Notes

Bisphenols That Stimulate Cells To Release Alkali Metal Cations: A Structure−Activity Study

Laszlo Hopp, Susan O. Megee, and John B. Lloyd*

Department of Pediatrics, Jefferson Medical College, Alfred I. duPont Hospital for Children, Wilmington, Delaware 19803

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The laxative action of phenolphthalein (**5**) is believed to result from induction of potassium and water efflux from the colon epithelium. In cultured cells, K^+ efflux is promoted by **5** and by a contaminant (**1**) present in commercial phenol red. Six compounds with chemical structures related to those of **5** and **1** were tested for ability to induce the release of ^{86}Rb from COS-7 cells preloaded with this isotope: 4,4′-(9-fluorenylidene)diphenol (**2**), 4,4′-(9-fluorenylidene)dianiline, 4,4′-(9-fluorenylidene)bisphenoxyethanol, 1,1′-bi-2-naphthol, 4,4′-biphenol, and bis(4-hydroxyphenyl)methane. With one exception these compounds were all inactive at a concentration of 10 μM. However, **2** caused profound ^{86}Rb efflux at concentrations as low as 100 nM. Concentrations of **5** 1−2 orders of magnitude higher were needed to achieve similar levels of activity. The three compounds known to be active in this experimental system share a common feature that is absent in all the inactive compounds: a five-membered ring structure, one of whose carbon atoms is disubstituted with *p*-hydroxyphenyl residues. Because **2** and **5** are readily available, comparative studies on the mechanism of action of these biphenols at the cellular level can now be undertaken.

Introduction

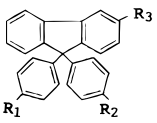
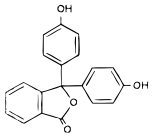
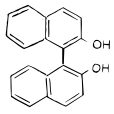
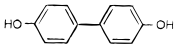
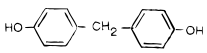
In many countries around the world phenolphthalein (**5**; Table 1) is used medicinally as a laxative. Phenolphthalein has effects on smooth muscle contractility and also promotes reversal of the normal process of absorption of water and electrolytes by the colon.[1] Bisacodyl and picosulfate sodium are prodrugs that are hydrolyzed in the intestine to a bisphenol that is believed to act in a manner similar to phenolphthalein.[2]

In 1993 Lubin[3] and Hopp and Bunker[4] independently reported that a contaminant present in commercial samples of phenol red induced several types of cells to release intracellular K^+. The chemical identity of this contaminant was subsequently elucidated:[5] it is a derivative of the tricyclic aromatic hydrocarbon fluorene with phenolic residues but no other functional groups (**1**; Table 1). The chemical structures of **1** and **5** have features in common. Moreover **5** also causes cation efflux from cultured cells.[6]

The mechanism by which these phenolic compounds act on cells is not yet known. The induction of monovalent cation release from cells by **1** is inhibited, at least partially, by verapamil and quinine.[3,5,6] Verapamil, best known as an inhibitor of L-type calcium channels, has direct effects on potassium channels.[7,8] Quinine is known to inhibit calcium-activated potassium channels, although it also affects other ion-transport processes.[9]

The more proximal effect of the active compounds may be mediated by nitric oxide. In vivo studies by Gaginella et al.[1] showed that the effect of **5** on water and electrolyte release from the colon was prevented by an inhibitor of nitric oxide synthase (L-NAME) but not by

Table 1. Chemical Structures of the Compounds Tested

Compound Number	Chemical Structure
	
1[a]	$R_1 = R_2 = R_3 = OH$
2	$R_1 = R_2 = OH$; $R_3 = H$
3	$R_1 = R_2 = NH_2$; $R_3 = H$
4	$R_1 = R_2 = OCH_2CH_2OH$; $R_3 = H$
5	
6	
7	
8	

[a] **1** is the structure of the active principle of commercial phenol red, as reported by Kym et al. (1996).

* Address correspondence to this author. Phone: +1 (302) 651-6819. Fax: +1 (302) 651-6876. E-mail: jlloyd@nemours.org.

