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Life Sciences, Vol. 61, No. 7, pp. PL 87-94, 1997

0024-3205/97 $17.00 + .00

PII S0024-3205(97)00543-2

PHARMACOLOGY LETTERS
Accelerated Communication

SCREENING FOR NOVEL DRUG EFFECTS WITH A MICROPHYSIOMETER: A POTENT EFFECT OF CLOFILIUM UNRELATED TO POTASSIUM CHANNEL BLOCKADE

Joshua D. Rabinowitz, Per Rigler, Cathy Carswell-Crumpton, Craig Beeson*, and Harden M. McConnell

Department of Chemistry, Stanford University, Stanford, CA 94305
*Current Address: Department of Chemistry, University of Washington, Seattle, WA 98195

(Submitted March 31, 1997; accepted May 7, 1997;
received in final form May 21, 1997)

Abstract. Changes in cellular metabolism in response to pharmacological compounds can be detected using a biosensor known as a microphysiometer, which measures the rate at which cells release acidic metabolites. We have applied this technique to screen for effects of cation channel blockers on the metabolism of a variety of human and murine cell lines. At concentrations sufficient for cation channel blockade, most of these drugs have little or no effect on cellular metabolism as measured by acid release. In contrast, the potassium channel blocker clofilium triggers sustained increases in acid release at low (≥3 μM) concentration. Acid release persists in media containing high (150 mM) extracellular potassium. This release is not triggered by chemically similar potassium channel blockers. Thus these metabolic effects reflect a potent and specific function of clofilium which is unrelated to potassium channel blockade. Attempts to identify physiological correlates to this response revealed that low concentrations of clofilium but not other potassium channel blockers cause lymphoma apoptosis. These findings demonstrate that effects of clofilium found in other studies may not be due to changes in plasma membrane potassium conductance.

Key Words: cytosensor, microphysiometer, potassium channel blocker, cation channel blocker, drug screen, cellular metabolism, clofilium, antiarrhythmic agent

Introduction

The Cytosensor microphysiometer is a biosensor which measures the rate at which cells acidify their surroundings (reviewed in 1). Cells are loaded into a pH sensing chamber perfused with media. When flow through the sensing chamber is stopped, the pH drops as the cells excrete acidic metabolites such as carbon dioxide and lactic acid. The rate of the decrease in pH during the pump-off period defines the acid release rate, which is measured every 90 seconds. The acid release rate is a useful measure of cellular biochemical responses to pharmacological stimuli because the activation of many signal transduction cascades can be detected within minutes of receptor triggering (1). In addition, while the microphysiometer does not directly measure a specific biochemical pathway, any stimulus which enhances cellular glycolysis will trigger increased acid release. Therefore the microphysiometer can be used to screen compounds for those which have major effects on cellular metabolism.

Several recent reports have suggested that some cation channel blockers and beta-blockers have effects on cellular metabolism beyond those caused by receptor and/or ion channel blockade. In particular, millimolar concentrations of hydrophobic beta blockers inhibit mitochondrial respiration

Corresponding Author: Harden M. McConnell, Department of Chemistry, Stanford University, Stanford, CA 94305; FAX (415) 723-4943; E-mail contact: J. D. R., rabino@leland.stanford.edu

(2) and directly activate G-proteins (3). Micromolar concentrations of the potassium channel blocker clofilium profoundly increase cellular phosphatidylserine synthesis (4), an effect mimicked by quinine but only minimally by another potassium channel blocker, tetraethylammonium (TEA) (5). Clofilium has also been reported to act as a calmodulin antagonist (6).

Motivated by these results, we studied the effects of different beta-blockers and cation-channel blockers on cellular metabolism as measured by the rate of acid release. Advantages of this technique are its kinetic resolution and sensitivity. A disadvantage is that the magnitude of the acid release response is sensitive to cell type and state, because both of these variables affect which biochemical pathways (e.g. aerobic versus glycolytic metabolism) generate most cellular acid release. For this reason, we screened a diverse panel of cell lines, including epithelial-type carcinomas, lymphomas, fibroblasts derived from fetal tissue, antigen-specific T lymphocytes, and Epstein-Barr virus (EBV) transformed B lymphocytes.

