

Substituted Benzyl Acetates: A New Class of Compounds That Reduce Gap Junctional Conductance by Cytoplasmic Acidification

D. C. SPRAY, J. NERBONNE, A. CAMPOS DE CARVALHO, A. L. HARRIS, and M. V. L. BENNETT

Marine Biological Laboratory, Woods Hole, Massachusetts 02543; Division of Cellular Neurobiology, Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461; and Division of Biology, California Institute of Technology, Pasadena, California 91125. Dr. Campos de Carvalho's present address is Instituto Biologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brasil. Dr. Harris's present address is Department of Biological Sciences, Stanford University, Palo Alto, California 94305.

ABSTRACT Conductance of gap junctions in many preparations has been shown to be sensitive to cytoplasmic pH, decreasing as pH decreases below 7.5 in fish and amphibian embryos and below 7.1 in crayfish septate axon. We have found a new class of compounds, benzyl acetate derivatives, that reversibly decrease junctional conductance, g_j , when applied in low concentration (~ 1 mM). Simultaneous intracellular pH (pH_i) measurements show that the ester effects are attributable to reduction in pH_i . The sensitivity of g_j to these compounds and the relative lack of side effects make these agents attractive for studies of the role played by gap junctions in normal tissue function. In addition, the finding of cytoplasmic acidification in response to cell exposure to esters suggests caution in interpretation of results obtained using esterified compounds for intracellular loading.

Gap junctions are common intercellular structures and the electrical coupling and molecular transfer that they mediate suggest that they play an important role in cell and tissue function (2, 17). To analyze their role, one would like to have a compound that specifically blocked gap junction channels. Such a compound might act directly or operate through an intermediate that affects gating processes of the channel (19).

Gap junction conductance (g_j)¹ is markedly decreased by treatments expected to lower intracellular pH (pH_i) in many tissues (e.g., liver, D. Meyer and J. P. Revel, personal communication; heart [12]; embryonic cells [15, 23]; crayfish axon [3, 6]; cortex [4]). (Junctions may be insensitive to lowering pH_i in adult lens [13; but see reference 11]; and retina [Griff and Pinto, personal communication].) For frog and fish embryos and crayfish septate axon, where the short term relation between intracellular pH (pH_i) and g_j has been measured, a simple titration curve has been found with an apparent pK

close to the normal cytoplasmic pH and a sufficiently steep slope that acidification by a few tenths of a pH unit significantly decreases g_j (3, 15). For cardiac Purkinje fibers (12) and ventricular myocytes (unpublished observations), normal pH_i is in a region of the curve in which small pH_i changes in either direction alter g_j . One mode of cytoplasmic acidification is to bathe the tissue in CO_2 -equilibrated saline or with salts of weak acids at or near neutrality. CO_2 or the undissociated acid permeates the cell membrane, dissociates, and thereby acidifies the cytoplasm. Although cytoplasmic acidification may have multiple effects, a drug that liberates protons intracellularly might be generally useful in the study of the significance of gap junctional communication.

We report here that a group of membrane permeant esters can be used to acidify the cytoplasm in several cell types. Because relatively low concentrations are effective, these compounds may be more specific in their action than the weak acids used previously. We noticed this method of cytoplasmic acidification during attempts to develop compounds that would release protons by flash photolysis, a process that would

¹ Abbreviations used in this paper: g_j , junctional conductance; o-NBA, o-nitrobenzyl acetate; pH_i , intracellular pH.

allow study of the kinetics of hydrogen ion action on gap junctions (7, 10). The ester *o*-nitrobenzyl acetate (*o*-NBA) and its derivatives are photosensitive, releasing acetic acid upon photolysis (9, 10). However, we find that these molecules also acidify the cytoplasm of several cell types without photolysis. Although we presume that acidification results from hydrolysis of these esters by endogenous esterases (see below), we have been unable to totally block the uncoupling with conventional esterase inhibitors.

A corollary of these findings is that esters used to achieve permeation of active molecular groups that are subsequently released by cytoplasmic esterases (e.g., calcium indicators and buffers [22] and fluorescent dyes [20]) will also acidify the cytoplasm, and this factor should not be ignored in subsequent analysis.

A preliminary account of these data has appeared (18).