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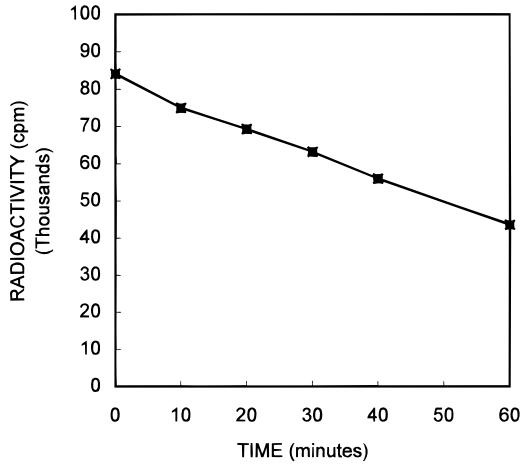

Figure 1. Cell-associated radioactivity in COS-7 cells preloaded with ^{86}RbCl, washed in ice-cold BSS, and then incubated in BSS for 60 min at 37 °C. See text for further details of the experimental method.

its inactive stereoisomer. Dexamethasone, an inhibitor of the inducible component of nitric oxide synthase, also antagonized the effect of **5**. To date these observations have not been followed up by studies at the cellular level.

As a preliminary to such studies on the mechanism(s) of action of **1** and **5** in causing monovalent cation loss from cells, we sought to discover the structural features a molecule requires for activity. A number of compounds closely and more distantly related in chemical structure to these two compounds were tested for their ability to induce the release of $^{86}Rb^+$ from cells preloaded with this cation.

Results

Figure 1 shows the results of a typical experiment in which COS-7 cells preloaded with ^{86}Rb were incubated for 60 min in balanced salts solution. Isotope is released at a steady rate over the 60-min period. In 42 control experiments 54 ± 8(SD)% remained in the cells at the end of the incubation.

Table 1 shows the chemical structures of the biologically active ingredient of commercial phenol red and of the compounds we investigated. Table 2 shows the effect of compounds **2**−**8** on the ability of the cells to retain ^{86}Rb. Even at a concentration of 100 nM, 4,4′-(9-fluorenylidene)diphenol (**2**), which has the structure most similar to that of **1**, the active component of commercial phenol red, causes the cells to release most of their ^{86}Rb. It was however inactive at 10 nM. Phenolphthalein (**5**) also promoted ^{86}Rb efflux, but at 1 μM **5** was less active than **2** at 100 nM. All the other compounds tested were inactive or marginally active even at 10 μM, despite their displaying many of the key structural features of **1**, **2** and **5**.

Figure 2 illustrates how the concentration of **2** affects the kinetics of release of ^{86}Rb from cells. As the concentration decreases, the extent of the ^{86}Rb release decreases, and there is a longer delay before the effect is observed.

Discussion

In these experiments the ability of the compounds under investigation to promote cation release from cells was measured by preloading the cells with ^{86}Rb and monitoring the release of this cation. In earlier experiments[4] the release of ^{86}Rb was shown to parallel the release of K^+, the dominant intracellular cation. The phenomenon being monitored is likely to involve an increase in the permeability of the plasma membrane to monovalent cations and the influx of Na^+ to compensate at least partially for the K^+ efflux.[4] Some anion release may also occur.[10]

We have shown that compound **2** is a powerful promoter of cation release from COS-7 cells. This com-

Table 2. Effect of Aromatic Compounds on the Release of ^{86}Rb from COS-7 Cells

compd added	concn (μM)	fraction of accumulated ^{86}Rb remaining[a]
control (no addition)		0.543 ± 0.078
2	10	0.003 ± 0.001
	1	0.006 ± 0.002
	0.1	0.147 ± 0.020
	0.01	0.532 ± 0.041
	0	0.556 ± 0.072
	0 (DMSO)	0.549 ± 0.051
3	10	0.419 ± 0.006
4	10	0.486 ± 0.010
5	10	0.091 ± 0.022
	1	0.234 ± 0.004
	0.1	0.472 ± 0.013
	0.01	0.461 ± 0.019
	0	0.503 ± 0.005
	0 (DMSO)	0.499 ± 0.024
6	10	0.565 ± 0.062
7	10	0.604 ± 0.032
8	10	0.581 ± 0.022

[a] The fraction of the total radioactivity remaining cell-associated after 40-min incubation with the compound shown. With the exception of the cumulative control value (line 1), which was for 42 experiments, values shown are mean ± SD of three experiments. The data for the two active compounds (**2** and **5**) include the controls for those experiments; the first value shown is with no addition, the second with the addition of DMSO (0.02% v/v).