None of the commonly prescribed drugs we studied triggers a microphysiometer response at concentrations used in clinical practice in any of these types of cells. However, in several types of cells the potassium channel blocker clofilium, which is currently in clinical trial (7), is a potent trigger of acid release. The mechanism by which clofilium triggers acid release is unrelated to its altering plasma membrane potassium conductance. These results argue for caution in interpretations of *in vitro* studies using clofilium, as well as in its clinical application.

Methods

Drugs. Clofilium tosylate and TEA chloride were purchased from LC Laboratories (Woburn, MA). All other compounds were purchased from Sigma (St. Louis, MO). All calcium channel blockers and beta blockers were dissolved as a 100 mM stock solution in dimethylsulfoxide. All sodium channel blockers were dissolved as a 1 M stock solution in dimethylsulfoxide. Bretylium tosylate was dissolved as a 100 mM stock solution in water, clofilium tosylate as a 4 mM stock solution in water, and TEA chloride as a 1 M stock solution in water. Clofilium tosylate was found to be > 99% pure by reverse phase high pressure liquid chromatography performed in our laboratory.

TABLE I

Compounds Studied

Compound	Class	Therapeutic Concentration[a]	Reference
Nifedipine	Ca++ channel blocker	125 nM	(8)
Verapamil	Ca++ channel blocker	250 nM	(9)
Carbamazepine	Na+ channel blocker	25 μM	(8)
Lidocaine	Na+ channel blocker	10 μM	(8)
Phenytoin	Na+ channel blocker	50 μM	(9)
Bretylium	K+ channel blocker	1 μM	(10)
Clofilium	K+ channel blocker	3 μM[b]	(7, 11)
Tetraethylammonium	K+ channel blocker	not in clinical use[c]	(12)
Propranolol	beta blocker	50 nM	(8)
other beta blockers[d]	beta blocker	20 - 400 nM	(9)

[a] Typical target concentration in human plasma.
[b] Studies in dogs and initial trials in humans suggest that clofilium is active at approximately 3 μM in the target tissue, with plasma concentrations peaking at ≤ 1 μM.
[c] Typical concentrations to achieve channel blockade in vitro are on the order of 1 mM
[d] Alprenolol, atenolol, metoprolol, nadolol, pindolol, and timolol

Microphysiometry. Acid release was measured as previously described (1). In brief, 1 - 3 x 10^6 cells which were grown according to American Type Tissue Culture (ATTC) instructions (with the exception of CHO cells and EAB1 cells, which were grown in RPMI 1640 medium with 10% fetal calf serum) were collected by centrifugation. The cell pellet was resuspended in 60 µL of medium, which was mixed with 15 µL of melted low temperature-melting agarose (Molecular Devices, Sunnyvale, CA) at 37°C. The agarose mixture (7 µL) was immediately spotted onto the membrane of a Cytosensor cell capsule (Molecular Devices, Sunnyvale, CA). After 5 minutes at 4°C to allow the agarose to solidify, the cell capsule was assembled and loaded in the microphysiometer chamber maintained at 37°C. The chamber was perfused (50 µL/minute) with the medium indicated in the text. All microphysiometer media contained 10 mM phosphate, 1 mg/mL endotoxin-free bovine serum albumin (Calbiochem, San Diego, CA) and no bicarbonate or serum (pH 7.4). The rate of acid release was determined with 20 s potentiometric rate measurements after a 58 s pump cycle and a 10 s delay (total cycle time, 90 s). Baseline acid release rates were between 60 - 200 µV s^{-1}.

Cell proliferation. 2 - 5 x 10^4 cells in 200 µL of the appropriate culture media (following ATTC instructions, with the exception of CHO cells and EAB1 cells, which were grown in RPMI 1640 medium with 10% fetal calf serum) were incubated with drug for 24 hours on a 96 well plate. After this time, 1 µCi per well of [^{3}H]thymidine was added and cell DNA was harvested 16 hours later. Proliferation in the absence of drug ranged between 50 - 500 x 10^3 cpm.

