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Irreversible Loss of [^{3}H]Forskolin Binding Sites in Human Platelets by α-Haloacetyl Analogs of Forskolin

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SUMMARY

The 7-bromoacetyl-7-desacetyl (BrAcFsk) and 7-chloroacetyl-7-desacetyl (ClAcFsk) analogs of forskolin were synthesized as alkylating agents to study the high affinity binding sites for forskolin. BrAcFsk and ClAcFsk activated adenylate cyclase in human platelet membranes with EC_{50} values of about 20 and 12 μM, respectively, Both analogs increased cyclic AMP in human platelets; however, they were less potent that forskolin. Forskolin inhibited [^{3}H]forskolin binding to human platelet membranes with an IC_{50} of 20 nM, whereas BrAcFsk and ClAcFsk inhibited [^{3}H]forskolin binding with IC_{50} values of 0.1 μM. Pretreatment of intact platelets with 10 μM BrAcFsk caused a 90% irreversible loss in [^{3}H]forskolin binding sites, whereas pretreatment with 10 μM ClAcFsk led to a loss of 55% of the binding sites. The loss of binding sites occurred within 5 min for BrAcFsk and within 30 min for ClAcFsk. The time required for the loss of binding sites produced by either alkylating agent was increased by the inclusion of 200 μM forskolin in the pretreatment buffer. The inactive bromoacetyl analog of forskolin 7-bromoacetyl-7-desacetyl-1,9-dideoxyforskolin (1,9-dideoxy-BrAcFsk) did not activate adenylate cyclase, inhibit [^{3}H]forskolin binding, or cause an irreversible loss of [^{3}H]forskolin binding sites. Adenylate cyclase was assayed in membranes from platelets treated with either 10 μM BrAcFsk or 10 μM 1,9-dideoxy-BrAcFsk. The stimulation of adenylate cyclase by prostaglandin E_1, guanosine-5′-*O*-(3-thio)triphosphate, and AlF_4 was inhibited by about 50% in membranes from platelets treated with BrAcFsk. However, the stimulation of adenylate cyclase by forskolin was unaffected by preincubation with BrAcFsk. Pretreatment of human platelets with 1,9-dideoxy-BrAcFsk had no effect on the stimulation of adenylate cyclase by prostaglandin E_1, AlF_4, or forskolin.

The diterpene forskolin activates adenylate cyclase in a variety of different tissues and preparations (1, 2). Forskolin elicits physiological effects that are generally attributed to the activation of adenylate cyclase and subsequent increase in intracellular cyclic AMP. Forskolin activates adenylate cyclase in the absence of the G_s protein and activates relatively pure preparations of the catalytic subunit of adenylate cyclase (3–6). These observations support the proposal that forskolin acts directly at the catalytic subunit of adenylate cyclase. However, the maximal activation of adenylate cyclase by forskolin requires the presence of a functional G_s protein (7, 8). This has led to speculation that forskolin may be acting at two sites, one site associated with the direct activation of adenylate cyclase and another unique site associated with the synergistic activation of adenylate cyclase by forskolin and agents that act through the G_s protein (9). An alternative model speculates that forskolin and the activated G_s protein can activate the catalytic subunit of adenylate cyclase through a cooperative interaction (10). In this model, forskolin and the activated G_s protein bind cooperatively to the catalytic subunit of adenylate cyclase, producing an activated state that is different from the state of the protein by either agent alone.

Specific binding sites for forskolin have been described in membranes from human platelets and bovine brain (10–13). These sites exhibited a structure-activity profile similar to that for forskolin activation of adenylate cyclase. However, the K_d for forskolin binding to these sites is about 10 nM, which is almost 3 orders of magnitude less than the EC_{50} for forskolin to activate adenylate cyclase. The number of forskolin binding sites is increased under conditions that promote the activation of adenylate cyclase by the G_s protein. It was proposed that the high affinity binding sites for forskolin might correspond to a ternary complex of forskolin, the catalytic subunit, and the G_s protein (11). However, the high affinity binding sites have not been unambiguously associated with adenylate cyclase.