MATERIALS AND METHODS

Experiments were performed on pairs of cleavage stage blastomeres mechanically isolated from amphibian (*Rana*), squid (*Loligo*), or fish (*Fundulus*) embryos and on crayfish (*Procambarus*) septate axons. *Fundulus* embryos were generally dissected into single cells and then reassociated into pairs, *Rana* and squid blastomeres were dissected as pairs, and lateral giant axons at the level of the third abdominal ganglion in the crayfish nerve cord were used after removal from the animal and desheathing the cord.

Normal physiological salines contained (in mM) the following: for *Fundulus*: NaCl (120), KCl (1.3), CaCl₂ (0.5), with 5 mM HEPES buffer at pH 7.6 (double strength Holtfreter's solution); for *Rana*: NaCl (60), KCl (0.65), CaCl₂ (0.25), with 5 mM HEPES at pH 7.6 (single strength Holtfreter's solution); for squid: filtered or artificial sea water; and for crayfish: NaCl (205), KCl (5.4), CaCl₂

(13.5), and HEPES (5), buffered to pH 7.4. To each of these solutions was added the appropriate volume of stock solutions of each drug in dimethyl sulfoxide, 95% ethanol or water, depending on solubility. Final concentrations of dimethyl sulfoxide or ethanol were 1% or less. By themselves in the appropriate salines, these organic solvents had no measurable action. Ammonium ions were applied as 10 mM NH₄Cl substituted for 10 mM NaCl, except that in artificial sea water, 50 mM NH₄Cl was used.

Each cell or axon segment was impaled with separate 3 M KCl-filled microelectrodes (5–10 MΩ) for current injection and potential measurement. Current pulses (0.1–0.3-s duration) were injected alternately in each cell and conductances of junctional (g_j) and nonjunctional membranes were calculated from measured input and transfer voltages (1, 16). These pulses were too short to decrease g_j appreciably in fish or amphibia (14, 19).

Intracellular pH in one cell or axon segment was measured with a recessed tip pH-microelectrode (21). Intracellular placement of this electrode was indicated if it recorded the same steady state voltage as the voltage electrode in the same cell in response to a long current pulse (3, 16). The pH_i was determined from the voltage differentially recorded between these two electrodes. Each pH electrode was calibrated (in a simple salt solution) over the pH range 6.0 to 7.5 before and after each experiment. Data were discarded if a calibration changed by >0.1 U.

RESULTS

We expected that an ultraviolet light flash applied to cell pairs loaded with appropriate membrane permeant esters (7–9) would create a pH jump (10), uncoupling the cells. In initial experiments on squid blastomeres, we were surprised to find that *o*-NBA uncoupled cells without a photolyzing light flash (Fig. 1). This uncoupling was due largely to decrease in g_j with only moderate changes in nonjunctional conductances.

The uncoupling by *o*-NBA alone might have been due to direct action of the compound on the channel or secondarily,