Flow cytometry and microscopy. Cells were fixed in 70% ethanol and stored at 4°C prior to analysis. Fixed cells were washed in phosphate buffered saline (PBS) and resuspended in PBS containing RNase (100 µg/mL) and propridium iodide (PI, 10 µg/mL). Following digestion of cellular RNA, the cells were pelleted and resuspended in fresh PBS + PI. Flow cytometric analysis was carried out on a Coulter EPICS 753 flow cytometer (Coulter Electronics, Hialeah, Florida) with 488 nm laser excitation and 635 nm band pass filter emission. Nuclear morphology was studied in > 100 cells per sample using a fluorescence microscope.

Results

We first tested the effect of 8 different cation channel blockers and 7 different beta-blockers on the metabolism of Chinese Hamster Ovary (CHO) cells using a microphysiometer. None of these compounds triggered a significant (> 10%) change in CHO cell acid release at less than 10 times the therapeutic concentration (table 1). Calcium channel blockers and some beta-blockers decreased CHO cell acid release if applied at high (100 µM) concentration. Similar to other investigators, we found that these metabolic effects correlated with the hydrophobicity of the compound (data not shown). This result provided a positive control that our system, like those used by other investigators (2, 3), could detect metabolic side-effects of high concentrations of these compounds. However, because similar responses had been previously reported and these responses did not occur at physiologically relevant concentrations, we did not pursue them further.

We next tested a smaller set of compounds (propranolol, verapamil, lidocaine, TEA, and clofilium) on the metabolism of the murine B cell lymphoma CH27. Of these compounds, only clofilium triggers a significant CH27 cell microphysiometer response at less than 10 times the therapeutic concentration (figures 1 and 2). The most remarkable aspect of this response is that it persists after wash-out of clofilium from the sample (figure 1). In contrast, the metabolic responses detected in response to high concentrations of other cation channel blockers never persist following washout of the compound. Similar sustained clofilium responses are also seen in 7 of 10 other cell lines we studied (table 2). In control experiments, propranolol, verapamil, and TEA all failed to produce significant sustained (> 10%) increases in the acid release of any of these cells lines at up to 30 times their therapeutic concentrations (tested concentrations: propranolol, 100 µM; verapamil, 100 µM; lidocaine, 100 µM; TEA, 30 mM; clofilium, 40 µM) .

To test if the acid release response to clofilium depends on extracellular potassium, we measured the clofilium response of lymphoma cells (either A20 or CH27) perfused with either phosphate buffered sodium chloride (PBNaCl) and glucose or phosphate buffered potassium chloride (PBKCl) and

glucose. Remarkably, the response to clofilium was qualitatively similar in these two media of different ionic composition, as well as in RPMI (figure 1). Because the extracellular potassium concentration in PBKCl matches typical intracellular potassium concentration, the clofilium response in this medium is probably not caused by alterations in plasma membrane potassium conductance. Moreover, even high concentrations of the potassium channel blockers bretylium (≤ 2 mM), which is similar in structure to clofilium, and the non-selective potassium channel blocker TEA (≤ 100 mM) do not recapitulate the clofilium response (data not shown). In addition, 30 mM TEA does not inhibit the acid release response to clofilium. Thus the clofilium response is specific and independent of changes in plasma membrane potassium conductance.

To investigate the effects of clofilium on cell physiology, we incubated CH27 and A20 cells overnight in 40 μM clofilium. The next day we noticed that most cells in the culture dish had died, and that primarily cell debris remained. Therefore, we were not surprised to find that low (<10 μM) concentrations of clofilium block the proliferation of both murine and human lymphoma cells (figure 2, table 2). None of the other drugs studied (table 1) blocked CH27 cell proliferation at ≤ 400-times their clinical concentration.