α-Haloketones have been used as alkylating groups on a variety of ligands to identify binding sites and proteins (14–19). The α-haloketones react very rapidly with nucleophilic groups and have been especially useful for affinity labeling of protein sulfhydryl groups. 7-Acyl derivatives of forskolin are potent in activating adenylate cyclase and in inhibiting forskolin binding (12, 20, 21). We have synthesized the 7-chloroacetyl and 7-bromoacetyl analogs of forskolin and have characterized

ABBREVIATIONS: G_s protein, stimulatory guanine nucleotide regulatory protein; BrAcFsk, 7-bromoacetyl-7-desacetylforskolin; ClAcFsk, 7-chloroacetyl-7-desacetylforskolin; 1,9-dideoxy-BrAcFsk, 7-bromoacetyl-7-desacetyl-1,9-dideoxyforskolin; PGE_1, prostaglandin E_1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTP-γ-S, guanosine-5′-*O*-(3-thio)triphosphate.

the ability of these compounds to activate adenylate cyclase and to irreversibly block forskolin binding.

Materials and Methods

Forskolin was from Calbiochem; ATP, creatine phosphokinase, phosphocreatine, cyclic AMP, and PGE_1 from Sigma; [^{3}H]adenine, [^{32}P]ATP, and [^{3}H]cyclic AMP were from New England Nuclear. Platelets were obtained from the Blood Bank of Navy Hospital (Bethesda, MD).

Preparation of Washed Platelets

Platelets were prepared as described by Siegl *et al.* (22). Briefly, freshly outdated platelets (25–45 ml in plasma) were chilled on ice for 10 min before the addition of ⅛ volume of 0.1 M EDTA, pH 7.5. The suspension was centrifuged at 3500 × *g* for 15 min at 4°. Platelets were resuspended in buffer (15 mM Tris·HCl, 134 mM glucose, 1 mM EDTA, pH 7.4) and centrifuged at 1000 × *g* for 5 min to sediment erythrocytes. The supernatant was transferred to another tube, centrifuged at 3500 × *g* for 15 min and resuspended in buffer.

Platelet Cyclic AMP

Washed platelets were incubated with 150 μCi of [^{3}H]adenine for 40 min at 37° in a water bath. Cold buffer was added and the cell suspension was centrifuged at 1000 × *g* for 10 min. The pellet was washed once with cold buffer, resuspended in buffer at a concentration of 1 × 10^7 cells/ml, and kept on ice until used. Labeled platelets (0.2 ml/assay) were allowed to equilibrate at 37° for 5 min, 50 μl of buffer containing the indicated drugs was added, and the incubation was continued for 5 min at 37°. The reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid. Cyclic AMP (0.25 ml of a 1.5 mM cAMP solution containing 5000 cpm of [^{32}P]cAMP) was added to monitor cyclic AMP recovery. The cyclic AMP was isolated by the method of Salomon *et al.* (23). Data were calculated as percentage of conversion, i.e., the percentage of total radioactive adenine taken up by the platelets that was converted to cyclic AMP.

Platelet Membranes

Washed platelets were resuspended in 12 ml of buffer and incubated for 10 min at 20° with 3.8 ml of a 60% glycerol solution. The suspension was then centrifuged at 5500 × *g* for 10 min and the resulting pellet was resuspended in 20 ml of ice-cold 50 mM Tris·HCl buffer at pH 7.4, containing 25 mM sucrose and 2 mM EDTA. Platelets were vortexed vigorously for 2 min and then homogenized with five strokes in a glass homogenizer. The membrane suspension was centrifuged twice at 25,000 × *g* for 25 min and resuspended in 10 mM Tris·HCl buffer, pH 7.4, containing 1 mM EDTA. Membrane proteins were determined by the method of Lowry *et al.* (24).

Platelet Adenylate Cyclase

Adenylate cyclase assays were carried out as described (3). Briefly, 25 μl of membrane suspension (100 μg/protein/assay) were incubated in 0.25 ml of a solution containing 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM dithiothreitol, 2 units of creatine phosphokinase, 2 mM creatine phosphate, 0.1 mM ATP containing 2 μCi of [α-^{32}P]ATP. Incubations were for 10 min at 30° and were terminated with 0.5 ml of 10% trichloroacetic acid and 0.25 ml of a 1 mM solution of cyclic AMP containing about 10,000 cpm of [^{3}H]cyclic AMP. Cyclic AMP was determined using the method of Salomon *et al.* (23).

Binding Experiments

Binding of [^{3}H]forskolin to membranes was determined as described (12). Membranes (0.5 mg of protein/tube), in a total volume of 0.4 ml with 50 mM Tris·HCl buffer at pH 7.4, were incubated for 1 hr at 20°. After the incubation, the membranes were rapidly filtered with 10 mM [^{3}H]forskolin. with a Brandel cell harvester using GF/C filters. The filters were washed three times with 4 ml of ice-cold buffer (50 mM Tris·HCl, pH 7.4).

