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# APROPHIT: AN IRREVERSIBLE ANTAGONIST FOR MUSCARINIC RECEPTORS*

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**Abstract**—The development of selective irreversible ligands has proven to be an invaluable technique for the isolation, purification and characterization of many receptor proteins. An isothiocyanato-derivative of the muscarinic antagonist aprophen was synthesized and evaluated as a potential irreversible ligand for muscarinic receptors. This compound (aprophit) displaced [$^3$H]*N*-methylscopolamine binding from rat cerebral cortex with a $K_i$ of $3.1 \times 10^{-7}$ M. The inhibition was concentration-dependent and could not be reversed by extensive washing. Aprophit inhibited the acetylcholine-stimulated release of catecholamines from isolated, perfused guinea pig adrenal glands in a concentration-dependent manner. This inhibition was not reversed by perfusing the tissue with Locke's solution and was not due to a non-selective acylation by the isothiocyanate function. The data suggest that aprophit is selectively acylating muscarinic receptor proteins and thus may be useful in their further characterization.

Structural studies and purification of the muscarinic receptor have been performed using site selective alkylating agents [1]. These ligands have been based on known muscarinic agonists and antagonists modified with functional groups that are capable of forming a covalent bond with an amino acid residue on or near the active site of the receptor protein. [$^3$H]Propylbenzylcholine mustard ([$^3$H]PCBM)§ was first used as an affinity alkylating agent to identify the polypeptide containing the acetylcholine (ACh) binding site [2, 3]. A single polypeptide was identified in rat, guinea pig and frog brain, in guinea pig intestinal smooth muscle [2, 3], and in porcine atrium [4] using this ligand. More recently, PCBM has been used to purify muscarinic receptors from rat forebrain, and the acidic residue in the transmembrane helix 3 has been identified as the site of covalent interaction [5]. A monoclonal antibody raised against the muscarinic ACh-affinity alkylated PCBM has allowed the immunoprecipitation of a portion of the muscarinic receptor that is at or near the ligand binding site [6]. Additionally, photoaffinity ligands have also been used successfully for the characterization of muscarinic receptors [7, 8].

Another approach to affinity ligands is chemically modifying a receptor selective agent with an isothiocyanate function. The ease of synthesis, stability of the agents, and their high reactivity toward amino and sulfhydryl groups [9, 10] play a major role in their successful application as affinity ligands. Moreover, these compounds do not react with hydroxyl groups and thus are stable in aqueous medium and do not have to be activated as do the mustards and photoaffinity labels. The development of irreversible ligands with the isothiocyanate function has been valuable in the isolation, purification and characterization of opiate [11–14], benzodiazepine [15–18] and phencyclidine [19] receptor systems.

Aprophen [$\alpha$-methyl-$\alpha$-phenylbenzeneacetic acid-2-(diethylamino)ethyl ester] is a potent antispasmotic and cholinolytic agent that has been used prophylactically for the treatment of nerve gas poisoning [20, 21]. Aprophen has been reported to displace [$^3$H]quinuclidinebenzilate ([$^3$H]QNB) and [$^3$H]*N*-methylscopolamine ([$^3$H]NMS) binding to N4TG1 neuroblastoma cells and NG108-15 neuroblastoma × glioma cells [22, 23]. In addition, the binding of aprophen to muscarinic receptors has been correlated with $ED_{50}$ values for the inhibition of carbachol-induced $\alpha$-amylase release from pancreatic acini cells and with $K_i$ values for inhibition of ACh-induced contractions in the guinea pig ileum [23]. Recently, carbamylation on one or both of the aromatic rings of aprophen has resulted in muscarinic receptor antagonists with anticholinesterase activity [24]. An aprophen analog with an isothiocyanate function in one of the aromatic rings (aprophit, see Fig. 1) was prepared, therefore, as a potential irreversible muscarinic ligand for the study of its possible interactions with muscarinic receptors.