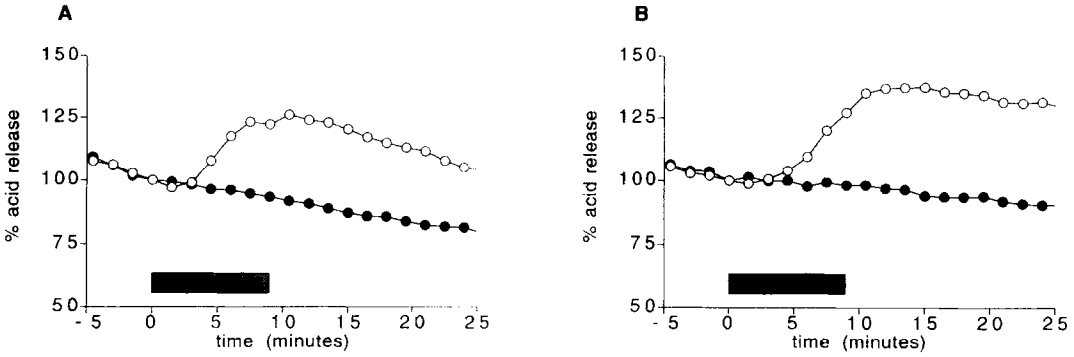

Fig. 1

Clofilium stimulates CH27 B cell lymphoma acid release via a mechanism independent of extracellular potassium. (A) Acid release response to 10 μM clofilium (open circles) compared to no drug (closed circles) in PBNaCl (150 mM NaCl) + glucose (10 mM). (B) Identical experiment in PBKCl (150 mM KCl) + glucose (10 mM). Similar results were obtained in three independent experiments, as well in RPMI media 1640.

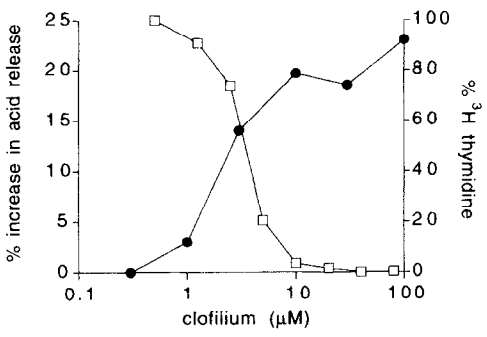

Fig. 2

Acid release (closed circles) and proliferation (open squares) dose response to clofilium. The acid release response is the maximum increase in acid release measured in response to a 9 minute exposure to clofilium in RPMI 1640. The time to the maximum response decreased with increasing clofilium concentration, ranging from 18 minutes at 0.3 μM clofilium to 90 seconds at 300 μM clofilium. Inhibition of proliferation by clofilium was measured as in the methods.

TABLE II

Sensitivity of Different Cell Lines to Clofilium

cell line	type	origin	acid release[a]	EC_{50} proliferation[b]
CHO	epithelial	hamster ovarian carcinoma	3%	80 μM
HeLa	epithelial	human cervical carcinoma	0%	80 μM
IMR90	fibroblast	human fetal lung	0%	10 μM
L929	fibroblast	mouse subcutaneous tissue	**55%**	80 μM
SW3T3	fibroblast	mouse embryo	**48%**	**3 μM**
A20	B lymphocyte	mouse lymphoma	**64%**	**3 μM**
CH27	B lymphocyte	mouse lymphoma	**30%**	**3 μM**
PJRP7.5	T lymphocyte	mouse lymph node[c]	**29%**	**6 μM**
5C.C7	T lymphocyte	mouse spleen[d]	**90%**	not done
CCRF	T lymphocyte	human ALL[e]	**24%**	**8 μM**
Daudi	B lymphocyte	human Burkitt's lymphoma	9%	**3 μM**
EAB1	B lymphocyte	human, EBV transformed	**15%**	not done

