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Halide and Alkyl Phenols Block Volume-Sensitive Chloride Channels in Human Glial Cells (U-138MG)

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Abstract. Swelling of cells in hypotonic media activates a volume-sensitive Cl channel with well-known characteristics, but its structure and its regulation are still largely undetermined. It also has many inhibitors and most of them are also blocking other types of Cl channels. The numerous inhibitors of Cl channels have apparently no structural relationship among them. The purpose of this study was to try to determine the most simple molecules that could block these channels and identify some common properties among inhibitors. From the 37 new molecules that were studied, it was found that simple halide phenols like trichloro and triiodophenols could block these channels in the micromolar range. Also alkyl phenols, like butylphenols, are very sensitive blockers, comparable to other well-known blockers. But acidic halide phenols or nitrophenols are poor blockers. Also neutral polyphenols are more sensitive than acidic polyphenols. All these results indicate that the common basis for blocking these Cl channels is a phenol with hydrophobic groups, like short alkyl chains or an additional phenyl ring, attached to some of its sites, preferably sites 3-4-5. These results identify a new family of Cl channel blockers and hopefully improve our understanding of the blocking mechanism.

Key words: Chloride channels — Volume regulation — Patch clamp — Ion channel blockers — Phenol derivatives

Introduction

Volume-sensitive chloride channels are present in most cell types and in a large variety of organisms. Their major role is to provide a rapid efflux pathway for Cl during regulatory volume decrease (RVD) (for recent reviews, *see* Strange, Emma & Jackson, 1996; Nilius et al., 1996; Okada, 1997). Single channel studies have shown that both a Cl and a K channel are activated by cell swelling in hypotonic media (Banderali & Roy, 1992*a*). It was also found that the Cl channel is not very selective among anions; it is permeable to organic anions as large as glutamate and also to most neutral amino acids (Roy & Malo, 1992, Banderali & Roy, 1992*b*, Roy, 1995) and to polyols like inositol (for a recent review, *see* Kirk, 1997).

This anion channel is blocked by a large variety of molecules; the best known are, NPPB (5-Nitro-2-(3-phenylpropylamino)benzoic acid), niflumic acid, DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid), DPC (Diphenylamine carboxylic acid (also known as N-phenylanthranilic acid) and 9-AC (9-anthracene carboxylic acid). Some years ago, Wangemann et al. (1986) studied a large variety of molecules (over 200), many of them having a composition similar to DPC or 9-AC, to determine those that could block Cl channels. That exhaustive study led to the discovery of NPPB, a very powerful blocker. The common property of the molecules studied by these authors was that they were all carboxylic anions. But later on Simchowitz et al. (1993) found that molecules made from aminomethylphenols were also efficient Cl channel blockers. Some lipoxygenase inhibitors like ketoconazole, gossypol and nordihydroguaiaretic acid (NDGA) were also found to be very sensitive inhibitors (Gschwentner et al., 1996, Jackson & Strange, 1993). Another unexpected very sensitive inhibitor of this channel was tamoxifen, a well-known antiestrogen (*see* Okada, 1997, for references). Quinine, a well-known K channel inhibitor was also observed to block the volume-sensitive Cl channel (Banderali & Roy,

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1992*a*, Voets et al., 1996). All these molecules do not have carboxylic groups. Recently, Bausch and Roy (1996) found three new blockers of these channels: Riluzole (2-Amino-6-(trifluoromethoxy)-benzothiazole, Nizofenone (2′-chloro-2-(2-diethylaminomethyl-1-imidazolyl)-5-nitrobenzophenone fumarate) and BW1003C87 (5-(2,3,5-trichlorophenyl)-2,4-diamino-pyrimidine). These three molecules do not have carboxylic groups and two of them have amino groups. When comparing the chemical composition of all these blockers, it is surprising that such a wide variety of molecules with apparently no structural relationship among them could block these channels. Is it possible that a common property among these molecules could be responsible for their blocking power? The objective of this study was to try a large variety of simple molecules and determine those that could block these Cl channels. One basic component of all the known blockers is a benzene ring. A starting molecule would be a phenol or a benzoic acid. Since this anionic channel has much affinity for halides, is it possible that the presence of halides on the benzoic ring would provide the appropriate atom configuration to block the channel? When examining the composition of some of the blockers, like niflumic acid and riluzole, it can be observed that these two molecules have a CF_3 group. Also BW1003C87 has three Cl on its benzene ring. Therefore it was thought that a starting point would be to test the blocking power of phenol, benzoic acid and their halide derivatives. It was found that many halide phenols could block the volume sensitive Cl channel, some with high affinity, while the benzoic acid halides could block only at very high concentrations. Also acidic nitrophenols were poor blockers, while nonpolar alkylphenols were high affinity blockers. A large variety of neutral polyphenols were also high affinity blockers.

