The effects of selected lichen extracts and purified compounds on the rat heart.

Richard C. Witt

Follow this and additional works at: https://digitalcommons.unomaha.edu/studentwork

Recommended Citation
https://digitalcommons.unomaha.edu/studentwork/3378

This Thesis is brought to you for free and open access by DigitalCommons@UNO. It has been accepted for inclusion in Student Work by an authorized administrator of DigitalCommons@UNO. For more information, please contact unodigitalcommons@unomaha.edu.
THE EFFECTS OF SELECTED LICHEN EXTRACTS AND PURIFIED COMPOUNDS ON THE RAT HEART

A Thesis

Presented to the

Department of Biology

and the

Faculty of the Graduate College

University of Nebraska

In Partial Fulfillment of the Requirements for the Degree

Master of Arts

University of Nebraska at Omaha

Submitted by

Richard C. Witt

July, 1996
THESIS ACCEPTANCE

Acceptance for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the degree of Master of Arts, University of Nebraska at Omaha.

Committee

<table>
<thead>
<tr>
<th>Name</th>
<th>Department/School</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chairperson

Date July 24, 1996
ABSTRACT

Extracts from Letharia vulpinia, Umbilicaria americana, Cladina subtenuis, Xanthoparmelia cumberlandia, Rimelia reticulata, and Parmotrema austrosinense were used to study their electrophysiological effects on rat heart ventricular tissue. Additionally, solutions of four purified lichen metabolites — vulpinic acid, usnic acid, stictic acid, and atranorin — were studied in the same manner. The action potential duration at 50% and 90% of repolarization was significantly reduced by two of the extracts and metabolites, those containing vulpinic acid and usnic acid and the purified forms of those compounds. A positive relationship between the effects of extracts and purified compounds was exhibited. These data suggest that the active metabolites may depress inward calcium current or enhance outward potassium currents. However, when single, dissociated rat myocytes were exposed to vulpinic acid, activation of an ATP-sensitive potassium channel was observed, and this was blocked with glibenclamide, a specific blocker of that channel. This ATP-sensitive potassium channel created an overlapping current which made it appear that an inward calcium current or outward repolarizing currents were affected, when in fact they were not.
ACKNOWLEDGEMENTS

This project was performed in partial fulfillment of an MA degree by the author. It was initially supported in part by a grant from the Department of Biology, University of Nebraska at Omaha. Final phases of research and writing of this thesis was supported by a Graduate Thesis/Dissertation Scholarship and a Reichenbach Summer Graduate Scholarship from the Office of Graduate Studies and Research, U.N.O.

The author would like to thank Dr. Robert Egan, U.N.O., for providing lichen specimens and purified compounds, and Dr. George Rozanski, U.N. Medical Center, for the use of his electrophysiology lab.

The author's graduate committee: Dr. Egan, Chairman, Dr. Rozanski, and Dr. Tom Weber, provided helpful insight and advice during the writing and revision of this thesis.

Dr. Sylvia Witt, the author's wife, and Mrs. Rosemary Witt, mother, also provided unselfish and immeasurable support.

For all of the above, the author is extremely grateful. Thank-you!
# TABLE OF CONTENTS

Introduction.................................................................................................................................1

Materials and Methods................................................................................................................
  - Extract Preparation..............................................................................................................2
  - Thin-layer Chromatography...............................................................................................3
  - Electrophysiology.................................................................................................................3
  - Isolation of Cardiac Myocytes............................................................................................6
  - Recording Techniques.........................................................................................................8
  - Patch-clamp Experimental Protocols..................................................................................9
  - Measurements....................................................................................................................9

Results......................................................................................................................................11
  - Effects of Lichen Extracts and Purified Compounds..........................................................11
  - Relationship of Purified Compounds and Extracts.............................................................14
  - Effects of Blocking Agents..................................................................................................14
  - Effects of Vulpinic Acid on Isolated Currents....................................................................17
  - Effects of Blocking Agents on Isolated Currents...............................................................19

Discussion................................................................................................................................25

Literature Cited.............................................................................................................................31
TABLES

Table I: Abbreviations...................................................................................................4
Table II: Metabolites Detected by Thin Layer Chromatography...............................4

FIGURES

Figure 1: Chemical Structures.......................................................................................5
Figure 2: Experimental Set-up.......................................................................................7
Figure 3: Measured Parameters of Rat Action Potentials .............................................10
Figure 4: Effects of Ethanol on Action Potential Duration..............................................12
Figure 5: Effects of Lichen Extracts.............................................................................13
Figure 6: Effects of Purified Compounds......................................................................15
Figure 7: Action Potentials with Control and Vulpinic Acid..........................................16
Figure 8: Comparison of Changes by Extracts and Compounds.................................16
Figure 9: Effects of Blocking Agents on Action Potentials............................................18
Figure 10: Vulpinic Acid Dose-Response Curves..........................................................20
Figure 11: Effect of Vulpinic Acid on Calcium Current................................................21
Figure 12: Effects of 4-Aminopyridine on Potassium Currents......................................22
Figure 13: Current Traces with and without 4-Aminopyridine......................................23
Figure 14: Effect of Glibenclamide on Steady State Current.......................................24

APPENDIX

Relationship Between Extracts and Compounds, APD$_{50}$ and $V_{\text{max}}$........................i
Relationship Between Extracts and Compounds, Amp and RMP.......................................ii
INTRODUCTION

Several lichen species have been utilized as medicines, curatives, and folk remedies since the Middle Ages. Certain American Indians and Chinese herbalists have used them as expectorants. