

# On the Inhibition of Muscle Membrane Chloride Conductance by Aromatic Carboxylic Acids

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**ABSTRACT** 25 aromatic carboxylic acids which are analogs of benzoic acid were tested in the rat diaphragm preparation for effects on chloride conductance ( $G_{Cl}$ ). Of the 25, 19 were shown to reduce membrane  $G_{Cl}$  with little effect on other membrane parameters, although their apparent  $K_1$  varied widely. This inhibition was reversible if exposure times were not prolonged. The most effective analog studied was anthracene-9-COOH (9-AC;  $K_1 = 1.1 \times 10^{-5}$  M). Active analogs produced concentration-dependent inhibition of a type consistent with interaction at a single site or group of sites having similar binding affinities, although a correlation could also be shown between lipophilicity and  $K_1$ . Structure-activity analysis indicated that hydrophobic ring substitution usually increased inhibitory activity while *para* polar substitutions reduced effectiveness.

These compounds do not appear to inhibit  $G_{Cl}$  by altering membrane surface charge and the inhibition produced is not voltage dependent. Qualitative characteristics of the I-V relationship for  $Cl^-$  current are not altered. Conductance to all anions is not uniformly altered by these acids as would be expected from steric occlusion of a common channel. Concentrations of 9-AC reducing  $G_{Cl}$  by >90% resulted in slight augmentation of  $G_T$ . The complete conductance sequence obtained at high levels of 9-AC was the reverse of that obtained under control conditions. Permeability sequences underwent progressive changes with increasing 9-AC concentration and ultimately inverted at high levels of the analog. Aromatic carboxylic acids appear to inhibit  $G_{Cl}$  by binding to a specific intramembrane site and altering the selectivity sequence of the membrane anion channel.

## INTRODUCTION

In skeletal muscle of many species the resting membrane ion conductance is predominantly that attributable to chloride (25, 37, 1). Although not directly involved in the genesis of an action potential, chloride conductance plays an important role in the maintenance of membrane potential stability. Recent studies in pathologically unstable muscle membranes have supported this concept (2, 38).

Myotonia, an example of such an unstable pathologic state, is a phenomenon characterized by repetitive action potential generation in the sarcolemma in

response to a brief stimulus (27). This phenomenon has been described in several naturally occurring diseases in man and animals (11, 27). In those that have been studied experimentally the repetitive activity is associated with an absolute reduction in membrane conductance to chloride (10, 30).

Myotonia can be produced in otherwise normal animals by various chemical agents, including inhibitors of cholesterol biosynthesis (40), certain herbicides (38), and in some species iodide (35). Many agents which reversibly produce a myotonic syndrome in normal animals are members of a class of substituted benzoic acids (15). In several cases these agents have been experimentally shown to reduce muscle membrane  $G_{Cl}$  under in vitro conditions (12).

We have previously reported the general characteristics of chloride conductance in the rat diaphragm (37), and evidence has been presented from this laboratory and others that a reduction in membrane  $G_{Cl}$  in muscle is sufficient grounds for repetitive electrical activity (6, 3, 8). In order to extend our understanding of chloride conductance mechanisms in normal and in pathologic states, we have studied in the rat diaphragm the mechanism of action of a series of aromatic acids similar to those known to produce myotonia in other species. We have documented that these compounds do indeed reversibly reduce membrane  $G_{Cl}$  and that direct correlations can be demonstrated between the physical properties of these analogs and their effectiveness in modifying chloride conductance.

Earlier reports have postulated that the observed reduction in membrane  $G_{Cl}$  produced by the benzoic acid derivatives is due to steric blocking of the anion permeation pathway (12). We show here, however, that this inhibition most probably involves a direct interaction with the anion permeation site in a manner affecting its ion selectivity rather than by steric blocking of these channels or by altering membrane surface charge.

#### MATERIALS AND METHODS

The majority of the 25 aromatic carboxylic acids studied were obtained from Aldrich Chemical Co., Milwaukee, Wis., from K & K Laboratories, Plainview, N. Y., or from Eastman Kodak Co., Rochester, N. Y. Several were the generous gift of Dr. R. B. Moffett of the Upjohn Pharmaceutical Co., Kalamazoo, Mich. Neuraminidase and phosphatidyl choline were obtained from Sigma Chemical Co., St. Louis, Mo.

Male Wistar rats of 200–300 g were used for all experiments. Ringer's solution with or without various concentrations of aromatic carboxylic acids was continuously perfused over sections of diaphragm which were obtained and handled as previously described (37). Temperature control, recording methods, and solution compositions were all as described in an earlier paper (37).

For routine determination of half-maximal blocking concentration ( $K_1$ ) several determinations of the percent of inhibition of  $G_{Cl}$  were made at three or more concentrations of each aromatic acid. For each determination the percent inhibition of  $G_{Cl}$  was calculated after three-point cable analysis of five or more fibers in each of the following solutions: (a) normal Ringer's; (b) normal Ringer's + carboxylic acid; and (c) chloride-free Ringer's + carboxylic acid. Control values for  $G_{Cl}$  were obtained by subtraction of an average  $G_K = 0.34$  mmho/cm<sup>2</sup> from the average  $G_m$  obtained in the control solution. Subtraction of the average  $G_m$  in solution (c) from that in solution (b) yielded a value for  $G_{Cl}$  in the presence of the carboxylic acid, allowing a percent inhibition to be calculated. Double reciprocal

plots of inhibition vs. analog concentration were then constructed and an observed  $K_1$  determined graphically.

Partition coefficients for the benzoic acids were calculated by summation of values for the benzoic acid nucleus and factors specific for the effect on partitioning of a substitution at a given location on the parent compound based on the experimental results and theoretical methods of Hansch and co-workers (24, 23, 19). Hammett  $\sigma$  values and values for steric hindrance were also obtained from the chemical literature (26, 32). (See Results for a discussion of these parameters.) Statistical analysis was performed by standard computer methods (36).