Materials and Methods

Cell Cultures

The U-138MG cell line was obtained from the American type Culture Collection starting at the 180th serial passage. Cells were used for patch-clamp experiments up to the 200th serial passage. Cells were seeded at medium density and grown until confluence in plastic bottles (Falcon 3023) with DMEM (Gibco). The medium contained 10% fetal bovine serum (Gibco) with Gentamycin as an antibiotic and the medium was changed every two days. Cultures were maintained at 37°C in a humid air atmosphere in closed Falcon bottles. Subculture was performed by detaching the cells from the bottles using a trypsin-EDTA solution containing 0.05% trypsin and 0.5 mM EDTA-Na. For patch-clamp experiments, cells were detached mechanically from a culture bottle by blowing the Earle medium on the cell layer with a pipette. The cells were maintained in suspension and a sample was introduced into the perfusion chamber. After a few minutes the cells settled on the bottom of the chamber and were ready for patch-clamp experiments.

Solutions and Reagents

The standard bathing solution was an Earle's medium containing (in mM): 121 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 6.0 $NaHCO_3$, 1.0 NaH_2PO_4, 5.5 glucose, 25.0 HEPES, 10.0 NaOH (pH: 7.3, osmolarity: 290 mosm/Kg). All the other experimental media were hypotonic at 200 mosm. The normal NMDG-Cl solution contained (in mM) 100 NMDG-Cl, 10 HEPES, 1 Mg-SO_4; the pH was adjusted at 7.3 with 4 NMDG base. All the drugs were added to the hypotonic NMDG-Cl medium from stock solutions (0.1 M or 0.5 M) in dimethyl sulfoxide (DMSO), except those used at 10 mM which were added directly into the medium. Adding the highest concentration of DMSO alone (1%) had no effect on the currents. All the drugs were obtained from Sigma-Aldrich Canada (Oakville, Ontario).

Patch-clamp Technique

Whole cell patch-clamp experiments were performed as previously described (Roy, 1995) with pipettes containing the hypotonic (200 mosm) NMDG-Cl pH 7.3 solution. Immediately after reaching the whole-cell configuration, the external isotonic Earle's solution was changed to hypotonic NMDG-Cl, which triggers cell swelling. During that time, the pipette solution dialized the cell and both internal and external media became identical, with the cell swollen to a new stable volume. The cell could not regulate its volume (RVD) because NMDG was not permeable. That method was more appropriate than using an isotonic solution in the pipette and a hypotonic external solution, which produced continuous swelling. It should be mentioned that the pipette solution did not contain ATP. It was found that ATP was not necessary when swelling was triggered immediately after reaching the whole-cell configuration. The currents were evoked similarly as previously described (Roy, 1995) and remained large for long periods (10–30 min). The patch-clamp amplifier was a PC-501, Warner Instrument (Hamden, CT). The pipettes were made from Pyrex glass capillaries and pulled using a David Kopf programmable puller (model 750). The pipette resistance ranged from 9 to 12 Megohm and the seal resistance was usually between 4–6 Gigaohm. The whole-cell current was recorded on a strip chart. The applied potentials were alternating + and − 30 mV step-pulses of 3-sec duration. There was no holding potential and the reversal potential was zero because symmetrical ionic solutions were used. A positive voltage produced a flow of anions into the cells and corresponded to the positive current. The effect of the drugs was measured by the change in the total amplitude of the current. The perfusion chamber had a small volume and the solutions could be changed in 15 sec. All experiments were performed at room temperature (22–24°C).

Results

Effects of Halide Phenols on Cl Currents in Hypotonic Media

Using pipettes filled with the hypotonic NMDG-Cl solution (200 mosm), a cell-attached patch was performed and the whole-cell configuration was reached after breaking the membrane. Immediately after, the external medium was changed from isotonic Earle's medium to hypotonic NMDG-Cl (*see* Materials and Methods). A slow increase of the whole-cell current was observed and reached a stable value, as shown in Fig. 1. This current

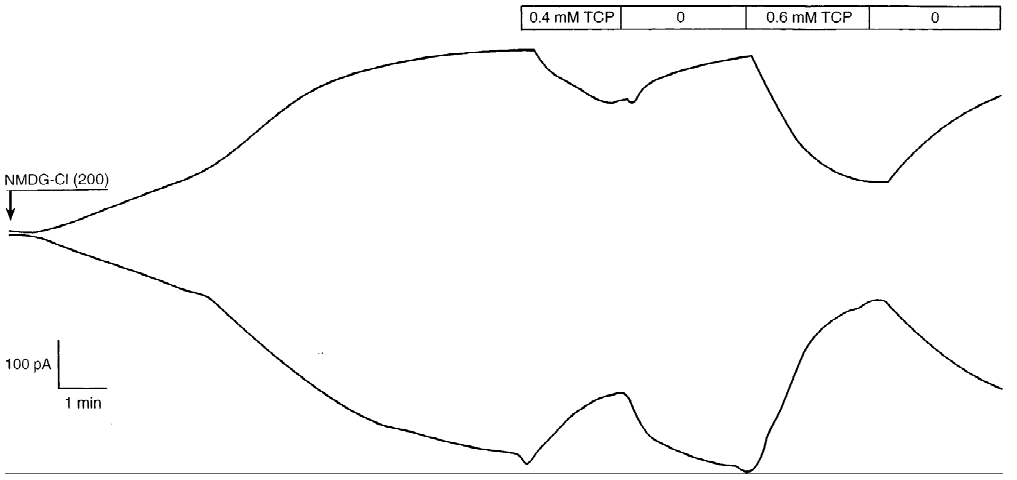


Fig. 1. Inhibition of Cl currents activated by osmotic swelling. Whole-cell currents produced by an alternating + and − 30 mV voltage pulse and measured on glial cells using a hypotonic NMDG-Cl solution (200 mosm) in both the pipette and the external medium (*see* Materials and Methods for details). Inhibition was produced by perfusing with the same NMDG-Cl solution containing trichlorophenol (TCP) at the indicated concentrations.