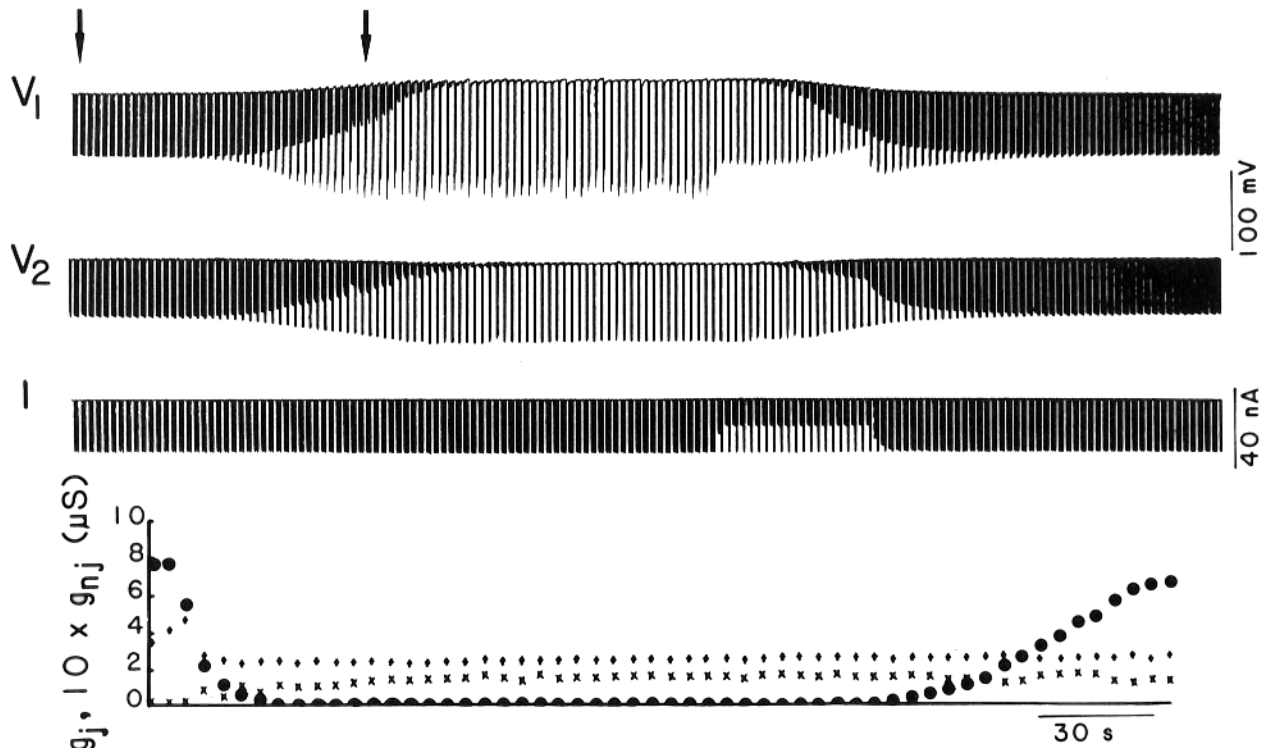


FIGURE 1 *o*-NBA reduces electrical coupling between squid blastomeres. V_1 , V_2 , and I traces show potentials in cells 1 and 2 and current pulses applied alternately in the two cells. In each cell the larger vertical deflections are a measure of the input resistance of that cell and the smaller deflections are a measure of the transfer resistance from the other cell. Beneath the chart recorder records are plotted calculated junctional (●) and nonjunctional (+’s and ×’s) conductances. 5 mM *o*-NBA was applied between arrows. The cells rapidly uncoupled and then recoupled during washing; in this experiment, junctional conductance decreased from ~8 μ S to <0.05 μ S. Initially coupling was too close for accurate measurement of the different conductances. The irregular size of the voltages in cell 1 when the cells were uncoupled was due to “breakdown” caused by the larger voltages associated with decreased input conductance.

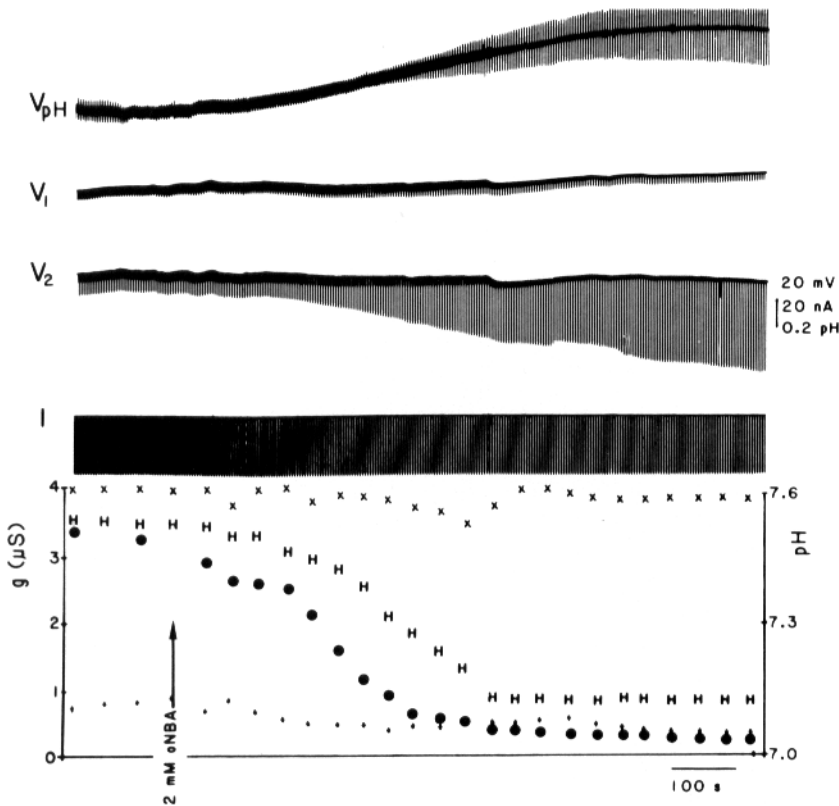


FIGURE 2 *o*-NBA reduces pH_i and electrical coupling between *Rana* blastomeres. Exposure to 2 mM drug starting at the arrow decreases electrical coupling and pH_i in parallel. (pH_i , measured with a recessed tip pH electrode in cell 1, is shown on the top trace; increased acidity is displayed upward in this and other figures; transients are due to capacitive coupling). Display as in Fig. 1 except that pH_i (H) is also shown in the graph below.

