
5 *p*-BrAc-fenamate	225	72.2	9.6

[a] Experiments were performed and the kinetic constants were derived as described in the text. [b] $K_{i(2)}$ for the reversible E·I complex. [c] $t_{1/2}$ extrapolated to saturation. [d] These values are estimates. Saturation kinetics were not observed.

inactivation rate constants observed with compounds **1–5** are summarized in Table II.

In the indomethacin series, 4′-BrAc-benzylindomethacin (**1**) inactivates 3α-HSD very rapidly with a $t_{1/2}$ value of <17 s at the limit of solubility. However, analysis of the inactivation data yields a Kitz–Wilson plot in which the line goes through the origin. This is indicative that saturation kinetics were not observed with this compound (Silverman, 1988), and solubility constraints prevented kinetic measurements at higher inactivation concentrations. As a consequence, the kinetic constants described for this compound are only estimates. By contrast to the rapid kinetics for enzyme inactivation observed with compound **1**, the 1-BrAc-benzylindomethacin ester (**2**) inactivates 3α-HSD over a much longer time scale (hours instead of seconds). Saturation kinetics were also not observed with compound **2**.

The *N*-phenylanthranilate analogues **3–5** generate Kitz–Wilson plots with positive *y*-intercepts, indicating that the inactivation process is saturable and therefore dependent on the total enzyme concentration. The results in Table II show that the *N*-phenylanthranilate analogues **3–5** all bind to free enzyme with similar affinity (140–220 μM) and inactivate on a similar rapid time scale (seconds), regardless of the site of substitution.

Site of Inactivation. Initial velocity studies indicate that the (bromoacetamido)-NSAID analogues **1** and **3–5** yield a kinetic profile identical to that observed with NSAIDs; they act as competitive inhibitors of androsterone and, therefore, compete for the steroid binding site. To corroborate that these analogues alkylate the NSAID binding site of 3α-HSD, protection studies were also performed. When 10 μM enzyme was preincubated with compounds **1** and **3–5** in the presence of 30–200 μM indomethacin, protection against inactivation was afforded. However, complete protection was not observed. This is a reflection of the lower affinity of indomethacin for free enzyme and the inability to saturate free enzyme with the concentrations of indomethacin used. Preincubation of free enzyme with NAD^+ also provided significant protection against activation by **1–5**, indicating that E·NAD^+ is resistant to covalent modification. Since both indomethacin and NAD^+ will protect 3α-HSD from inactivation and since both of these ligands bind at the active site, it is presumed that the (bromoacetamido)-NSAID analogues also bind at this site. It should be emphasized that inactivation of 3α-HSD by 5 mM bromoacetic acid is not observed. This implies that the inactivation observed with compounds **1–5** has an obligatory requirement for an NSAID structure so that alkylation is directed toward the NSAID binding site of 3α-HSD.

Several lines of evidence suggest that compounds **1–5** covalently modify 3α-HSD. First, when the enzyme is inactivated by these agents to less than 5% of its initial activity, no activity is recovered after extensive dilution (200-fold) into the assay system. Second, enzyme activity is not restored after excess inactivator is removed by extensive dialysis against 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. Third, enzyme activity is not restored after excess inactivator is removed by gel-exclusion chromatography. Control enzyme retains full activity during these manipulations.

Stoichiometric Incorporation. The indomethacin analogues, 4′-BrAc-benzylindomethacin (**1**) and 1-BrAc-benzylindomethacin ester (**2**), and 4-BrAc-mefenamate (**3**) were radiolabeled using [^{14}C]bromoacetic acid. Inactivation of homogeneous 3α-HSD with these compounds, followed by the removal of excess label by gel-exclusion chromatography, showed that each monomer of inactivated enzyme had incorporated 1 molecule of the (bromoacetamido)-NSAID analogue. Together our data imply that each analogue will label a discrete nucleophilic amino acid at the NSAID binding site of 3α-HSD.

Discussion

Our knowledge of the structure of the NSAID binding sites of target enzymes (cyclooxygenase, hydroxyprostaglandin dehydrogenase, and 3α-HSD) is ill-defined. One approach to identify amino acid residues that are of importance in binding NSAIDs is to synthesize a series of affinity labeling analogues based on NSAIDs. Previous work in this area has concentrated on the identification of the site on cyclooxygenase that is acetylated by aspirin. It is now known that aspirin acetylates a serine residue 70 amino acids in from the C-terminus (Roth et al., 1983). However, evidence has been presented that this serine residue is not located at the NSAID binding site (Kulmacz, 1989; DeWitt et al., 1990).