Electrophoretic mobilities of erythrocytes and liposomes were determined in a cylindrical microelectrophoresis cell 10 cm long with large volume electrode vessels at either end. The design was that of Bangham et al. (4). Constant temperature was maintained by immersion in a regulated water bath. Measurements were made with frequent reversals of field direction. Erythrocytes were prepared from human blood by dilution with 0.145 M NaCl and repeated centrifugation. Liposomes were prepared after the method of Bangham et al. (5). Phosphatidyl choline was dissolved in hexane-chloroform 1:10 and deposited on the sides of a round-bottom flask by flash evaporation. Liposomes were formed by the addition of 100  $\mu$ l of 0.145 M NaCl (pH 7.35) to the dried residue and sonication until vesicular formation was optimized.

#### RESULTS

Anthracene-9-COOH (9-AC) may be considered representative of the aromatic carboxylic acids studied in its effects on the rat diaphragm. The addition of  $10^{-5}$  M 9-AC to the Ringer's solution surrounding a section of diaphragm being maintained in vitro results in a rapid increase in the membrane resistance. This increase begins almost immediately and reaches a new steady value within 10 min. When normal Ringer's solution is reperfused over the diaphragm, membrane resistance gradually returns to near control levels over a 30–60-min period. When similar experiments are performed in methylsulfate-substituted chloride-free Ringer's, membrane resistance is unaffected by this aromatic acid at the same concentration, suggesting that the compound acts specifically to reduce the membrane conductance to chloride ( $G_{Cl^-}$ ).

When a complete series of steady-state conductance measurements was made on diaphragm fibers equilibrated in various concentrations (1–100  $\mu$ M) of 9-AC in normal Ringer's and  $Cl^-$ -free Ringer's, a sigmoid relationship could be demonstrated between the measured membrane  $G_{Cl}$  and the logarithm of the concentration of 9-AC (Fig. 1). When the average  $G_{Cl}$  at each 9-AC concentration is calculated and replotted according to the Hill equation, a diagnostic plot for binding characteristics, a linear relationship is obtained (Fig. 2). (9, see reference 31 for recent discussion). The Hill coefficient calculated from these data is not significantly different from one, suggesting that the inhibitory action of the anthracene derivative is mediated via the binding at a single class of noninteracting sites. A dissociation constant of  $1.1 \times 10^{-5}$  M can be calculated for the apparent ligand-site interaction. This constant may be more conveniently considered to be an inhibition constant for 9-AC with respect to measured  $G_{Cl}$  and for this and subsequent analogs will be referred to as  $K_1$ .

As would be anticipated from the nature of the Hill plot for the above data, the relationship between the percent inhibition of  $G_{Cl}$  and the concentration of 9-

AC is hyperbolic in nature, and a double reciprocal plot of data in this form is linear. A value for  $K_i$  identical to that obtained from the Hill plot can be determined more conveniently from this graph, and this method was used subsequently to evaluate the apparent inhibition constants with respect to  $G_{Cl}$  of 24 other related derivatives of the benzoic acid nucleus. For each analog determinations of percent inhibition were made at at least three analog concentrations (see Materials and Methods) and values of  $K_i$  determined graphically.

Significant reduction of membrane  $G_{Cl}$  was produced by 19 of these compounds. In all cases in which inhibition of chloride conductance was observed, a

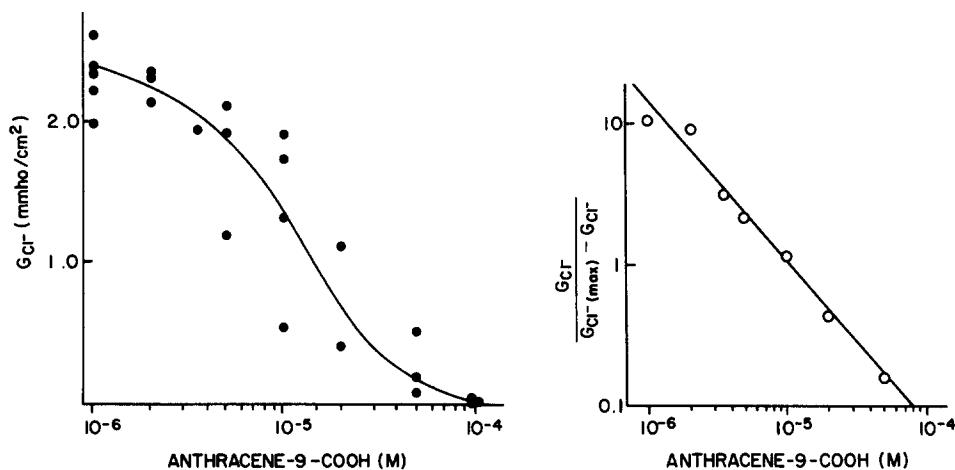


FIGURE 1. Membrane chloride conductance ( $G_{Cl}$ ) observed after incubation of rat diaphragm preparation in various concentrations of 9-AC for 30 min at 35°C. Each point at a given 9-AC concentration represents the average of fibers measured in a different diaphragm. Measurements were made in  $Cl^-$ -containing and  $Cl^-$ -free Ringer's solution for conductance calculations (see Materials and Methods).

FIGURE 2. The average values of  $G_{Cl}$  at each concentration of 9-AC from Fig. 1 are plotted according to the Hill equation. The average  $G_{Cl}$  of all preparations before addition of 9-AC was taken as  $G_{Cl(max)}$ . The Hill coefficient of the regression line to this data is not significantly different from one. The dissociation constant for the apparent 9-AC site complex calculated from this data is  $1.1 \times 10^{-5}$  M.

hyperbolic relationship between analog concentration and percent inhibition could be demonstrated and the plots of (1/percent inhibition) vs. (1/[aromatic acid]) were linear. Inhibition constants determined from the double-reciprocal plots are listed in Table I. It can be seen that the concentrations required to produce 50% inhibition of  $G_{Cl}$  varied among these compounds over a range of three orders of magnitude.