as a result of action through an intermediate. Because *o*-NBA uncoupling was sometimes more rapidly reversed by bathing in ammonium salts, it seemed likely that increase in cytoplasmic acidity was involved. Although stable to hydrolysis in solution, these membrane permeant esters might liberate protons intracellularly by action of intrinsic esterases. To test whether the change in g_j was associated with a decrease in pH_i , we inserted a recessed tip pH-sensitive microelectrode into one cell of the pair, here shown for *Rana* blastomeres (Fig. 2). Uncoupling the cells by exposure to the drug was accompanied by dramatic decreases in cytoplasmic pH and g_j .

When g_j was plotted as a function of pH_i , the relation resembled that previously seen with acidification by exposure to CO_2 or the weak acids acetate, lactate, and propionate (Fig. 3). However, a major difference in using the ester as an uncoupling agent is the much lower concentration required, 1–5 mM compared with ~100 mM weak acid or solutions equilibrated with 100% CO_2 . Presumably weak acids are less effective in decreasing pH_i because the membrane permeant undissociated forms are present in low concentrations near neutral pH.

The ester acted on other kinds of coupled cells, suggesting that it might be generally useful. Fig. 4 illustrates the effect of the ester on pH_i and g_j in the septate axon of crayfish. A comparison of the g_j - pH_i curves for crayfish axon treated with the esters and various weak acids shows similar dependence (Fig. 5). This similarity indicates that the action of the ester is the same as that of the weak acids: acidification of the cytoplasmic face of the gap junction. Furthermore, the ester uncoupled blastomeres of *Fundulus* embryos (not shown). This uncoupling was reversed by NH_4 ions suggesting mediation by H ions (not shown), an inference that was supported by photometric determinations on these cells using intracellularly injected phenol red (18).

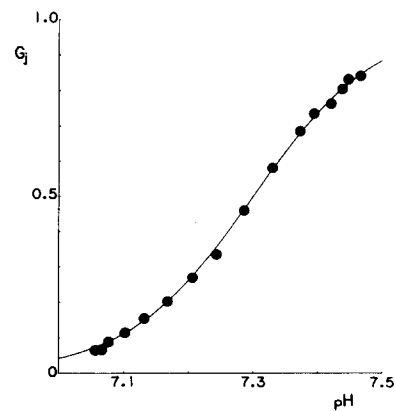


FIGURE 3 The relation of normalized g_j (G_j) to pH_i for the experiment of Fig. 2 is similar to that obtained previously for amphibian and fish blastomeres (solid line) with a pK around 7.3 and a Hill coefficient of about 4.5. This similarity indicates that the effect of the ester on g_j is through acidification of the cytoplasm.

A number of derivatives of *o*-NBA were evaluated for their ability to uncouple cells (Table I). All, except the possibly membrane-impermeant nitroaniline derivative, acted quickly and reversibly, without the need for photolysis. We also tested methyl and ethyl esters of acetic acid in at least two experiments, but neither of these other compounds significantly acidified the cytoplasm or uncoupled the cells at external concentration <10 mM in amphibian or 20 mM in squid. These simpler esters are likely to be poorer substrates for the intrinsic esterases. The aromatic esters, benzyl and phenyl acetate, acidify crayfish axons, but either the esters themselves or products of hydrolysis are toxic, since the decreases in g_j were irreversible and the axons were depolarized.