Specific forskolin binding was calculated as the difference between total binding in the absence of unlabeled forskolin and nonspecific binding in the presence of 20 μM forskolin. Binding parameters were analyzed using the LIGAND program of Munson and Rodbard (25).

Pretreatment with Alkylating Agents

Suspensions of washed intact platelets were incubated at 37° with α-haloacetyl derivatives of forskolin[1] at the indicated concentrations and times. The reaction was stopped by the addition of 50 volumes of ice-cold buffer and centrifugation at 3500 × *g* for 10 min. This washing was carried out five times. Membranes were prepared and [^{3}H]forskolin binding was determined.

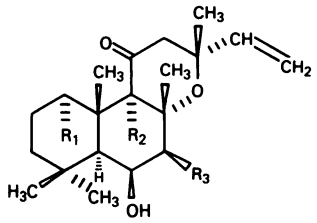
Synthesis of Forskolin Analogs

7-Desacetylforskolin. Forskolin (500 mg) was dissolved in 17 ml of methanol. Water (6 ml) containing 1.2 g of K_2CO_3 was added to the forskolin solution and allowed to react at room temperature overnight. The water and methanol were removed by evaporation under vacuum and the residue was dissolved in 5 ml of methylene chloride. The methylene chloride was washed three times with 15 ml of water. The organic layer was then dried over Na_2SO_4 and removed by evaporation. The 7-desacetylforskolin was left as a white powder at an average yield of about 90%. The same procedure was used for the synthesis of 7-desacetyl-1,9-dideoxyforskolin.

BrAcFsk. 7-Desacetylforskolin (150 mg) was dissolved in 5 ml of methylene chloride. Bromoacetic acid (55 mg) and dicyclohexylcarbodiimide (0.5 g) were added to the 7-desacetylforskolin. Dimethylaminopyridine (2 mg) was added to the mixture and the reaction was allowed to proceed at room temperature for 1 hr. The solvent was removed by rotary evaporation under reduced pressure. BrAcFsk was purified by flash chromatography on silica gel with methylene chloride/ethyl acetate (9:1) and was recrystallized from petroleum ether. The overall yield of the reaction was 35%. ClAcFsk and 1,9-dideoxy-BrAcFsk were synthesized using the same procedure as that for BrAcFsk.

Results

Activation of adenylate cyclase. The haloacetyl analogs of forskolin (Fig. 1) were tested for their ability to activate adenylate cyclase in membranes from human platelets (Fig. 2).



R_1	R_2	R_3	Compound
OH	OH	$OCOCH_3$	forskolin
OH	OH	$OCOCH_2Br$	7-bromoacetyl-7-desacetyl-forskolin
OH	OH	$OCOCH_2Cl$	7-chloroacetyl-7-desacetyl-forskolin
H	H	$OCOCH_3$	1,9-dideoxyforskolin
H	H	$OCOCH_2Br$	1,9-dideoxy-7-bromoacetyl-7-desacetyl-forskolin

Fig. 1. Structure of forskolin and forskolin analogs.

[1] The α-haloketones have been studied for their stability in aqueous solution. There is no hydrolysis of ClAcFsk or BrAcFsk to the 7-desacetyl derivatives of forskolin nor is there any acyl group migration to the 6-position when incubations are carried out at pH 8.5 or 7.5 with a Tris buffer or a HEPES buffer (K. B. Seamon, unpublished results).

Forskolin activated adenylate cyclase in platelet membranes approximately 10-fold with 50% of the forskolin activation occurring at a concentration of 8 μM. The same level of activation required higher concentrations of ClAcFsk and BrAcFsk, 12 and 20 μM, respectively. None of the compounds produced a saturable stimulation of adenylate cyclase. 1,9-Dideoxy-BrAcFsk did not stimulate adenylate cyclase even at 100 μM, consistent with the inability of 1,9-dideoxyforskolin to stimulate adenylate cyclase (20).

The α-haloacetyl analogs were less effective than forskolin in increasing cyclic AMP in intact platelets. Forskolin increased cyclic AMP 10-fold over basal levels, with an EC_{50} of approximately 20 μM (Fig. 3A). In contrast, BrAcFsk and ClAcFsk increased cyclic AMP levels only 5-fold at 200 μM (Fig. 3A). Forskolin, BrAcFsk, and ClAcFsk produced synergistic increases in cyclic AMP with PGE_1 (Fig. 3B). PGE_1 (10 μM) increased cyclic AMP 4-fold to 0.85% conversion in the absence of forskolin and to 6% in the presence of 100 μM forskolin. PGE_1 increased cyclic AMP to 4% conversion in the presence of BrAcFsk (100 μM) and ClAcFsk (100 μM). 1,9-Dideoxy-BrAcFsk had no effect on the increase in cyclic AMP produced by PGE_1.