was shown previously by Roy (1995) to be produced by chloride ions and to have all the known characteristics of the volume-sensitive chloride current: the current-voltage curve had an outward rectification and the current inactivated at positive potentials larger than 50 mV. The well-known inhibitors of Cl channels, NPPB and niflumic acid, were found to block these currents (Bausch & Roy, 1996). The first molecules introduced into the hypotonic medium were phenol or benzoic acid. Neither of them had any inhibitory effect at concentrations up to 10 mM. The next molecule to be studied was trichlorophenol. When it was introduced into the external hypotonic medium, the current rapidly decreased, as shown on Fig. 1, to reach a new stable value depending on the concentration of the inhibitor used. As shown on Fig. 1, trichlorophenol could block 30% of the current at 0.4 mM and 50% at 0.6 mM. An almost complete block (90%) was obtained with 1.0 mM. The effect was rapid and reversible, and could be reproduced similarly on many different cells. Also inhibition did not seem to be voltage dependent because the current reduction was the same for +30 and −30 mV. But we cannot rule out, however, that some voltage-dependent blocking could appear at higher voltages. The percentage of inhibition was calculated for different inhibitor concentrations from the amplitude of the current with and without inhibitor. Dose-inhibition data values were obtained, as shown on Fig. 2; the continuous curve was obtained by curve-fitting of the data values with the Hill equation, which yielded a half-inhibition concentration of 0.57 mM for trichlorophenol. On the basis of these results, it was interesting to determine if phenol molecules with only one or two Cl could also block the channel. Dichlorophenol was studied and was also found to block the channel.

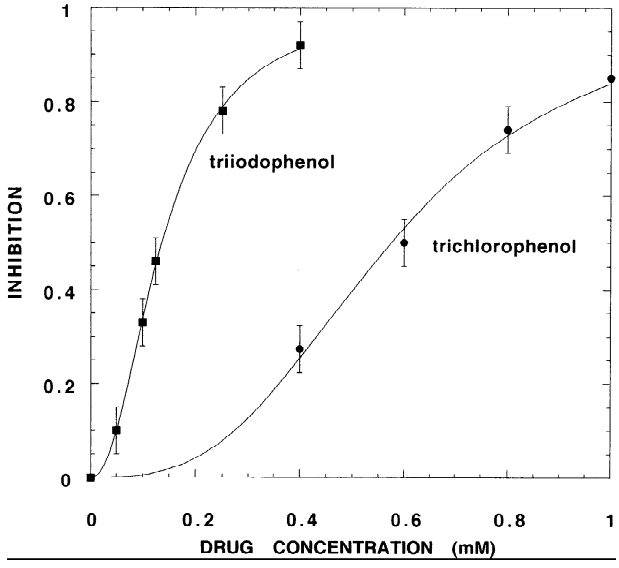
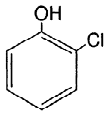
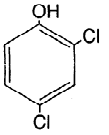
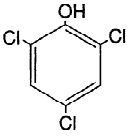
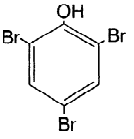
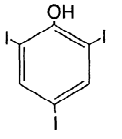
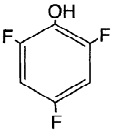
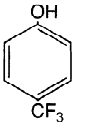
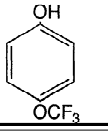


Fig. 2. Dose-Inhibition curves. Data points represent the average and the standard deviation obtained from $n = 3$ values. Each value is calculated from the ratio of the stable current during inhibition to that before inhibition, using data as shown on Fig. 1. Curves were obtained by least-square fitting of the data points, using the Hill equation.

The results were similar to those obtained with trichlorophenol, but a slightly larger concentration was required to reduce the current; the IC_{50} for dichlorophenol, as given in Table 1, was 0.77. It was also possible to block the Cl current with chlorophenol. Even though the results were similar to those shown on Fig. 1 for trichlorophenol, much larger concentrations of chlorophenol were required. About 5 mM was necessary to produce only a half inhibition of the current, as shown on Table

Table 1. Drug structure, name and IC_{50} value

Structure	Name	IC_{50} (mM)
	2-CHLOROPHENOL	4.9
	2-4-DICHLOROPHENOL	0.77
	2-4-6-TRICHLOROPHENOL	0.57
	2-4-6-TRIBROMOPHENOL	0.46
	2-4-6-TRIIODOPHENOL	0.14
	2-4-6-TRIFLUOROPHENOL	1.0
	4-TRIFLUOROMETHYLPHENOL	1.0
	4-TRIFLUOROMETHOXYPHENOL	0.56