[a] The indicated cell line was treated with 40 μM clofilium for 9 minutes in RPMI 1640, and the % increase in acid release 30 min. after drug wash-out was recorded; significant responses are in bold; in cases where the same experiment was repeated several times on a single day, the standard deviation of the acid release response was always less than ± 10%; between days the magnitude of the response varied by up to a factor of two, while the potency remained nearly constant
[b] The concentration of clofilium which caused a 50% decrease in the proliferation of the indicated cell line was measured as described in the methods; EC_{50} < 10 μM marked in bold
[c] *in vitro* transformed T cells from a mouse immunized with myelin basic protein peptide
[d] from a 5C.C7 T cell receptor transgenic mouse; grown *in vitro* by weekly antigenic stimulation
[e] ALL, acute lymphoblastic leukemia

The morphology of cells dying from clofilium treatment is consistent with apoptotic cell death. Typical characteristics of apoptosis are chromatin condensation, cell shrinkage, and formation of cytoplasmic blebs and apoptotic bodies (13). As shown in figure 3, approximately 20% of CH27 cells treated overnight with 10 μM clofilium show decreased DNA staining characteristic of chromatin condensation (compare areas A and C) and decreased cell size as measured by forward angle light scatter (compare areas B and D). In addition, there is a 2-fold reduction in the fraction of cells in the S and G2 phases of the cell cycle following clofilium treatment (left hand panels, large peak is cells in G0/G1 and area to the right of this peak is S followed by G2), consistent with some decrease in overall mitotic activity without blockade of any particular point in the cell cycle. Apoptosis was confirmed by microscopic analysis of nuclear morphology of cells in this sample, which revealed chromatin condensation and nuclear fragmentation in 26% of cells, compared to <1% of cells in the control sample. In dose response studies with CH27 cells, apoptosis occurs at concentrations of clofilium similar to those required to block CH27 cell proliferation (figure 2).

Discussion

The Cytosensor microphysiometer can detect activation of a wide variety of cell signaling pathways by monitoring changes in cellular acid release (1). In this report we describe the microphysiometer response to various compounds which block cation channels, most in clinical use as antiarrhythmic or antihypertensive drugs. The effect of these cation channel blockers was studied in the absence of stimuli (e.g. receptor ligands) known to cause significant cation fluxes across the plasma membrane. Thus, it is not surprising that we find that in general these compounds have only a small effect on cellular acid release at concentrations sufficient for ion channel blockade.

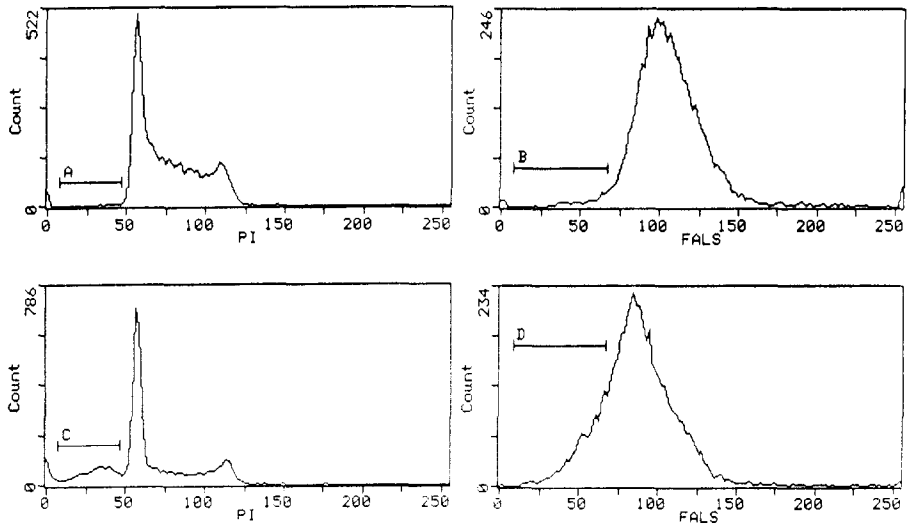

Fig. 3

Clofilium triggers CH27 B cell lymphoma apoptosis. Shown is flow cytometric analysis of CH27 cell DNA staining (left hand panels, A and C) and size (right hand panels, B and D) after clofilium treatment. Upper panels (A and B) are negative control (no clofilium). Lower panels (C and D) received 10 μM clofilium for 18 hours. Cells with sub-G0/G1 DNA staining (propridium iodide, PI) or decreased cell size (forward angle light scatter, FALS) suggestive of apoptosis are indicated by the bars. The fraction of cells in the marked areas are A = 0.7%, B = 2.8%, C = 18.3%, and D = 17.4%.