Inhibition of forskolin binding to human platelet membranes. [^{3}H]Forskolin binds to human platelet membranes with a K_d of 18 nM and a B_{max} of 400 fmol/mg of protein in the presence of 5 mM $MgCl_2$ and 10 mM NaF (10). Forskolin inhibited the binding of [^{3}H]forskolin to human platelet membranes with an IC_{50} of 20 nM (Fig. 4). ClAcFsk and BrAcFsk were less potent than forskolin in inhibiting [^{3}H]forskolin binding, with IC_{50} values of approximately 100 nM. The 1,9-dideoxy analog of BrAcFsk, at 10 μM, produced only a small inhibition of [^{3}H]forskolin binding. This is similar to the lack of inhibition of [^{3}H]forskolin binding to brain membranes by 1,9-dideoxyforskolin (12).

Irreversible loss of forskolin binding sites. Intact human platelets were preincubated with different concentrations of the α-haloacetyl analogs for 15 min at 37°. The platelets were then washed five times with a 50-fold volume of buffer to remove noncovalently bound analogs. Membranes were prepared and [^{3}H]forskolin binding was determined in the presence of 10 nM [^{3}H]forskolin.

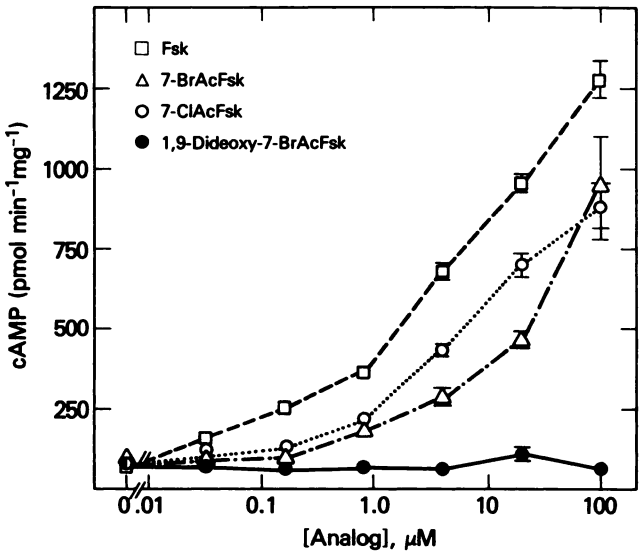


Fig. 2. Stimulation of human platelet adenylate cyclase by forskolin analogs. Platelet membranes were stimulated with the indicated concentrations of forskolin analogs for 10 min at 30°. The data are the mean of triplicate determinations and the standard error is indicated. *Fsk*, forskolin.

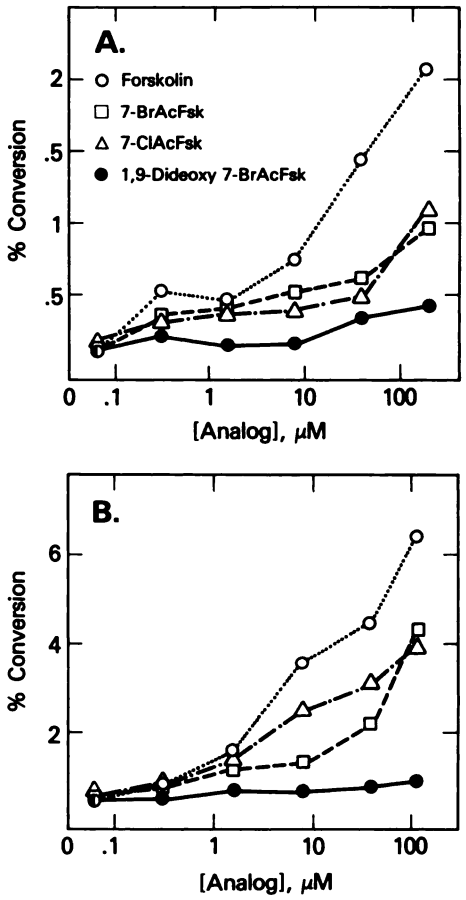


Fig. 3. Effect of forskolin analogs on intracellular cyclic AMP in intact platelets. Intact platelets were incubated with the indicated concentration of forskolin (○), BrAcFsk (□), ClAcFsk (△), or 1,9-dideoxy-BrAcFsk (●) for 5 min in the absence (A) or presence of 10 μM PGE_1 (B) and then [^{3}H]cAMP was determined as described in Materials and Methods. The data are the mean of triplicate determinations. The standard error is less than 10% for each data point and is not indicated on the graph.