1; these inhibitions were as rapid, reversible and reproducible as with tri or dichlorophenols. Therefore, these results indicate that tri or dichlorophenols are moderately sensitive blockers of the volume-sensitive anion channels, while chlorophenol is much less effective. To explore the possibility that other halide phenols could also be interesting inhibitors of this channel, tribromophenol, trifluorophenol and triiodophenol were studied. It should be noted, as shown on Table 1, that all these halides were in the same positions on the phenol molecule (2-4-6). The inhibitory effects that were obtained with these three halide phenols were similar to those obtained above (Fig. 1), although the required concentrations were different. With tribromophenol, the IC_{50} was about the same as with trichlorophenol, as seen on Table 1, but with trifluorophenol, it was about twice that of trichlorophenol. But the most surprising result was the low concentration of triiodophenol that could block the current, only 0.14 mM was sufficient for half inhibition (Fig. 2). This result makes triiodophenol one of the most sensitive blockers of this channel in our glial cells, with a IC_{50} comparable to that of NPPB (Bausch & Roy, 1996). Two other fluorophenols were also studied, trifluoromethylphenol (CF_3) and trifluoromethoxyphenol (OCF_3). These molecules, as shown on Table 1, have their three fluoride on the same phenol site (4). The half inhibition value was the same for trifluoromethylphenol (CF_3) and 2-4-6-trifluorophenol (IC_{50} = 1 mM), but it was lower for trifluoromethoxyphenol (IC_{50} = 0.56).

Because many of the well-known Cl channel blockers are carboxylic acid, it was important to study acidic molecules. The best candidate to be compared with trichlorophenol was trichlorobenzoic acid. To our surprise, trichlorobenzoic acid did not produce any inhibition until a 5 mM concentration was used and 10 mM was necessary to inhibit half of the current (Table 2). This was in contrast with trichlorophenol, which could completely block the current at 1 mM, indicating that the presence of a carboxylic group on the benzene ring was not favorable for inhibition of this channel. To verify if an acidic configuration on the whole phenol molecule had similar effects, trinitrophenol was studied. This molecule, as shown on Table 2, has the same configuration as trichlorophenol, but it is a well-known strong acid (picric acid); it was used in a buffered solution at pH 7. No inhibition of the current could be measured at concentrations up to 10 mM. Dinitrophenol was also studied for comparison and it was found that it could inhibit half of the Cl current with a concentration of 5 mM. Dinitrophenol is less acidic than picric acid. To verify if the anionic character of these phenols had a role in reducing their inhibitory power, trichlorophenol (1 mM) was introduced into the hypotonic medium at a pH of 9.1. Since the pK of trichlorophenol is 9.1, half of the molecules are in their anionic configuration in these pH conditions. Perfusing the cells with that high pH solution, it was found that the current was inhibited by only 30%, while in the same cell, the current was inhibited by 90%, with a solution containing the same trichlorophenol concentration at pH 7. This result clearly established that the anionic character of the halide phenols was not favorable for inhibition of the Cl channels.

It was also interesting to determine the effect of polar groups like NO_2 or NH_2 on the inhibitory power of dichlorophenol. As shown on Table 2, the presence of either NO_2 or NH_2 on dichlorophenol increased the IC_{50}, indicating that both these polar groups reduced the binding on the Cl channel. Another molecule having some similarity with trichlorophenol was introduced into the hypotonic medium, trichloropyrimidine. As seen on

Table 2. Drug structure, name and IC_{50} value

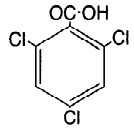
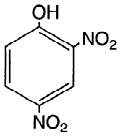
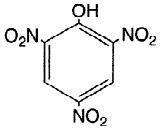
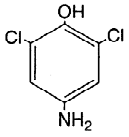
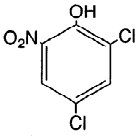
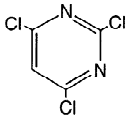
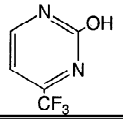
Structure	Name	IC_{50} (mM)
	2-4-6-TRICHLOROBENZOIC ACID	10.0
	2-4-DINITROPHENOL	5.0
	2-4-6-TRINITROPHENOL	>10.0
	4-AMINO-2-6-DICHLOROPHENOL	2.5
	2-4-DICHLORO-6-NITROPHENOL	1.5
	2-4--6-TRICHLOROPYRIMIDINE	>5.0
	4-TRIFLUROMETHYL-2-PYRIMIDINOL	>3.0

Table 3. Drug structure, name and IC_{50} value

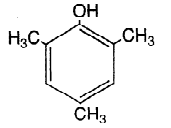
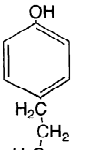
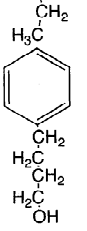
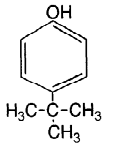
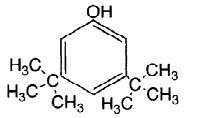
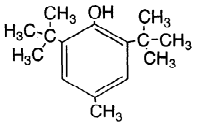
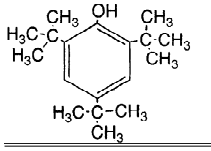
Structure	Name	IC_{50} (mM)
	2-4-6-TRIMETHYLPHENOL	1.1
	4-PROPYLPHENOL	1.5
	4-PHENYL-1-BUTANOL	>5.0
	4-TERT-BUTYLPHENOL	0.42
	3-5-DITERTBUTYL PHENOL	0.10
	2-6-DITERTBUTYL 4-METHYLPHENOL (BHT)	>0.5
	2-4-6-TRITERTBUTYL PHENOL	>0.5