## MATERIALS AND METHODS

*Chemistry*. 4′-Aminoaprophen was synthesized in one step from methyl-2-phenyl-2-(4-aminophenyl)-propionate by a general method described recently

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§ Abbreviations: PCBM, propylbenzylcholine mustard; ACh, acetylcholine; NMS, *N*-methylscopolamine; QNB, quinuclidinebenzilate; IR, infrared spectrum; $^1$H-NMR, nuclear magnetic resonance spectrum; m, multiplet; s, singlet; t, triplet; MS, mass spectrum; M+1, mass plus 1; *m/z*, mass to charge ratio; KCl, potassium chloride, KNCS, potassium isothiocyanate; and nH, Hill coefficient.

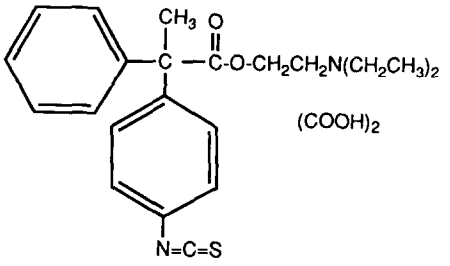


Fig. 1. Chemical structure of aprophit.

[24]. Racemic aprophit was prepared from 4'-aminoaprophen (120 mg, 0.35 mmol) using an established procedure [15] involving treatment with thiophosgene (40 μL, 0.24 mmol) in a biphasic chloroform (7.5 mL)/aqueous sodium bicarbonate (100 mg/3.0 mL) system. The crude product was isolated by extraction and purified by column chromatography (silica gel, chloroform: methanol, 95:5) to give 130 mg (97%) of the product as a golden oil. Treatment of the free base with oxalic acid and recrystallization in acetone/ether gave 110 mg (69%) pure aprophit as the oxalate salt. IR (potassium bromide) (broad 2100 $cm^{-1}$); $^1$H-NMR (deuterochloroform) δ7.32–7.13 (m, 9H), δ4.22 (t, 2H, $J$ = 6 Hz), δ2.63 (t, 2H, $J$ = 6 Hz), δ2.49 (q, 4H, $J$ = 7 Hz) δ1.90 (s, 3H) δ0.95 (t, 6H, $J$ = 7 Hz); MS (chemical ionization) M+1 383 $m/z$.

*Radioligand binding assay*. Male Charles River rats (Wilmington, MA) weighing 200–250 g were killed by decapitation, and brains were rapidly removed and placed into ice-cold 50 mM potassium phosphate (pH 7.4) buffer. Cerebral cortices were dissected and homogenized in 50 vol. (v/w) buffer and centrifuged twice at 20,000 $g$, at 4°. The final pellet was resuspended in 50 vol. of buffer and used for the binding assays. In some experiments, the tissue suspensions were incubated with aprophit at various concentrations, for 5–60 min at 25°, and then the tissue was washed four times by centrifugation as described above. The tissue was diluted further to a final suspension in 300 vol. of buffer. Control tissues were treated in a parallel fashion without the addition of aprophit. Binding experiments were carried out at a total volume of 1 mL consisting of 0.1 mL [$^3$H]NMS (New England Nuclear, Boston, MA; sp. act. 72 Ci/mmol), 0.1 mL drug, 0.25 mL of tissue suspension (50–75 mg protein), and the appropriate volume of buffer. Tubes were incubated at 25° for 60 min, and the incubation was terminated by rapid filtration using a Brandel MR-24 cell harvester (Gaithersburg, MD) with Whatman GF/B filters, and two 5-mL washes with ice-cold buffer. Nonspecific binding was determined in the presence of 10 μM atropine. Radioactivity was measured using a Packard scintillation spectrophotometer model 2000 CA/LL. The $IC_{50}$ values and Hill coefficients were determined by the GRAFCAL IBM-PC program (D. Kaplan, Israel Institute for Biological Research, Israel). The $K_i$ values were derived from the $IC_{50}$ values by correcting for receptor occupancy [25] by the [$^3$H]NMS; the $K_D$ value in cerebral cortex was 0.48 nM. All experiments were performed in triplicate.