We then asked whether cytoplasmic acidification by action of intrinsic esterases also occurs following administration of

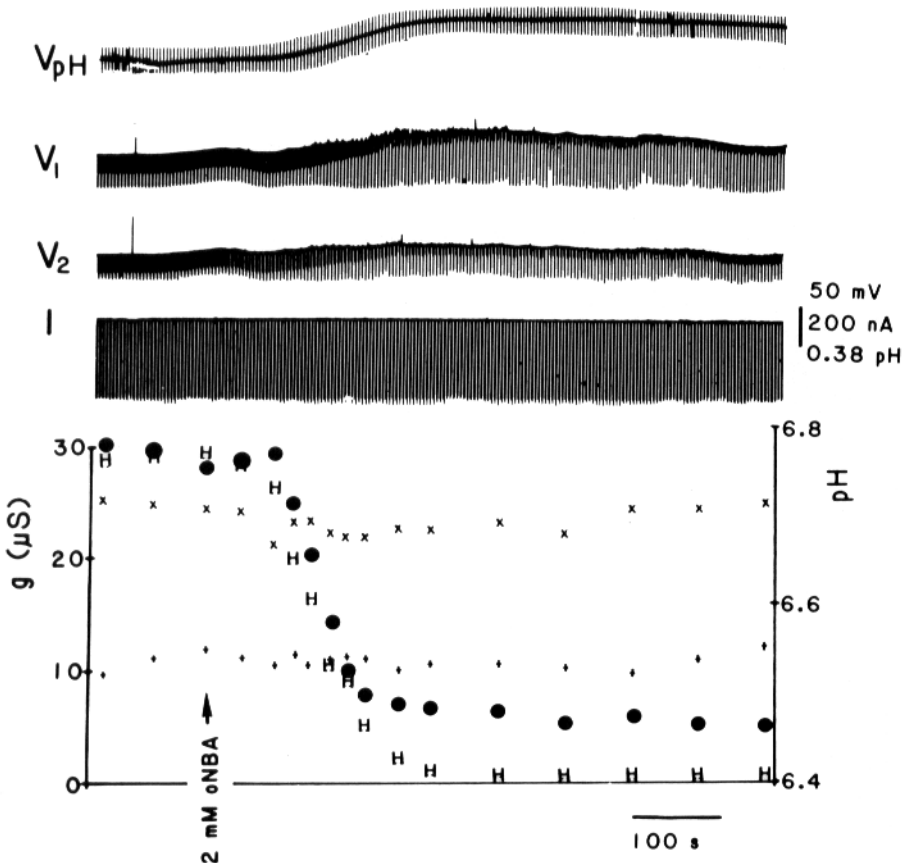


FIGURE 4 *o*-NBA acidifies the interior of the crayfish septate axon and reduces electrotonic coupling. Display as in Fig. 2. Administration of 2 mM *o*-NBA at the arrow decreases pH_i to about 6.4 and decreases g_j by ~80%. Conductance of nonjunctional membrane is little affected.

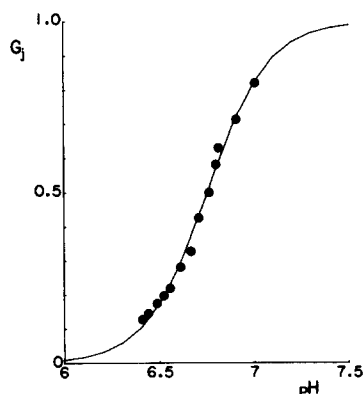


FIGURE 5 The pH conductance relation obtained in the experiment of Fig. 4 for *o*-NBA (●) was similar to that obtained in other axons with exposure to CO_2 equilibrated saline or weak acids (—). The normalized g_j (G_j) value corresponding to pH_i near 7 was obtained earlier in the experiment of Fig. 4.

any of the esterified compounds used to load cells with optical probe molecules or buffers or intracellular messengers. The acetoxy ester of QUIN 2, a calcium indicator developed by Tsien (22), and the dibutyl ester of cAMP, used to deliver cAMP within the cell, can decrease pH_i a few tenths of a pH unit (unpublished observations). The ester 6-carboxyfluorescein diacetate (6-CFA), used to generate internal 6-carboxyfluorescein as an indicator of pH_i (20), decreased pH_i in the crayfish septate axon by several tenths of a pH unit when applied at 0.5 mM (Fig. 6). This compound also reduced g_j . The effect of 6-carboxyfluorescein diacetate illustrated here is especially insidious; both junctional and nonjunctional conductances were decreased so that the coupling coefficient

TABLE I
Compounds Related to *o*-NBA Tested as Uncoupling Agents

Compound	<i>n</i>	Effective concentration* (mM)
<i>o</i> -NBA	>40	1–5
2,6-Dinitrobenzyl acetate	6	2.5
<i>o</i> -Nitro- α,α' -toluene diacetate	3	5
<i>N</i> -Acetyl- <i>N</i> -methyl- <i>o</i> -nitroaniline	5	10 (Never effective)

* At these concentrations, g_j was reduced at least 50%.

changed little. Such effects could go unnoticed in a more complex system in which junctional and nonjunctional conductances could not be unambiguously determined. The effect of these esters was fully reversible by prolonged rinsing in normal saline.