Table 2, this molecule has two nitrogen replacing two carbon atoms into the benzene ring and it does not have a hydroxyl. There was no inhibition of the Cl current produced by trichloropyrimidine, with concentrations up to 5 mM. Another pyrimidine was also tested because of its similarity with trifluoromethylphenol; it was trifluoromethylpyrimidinol. It had no effect on the current at a concentration of 3 mM. These two results indicate that the presence of nitrogen atoms in the benzene ring are not favorable for binding with the Cl channel. A highly polar molecule, inositol, having a structure similar to a phenol, but with six hydroxyl instead of one, was tested; it had no inhibitory effect at 10 mM.

Effects of Alkyl Phenols on Cl Currents in Hypotonic Media

The inhibitory effects of halide phenols on the volume-sensitive chloride current indicate that nonpolar molecules are more appropriate for blocking than polar molecules. To test that possibility, nonpolar phenol derivatives were studied. Trimethylphenol was the first one. It could block the Cl current rapidly and reversibly, having a IC_{50} of about 1 mM, as shown on Table 3. This value is twice that of trichlorophenol. Two other nonpolar phenol derivatives were also tested: propylphenol and butylphenol. Both could inhibit the current, with butylphenol having a $IC_{50} = 0.42$ mM, a value comparable with that of trichlorophenol, as shown on Table 3. Another molecule similar to propyl and butylphenol was tested, phenylbutanol. As seen on Table 3, the hydroxyl on this molecule is at the end of the alkyl chain rather than on the benzene ring. In this case, there was no inhibition, indicating that the presence of a hydroxyl on

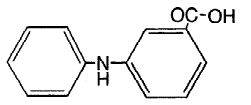
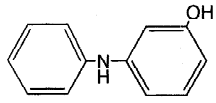
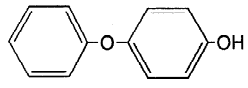
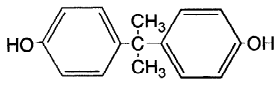
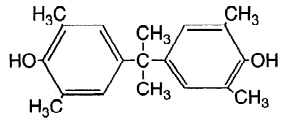
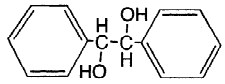
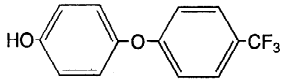
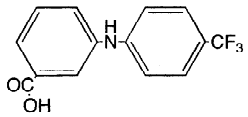
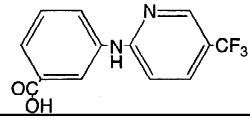
the benzene ring was necessary for inhibition. There are also naturally occurring phenol derivatives similar to propylphenol, such as tyrosine and dopamine. Their main difference is the addition of a carboxyl and an amino group at the end of the alkyl chain for tyrosine and only an amino group for dopamine. Neither of these two molecules at 5 mM had any inhibitory effect, indicating that the presence of a carboxyl or an amino group on the short alkyl chain is not favorable for inhibitory interaction with the Cl channel. An important natural derivative of tyrosine, diiodotyrosine was also studied because triiodophenol was found to be a very powerful blocker (Table 1). But diiodotyrosine at a 1 mM concentration had no effect on the Cl current, indicating again that the presence of carboxyl and amino groups reduced the inhibitory power.

To determine if additional butyl groups on the phenol ring could increase its inhibitory power, 3-5-dibutylphenol was tested. As shown on Table 3, this molecule had an IC_{50} = 0.10 mM, a value much lower than that of butylphenol. In fact, it was one of the most sensitive blockers that we have found. Its blocking action was rapid and reversible, and a complete block of the current was reached with 250 μM. Another very similar molecule was also tested, 2-6-dibutyl-4-methylphenol, usually known as dibutylhydroxytoluene (BHT). The main difference with the preceding molecule is the position of the dibutyl groups. As shown on Table 3, BHT did not block the current, as 3-5-dibutylphenol did, using a concentration of 0.5 mM, its maximum solubility. Similarly 2-4-6-tributylphenol was tested and could not block the current at 0.5 mM, its maximum solubility. The main difference between these last two compounds and 3-5-dibutylphenol is the position of the butyl groups on the phenol ring. In the former, the butyl groups are very close to the hydroxyl, while in the latter they are farther away. It seems that if the two butyl groups are too close to OH, they could occlude it and reduce its binding to the channel. These results indicate that the interaction of a hydroxyl with the channel is necessary and the hydroxyl must be on the benzene ring. To determine if a longer alkyl chain attached on site 4 on the phenol ring could reduce the IC_{50} further, 4-octylphenol was tested on the Cl current. It had no inhibitory effect at 1 mM, its maximum solubility. This indicates that a long alkyl chain on the phenol is not appropriate for inhibitory interaction with the channel. Two short alkyl groups attached on site 3 and 5 are much better.