TABLE III

Selected *In Vitro* Studies Involving Clofilium.

Effect	Target	Concentration	Ref.
increase action potential duration	canine Purkinje fibers	20 nM	15
increase effect refractory period	ischemically injured canine epicardium	300 nM	16
use-dependent block of transient outward K+ current	rat ventricular myocytes	500 nM	17
increase acid release	murine B cell lymphoma	3 μM	this report
block proliferation	human B cell lymphoma	3 μM	this report
block glibenclamide-sensitive K+ channel	transfected Xenopus oocytes	6 μM	18
block delayed rectifier K+ current	neuroblastoma x glioma hybrid cells	6 μM	19
cause apoptosis	murine B cell lymphoma	10 μM	this report
block proliferation	human peripheral blood T cells	10 μM	14
prevent ventricular fibrillation	isolated rabbit heart	10 μM	20
block activation of phosphodiesterase	partially purified calmodulin	20 μM	6
block $K_{V1.2}$ and $K_{V1.4}$ current	transfected Xenopus oocytes	40 μM	21
block delayed rectifier current	guinea pig ventricular cells	50 μM	22
block HIsK current	transfected Xenopus oocytes	80 μM	14
enhance transient outward K+ current	transfected Xenopus oocytes	100 μM	23
reduce excitatory postsynaptic potentials	frog semicircular canals	1 mM	24

While most beta-blockers and cation-channel blockers have no detectable effect on cellular metabolism as measured by acid release at typical therapeutic concentrations, the potassium blocker clofilium triggers sustained increases in acid release. The clofilium response is particularly interesting for several reasons: (1) the response persists in media containing no extracellular potassium and in media with high extracellular potassium i.e. the response is likely unrelated to potassium, (2) half-maximal response occurs at 3 μM, a lower concentration than required to block many potassium channels (table 3), (3) other quaternary amines, including ones with similar structure, do not mimic this effect of clofilium, and (4) similar concentrations of clofilium block T cell proliferation (14; J.R. and C.B. unpublished results) and cause lymphoma apoptosis, suggesting that clofilium derivatives may have potential as chemotherapeutic agents. Our finding that clofilium causes both acid release and apoptosis is consistent with recent work demonstrating that in a variety of cases apoptosis is preceded by enhanced cellular acid release (25, 26). However, many stimuli which trigger acid release do not cause apoptosis (1), and our data are not sufficient to prove a direct correlation between the acid release and apoptosis responses to clofilium.

Further work is required to identify the biochemical target(s) responsible for these new effects of clofilium. Preliminary studies in our laboratory suggest that the increases in glycolytic metabolism described in response to clofilium in this report may occur in response to a more direct effect of clofilium on redox metabolism. Our preliminary data are most consistent with clofilium inhibiting either the pentose phosphate pathway (which produces NADPH) or electron transfer between NADPH and a quinone substrate. Inhibition of redox metabolism may play a role in clofilium's triggering of apoptosis.

Acknowledgements

The authors thank Brett Premack for his valuable advice concerning potassium channel blockers and experimental design, Steve Sherwood for help determining the mechanism of clofilium-induced cell death, Keri Tate for her experimental contributions, and Patricia Jones and the Stanford University Department of Biological Sciences for use of the flow cytometry facilities. This work was supported by grant AI13587 to H. M. M. from the National Institutes of Health. Funding for J. R. was provided by the Medical Scientist Training Program and C. B. by the Cancer Research Institute.

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