*Adrenal perfusion assay*. Male guinea pigs, weighing 400–500 g, were anesthetized with sodium pentobarbital (60 mg/kg) intraperitoneally. Both adrenal glands were isolated and perfused with a modified Locke's solution through a cannula inserted into the abdominal aorta below the renal artery, at a flow rate of 0.6 mL/min, at 25° [26]. The adrenal perfusate was drained through a cannula inserted into the posterior vena cava below the renal vein and collected continuously in 5-min aliquots, in test tubes cooled on ice. The administration of drugs began 30–40 min after isolation of the adrenal glands. The samples were acidified with 8 M acetic acid to a final concentration of 0.4 M and stored on ice until assayed.

The experimental design consisted of 2-min infusions of ACh ($10^{-5}$ M) at 15-min intervals. Values for ACh-stimulated catecholamine secretion (norepinephrine and epinephrine) represent the 5-min period beginning 30 sec after ACh infusion minus the level of secretion for 5 min before infusion of ACh. We have demonstrated previously that catecholamine secretion to the second and third infusion of ACh was 93 and 88% of the first (control) response to ACh [27]. Various concentrations of the antagonist were infused for 15 min subsequent to the control response (see Fig. 3). To estimate the net effect of each antagonist, the response to ACh in the presence or following washout of the inhibitory drug was corrected for the expected decline in secretion seen on repeated administration of ACh and then compared with the control response for the experiment. The second response to ACh represents the effect of the antagonist, while the third response demonstrates reversibility or irreversibility. All experiments were repeated at least twice, and the results for each dose of antagonist tested varied less than 10% KCl (56 mM) was infused at the end of each experiment to ensure that the chromaffin cells remained functionally responsive.

Total catecholamine release in each sample was obtained by a standard fluorometric method [28] and verified by high performance liquid chromatography with electrochemical detection [29].

*Materials*. Aprophen [22] was synthesized in the Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC; and azaprophen [30] was synthesized at Research Triangle Park, NC. Atropine, pirenzepine hydrochloride, ACh, norepinephrine, and epinephrine were obtained from the Sigma Chemical Co., St. Louis, MO.

## RESULTS

*Radioligand binding studies*. Substitution in one of the phenyl rings of aprophen with an isothiocyanate group resulted in a diminished affinity for muscarinic receptors, as determined in the rat cerebral cortex. The $K_i$ values of aprophen and aprophit were $2.12 \times 10^{-8}$ M and $3.11 \times 10^{-7}$ M respectively. Under the same conditions, other muscarinic ligands tested demonstrated high affinity for these receptors, as previously reported [31] (Table 1). In tissues preincubated with $10^{-6}$ M aprophit, the $K_i$ of aprophen

Table 1. Effects of muscarinic antagonists on the binding of [$^3$H]NMS to rat cerebral cortex

| Antagonist | $K_i$ (M) | nH* | N |
|---|---|---|---|
| Aprophen | $2.12 \pm 0.34 \times 10^{-8}$ | $1.01 \pm 0.08$ | 6 |
| Aprophit | $3.11 \pm 0.37 \times 10^{-7}$ | $0.93 \pm 0.16$ | 6 |
| Atropine | $3.18 \pm 0.02 \times 10^{-9}$ | $1.33 \pm 0.19$ | 3 |
| Pirenzepine | $3.21 \pm 0.35 \times 10^{-7}$ | $0.65 \pm 0.05$ | 4 |

Data are means ± SE. Binding experiments were carried out as described in Materials and Methods with a final ligand concentration of 1 nM.
* Hill coefficient.

was $1.69 \pm 0.23 \times 10^{-7}$ M, with a Hill coefficient of $1.07 \pm 0.04$ (N = 3). Incubating cerebral cortical membranes in the presence of $2.5 \times 10^{-7}$ M aprophit at 25° for 30 min caused a reduction of [$^3$H]NMS binding to 38% of control. Preincubating the tissue with $10^{-7}$ M aprophit resulted in a reduction of [$^3$H]NMS binding to 45% of control. After incubating cerebral cortical membranes in the presence of $10^{-7}$ M aprophit with $10^{-6}$ M atropine, a reduction of [$^3$H]NMS binding to 42% of control was observed. The marked inhibition of binding was persistent following extensive (× 4) washing of the tissue. A time course was performed with $10^{-6}$ M aprophit. The percent inhibition observed at the following time points was: 27.3 ± 2.7% at 20 min, 26.8 ± 1.5% at 10 min, and 27.7 ± 1.7% at 5 min. Complete inhibition took place by the first measured time point and remained constant thereafter; thus, essentially no measurable time course of inhibition could be obtained under standard incubation conditions.