DISCUSSION

The results reported here show that certain esters very effectively reduce pH_i . As a result of the pH change, g_j of treated cells falls and the cells uncouple. The related esters, benzyl and phenyl acetate, also acidify the cytoplasm, but they have much more severe toxic side effects. Further study may reveal other esters at least as well tolerated as *o*-NBA.

The cytoplasmic acidification produced by these esters is unlikely to be the result of either acid or base catalyzed hydrolysis, to which these compounds are extremely resistant. Intracellular esterases exist in high concentrations in certain cell types, an illustrative example being acetylcholinesterases in vertebrate embryos (5). It is plausible, therefore, that these enzymes are largely responsible for the proton release produced by esters. We tested whether certain esterase inhibitors

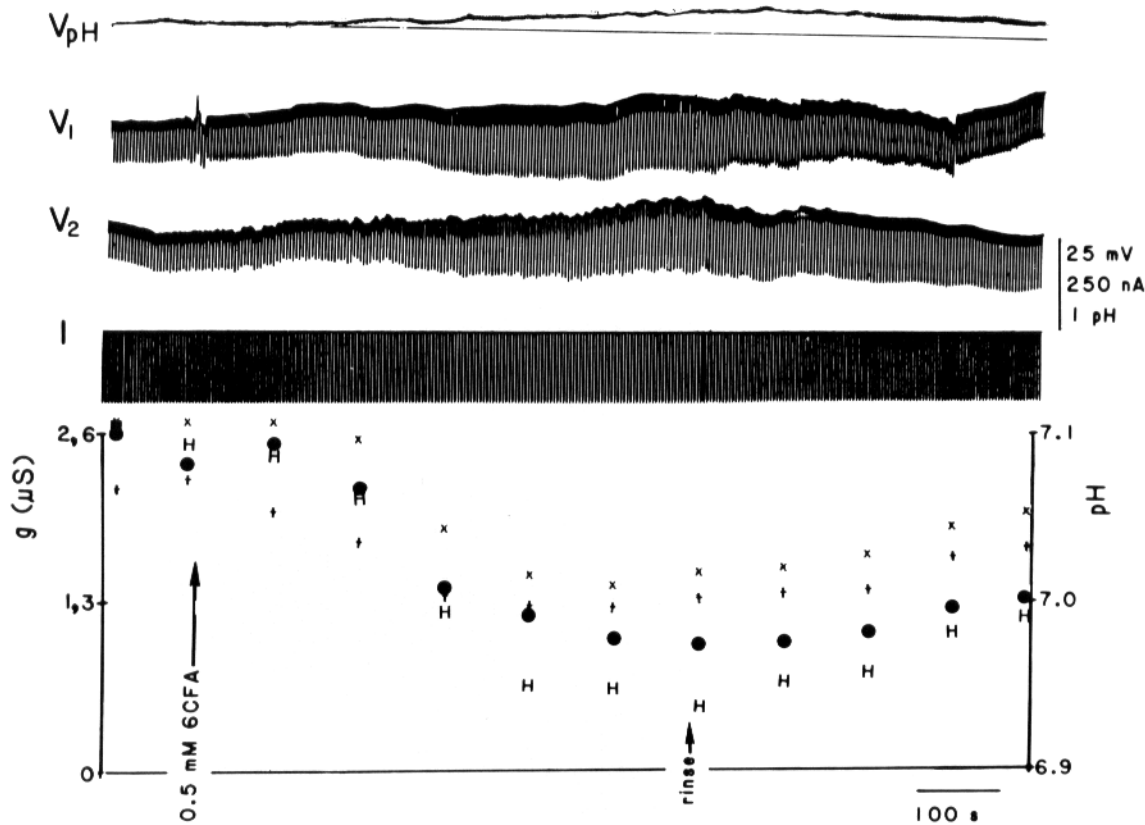


FIGURE 6 6-Carboxyfluorescein diacetate (6CFA) reduces pH_i and electrical coupling between segments of the crayfish septate axon. The drug at 0.5 mM was applied between arrows. (Display as in Fig. 2 except that a horizontal reference line is shown for the recording of pH_i .) As pH_i fell ~ 0.15 U nonjunctional conductances (+’s and x’s, larger values on the ordinate) and g_j (filled circles, smaller values on the ordinate) decreased and partially recovered as pH_i returned towards normal.