Resting membrane potential (RMP) changed little after exposure to 9-AC. Thus the average RMP of all fibers before exposure to 9-AC was  $70.3 \pm 5.2$  (SD) mV while fibers exposed to  $2 \times 10^{-6}$  M,  $1 \times 10^{-5}$  M, and  $5 \times 10^{-5}$  M 9-AC had average RMP of  $68.3 \pm 3.8$ ,  $67.8 \pm 4.9$ , and  $67.7 \pm 4.8$  mV, respectively. The small decrement observed may represent the inherent "rundown" of the dia-

phragm fibers at 35°C since all measurements on control fibers were carried out within the first 30 min of an experiment while determinations in 9-AC were made from 30 min to 2 h after dissection of the diaphragm. Overall, no correlation was noted between RMP and concentration of 9-AC in the perfusion medium over the range indicated in Fig. 1.

For each compound, change in residual conductance in chloride-free solution

TABLE I  
INHIBITION OF  $G_{Cl}$  IN RAT DIAPHRAGM BY AROMATIC ACIDS

Compound	$K_1(M)$	Relative potency	Calc. log P	$G_K$
Anthracene-9-COOH	$1.1 \times 10^{-5}$	909	0.26	0
2,4,5-Trimethyl benzoic acid	$6.6 \times 10^{-5}$	152	-0.47	+
Pentachloro BA	$8.0 \times 10^{-5}$	125	0.53	+
3,5-Dichloro-2-amino BA	$1.3 \times 10^{-4}$	76	-1.25	+
2,3,5,6-Tetrachloro BA	$2.1 \times 10^{-4}$	47	-0.35	+
3,5-Dichloro BA	$2.5 \times 10^{-4}$	40	-0.59	0
3,4-Dichloro BA	$2.6 \times 10^{-4}$	38	-0.63	0
2,5-Dichloro BA	$3.0 \times 10^{-4}$	33.3	-1.29	0
2,5-Dimethyl BA	$3.1 \times 10^{-4}$	32	-0.89	0
2,3,5,6-Tetramethyl BA	$6.0 \times 10^{-4}$	16.7	0.15	0
2,3,6-Trichloro BA	$9.5 \times 10^{-4}$	10.5	-1.17	0
3,5-Dimethyl BA	$1.5 \times 10^{-3}$	6.7	-1.21	0
2,4-Dimethyl BA	$1.6 \times 10^{-3}$	6.2	-1.14	+
3,6-Dichloro-2-nitro BA	$1.7 \times 10^{-3}$	6.0	-1.63	0
3,4-Dimethyl BA	$2.0 \times 10^{-3}$	5.0	-1.31	0
2,4-Dichloro phenoxyacetic acid	$2.0 \times 10^{-3}$	5.0	-1.56	?
2,6-Dichloro BA	$2.0 \times 10^{-3}$	5.0	-1.88	0
3,5-Dimethyl-4-nitro BA	$2.8 \times 10^{-3}$	3.6	-1.19	0
2,4,6-Trimethyl BA	$4.0 \times 10^{-3}$	2.5	-0.47	0
Benzoic acid (BA)	$>1.0 \times 10^{-2}$	<1.0	-2.21	0
4,5-Dichloro-2-COOH BA	$1.6 \times 10^{-2}$	0.63	-2.09	0
3,5-Dimethyl-4-amino BA	$>3.0 \times 10^{-2}$	<0.3	-2.40	-
2,5-Dimethyl benzene $SO_3H$	$>3.0 \times 10^{-2}$	<0.3	-4.87	0
2,3,5,6-Tetramethyl-4-COOH-BA	$>1.0 \times 10^{-1}$	<0.1	-1.62	0
3,5-Dimethyl-4-hydroxy BA	$>1.0 \times 10^{-1}$	<0.1	-1.51	0

The 25 aromatic acids tested listed in order of decreasing effectiveness in reducing  $G_{Cl}$  in the rat diaphragm. The  $K_1$  value represents analog concentration required for 50% inhibition of  $G_{Cl}$ . Relative potency is calculated assuming a potency of 1 for a compound having a  $K_1$  of  $1 \times 10^{-2}$  M. Calculated Log P is the logarithm of the octanol-water partition coefficient of the dissociated form of the acid calculated according to Hansch and Glave (24). Effects on residual conductances ( $G_K$ ) are noted in the final column.

(largely attributable to  $K^+$ ) was also evaluated. Most compounds tested had no effect on  $G_K$ , but several increased this residual conductance to a minor degree (see Table I). Those compounds reproducibly increasing  $G_K$ , however, usually did so only at concentrations well above their  $K_1$  for inhibition of  $G_{Cl}$ .

Several of these compounds have previously been shown to be reversible in their effects on conductance in goat muscle and in their in vivo effects on mice (12, 34). We have found that these compounds when tested in vitro on the rat diaphragm are usually reversible with sufficient washing after exposure at initial

concentrations which significantly reduce  $G_{Cl}$ . However, with higher concentrations and longer exposure times some analogs appear to be cytotoxic, and irreversible decreases in muscle resting potential occur. Table II indicates typical membrane conductances before, during, and after exposure of rat diaphragm fibers to 9-AC, demonstrating nearly complete reversal of inhibition after 30 min of wash. It can also be seen that when small test pulses are used during cable analysis, the direction of current flow has no discernible effect on the magnitude of the block in  $G_{Cl}$  observed.

#### Structure-Activity Correlations

A theoretical octanol-water partition coefficient was calculated for each of the 25 compounds tested after the method of Hansch and coworkers (19, 22, 24). These investigators have studied the partitioning characteristics of related benzoic acid derivatives, as well as other compounds, in an octanol-water system and have

TABLE II  
REVERSIBILITY OF AND EFFECT OF DIRECTION OF APPLIED CURRENT ON  
OBSERVED INHIBITION OF  $G_{Cl}$  BY ANTHRACENE-9-COOH

	$G_{Cl}$		Reduction of $G_{Cl}$ %	$G_{Cl}$ Recovery (wash-out) $mmho/cm^2$
	Control	Anthracene-9-COOH $10^{-5}$ M $mmho/cm^2$		
Hyperpolarizing pulses	$2.53 \pm 0.39$	$1.07 \pm 0.16$	58	$2.09 \pm 0.43$
Depolarizing pulses	$2.36 \pm 0.39$	$1.07 \pm 0.14$	54	$1.84 \pm 0.15$

$G_{Cl}$  values determined before and 30 min after exposure of diaphragm to  $1 \times 10^{-5}$  M 9-AC as well as 30 min after return to normal Ringer's. In each case either small hyperpolarizing or depolarizing pulses were used for conductance measurements. Significant recovery is noted during the washout period.

quantified the effects of various ring substituents on the relative lipophilicity of a given derivative (22). Values derived from this work for various substituents at each ring position allow a theoretical partition coefficient to be calculated for any related compound. These theoretical coefficients can be shown to correlate closely with subsequently determined experimental values. Such partition coefficients have since been used by numerous investigators in studies of structure-activity relationships in chemical and biological systems (7, 24).