In this paper we describe the synthesis of (bromoacetamido)-NSAID analogues which may have general utility in affinity labeling NSAID target enzymes. Although a series of (bromoacetamido)benzoylindomethacin analogues were previously reported by Lui et al. (1977), their synthesis in our hands was difficult to reproduce and offered limited flexibility in the incorporation of affinity labeling moieties around the perimeter of the indomethacin nucleus. Also, these earlier compounds were only evaluated as tags for the tryptophan binding site of defatted human serum albumin, and no attempts were made to utilize these compounds to affinity label NSAID target enzymes. The (bromoacetamido)benzylindomethacin and *N*-phenylanthranilate analogues **1–5** described in this paper have not been previously reported. We have also evaluated these compounds (i) as affinity labeling agents of 3α-HSD, which displays a high degree of sequence homology with prostaglandin F synthase, and (ii) as affinity labeling agents of cyclooxygenase (Askonas et al., 1990).

Evidence is provided in this paper that compounds **1–5** affinity label the NSAID binding site of 3α-HSD in a stoichiometric manner. Because these compounds can inactivate 3α-HSD on very different time courses, it is unlikely that a single reactive amino acid is being labeled by these agents at the active site of 3α-HSD. Data is also presented which shows that the NSAID analogues **1–5** inhibit the enzyme by two distinct mechanisms. Under initial velocity conditions in the presence of saturating NAD^+, these compounds act as competitive inhibitors against androsterone, binding reversibly and

with high affinity to the E·NAD$^+$ complex. This mechanism of inhibition is observed with all the NSAIDs and provides evidence that these NSAID analogues bind at the drug binding site. The NSAID binding site of 3α-HSD appears to be relatively tolerant to changes in NSAID structure as determined by structure–activity relationships. Only the addition of the bromoacetamido group to the A ring of the *N*-phenylanthranilate nucleus or the removal of the acid moiety from benzylindomethacin analogues seriously perturbs reversible binding. The second mechanism of inhibition of 3α-HSD by NSAID analogues **1**–**5** is time-dependent inactivation of free enzyme, resulting in covalent bond formation. For the analogues **1** and **3**–**5**, this inactivation is accomplished on a very short time scale. In this second mechanism, the compounds bind to free enzyme with much lower affinity than to the E·NAD$^+$ complex. Protection studies have shown that indomethacin is able to slow the inactivation process through the formation of its low-affinity complex with free enzyme and that NAD$^+$ is also able to protect free enzyme as the E·NAD$^+$ complex. These and other data suggest that alkylation occurs at the active site. These studies are consistent with the established kinetic mechanism of 3α-HSD in which indomethacin binds either to E·NAD$^+$ with high affinity or to free enzyme with low affinity (Askonas et al., 1991). It is of interest that (bromoacetamido)-NSAID analogues can perturb the "normal" equilibrium between these enzyme complexes, since they can effectively trap free enzyme as a covalent E·NSAID complex.

Preliminary evaluation of these analogs on a solubilized microsomal preparation of cyclooxygenase has shown that compounds **1** and **3** inactivate this enzyme in a time-dependent manner (Askonas et al., 1990). Interpretation of this finding is complicated by the observation that indomethacin will cause time-dependent inactivation of cyclooxygenase by a mechanism that does not involve covalent incorporation of the drug (Stanford et al., 1977). We have replicated these findings and found that 4 μM indomethacin will inactivate cyclooxygenase with a $t_{1/2}$ value of 30 s. These data are to be contrasted with those obtained with compounds **1** and **3** where the $t_{1/2}$ values for the inactivation of cyclooxygenase are on the order of 4 h (Askonas et al., 1990). These differences in time courses imply that compounds **1** and **3** inactivate cyclooxygenase by a mechanism fundamentally different from that observed with indomethacin. Evaluation of these compounds to serve as affinity labeling agents of a homogeneous cyclooxygenase preparation is currently in progress.

Compounds **1**–**5** described in this paper have the potential to affinity label the NSAID binding sites of three target enzymes, 3α-HSD, cyclooxygenase, and 15-hydroxyprostaglandin dehydrogenase. These compounds should permit the identification of amino acid residues that are of functional importance in binding NSAIDs. It should be possible via peptide mapping to locate the position of the individual carboxymethylated amino acid within the primary structure of these targets. This information will be useful in modeling NSAID–target enzyme interactions and may be useful in the rational design of superior NSAIDs.