Pretreatment with either BrAcFsk or ClAcFsk produced a dose-dependent decrease in the number of forskolin binding sites (Fig. 5). Pretreatment with 10 μM BrAcFsk and 1 μM BrAcFsk resulted in a loss of over 75 and 50%, respectively, of the forskolin binding sites. Higher concentrations of ClAcFsk were required to block binding than were required for BrAcFsk (Fig. 5). The decrease in forskolin binding at intermediate stages of inhibition by either ClAcFsk or BrAcFsk was due to a decrease in B_{max} and was not due to a change in K_d (data not shown). Pretreatment with 1,9-dideoxy-BrAcFsk did not produce any inhibition of binding (Fig. 5). Platelets were pretreated with 10 μM forskolin and washed in order to determine the potential effect of noncovalent association of forskolin with the high affinity binding sites. Membranes from platelets pretreated with forskolin had the same K_d and B_{max} for [^{3}H]forskolin (data not shown).

The loss of binding sites produced by pretreatment of platelets with the alkylating analogs was dependent on the time of preincubation (Fig. 6). The loss of binding due to pretreatment with 10 μM BrAcFsk was maximal within 5 min. The loss of

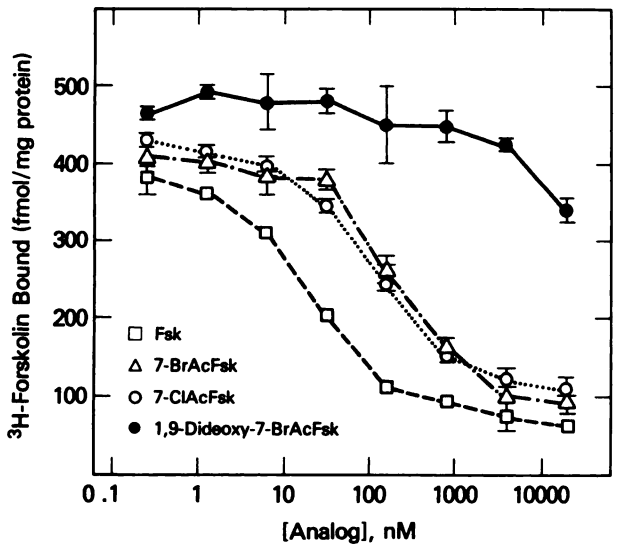


Fig. 4. Inhibition of [^{3}H]forskolin binding to human platelet membranes by forskolin and analogs. Platelet membranes were incubated with the indicated concentrations of analogs for 1 hr at 20° in the presence of 10 nM [^{3}H]forskolin. Nonspecific binding was determined in the presence of 20 μM unlabeled forskolin. The data are the mean of triplicate determinations and the standard error is indicated. *Fsk*, forskolin.

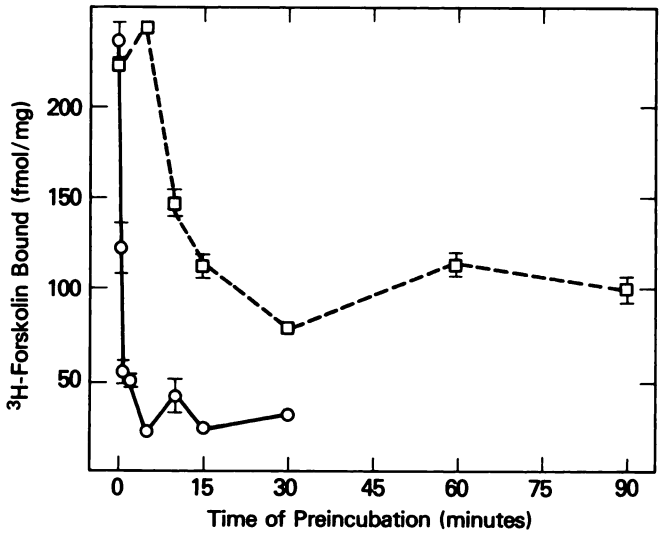


Fig. 6. Time dependence for the loss of [^{3}H]forskolin binding by alkylating analogs of forskolin. Intact platelets were incubated at 37° for the indicated times in the presence of 10 μM BrAcFsk (○) or 10 μM ClAcFsk (□). The platelets were then washed, membranes were isolated, and [^{3}H]forskolin was measured as described in Materials and Methods. The data are the mean of triplicate determinations and the standard error is indicated.