In another set of experiments, the binding of [$^3$H]flunitrazepam to cortical membranes, preincubated with aprophit ($10^{-6}$ M) for 30 min, was examined. Under these conditions, aprophit had no effect on central benzodiazepine receptors (results not shown).

*Inhibitory effect of aprophit on ACh-induced catecholamine secretion.* Catecholamine secretion from isolated, perfused guinea pig adrenal glands, stimulated by $10^{-5}$ M ACh or lower, has been shown to be mediated primarily by muscarinic receptors [29]. Therefore, the effects of aprophit and other muscarinic antagonists were studied at this concentration of ACh. As shown in Fig. 2, aprophit inhibited ACh-induced catecholamine release in a concentration-dependent manner with an $EC_{50}$ of $1.2 \times 10^{-6}$ M compared to the parent compound aprophen, which had an $EC_{50}$ of $2.1 \times 10^{-7}$ M. The muscarinic antagonists atropine (Fig. 3) and pirenzepine (Fig. 4) caused an inhibition of ACh-induced catecholamine secretion that was concentration-dependent and reversed upon further perfusion with Locke's solution. The ability of aprophit to inhibit ACh-induced catecholamine release persisted after washing for 10 min with Locke's solution, as shown in Fig. 5. Prolonged inhibition of the ACh-induced catecholamine release was also observed with structurally similar aprophen and azaprophen [30], as seen in Figs. 6 and 7, respectively. However, the inhibition by aprophen and azaprophen was reversed after 25 min of perfusion with Locke's solution. Additional experiments with aprophit ($10^{-5}$ M) showed that despite greater than 30 min of perfusion with Locke's solution, the ACh-induced response did not return, i.e. 0.49 nmol/5 min, (16%) compared to control response of 2.64 nmol/5 min and a first response to aprophit of 0.64 nmol/5 min (13%). However, the tissue was still functionally responsive as demonstrated by its response to KCl (3.10 nmol/5 min, 118%). The irreversible antagonism of this response by aprophit was not due to a non-specific acylation by the isothiocyanate function since $10^{-4}$ M KNCS had no effect (data not shown).

## DISCUSSION

An isothiocyanato-derivative of aprophen was synthesized as a potential irreversible ligand for muscarinic receptors. This compound, referred to as aprophit, was prepared in two steps from methyl-2-phenyl-2-(4-aminophenyl)propionate [24] and purified as the oxalate salt. Aprophit was tested in radioligand binding studies to determine its affinity for muscarinic receptors labeled with [$^3$H]NMS in rat cerebral cortex. Aprophit inhibited the binding of [$^3$H]NMS with a $K_i$ value of $3.11 \times 10^{-7}$ M. This represents a 14.7-fold decrease in affinity to the muscarinic receptors compared to the parent compound, aprophen ($K_i$ $2.12 \times 10^{-8}$ M). However, unlike the reversible binding of aprophen to muscarinic receptors, the inhibition of [$^3$H]NMS binding by aprophit could not be reversed by extensive washing of the issue. The inhibition was concentration-dependent, and aprophit caused a significant reduction in aprophen and atropine binding to muscarinic receptors labeled with [$^3$H]NMS. In addition, $10^{-5}$ M aprophit did not displace [$^3$H]flunitrazepam binding from central benzodiazepine receptors, further substantiating that this is a selective muscarinic receptor interaction. No measurable time course of inhibition could be obtained under standard incubation conditions, suggesting that the reaction leading to the irreversible complex between aprophit and the protein may be too fast to be detected due to the washing techniques employed. The inability to measure a time course of inhibition corresponds to another reported time study using an isothiocyanate agent [16]. These results suggest that aprophit is acylating the muscarinic receptor and forming a covalent bond between the isothiocyanate function of the ligand and a primary amino or sulfhydryl group on the receptor protein.