Effects of Neutral Polyphenols on Cl Currents in Hypotonic Media

It was demonstrated in the above experiments that the presence of anionic groups dramatically reduced the sensitivity of phenol derivatives. It seemed therefore necessary to determine if the presence of a carboxylic group on well-known Cl channel blockers had a similar effect. The first example is DPC, which is a diphenyl molecule with a carboxylic group. It was found to block the Cl current rapidly and reversibly with a IC_{50} of 1.1 mM, as shown on Table 4. A molecule having the same composition as DPC, except that the carboxylic group is replaced by a hydroxyl, hydroxydiphenylamine, was tested. It was found to block the current similarly, but with a IC_{50} = 0.55 mM. This result shows that a hydroxyl is more appropriate than a carboxyl for inhibitory interaction of polyphenols with the Cl channel, although the effect is not as important as above with single phenol derivatives. To determine if the group of atoms between the two phenyl rings has some importance for inhibition,

Table 4. Drug structure, name and IC_{50} value

Structure	Name	IC_{50} (mM)
	DIPHENYLAMINE-2-CARBOXYLIC ACID	1.1
	3-HYDROXY DIPHENYLAMINE	0.55
	PHENOXYPHENOL	0.20
	BISPHENOL	0.23
	4-4-ISOPROPYLDENE BIS(2-6-DIMETHYL PHENOL)	0.20
	HYDROBENZOIN (DIPHENYL ETHANEDIOL)	5.0
	4-4-TRIFLUROMETHYL PHENOXYPHENOL	0.10
	2-TRIFLUOROMETHYL DIPHENYLAMINE CARBOXYLIC ACID (FLUFENAMIC ACID)	0.24
	2-TRIFLUROMETHYL PHENYLAMINE NICOTINIC ACID (NIFLUMIC ACID)	0.21

a few other molecules with different groups were tested. One was phenoxyphenol, where NH was replaced by O as a link between the phenols (Table 4). This molecule could block the channel with a $IC_{50} = 0.2$ mM, which is 5 times less than DPC (Table 4). Another interesting molecule was bisphenol, where NH between the phenyl rings was replaced by a isopropyl group and with each phenyl ring having a OH, as shown on Table 4. It also had a low $IC_{50} = 0.23$ mM, making it a more sensitive blocker than DPC. Another one with much similarity to bisphenol had methyl groups added on the phenols, isopropyldene-bis(dimethylphenol); its IC_{50} was similar to that of bisphenol (Table 4), indicating that the added methyl groups did not have any influence. To determine if hydroxyl groups on the phenyl rings were important, a molecule with hydroxyl groups only between the phenyls was tested, diphenyl ethanediol, known as hydrobenzoin. As shown on Table 4, it had a high $IC_{50} = 5$ mM, a value 20 times higher than the three above molecules. This indicates that hydroxyls are more effective for blocking when they are situated on the phenyl rings rather than between them.

Two well-known and very similar blockers are niflumic and flufenamic acids. As shown in Table 4, these two molecules are similar to DPC, but they have in addition a CF_3 group, which reduces their IC_{50} by about five times, compared with that of DPC. Another molecule similar to these two, but without the carboxyl group, was tested, trifluoromethylphenoxyphenol. As shown on Table 4, this compound had a $IC_{50} = 0.1$ mM, half that of niflumic acid and among the lowest values obtained.

The next class of polyphenols that was studied included molecules made of two or three adjoining phenyl rings. A well-known member of this class is 9-AC, with three adjoining phenyl rings. This molecule was found to block the Cl current rapidly and reversibly, but required very large concentrations, having a $IC_{50} = 5$ mM, as shown on Table 5. A few molecules having a similar structure but without the carboxylic group were tested. The one most closely related to 9-AC was anthrarobin. As shown on Table 5, it had three hydroxyls instead of one carboxyl, and that gave a $IC_{50} = 0.5$ mM, ten times less than that of 9-AC. Another molecular similar to anthrarobin was tested; it had only two phenyl rings instead of three, dihydroxynaphthalene. As shown on Table 5, the $IC_{50} = 2.5$ mM, a much larger value. A molecule similar to dihydroxynaphthalene, biphenol, was tested; its IC_{50} was about the same. These results indicate that molecules made of adjoining phenyl rings with hydroxyl groups on them are not the most sensitive inhibitors of the Cl channel. Three other molecules with a structure similar to dihydroxynaphthalene were also tested, hydroxyquinoline, dihydroxyquinoxaline and trifluoromethylquinolinol. As seen on Table 5, these molecules had one or two nitrogen atoms in place of carbon atoms on the phenyl rings. That eliminated the inhibitory power, at least for concentrations up to 3 mM. This result is similar to the one observed for trichloropyrimidine and confirms that the presence of N atoms in the phenyl rings reduces the inhibitory power.