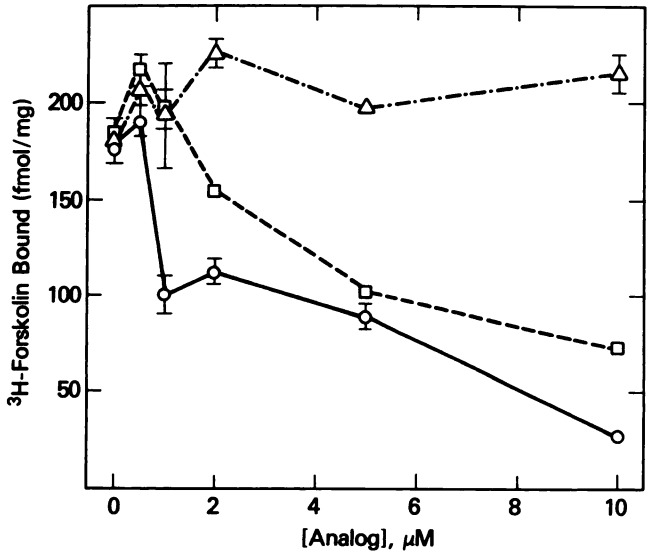


Fig. 5. Concentration dependence of the loss of [^{3}H]forskolin binding by alkylating analogs of forskolin. Intact platelets were incubated at 37° for 15 min in the presence of the indicated concentrations of BrAcFsk (○), ClAcFsk (□), or 1,9-dideoxy-BrAcFsk (△). The platelets were extensively washed, membranes were isolated, and [^{3}H]forskolin binding was measured as described in Materials and Methods. The data are the mean of triplicate determinations and the standard error is indicated.

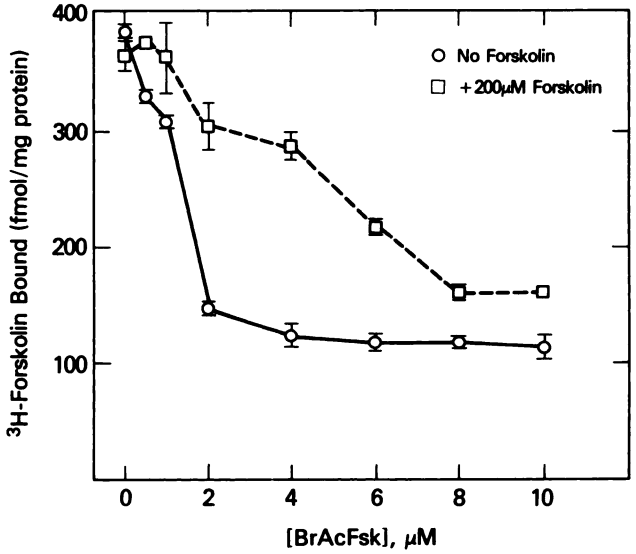


Fig. 7. Effect of forskolin on the loss of [^{3}H]forskolin binding sites by BrAcFsk. Intact platelets were incubated in the presence of the indicated concentrations of BrAcFsk with no additions (○) or with 200 μM forskolin (□) for 1 min. The platelets were then washed, membranes were made, and [^{3}H]forskolin was measured as described in Materials and Methods. The data are the mean of triplicate determinations and standard error is indicated.

binding due to pretreatment with ClAcFsk required 30 min of incubation in order to produce a maximal loss of binding sites.

The loss of binding sites observed after preincubation with BrAcFsk could be partially prevented by the presence of high concentrations of forskolin during the preincubation (Fig. 7). Intact platelets were incubated with increasing concentrations of BrAcFsk for 1 min at 37° in the presence and absence of 200 μM forskolin. The decrease in [^{3}H]forskolin binding sites is almost maximal at 2 μM BrAcFsk in the absence of forskolin (Fig. 7). However, a maximal decrease in binding sites was not observed until 8 μM BrAcFsk in the presence of 200 μM forskolin (Fig. 7). It was more difficult to detect a protective effect of forskolin on the irreversible loss of binding sites when incubations were carried out for longer than 5 min. A protective effect of forskolin on the loss of binding sites due to pretreatment with ClAcFsk was also observed (data not shown).