Acknowledgments

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Supplementary Material Available

Detailed descriptions of the syntheses outlined in Schemes I–IV including spectral data on the intermediates (6 pages). Ordering information is given on any current masthead page.

Registry No. **1**, 134312-68-8; **2**, 134312-69-9; **3**, 134312-70-2; **4**, 134312-72-4; **5**, 134312-71-3; **6**, 24730-11-8; **7**, 136881-90-8; **8**, 136881-91-9; **9**, 136881-92-0; **10**, 136881-93-1; **11**, 136881-94-2; **12**, 1601-18-9; **13**, 136881-95-3; **14**, 59748-75-3; **15**, 27693-70-5; **16**, 7221-31-0; **17**, 25293-29-2; **18**, 41139-95-1; 3α-HSD, 37250-75-2; *p*-nitrobenzoyl chloride, 122-04-3; *p*-anisidine, 104-94-9; but-2-ynyl bromide, 3355-28-0; *m*-chloroperbenzoic acid, 937-14-4; indomethacin, 53-86-1; bromoacetic acid, 79-08-3; 2-chloro-4-nitrobenzoic acid, 99-60-5; 2,3-dimethylaniline, 87-59-2; 4-amino-*N*-(2,3-dimethylphenyl)anthranilic acid, 136881-96-4; diphenyliodonium-2-carboxylate, 1488-42-2; *m*-nitroaniline, 99-09-2; *p*-nitroaniline, 100-01-6; meclofenamate, 644-62-2; mefenamate, 61-68-7; bromoacetic anhydride, 13094-51-4.

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Transport and Metabolism of Glucose in an Insulin-Secreting Cell Line, βTC-1†

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ABSTRACT: Kinetic characteristics of glucose transport and glucose phosphorylation were studied in the islet cell line βTC-1 to explore the roles of these processes in determining the dependence of glucose metabolism and insulin secretion on external glucose. The predominant glucose transporter present was the rat brain/erythrocyte type (Glut1), as determined by RNA and immunoblot analysis. The liver/islet glucose transporter (Glut2) RNA was not detected. The functional parameters of zero-trans glucose entry were K_m = 9.5 ± 2 mM and V_{max} = 15.2 ± 2 nmol min^{-1} (μL of cell water)$^{-1}$. Phosphorylation kinetics of two hexokinase activities were characterized in situ. A low-K_m (0.036 mM) hexokinase with a V_{max} of 0.40 nmol min^{-1} (μL of cell water)$^{-1}$ was present along with a high-K_m (10 mM) hexokinase, which appeared to conform to a cooperative model with a Hill coefficient of about 1.4 and a V_{max} of 0.3 nmol min^{-1} (μL of cell water)$^{-1}$. Intracellular glucose at steady state was about 80% of the extracellular glucose from 3 to 15 mM, and transport did not limit metabolism in this range. In this static (nonperifusion) system, 2-3 times more immunoreactive insulin was secreted into the medium at 15 mM glucose than at 3 mM. The dependence of insulin secretion on external glucose roughly paralleled the dependence of glucose metabolism on external glucose. Simulations with a model demonstrated the degree to which changes in transport activity would affect intracellular glucose levels and the rate of the high-K_m hexokinase (with the potential to affect insulin release).

Much evidence has been advanced that the signal for insulin secretion is generated by the metabolism in β cells of the fuels which act as secretagogues (Meglasson et al., 1986). In isolated islets, it appears that transport of glucose does not significantly limit glucose metabolism, so the responsiveness of insulin secretion to extracellular glucose concentration would reflect the kinetics of the hexokinases present, which include the high-K_m glucokinase. In HIT-T15 cells, the response of insulin secretion to external glucose was inhibited 90% by the glucose-transport inhibitor phloretin, suggesting that transport might be a limiting step influencing the response to external glucose (Ashcroft & Stubbs 1987). The existence of β-cell lines with various glucose transport characteristics and glucose phosphorylation characteristics affords further opportunity to test the hypothesis that metabolism of the secretagogue fuel generates the secretory signal and to investigate the roles of early steps as determinants of the response to external glucose.

We have been developing techniques for characterizing glucose transport, glucose phosphorylation, and the interaction between them in various cell preparations including cultures (Whitesell et al., 1989a,b, 1990). In the present studies, those techniques were applied to the βTC-1 cell line which is known to increase its insulin release in response to increased medium glucose.

Experimental Procedures

Cell Culture and Incubations. βTC-1 cells (obtained from Dr. Doug Hanahan; Efrat et al., 1988) were grown in 35-mm culture dishes in Dulbecco's minimum essential medium (4.5

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