Partition coefficients were calculated for the dissociated form of each of the analogs studied. Since all compounds tested had pK values two or more pH units below the test pH, more than 99% of each compound was present as the dissociated ionic species under our experimental conditions. Similar calculations for the undissociated forms yield a series of coefficients shifted toward more positive values by a constant amount for all compounds, and hence correlation analysis generates comparable results with either data set. The logarithm of the partition coefficient for the dissociation form of each analog is given in Table I. A simple linear correlation analysis was performed between Log P and relative potency for each of the 19 analogs producing quantifiable inhibition of  $G_{Cl}$  (Fig.

3). The correlation coefficient ( $R$ ) for the regression line calculated for these data was 0.77. Statistical analysis of the average deviation of experimental points from the calculated regression line in the correlation analysis revealed that the confidence with which a positive correlation between the two tested variables can be stated (confidence of correlation) is better than 0.001 (36). The values of 0.77 obtained for the regression coefficient, however, indicates that only 59% of the variance in potency observed between analogs can be explained by variations in their respective partition coefficients.

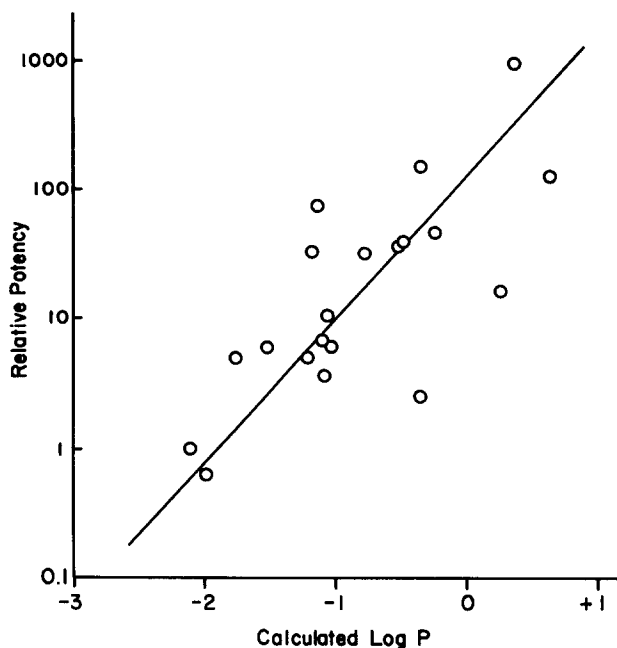


FIGURE 3. Correlation between relative potency of benzoic acid derivatives producing measurable inhibition of  $G_{Cl}$  and the calculated octanol-water partition coefficient for that derivative. Note that the scales extend over a range of four orders of magnitude. The regression coefficient for the line fitted to the data is 0.77.

In related structure-activity studies of aromatic acids which stimulate plant growth, it has been demonstrated that physical characteristics other than the partition coefficient can be important in determining activity (20). These factors include the Hammett  $\sigma$  value for each analog as well as parameters related to steric hindrance introduced by substituents at various ring positions. The Hammett  $\sigma$  value is a factor relating the relative dissociability of a functional group of an analog to that of the parent compound (reference 21, chapter 11). In the present case,  $\sigma$  for an analog indicates the dissociability of the carboxyl function relative to benzoic acid, and for each analog can be precisely calculated on the basis of electron orbital theory if the ring substituents are known. Hammett  $\sigma$  values for a wide range of small molecules have been determined (for example,

reference 32) and are widely used in structure-activity correlations for organic and bioorganic reactions as well as biologic phenomena (23; for a review of the Hammett  $\sigma$  function see reference 26).

Since differences in partition coefficients could not adequately account for all the variance in potency noted with our aromatic acids, a multivariate analysis incorporating these additional parameters was carried out. For this analysis, potency was considered to be the dependent variable and log P, Hammett  $\sigma$  value, and weighted values for steric hindrance of ring substituents at each position were considered as independent variables. Hammett  $\sigma$  values experimentally determined for these or similar compounds were obtained from the chemical literature (26, 24, 32). The coefficient of multiple regression of this data was 0.94 indicating that greater than 88% of the observed variance in potency between analogs could be explained by simultaneous contributions from the three variables considered. In this analysis, each of the variables is assumed to function independently and their effects on potency are assumed to be linear and noninteractional. Although these assumptions represent potential limitations on our analysis, several conclusions can be drawn. Statistical evaluation of the significance of the partial correlations obtained for each variable (an adaptation of the standard F test was used) suggest that only log P,  $\sigma$  value, and steric considerations at the *ortho* position contribute significantly to variance (reference 36, see especially pp. 260–310). Ring substitutions which result in a net positive Hammett  $\sigma$  value (carboxyl group electron-deficient relative to benzoic acid) usually increase potency. Bulky substituents attached *ortho* to the carboxyl group, especially when both *ortho* positions are occupied, usually decrease potency. The net effect of any set of substituents involves summation of all individual contributions.