TABLE II
Esterase Inhibitors Tested and Found without Effect on *o*-NBA
Uncoupling

Compound	Concentration (mM)	<i>n</i>
Acetazolamide	0.5	2
Phenylmethylsulfonyl fluoride	1	3
Diisopropyl-fluorophosphate	1	3
Eserine	1	3
Neostigmine	20–40*	6

* The drug was added to 3 M KCl in all the intracellular electrodes; all other drugs were added to the extracellular medium.

(Table II) reduced the uncoupling produced by *o*-NBA at concentrations effective in other systems. The results were highly variable: in some cases, the time course of the uncoupling was apparently prolonged but in no case was the uncoupling completely prevented. Among the possible explanations of the lack of blockade of acidification by the esters are low membrane permeability and inappropriate specificity of the esterase inhibitors examined.

The lack of side effects and low concentration at which substituted benzyl esters are effective offer considerable advantage to their use in studies linking chronic junctional uncoupling with physiological response. Conductance of the nonjunctional membrane is little changed by exposure to these compounds (cf. Figs. 1, 2, and 4), and cells are depolarized to a lesser extent than when exposed to high pCO_2 or high extracellular concentrations of weak acids. Moreover, junctional conductance generally recovers after exposures to

these compounds lasting an hour or longer.

Although intrinsic esterases that act on *o*-NBA appear widespread, not every cell type may have them in sufficient concentration to acidify the cytoplasm rapidly. In salivary gland cells of the midge *Chironomus* brief exposure to *o*-NBA does not uncouple without application of photolyzing flashes (10). Complete uncoupling in response to photolysis requires on the order of 30 s, rendering it difficult to correlate uncoupling with changes in pH_i ; long exposures to *o*-NBA uncouple in the absence of light flashes.

Although it is obvious that loading cells with hydrolyzable esters of impermeant molecules tends to acidify the cytoplasm, the degree of acidification may be greater than sometimes appreciated. Recovery of pH_i occurs as would be expected because the acid liberated by hydrolysis can escape from cells in its undissociated form. Our findings also suggest that other esters may have intracellular acidification as a primary or secondary effect. One especially potent category of biologically active compounds are the tumor promoting phorbol esters, of which tetradecanoyl phorbol acetate is the most active. We will report elsewhere that even low doses of these agents but not nonpromoting esters acidify the cytoplasm and reduce g_j (unpublished observations).

We are continuing our search for compounds that liberate protons when irradiated (9) with a clearer recognition that such a compound must not be sensitive to cytoplasmic enzymes. We are now investigating amides, carbamates, and carbonates. When found, such agents will be useful for kinetic studies of gap junction gating by H ions and could, by localized illumination, allow the metabolic and electrical iso-

lation of individual cells in a coupled network. As of now the generality of pH gating of g_j and the effectiveness of α -NBA and its analogues at low concentrations suggest that this class of agents may provide a useful group of uncoupling agents with which one could probe the function of gap junctions in various tissues.

We are grateful to H. A. Lester for support, technical advice, and discussions of the manuscript, to J. Zvilowitz for superb technical assistance and to J. A. Connor for preliminary data showing ester-induced cytoplasmic acidification indicated by absorbance changes of intracellularly injected phenol red.

This study was supported in part by NS-07512 and HD-04248 (to M. V. L. Bennett), NS-16524 and NS-19830 (to D. C. Spray), GM-29836 (to Dr. H. A. Lester), a fellowship from the Los Angeles Affiliate of the American Heart Association (to J. M. Nerbonne), a fellowship from the Fogarty Foundation (A. Campos de Carvalho) and a McKnight Development Award (D. C. Spray).

Received for publication 9 January 1984, and in revised form 7 March 1984.