A very sensitive blocker, riluzole, was found recently by Bausch and Roy (1996); its IC_{50} was 0.14 mM. That molecule has a rather simple composition; it is an amino-benzothiazole with a OCF_3 attached to the phenyl ring, as shown on Table 5. It has some similarity with one of the above molecules, trifluoromethylquinolinol,

Table 5. Drug structure, name and IC_{50} value

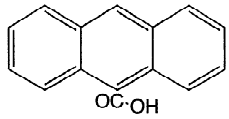
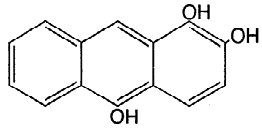
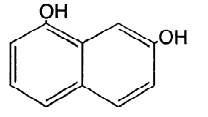
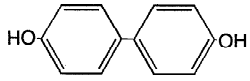
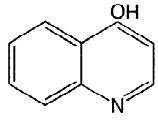
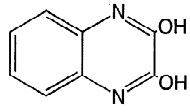
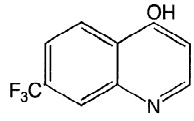
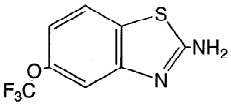
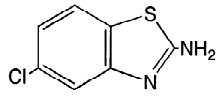
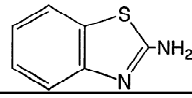
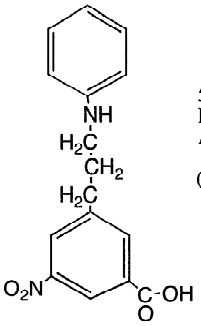
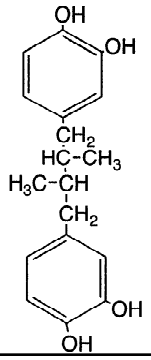
Structure	Name	IC_{50} (mM)
	9-ANTHRACENE CARBOXYLIC ACID	5.0
	ANTHRAROBIN	0.5
	2-7-DIHYDROXY NAPHTALENE	2.5
	BIPHENOL	2.0
	4-HYDROXY QUINOLINE	>3.0
	2-3-DIHYDROXY QUINOXALINE	>3.0
	8-TRIFLUOROMETHYL 4-QUINOLINOL	>3.0
	2-AMINO-6-TRIFLUORO METHOXY-BENZOTHIAZOLE (RILUZOLE)	0.14
	2-AMINO-6-CHLORO BENZOTHIAZOLE	>1.0
	2-AMINO BENZOTHIAZOLE	>5.0

Table 6. Drug structure, name and IC_{50} value

Structure	Name	IC_{50} (mM)
	5-NITRO-2-(3-PHENYL PROPYLAMINO)BENZOIC ACID (NPPB)	0.09
	NORDIHYDRO GUAIARETIC ACID (NDGA)	0.05

but the IC_{50} of riluzole for blocking the Cl current is much lower. The main difference between these two molecules is the presence of a sulfur atom in place of a carbon and the addition of a NH_2. As shown above for halide phenols, a NH_2 group does not usually improve inhibition, it rather decreases it. Therefore the S atom might play an important role. To determine if the presence of the OCF_3 group was important on riluzole, a molecule without it was tested, aminobenzothiazole. As shown on Table 5, there was no measurable inhibition, indicating that the OCF_3 group was absolutely required. But the benzothiazole structure had an important modulating effect; it provided a more sensitive inhibition than phenol alone, since trifluoromethoxyphenol (Table 1) had a IC_{50} four times larger. Also a molecule with a Cl instead of a OCF_3 was tested; no inhibition could be measured with 1 mM, its maximum solubility.

A molecule that had some similarity to NPPB was also studied, nordihydroguaiaretic acid (NDGA). As seen on Table 6, the main difference between these molecules is the presence of two hydroxyls on each phenyl ring for NDGA, instead of a carboxyl and a nitrate on one of the phenyl rings for NPPB. NDGA is widely used as a lipoxygenase inhibitor and was found recently to be a very sensitive Cl channel blocker (Gschwentner et al., 1996) with a IC_{50} of 4 μM in NIH3T3 cells. NDGA was tested on the Cl current of our glial cells and was found to block it with a very low concentration; its IC_{50} was 50 μM. Again it seems that the replacement of a carboxyl by a hydroxyl on the phenyl rings reduced the IC_{50} for blocking the volume sensitive Cl current. In this case the effect is rather small, probably because NPPB has a long hydrophobic portion.

As mentioned in the introduction, tamoxifen was found to be a very sensitive blocker in a few cell types, requiring only 1 μM for 90% inhibition (Okada, 1997). Tamoxifen was tested on the Cl current of our glial cells; using concentrations of 10 and 100 μM, no inhibition was observed (A.R. Bausch and G. Roy, *unpublished data*). Also quinine was found to be a blocker of the Cl current in some cell types (Voets et al., 1996). It has no inhibitory effect on the Cl current of our glial cells, at a concentration of 1 mM.