In the isolated guinea pig adrenal perfusion assay, aprophit irreversibly inhibited ACh-induced catecholamine release in a concentration-dependent manner with $EC_{50}$ value of $1.2 \times 10^{-6}$ M. Similar to the radioligand binding experiments, this value represents a 5.7-fold decrease in potency compared to the parent ligand, aprophen ($EC_{50}$ $2.1 \times 10^{-7}$ M). This inhibition could not be reversed despite an extended perfusion of Locke's solution of over 25 min. Prolonged inhibition of the ACh-induced catecholamine release was also observed after washout of the diphenyl substituted muscarinic antagonists aprophen and azaprophen, but this inhibition

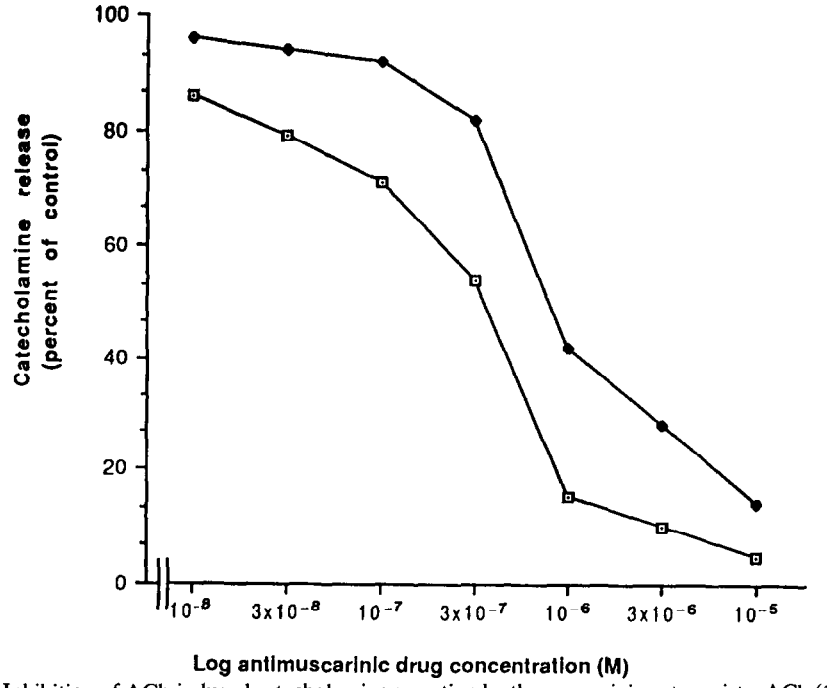


Fig. 2. Inhibition of ACh-induced catecholamine secretion by the muscarinic antagonists. ACh ($10^{-5}$ M) was sequentially applied for 2 min at 15-min intervals in the presence and absence of various concentrations of aprophen (-□-) and aprophit (-◆-). Mean values of at least two separate experiments are shown. The ordinate scale represents the percent of control values (control catecholamine responses varied from 1.39 to 4.44 nmol/5 min; all other values are proportional to this). The abscissa scale represents the concentration of muscarinic antagonists on a logarithmic scale.