REFERENCES

- Bennett, M. V. L. 1966. Physiology of electrotonic junctions. *Ann. NY Acad. Sci.* 137:509-539.
- Bennett, M. V. L., and D. A. Goodenough. 1978. Gap junctions, electrotonic coupling and intercellular communication. *Neurosci. Res. Prog. Bull.* 16:377-486.
- Campos de Carvalho, A., D. C. Spray, and M. V. L. Bennett. 1984. pH Dependence of transmission at electrotonic synapses of the crayfish septate axon. *Brain Res.* In press.
- Connors, B. W., and B. R. Ransom. 1982. Electrophysiological studies on ependymal cells of the turtle cortex. *Soc. Neurosci. Abstr.* 8:237.
- Fluck, R. A. 1978. Acetylcholine and acetylcholinesterase activity in early embryos of the medaka *Oryzias latipes*, a teleost. *Dev. Growth Differ.* 20:17-25.
- Giaume, C., M. E. Spira, and H. Korn. 1980. Uncoupling of invertebrate electrotonic synapses by carbon dioxide. *Neurosci. Lett.* 17:197-202.
- Lester, H. A., and J. M. Nerbonne. 1982. Physiological and pharmacological manipulations with light flashes. *Annu. Rev. Biophys. Bioeng.* 11:151-175.
- Nargeot, J., J. M. Nerbonne, J. Engels, and H. A. Lester. 1983. Time course of the decrease in the myocardial slow inward current after a photochemically generated concentration jump of intracellular cAMP. *Proc. Natl. Acad. Sci. USA.* 80:2395-2399.
- Nerbonne, J., and H. A. Lester. 1983. The design of photoactivated molecules: chemical and structural control of reaction rates and efficiencies. *Biophys. J.* 41(2, Pt. 2):3a. (Abstr.)
- Nerbonne, J., H. A. Lester, and J. A. Connor. 1982. Photolabile proton donors and pH control of gap junctions. *Soc. Neurosci. Abstr.* 8:945.
- Rae, J. L., R. D. Thomson, and R. S. Eisenberg. 1982. The effect of 2-4 dinitrophenol on cell to cell communication in the frog lens. *Exp. Eye Res.* 35:597-609.
- Reber, W. R., and R. Weingart. 1982. Ungulate cardiac Purkinje fibres: the influence of intracellular pH on the electrical cell-to-cell coupling. *J. Physiol. (Lond.)* 328:87-104.
- Schuetze, S. M., and D. A. Goodenough. 1982. Dye transfer between cells of embryonic chick becomes less sensitive to CO₂ treatment with development. *J. Cell Biol.* 92:694-705.
- Spray, D. C., A. L. Harris, and M. V. L. Bennett. 1981. Equilibrium properties of a voltage dependent junctional conductance. *J. Gen. Physiol.* 77:77-93.
- Spray, D. C., A. L. Harris, and M. V. L. Bennett. 1981. Gap junctional conductance is a simple and sensitive function of intracellular pH. *Science (Wash. DC)* 211:712-715.
- Spray, D. C., A. L. Harris, and M. V. L. Bennett. 1982. Comparison of pH and Ca dependence of gap junctional conductance. In *Intracellular pH*. R. Nuccitelli and D. Deamer, editors. Alan R. Liss, New York. 445-461.
- Spray, D. C., A. L. Harris, and M. V. L. Bennett. 1982. Control of communication via gap junctions. In *Cellular Communication during Development*. R. Hilfer and J. Sheffield, editors. Springer-Verlag, New York. 57-84.
- Spray, D. C., J. Nerbonne, J. A. Connor, A. L. Harris, A. C. Carvalho, and M. V. L. Bennett. 1982. Nitrobenzyl esters reversibly decrease gap junctional conductance. *J. Cell Biol.* 95(2, Pt. 2):109a. (Abstr.)
- Spray, D. C., R. L. White, A. C. Carvalho, A. L. Harris, and M. V. L. Bennett. 1984. Gating of gap junctional channels. *Biophys. J.* 45:219-230.
- Thomas, J. A., P. C. Kolbeck, and T. A. Langworthy. 1982. Spectrophotometric determination of cytoplasmic and mitochondrial pH transitions using trapped pH indicators. In *Intracellular pH*. R. Nuccitelli and D. Deamer, editors. Alan R. Liss, New York. 105-124.
- Thomas, R. C. (1978). *Ion-sensitive Intracellular Microelectrodes*. Academic Press, Inc., New York.
- Tsien, R. Y. 1981. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature (Lond.)* 290:527-528.
- Turin, L., and A. E. Warner. 1980. Intracellular pH in early *Xenopus* embryos: its effect on current flow between blastomeres. *J. Physiol. (Lond.)* 300:489-504.