It should be noted that substitution of a polar residue at the *para* position usually results in complete loss of inhibitory activity in spite of a partition coefficient which should not in itself preclude observable effect. Thus 2,3,4,6-tetramethyl benzoic acid has an observed  $K_1$  of  $6 \times 10^{-4}$  M, while the 4-carboxy-substituted tetramethyl derivative is without detectable effect on  $G_{Cl}$ . Similar loss of activity is seen with *para*-hydroxy and *para*-amino substitution (Fig. 4). Such compounds could not be included in the above statistical analysis because values for potency could not be defined.

The observed correlation between log P and potency suggests that the action of these analogs requires their partial or complete partitioning into the membrane hydrophobic phase before interaction with a specific site. A later ligand-site interaction can be inferred from the nature of the inhibition vs. concentration curve, and this interaction may be reflected in the dependency of potency on ring electron density and steric factors in the neighborhood of the carboxyl residue.

#### *Aromatic Acids and Surface Charges*

Since partitioning into the membrane appears to be of importance in the mechanism of action of these aromatic acids in the rat diaphragm, the possibility that their effect might be due to a nonspecific increase in outer membrane fixed



negative charge density was examined. The effect of various concentrations of 9-AC and 2,4,5-trimethyl benzoic acid (MBA) on the electrophoretic mobility of red cells and artificial liposomes was studied in a microelectrophoresis cell. The rates of migration of the cells or liposomes were observed under constant temperature at the critical depth of the cylindrical microcell. In each sample average rates of migration of a total of 20 particles were each measured before and after reversing the electrical field to minimize bulk flow errors. Concentra-

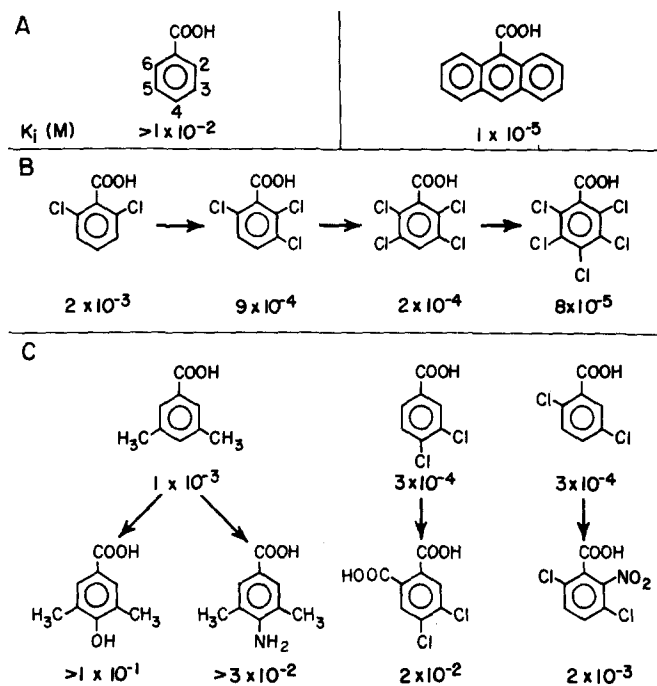


FIGURE 4. General relationships between derivative structure and  $K_1$ . A, Addition of hydrophobic residues at *ortho* and *meta* positions generally increases effectiveness with little dependence on group size. B, Successive halogenation or methylation of the ring usually results in a progressive increase in inhibitory efficacy. C, *Para* substitution of a polar residue abolishes inhibitory activity; *ortho* substitution of similar residues reduces activity in a manner partially related to the size of the substituting group.

tions ranging from 0.1 to 100 times the  $K_1$  for each acid were used. No change was noted in red blood cell (RBC) mobility at any concentration of 9-AC or 2,4,5-MBA studied. Further experiments were performed after treatment of RBC with neuraminidase to reduce their intrinsic surface charge, but again, no change in observed mobility was seen after equilibration with either compound. Liposomes prepared from phosphatidyl choline were examined after equilibration with each analog over the same concentration range. Again, no significant alteration in mobility reflecting a change in surface charge could be detected.

The effects of variations in  $[Ca^{++}]$  on membrane electrical phenomena have

been related to alterations in membrane surface charge produced by this cation (18, 33, 39). In membranes with a net negative surface charge, addition of  $\text{Ca}^{++}$  could be expected to reduce the effective surface charge experienced by other ions in the vicinity of the membrane (13, 14). Such a reduction might be reflected in an increased anion conductance. We have observed that in the rat diaphragm at pH 7.4 increasing external  $[\text{Ca}^{++}]$  from 2 mM to 10 mM increases the measured  $G_{\text{Cl}}$  by  $37\% \pm 6\%$  (average of five preparations). In seven preparations exposed to 20 mM external  $[\text{Ca}^{++}]$  under otherwise constant conditions,  $G_{\text{Cl}}$  was increased  $43\% \pm 8\%$ . These results are consistent with the presence of a fixed negative surface charge affecting anion flux. When these experiments were repeated in the presence of  $10^{-5}$  M 9-AC, the percent reduction in observed  $G_{\text{Cl}}$  was the same when external  $[\text{Ca}^{2+}]$  was 2 mM, 10 mM, and 20 mM, although the absolute magnitude of the  $G_{\text{Cl}}$  varied as described above. This observation again suggests that the inhibitory effect of this compound is not mediated by a change in membrane surface charge.

#### *Current-Voltage Relationships*

Current-voltage relationships were determined in a group of fibers of similar diameter under control conditions and in the presence of  $2 \times 10^{-5}$  M 9-AC. Values of membrane potential were determined 20 ms (early) and 700 ms (late) after onset of hyperpolarizing current pulses as previously described (37). Early I-V relationships were linear in the presence and absence of 9-AC; those in the presence of 9-AC were proportionately reduced at all voltage levels to about 30% of control values. Under control conditions I-V plots of late voltages showed marked deviation from linearity as previously described. In the presence of 9-AC this deviation was still apparent and present to about the same extent in relation to current amplitude (Fig. 5). Over the potential range extending to 70 mV in a hyperpolarizing direction from resting potential, no significant voltage dependence of the block produced by 9-AC could be detected.