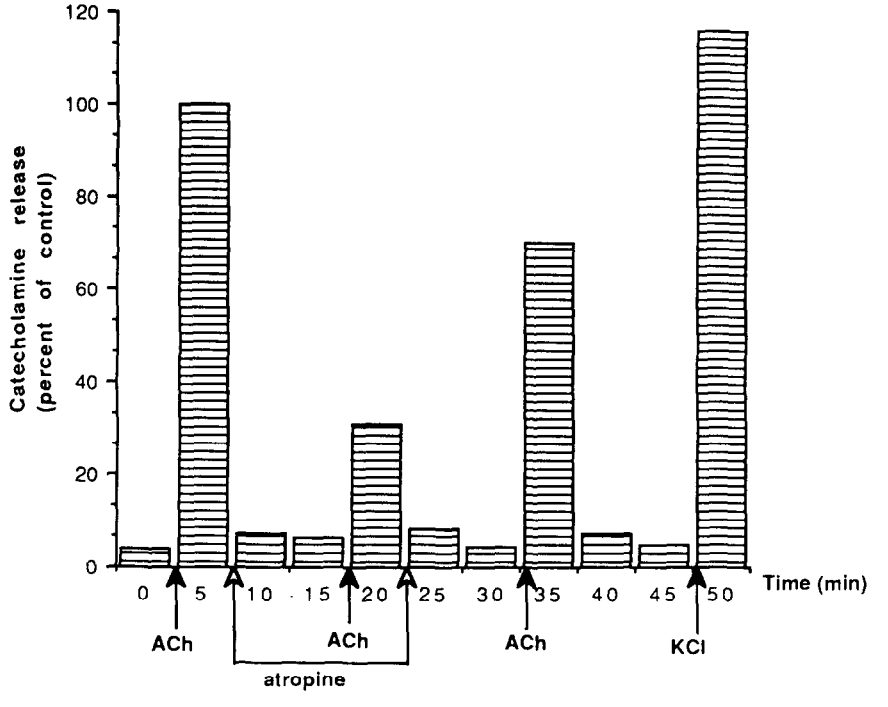


Fig. 3. Representative inhibitory effect of atropine on ACh-induced catecholamine secretions. ACh ($10^{-5}$ M) was sequentially infused three times for 2 min at 15-min intervals. Atropine ($10^{-8}$ M) was added from the end of the first response to the end of the second response. Columns represent percent of control catecholamine release evoked by ACh (as described in Materials and Methods). The absolute values for catecholamine release (nmol/5 min) are: 0.01, 2.34, 0.16, 0.15, 0.69, 0.17, 0.10, 1.26, 0.12, 0.09, and 2.73. KCl (56 mM) was infused after the last 15-min interval to ensure that the chromaffin cells remained functionally responsive.

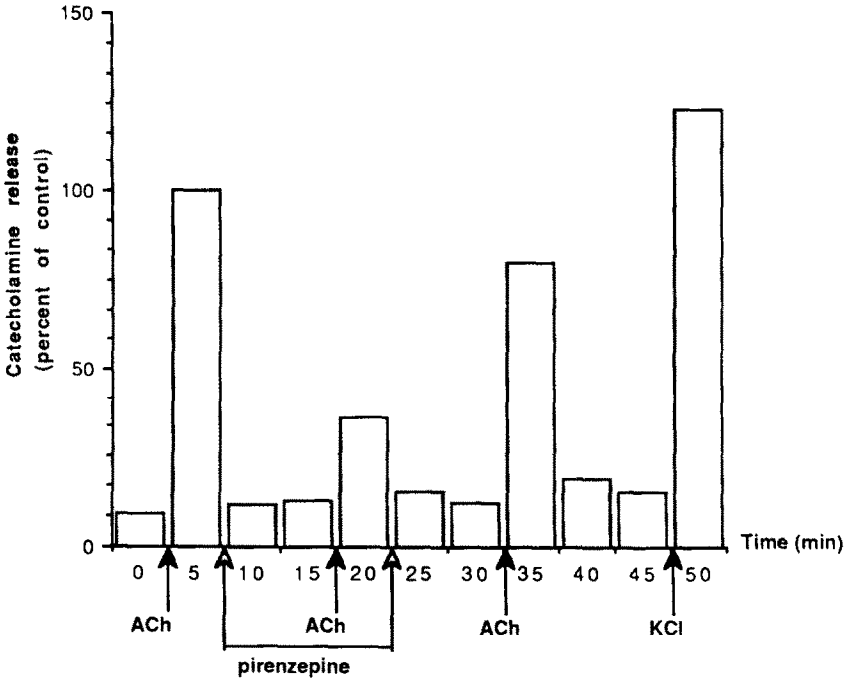


Fig. 4. Representative inhibitory effect of pirenzepine on ACh-induced catecholamine secretions. ACh ($10^{-5}$ M) was sequentially infused three times for 2 min at 15-min intervals. Pirenzepine ($10^{-7}$ M) was added from the end of the first response to the end of the second response. Columns represent percent of control catecholamine release evoked by ACh (as described in Materials and Methods). The absolute values for catecholamine release (nmol/5 min) are: 0.10, 1.14, 0.13, 0.14, 0.39, 0.16, 0.13, 0.70, 0.16, 0.13, and 1.41. KCl (56 mM) was infused after the last 15-min interval to ensure that the chromaffin cells remained functionally responsive.