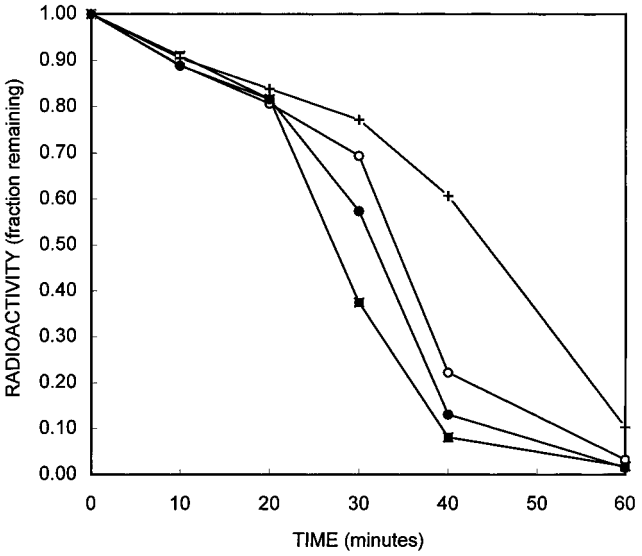

Figure 2. Cell-associated radioactivity in COS-7 cells preloaded with ^{86}Rb, washed in ice-cold BSS, and then incubated at 37 °C in BSS. After 20 min the BSS was replaced by BSS containing 4,4′-(9-fluorenylidene)diphenol (**2**). Incubation at 37 °C was continued for a further 40 min. The radioactivity in the cells at each time point is expressed as a fraction of the total radioactivity initially present in the cells. Concentrations of **2** are 10 (■), 5 (●), 1.25 (○), and 0.313 (+) μM. See text for further details of the experimental method.

pound differs from **1**, the published structure of the active contaminant of phenol red,[5] only in that the latter's fluorene moiety has an additional phenolic hydroxy group. Since **1** was not available to us to test, we compare our data with its potency as reported by Kym et al.[5] Although the two experimental systems differ in detail, it appears that **2** is approximately as active as **1**.

Compound **2** lost activity if its phenol moieties were replaced by aniline moieties (**3**) or derivatized to the 2-hydroxyethyl ether (**4**). This suggests that phenolic moieties are essential for activity. Phenolphthalein (**5**), although much less active than **2**, also contains two phenolic structures. However 1,1′-bi-2-naphthol (**6**), 4,4′-biphenol (**7**), and bis(4-hydroxyphenyl)methane (**8**), all of which contain two aromatic moieties each bearing a phenolic hydroxy group, were inactive.

The three compounds known to be active (**1**, **2**, and **5**) all share a common feature that is absent in all the inactive compounds: a five-membered ring structure, one of whose carbon atoms is disubstituted with *p*-hydroxyphenyl residues.

Because compounds **2** and **5** are readily available, unlike the phenol red contaminant **1**, a comparative study on the mechanism of action of biphenols at the cellular level can now be undertaken. Our identification of several structurally similar compounds that are nevertheless inactive will aid the investigation. The mechanistic study should inform understanding of the underlying cell biology of laxative action and may lead to the development of laxatives with fewer side effects than phenolphthalein.

Experimental Section

^{86}RbCl (product NEZ-072; >1 Ci/g) was from NEN Research Products. The HEPES-buffered balanced salts solution contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM HEPES, adjusted to pH 7.2, and is referred to below as balanced salts solution (BSS). Compounds **2**−**8** were obtained from Aldrich and dissolved in DMSO, prior to dilution at least 5000-fold with BSS.

The ability of compounds to elicit the efflux of monovalent cation from cells was measured as described by Hopp et al.[6] COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 ng/mL each of penicillin and streptomycin, under air:CO_2 (95:5). For the uptake and release experiments, cells were inoculated into 12-well tissue culture plates and allowed to reach 85−98% confluence. To each well were added 1.0 mL of BSS and approximately 4 μCi of ^{86}RbCl. After 90 min of incubation at 37 °C extracellular isotope was removed by washing the cells three times with 3-mL portions of ice-cold BSS. The cells were then reincubated with 1.0 mL of BSS at 37 °C for 10 min, at which time the incubation medium was carefully removed and replaced by a fresh 1.0 mL of BSS. This removal and replacement of the incubation medium was repeated at 20, 30, and 40 min, the fresh medium being either 1.0 mL of BSS or 1.0 mL of BSS containing one of the compounds being tested. At 60 min the incubation medium was removed, and the cells were dissolved in 1.0 mL of 200 mM NaOH. All the incubation media and the solution of the cells were counted for ^{86}Rb radioactivity in a Beckman LS 7500 liquid scintillation spectrometer.

The results were processed as follows. After correction for background radioactivity, the radioactivity present in the cells at the end of the 60-min incubation was expressed as a fraction of the radioactivity present in the cells at the start of the incubation. This total was calculated by summing the radioactivity in the cells at the end of the incubation and the radioactivity in the five samples of culture media removed during the course of the 60 min.

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