#### *Anion Conductance Sequences*

A molecule which blocks an ion channel by sterically preventing entrance of usually permeant ions in a manner similar to the block produced by tetrodotoxin in the sodium channel should prevent the movement of all ions to which that channel is permeable. Since the normal anion conductance and permeability sequences have been documented in the rat diaphragm (37), the effects of various levels of aromatic carboxylic acids on these sequences was investigated.

The anion conductance sequence obtained in control fibers was  $\text{Cl}^- > \text{Br}^- > \text{NO}_3^- > \text{I}^-$ , the same as that reported earlier from this laboratory. 9-AC at  $8 \times 10^{-6}$  M reduced  $G_{\text{Cl}}$  by approximately 50%, but  $G_{\text{Br}}$  changed little while  $G_{\text{I}}$  actually appeared to increase. At  $5 \times 10^{-5}$  M 9-AC,  $G_{\text{Cl}}$  was reduced by more than 90% while  $G_{\text{I}}$  again appeared increased relative to control values. In the presence of  $5 \times 10^{-5}$  M 9-AC, the conductance sequence was completely reversed and was determined to be  $\text{I}^- > \text{NO}_3^- > \text{Br}^- > \text{Cl}^-$  (Table III).

Reduction in pH from 7.0 to 5.0 also reduces  $G_{\text{Cl}}$  by 50–60%, presumably by partial protonation of a site within the channel which is necessary for ion movement (37). When complete conductance sequence is determined under

these conditions, however, it is found that conductance to all ions has been reduced by approximately the same amount and that the overall conductance sequence remains unchanged.

Since we have shown previously that conductance and permeability sequences

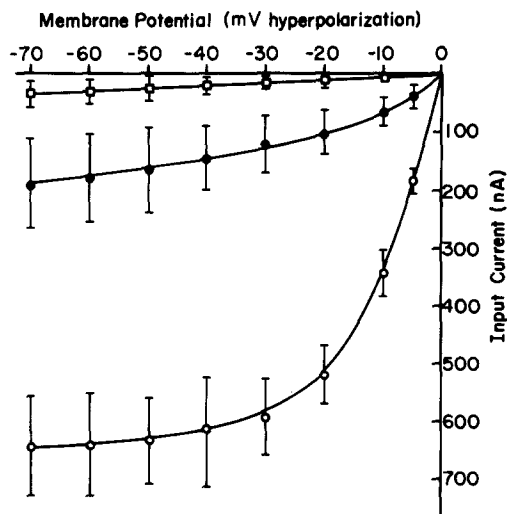


FIGURE 5. Current-voltage relationships for a group of fibers having similar diameters as determined in normal  $\text{Cl}^-$ ,  $\text{Rb}^+$  Ringer's ( $\circ$ );  $\text{Cl}^-$ -free,  $\text{Rb}^+$  Ringer's ( $\square$ ); and normal  $\text{Cl}^-$ ,  $\text{Rb}^+$  Ringer's containing  $8 \times 10^{-6}$  M 9-Ac ( $\bullet$ ) at pH 7.5. The nonlinearity normally observed in  $\text{Cl}^-$  current with membrane hyperpolarization is also present after exposure to 9-AC. No voltage-dependent changes in inhibition could be demonstrated. Early current-voltage relationships remained linear in all cases.

TABLE III  
ANION CONDUCTANCE SEQUENCE IN THE PRESENCE OF 9-AC

	$G_{\text{Cl}}$	$G_{\text{Br}}$	$G_{\text{NO}_3}$	$G_{\text{I}}$	Sequence
Control, pH 7.5	$2.35 \pm 1.3$	$0.49 \pm 0.1$	$0.33 \pm .1$	$0.27 \pm .09$	$\text{Cl} > \text{Br} > \text{NO}_3 > \text{I}$
$8 \times 10^{-6}$ M 9-AC	$0.89 \pm .16$	$0.31 \pm 0.17$	$0.52 \pm .14$	$0.60 \pm .2$	$\text{Cl} > \text{I} > \text{NO}_3 > \text{Br}$
$5 \times 10^{-5}$ M 9-AC	$0.10 \pm .08$	$0.26 \pm .07$	$0.30 \pm .05$	$0.39 \pm .07$	$\text{I} > \text{NO}_3 > \text{Br} > \text{Cl}$
Control, pH 5.0	$1.03 \pm .11$	$0.18 \pm .07$	$0.11 \pm .06$	$0.05 \pm .05$	$\text{Cl} > \text{Br} > \text{NO}_3 > \text{I}$

Conductance sequences obtained after equilibration of diaphragm with Ringer's containing the specified anion. Residual conductance in  $\text{MeSO}_4^-$  Ringer's has been subtracted in each case. Each value given as mean  $\pm$  SEM.

follow the same order in the rat diaphragm, and since relative permeability measurements can be made with more facility after introduction of a test acid than can conductance measurements, further experiments were carried out examining permeability sequences in the presence of these acids. Bi-ionic potential shift measurements were made with intracellular microelectrodes during rapid changes of extracellular anion composition before and after exposure to

varying concentrations of aromatic carboxylic acids. The most complete data were obtained again with 9-AC. In control solutions both  $I^-$  and  $Br^-$  were less permeable than  $Cl^-$ , with the usual sequence being  $Cl^- > Br^- > I^-$ . After exposure to  $5 \times 10^{-5}$  M 9-AC, the potential shift on changing to  $I^-$ -containing solution reversed sign, indicating that under these conditions the membrane is now more permeable to  $I^-$  than to  $Cl^-$  (Fig. 6). Increasing [9-AC] to  $2.5 \times 10^{-4}$  M resulted in both  $I^-$  and  $Br^-$  solutions producing a hyperpolarizing response, the overall permeability sequence now being inverted to  $I^- > Br^- > Cl^-$  in a manner analogous to that observed for the conductance sequence. Such sequence inversions could also be demonstrated in  $K^+$ -free medium with  $Rb^+$  substitution,