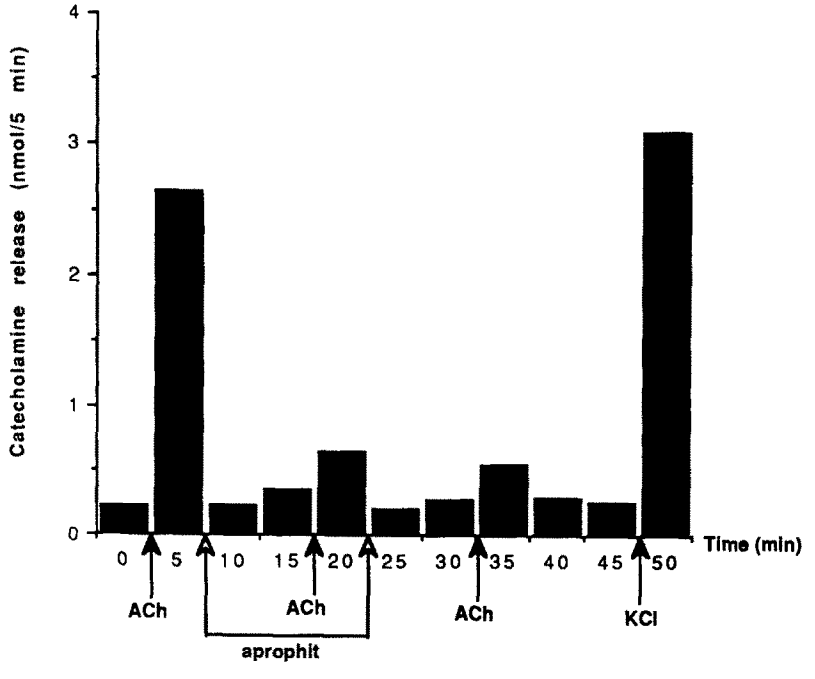


Fig. 5. Representative inhibitory effect of aprophit on ACh-induced catecholamine secretions. ACh ($10^{-5}$ M) was sequentially infused three times for 2 min at 15-min intervals. Aprophit ($10^{-5}$ M) was added from the end of the first response to the end of the second response. Columns represent catecholamine (norepinephrine and epinephrine) release (nmol/5 min) evoked by ACh. KCl (56 mM) was infused after the last 15-min interval to ensure that the chromaffin cells remained functionally responsive.