Effect of BrAcFsk on adenylate cyclase. Intact platelets were treated for 10 min with 10 μM BrAcFsk, 10 μM 1,9-dideoxy-BrAcFsk, or no additions (control). Membranes were isolated and assayed for adenylate cyclase activity in the presence of no additions, 100 μM GTP-γ-S, 10 μM AlF_4, 10 μM PGE_1, or 100 μM forskolin (Fig. 8). Adenylate cyclase activity in the presence

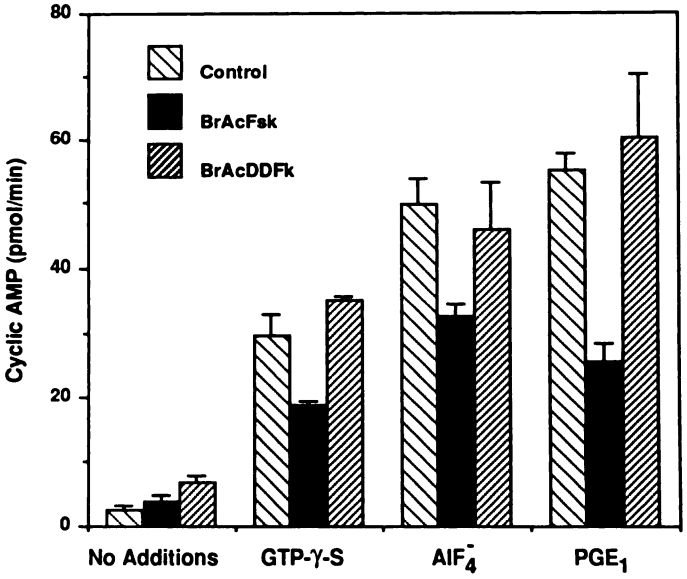


Fig. 8. Effect of pretreatment with BrAcFsk and 1,9-dideoxy-BrAcFsk on adenylate cyclase activity in platelet membranes. Intact platelets were incubated with 10 μM BrAcFsk, 10 μM 1,9-dideoxy-BrAcFsk (BrAcDDFe), or no additions (control) and were washed extensively. Membranes were isolated as described in Materials and Methods and assayed with no additions or with 100 μM GTP-γ-S, 10 μM AlF_4, 10 μM PGE_1, or 100 μM forskolin, as indicated in the figure. Adenylate cyclase activity in the presence of forskolin was 88 pmol/min for control membranes, 83 pmol/min for BrAcFsk-treated membranes, and 90 pmol/min for 1,9-dideoxy-BrAcFsk-treated membranes. The data are the mean of triplicate determinations and the standard error is indicated.

of GTP-γ-S or AlF_4 was inhibited 35% in membranes from BrAcFsk-treated platelets, when compared with the activity in membranes from either control platelets (not treated with alkylating agent) or platelets treated with 1,9-dideoxy-BrAcFsk. Adenylate cyclase activity in the presence of PGE_1 was inhibited by 50% in membranes from BrAcFsk-treated platelets, when compared with membranes from either control platelets or platelets treated with 1,9-dideoxy-BrAcFsk. In contrast, there was no effect of BrAcFsk pretreatment on adenylate cyclase activity in the presence of forskolin. The adenylate cyclase activity in the presence of 100 μM forskolin was 88 pmol/min for control membranes, 83 pmol/min for BrAcFsk-treated membranes, and 90 pmol/min for 1,9-dideoxy-BrAcFsk-treated membranes.

Discussion

A large body of evidence indicates that forskolin can act directly at the catalytic subunit of adenylate cyclase (3–6). It has also been shown that forskolin can directly affect the glucose transporter of erythrocytes and adipocytes and it has been demonstrated that forskolin affects other membrane-associated proteins (26–32). Specific high affinity binding sites for forskolin have been described in membranes from rat brain, human platelets, and solubilized proteins from rat brain and it was suggested that the binding sites may be related to adenylate cyclase (10–13, 33). We have now attempted to develop affinity ligands to directly identify the forskolin binding sites.

The ClAcFsk and BrAcFsk analogs of forskolin exhibit properties similar to those of other 7-acyl derivatives of forskolin. Both analogs activate adenylate cyclase and inhibit [^{3}H]forskolin binding to human platelet membranes and rat brain membranes,[2] although they are less potent than forskolin. The inactive analog of forskolin, 1,9-dideoxy-BrAcFsk, was synthesized as a control and did not activate adenylate cyclase or inhibit the binding of [^{3}H]forskolin.