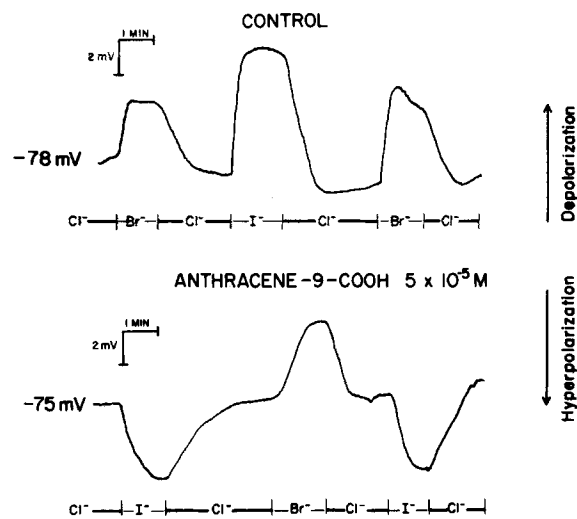


FIGURE 6. A, Continuous traces of membrane potential demonstrating depolarizing shifts when external  $Cl^-$  is rapidly replaced by  $Br^-$  or  $I^-$ . In each case return to normal  $Cl^-$  solution results in membrane repolarization. B, After exposure to 9-AC ( $5 \times 10^{-5}$  M) for 30 min, similar recordings in the same diaphragm demonstrate a hyperpolarization when  $I^-$  is rapidly substituted for  $Cl^-$  and depolarization when  $Br^-$  is substituted for  $Cl^-$ . Figures represent ink trackings of original records.

eliminating changes in  $K^+$  permeability as a determinant factor in the observed responses. Similar results in the presence of  $S_2O_3$  (42) (5 mM) rule out contribution from an  $I^-_3$  carrier system. 13 experiments were done comparing control permeability sequences to sequences obtained after equilibration in Ringer's solution containing 9-AC in concentrations between 1 and  $100 \times 10^{-5}$  M. The permeability sequences obtained are shown graphically in Fig. 7. A progressive and reproducible shift in sequence is seen with increasing concentrations of the aromatic acid. The same sequence inversions were observed with 2,4,5-trimethyl benzoic acid. Analogs with lower potencies produced partial sequence inversions in similar experiments, but concentrations in solution proportionately higher than their  $K_1$  could not be obtained due to their limited solubility.

Again, permeability sequences determined at reduced pH showed a pattern identical to that obtained at pH 7.

## DISCUSSION

In rat diaphragm, halogenated and methylated derivatives of benzoic acid increase membrane resistance by specifically reducing membrane conductance to  $\text{Cl}^-$ . Such a reduction in  $G_{\text{Cl}}$  has previously been suggested as the primary physiologic effect of several of these compounds (12) of which 2,4-dichlorophenoxyacetic acid (2,4-D) is the best known example (38). This behavior is now shown to be characteristic of this class of compounds in general. In vivo studies have demonstrated a transient myotonic syndrome in mice associated with a single injection of analogs similar to those tested here (34). We confirm that the effects of these compounds on  $G_{\text{Cl}}$  in vitro is indeed reversible if the concentra-

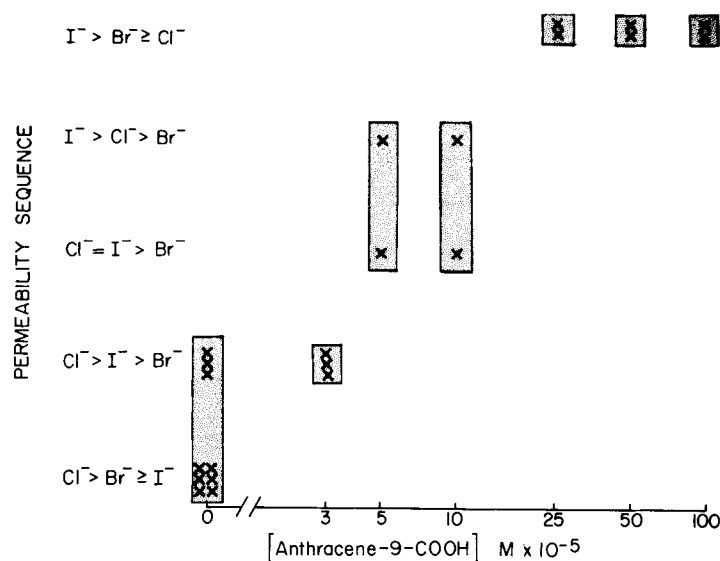


FIGURE 7. Summary of average permeability sequences determined from experiments similar to that shown in Fig. 6 after exposure of diaphragm preparations to 9-AC at various concentrations for 30 min at 35°C and pH 7.5.

tion of the analog is kept below cytotoxic levels. By extrapolation from other work; it would appear likely that these compounds induce a myotonic syndrome in vivo by virtue of the reduction in  $G_{\text{Cl}}$  which they produce (6, 38).

The benzoic acid derivatives reported here reversibly depress  $\text{Cl}^-$  conductance at extracellular concentrations which can be directly related to their relative solubility in a nonpolar environment. This observation suggests that the compounds must partition into the lipid phase of the muscle membrane before producing their effect. The hyperbolic relationship observed between extracellular analog concentration and percent reduction in  $G_{\text{Cl}}$ , however, suggests that the inhibition of  $G_{\text{Cl}}$  is not simply related to intramembrane analog concentration. The nature of the observed relationship implies that binding at a specific site or class of sites having similar affinities is a critical step leading to inhibition. The dependence on partition coefficient would suggest that the specific site of action is located either within or across the surface membrane.

When calculated inhibition constants are corrected for intramembrane concentration based on partition coefficients for each analog they are still found to vary considerably among the analogs tested. This suggests that structural features related to specific binding also play a role in determining the effectiveness of a given analog. Examination of these factors should yield information about the local environment of the benzoic acid binding site. In general this site appears to have two regions, one relatively polar and one nonpolar. The unsaturated hydrophobic ring structure common to all effective analogs must associate with a rather loosely defined nonpolar region since this region can accommodate simple methyl groups as ring substituents as well as complete additional 6-carbon rings as in 9-AC without significantly affecting the ability of the compound to inhibit  $G_{Cl}$ . Attachment of a polar residue to the ring at sites removed from the carboxyl residue, on the other hand, drastically reduces activity although steric considerations should be minimal. This is particularly true of substitutions at the *para* position.