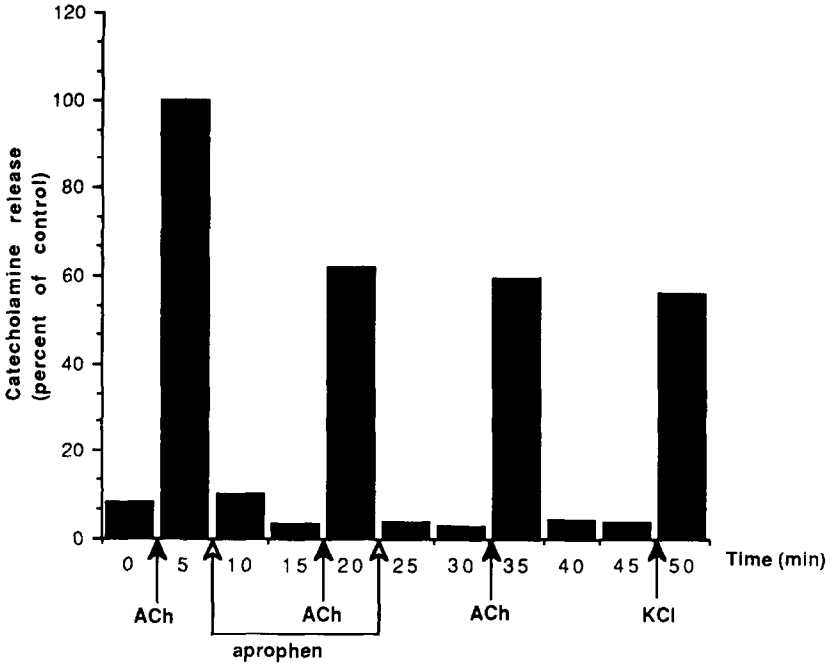


Fig. 6. Representative inhibitory effect of aprophen on ACh-induced catecholamine secretions. ACh ($10^{-5}$ M) was sequentially infused three times for 2 min at 15-min intervals. Aprophen ($10^{-6}$ M) was added from the end of the first response to the end of the second response. Columns represent percent of control catecholamine release evoked by ACh (as described in Materials and Methods). The absolute values for catecholamine release (nmol/5 min) are: 0.35, 4.66, 0.46, 0.16, 2.75, 0.19, 0.15, 2.11, 0.16, 0.13, and 2.63. KCl (56 mM) was infused after the last 15-min interval to ensure that the chromaffin cells remained functionally responsive.

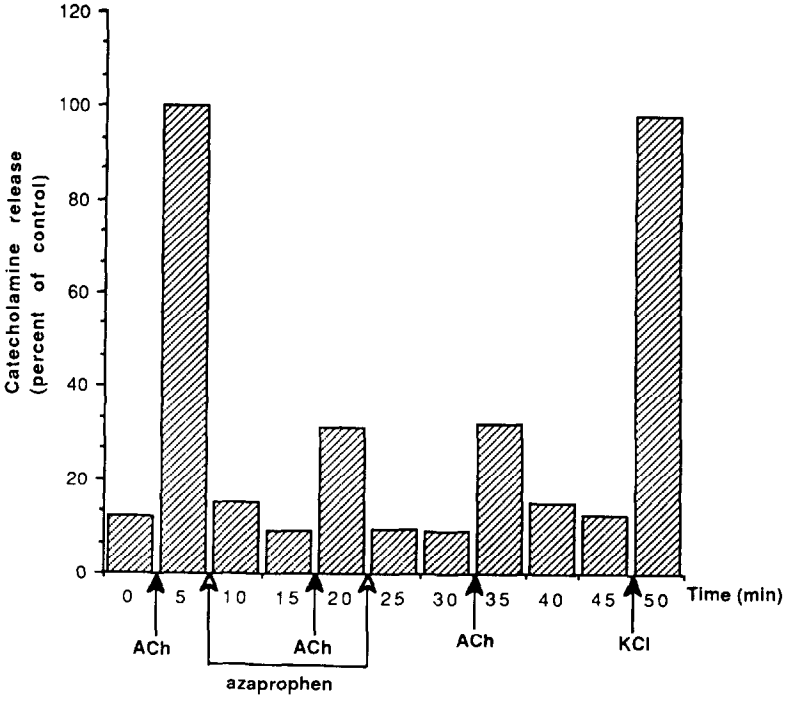


Fig. 7. Representative inhibitory effect of azaprophen on ACh-induced catecholamine secretions. ACh ($10^{-5}$ M) was sequentially infused three times for 2 min at 15-min intervals. Azaprophen ($10^{-8}$ M) was added from the end of the first response to the end of the second response. Columns represent percent of control catecholamine release evoked by ACh (as described in Materials and Methods). The absolute values for catecholamine release (nmol/5 min) are: 0.25, 2.07, 0.31, 0.19, 0.61, 0.19, 0.18, 0.50, 0.24, 0.20, and 2.04. KCl (56 mM) was infused after the last 15-min interval to ensure that the chromaffin cells remained functionally responsive.

was reversed after 25 min of perfusion with Locke's solution. The muscarinic antagonists atropine and pirenzepine, compounds without diphenyl substitution showed reversible inhibition of ACh-induced catecholamine release, after the standard 10-min perfusion with Locke's solution. These data suggest that the lipophilic nature of the diphenyl substituted muscarinic antagonists results in prolonged inhibition of ACh-induced catecholamine secretion in guinea pig adrenal glands, but that the covalent bond formed between the isothiocyanate function of aprophit and an amino acid residue on or near the muscarinic receptor protein causes an irreversible inhibition that cannot be washed out.

Aprophit represents a novel, irreversible ligand for the study of muscarinic receptors. Results from radioligand binding experiments and the isolated guinea pig adrenal perfusion assay indicate that aprophit is selectively acylating muscarinic receptors. This compound may be useful in further characterizing muscarinic receptors.

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