Pretreatment of intact platelets with either ClAcFsk or BrAcFsk led to an irreversible loss of forskolin binding sites. This loss of binding was time dependent and concentration dependent. The loss of binding occurred much faster with BrAcFsk than with ClAcFsk, consistent with the greater reactivity of the bromoacetyl group as compared with the chloroacetyl group. The concentration dependence for the loss of binding was similar to the concentration dependence for the activation of adenylate cyclase, suggesting that the two actions may be related. The loss of binding was not due to nonspecific alkylation, because pretreatment of intact human platelets with 1,9-dideoxy-BrAcFsk did not produce an irreversible loss of binding sites.

The irreversible loss of binding sites caused by BrAcFsk and ClAcFsk could be partially prevented by the presence of forskolin during the pretreatment. This protection was observed at short incubation times with BrAcFsk and was difficult to observe when platelets were incubated for longer times. Protection of labeling with chemical affinity labels is sometimes difficult to observe because of the long-lived nature of the alkylating species.

The irreversible loss of [^{3}H]forskolin binding sites as a result of treatment with ClAcFsk or BrAcFsk requires an active forskolin analog and a reactive group. Thus, there is no decrease in binding sites in membranes from platelets that were pretreated with forskolin (data not shown). Therefore, the loss of binding sites is not due to a noncovalent association of the forskolin analogs with the binding sites, which would produce an apparent decrease in binding as a result of competition. The loss of binding also requires an active forskolin analog, inasmuch as the inactive α-haloketone analog of forskolin 1,9-dideoxy-BrAcFsk does not lead to a loss of binding sites. These results indicate that the alkylating analogs of forskolin are probably binding at the forskolin binding site, reacting with an active nucleophilic group at the site, and blocking the site. However, the high affinity binding sites for forskolin appear to require the G_s protein and, therefore, the alkylating groups could be covalently modifying the G_s protein and inhibiting its interaction with the catalytic subunit. It is, therefore, premature to conclude that the loss of binding sites is due to a covalent modification of the catalytic subunit of adenylate cyclase.

It is very surprising that pretreatment of platelets with BrAcFsk inhibited adenylate cyclase activity in the presence of GTP-γ-S, AlF_4, and PGE_1. These results indicate that BrAcFsk has a marked inhibitory effect on adenylate cyclase activity, which is stimulated by agents acting through the G_s protein. However, it is intriguing that treatment of platelets with BrAcFsk under conditions that eliminate about 80% of the high affinity forskolin binding sites had no effect on the direct stimulation of adenylate cyclase by forskolin. It has been suggested that forskolin may have two binding sites on adenylate cyclase (9). One site is associated with the synergistic stimulation of adenylate cyclase by forskolin and hormones and has a high affinity for forskolin. A second site would be responsible

[2] Unpublished data.

for the direct activation of adenylate cyclase by forskolin and would have a lower affinity for forskolin. The site responsible for the synergistic interactions of forskolin might be associated with the high affinity binding sites measured using a filtration binding assay. These high affinity sites are dependent on the presence of the G_s protein (10–13). BrAcFsk might be binding to this site and alkylating a region on the catalytic subunit that is important for interacting with the G_s protein. BrAcFsk and ClAcFsk can bind at the low affinity site responsible for direct activation; however, this site might not have a reactive nucleophilic group necessary for the covalent reaction of the α-haloketone. However, the data are also compatible with adenylate cyclase having only one site for forskolin. BrAcFsk could be binding to this site located at an interface between G_s and the catalytic subunit and alkylating an essential residue on G_s (or the catalytic subunit). Alkylation of this residue would inhibit both the high affinity binding of forskolin and the activation of the catalytic subunit of G_s but could leave the forskolin binding site intact for low affinity activation at adenylate cyclase.

Many of the components of the adenylate cyclase enzyme complex have been identified and cloned. Proteins covalently labeled with alkylating derivatives of forskolin can be identified by immunodetection with antiforskolin antibodies.[3] These studies should allow an identification of the specific proteins associated with adenylate cyclase that are labeled with alkylating derivatives of forskolin as well as the specific binding sites for these agents.

Conclusion

Alkylating analogs of forskolin have been synthesized and are effective in activating adenylate cyclase and inhibiting [^{3}H] forskolin binding to human platelet membranes. These analogs irreversibly inhibit the high affinity binding of [^{3}H]forskolin, probably by alkylation of a sulfhydryl group or other reactive nucleophilic group. These compounds should prove useful in identifying the protein(s) associated with adenylate cyclase that interact with forskolin, as well as with other proteins that are unrelated to adenylate cyclase but that bind forskolin.

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[3] McHugh-Sutkowski and Seamon, unpublished data.

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