Steric effects alone appear to be most significant in the positions *ortho* to the carboxyl residue, suggesting that conformational requirements for binding in the vicinity of this residue are more restrictive than elsewhere on the ring.

The data presented in this paper allow us to differentiate between several potential mechanisms by which these carboxylic acids might have produced the observed inhibition of  $G_{Cl}$ . Initially, the amphipathic nature of the derivatives suggested that they might intercalate their hydrophobic ring structure into the membrane lipid phase while the polar carboxyl residues remained at the membrane-water interface. The net effect of such partitioning would be to increase membrane fixed negative surface charge density; such an increase would be expected to retard transmembrane anion movement. Failure to find any effect of these analogs within their physiologically active concentration range on the electrophoretic mobilities of several test systems, as well as the lack of competition by  $Ca^{2+}$ , would seem to preclude the surface charge mechanism in this case.

A second hypothesis which had been proposed earlier for several of these compounds suggested that they interact in the membrane in such a way that their charged carboxyl residue sterically blocks access to the anion channel. This would produce inhibition of  $G_{Cl}$  in a manner similar to that observed in the sodium system with tetrodotoxin. A testable consequence of this hypothesis is that conductance to all ions passing through this channel ought to be equally affected. Evidence to date suggests that in normal muscle all the halide ions pass through the membrane predominantly along the same permeation pathway. In light of the trace amounts of  $I^-$  outside the thyroid and of  $Br^-$  normally present in the rat, it would seem teleologically unlikely that separate pathways of relatively high capacity would have developed for each anion in rat sarcolemma, and several points of experimental evidence support the concept that indeed a single channel serves for all these anions. We have shown that changes in pH affect the movement of all three halides in the same reversible manner and that the decrease in conductance observed with decreasing pH shows the same apparent inflection point in each case (37). Further, significant interaction is observed between  $Cl^-$  and  $I^-$  and to a lesser extent between  $Cl^-$  and  $Br^-$  when

conductance measurements are made with varying mole fractions of these ions. Low concentrations of  $I^-$  markedly inhibit movement of  $Cl^-$  through the membrane. Thus at least a portion of these ions must move through the same pathway. Although absolute proof is lacking, it seems unlikely that multiple noninteracting channels specific for each anion are present in the membrane.

If the premise of a single predominant anion channel is accepted, then the observation that  $5 \times 10^{-5}$  M 9-AC reduces  $G_{Cl}$  by 80% while  $G_{Br}$  remains nearly constant and  $G_I$  apparently increases cannot be reconciled with a mechanism which implies simple steric blocking of the channel by the analog.

Most of the evidence presented here would suggest that the benzoic acid derivatives exert their effect by interacting with the anion channel in such a way as to alter its ion selectivity sequence. Similar changes in ion selectivity have been reported as a function of proton concentration in the gall bladder epithelium (41) and were attributed by those authors to an alteration in the field strength of a polar site or sites controlling selectivity in a manner analogous to that described by Eisenman in his classical studies with glass electrodes (16).

Levitan and Barker have studied the effects of a series of salicylates and other benzoic acid derivatives on the ion permeability characteristics of molluscan neurons. They find that these compounds alter the cation selectivity series in this preparation in a manner similar to that observed for anion permeability and conductance sequences in the present report (29). Simultaneous effects were also seen in anion permeability. They report a very high correlation between effective aromatic acid concentration and octanol-water partition coefficient and conclude that in their system the effects on ion permeability are due to a nonspecific increase in membrane anion field strength produced by partitioning of these compounds into the membrane. They conclude that in their system a change in surface charge alone could not produce the observed effects (28, 7). We also present evidence that membrane surface charge is not significantly altered by concentrations of our analogs which affect  $G_{Cl}$ . However, while partitioning into the membrane appears to be of importance in the effects which we report, there is evidence suggesting further specific interaction with intramembrane sites. The observed linearity of the Hill plot as well as the nature of the alterations in anion selectivity sequence suggest that in the rat diaphragm direct interaction with the anion conductance channel is probably involved in the inhibitory effect of these aromatic carboxylic acids. Such interaction might well act to alter the field strength of a channel region involved in producing ion selectivity and result in alterations in ion selectivity sequence of a type predicted by Eisenman on the basis of his field strength theory (17).

The progressive alteration in permeability sequence demonstrated with increasing concentrations of 9-AC may be interpreted in several ways. If one assumes the interaction of a single molecule of 9-AC with a single channel to convert the selectivity of that channel from  $Cl^- > Br^- > I^-$  to  $I^- > Br^- > Cl^-$  then the observed intermediate permeability sequences found in Fig. 7 may represent the average permeability of a membrane containing various ratios of the two channel types. The sequence obtained at any one concentration of 9-AC would reflect the percent of anion channels whose sites are occupied by the analog and would

relate directly to the affinity of this site for the analog. Although the intermediate sequences reported in Fig. 7 are not among those predicted by Eisenman on the basis of his field strength theory of ion selectivity (16), they are precisely those which would be anticipated from a membrane having varying ratios of the two channel types described. Alternatively, the permeability sequence of a single channel might undergo a progressive change due to the binding of a number of molecules of the benzoic acid derivative. The measured membrane permeability sequence would then represent a more complex ensemble average of channels having multiple intermediate sequences. The agreement between the shape of the saturation curves for the analogs studied and that expected from binding to a single active site would tend to support the first hypothesis but cannot be considered conclusive at this point.

The major question raised by these data is how the interaction of an amphipathic molecule such as 9-AC changes the selectivity of a single channel. Mechanisms based on alterations in field strength of an ion-exchange site within the channel or on alterations in critical channel geometry can be postulated, but there clearly is at present insufficient information on the basic selectivity mechanism in the anion channel to make such hypotheses more than speculation.

This work was supported in part by National Institutes of Health grant NS 08075 and by a grant from the Muscular Dystrophy Society.

Received for publication 11 January 1977.

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