Discussion

There are numerous chloride channel blockers with apparently no structural relationship. They differ in their sensitivity for blocking the channel in a particular cell type and their sensitivity varies from one cell type to another. Besides, many of them have additional effects, such as lipoxygenase inhibition (Nilius et al., 1996). It would be interesting if some basic characteristics of chloride channel blockers could be established. It could help in designing more specific blockers and possibly indicate how inhibition takes place on the channel. The purpose of this study was to try to determine such basic characteristics. One of the main results was to demonstrate that simple hydrophobic phenol molecules, like triiodophenol and dibutylphenol, were quite sensitive blockers and that anionic phenol compounds were much less effective. As shown with the chlorophenols, increasing the number of Cl on the phenol ring increased the blocking sensitivity and adding a carboxylic group decreased it dramatically. In addition, when trichlorophenol was made anionic by increasing the pH, blocking was largely reduced. Also other anionic phenol molecules, like trinitrophenol, had a poor blocking power, while the hydrophobic ones, like 3-5-dibutylphenol, were among the best blockers. This conclusion is in contrast with previous studies, for example that of Wangemann et al. (1986), who concluded that the blockers should be anionic. Other simple neutral phenol derivatives, aminomethylphenols, were also found to be sensitive Cl channel blockers by Simchowitz et al. (1993). One of their molecules, MK447, is similar to 4-tert-butylphenol and another one, MK447A, is similar to bisphenol. These authors found an IC_{50} of 90 μM for MK447 and 16 μM for MK447A. These two blockers were also tested on our glial cells and their IC_{50} were equal to 300 μM and 180 μM respectively (A.R. Bausch

and G. Roy, *unpublished data*). These values are much larger than those of Simchowitz et al. (1993), but similar to those of 4-tert-butylphenol and bisphenol (Tables 3 and 4).

Comparing molecules that were similar to well-known blockers, like DPC, 9-AC, niflumic acid and NPPB, it was found that replacing the carboxylic group by a hydroxyl improved the sensitivity of the blocker. Also, comparing the IC_{50} of bisphenol and hydrobenzoin, it can be concluded that the hydroxyl group has to be on the phenol rings of the polyphenols, rather than between them. A similar result was obtained with the single phenol derivatives, where the hydroxyl had to be on the phenol ring. The presence of polar or nonpolar groups between the phenol rings were more favorable for inhibition than without them. For example, bisphenol was ten times more sensitive than biphenol or other molecules with adjoining phenol rings, like dihydroxynaphthalene. When comparing the molecules of these two groups (Tables 4 and 5), it can be observed that those of Table 4 are more flexible than those of Table 5. The latter remain flat, while the former can bend at an angle of about 45° through the center group. This is easily seen with a space-filling (CPK) model of these molecules, comparing for example bisphenol and biphenol. It is also interesting to compare the three dimensional shape of a space-filling model of NDGA and NPPB. Because of the longer chain between the phenol rings, these molecules can be twisted much more and the two phenols can take many different positions. Could that flexibility give them a shape and a size more favorable for binding to the Cl channel? This is a likely possibility. The phenol derivatives shown on Tables 1 and 3 appear smaller than these polyphenols, but in some cases they are equally sensitive. It is possible that the hydrophobic character of all these molecules, as well as their size, are playing a major role in blocking these channels.

It is usually assumed that anion channel blockade is due to the binding of a large anion in the channel pore. But our results suggest that the blocking site for inhibition might not be a binding site for anion permeation in the channel, since hydrophobic molecules are more sensitive blockers than hydrophilic or anionic molecules. For example, inositol is a large permeable molecule in C6 glioma cells (Jackson & Strange, 1993) and it is not a blocker in our glial cells, while smaller molecules like trimethyl or butylphenol are efficient blockers. It is possible that some amphiphilic domain in the channel protein could interact more strongly with hydrophobic phenols and modify the shape and the permeability of the channel. Such a possibility remains to be demonstrated.

From the results we have obtained, it seems possible to determine the characteristics of the molecules that would be appropriate blockers. It should be a phenol with one, two and possibly three short hydrophobic chains attached preferably at sites 3-4-5. A hydroxyl group at site 1 on the benzene ring is necessary and is more appropriate than a carboxyl. The blocker could also be made of two phenols linked with a short alkyl chain, like propyl or butyl. Again hydroxyl groups on the benzene rings are more appropriate than carboxyl groups. Although these two types of molecules are apparently different, they might have a similar interaction with the blocking site. We cannot state yet what is the optimum size and configuration for such molecules to provide the lowest IC_{50}. It should be pointed out here that the sensitivity of a blocker is relative and not the same in all cell types, and even very sensitive blockers in some cells, like tamoxifen, have no effect on other cells. With our glial cells, no inhibitor could block the Cl channel at a concentration of 1 μM. The lowest concentration found to block half of the current was 50 μM, with NDGA. That inhibitor could block the Cl channel in fibroblasts with only 1 μM (Gschwentner et al., 1996). Therefore, when an inhibitor is considered very sensitive in our cells, it might not appear really sensitive when compared to other very sensitive inhibitors in other cells. It is possible that all the inhibitors shown in Tables 1 to 6 are effective at lower concentrations in other types of cells. We considered in this study that inhibiton at concentrations up to 10 mM were valid. The most important desirable aspect of volume-sensitive anion channel blockers is their specificity rather than their sensitivity. It should be recalled that TEA (tetraethylammonium) is a well-known selective K channel blocker requiring between 0.4 and 8 mM for half inhibition depending on cell types (Hille, 1992).

Among the numerous new molecules that were tested (37), some could be potentially interesting blockers. Until now the role of most of them as Cl channel blockers was not known. It remains to be determined if this effect is specific at the concentrations used. Further experiments should determine if they block other channels and if they have other effects on cell functions. Their effects on other types of cells should also be studied. These results could provide new guidelines for the synthesis of more specific anion channel blockers.

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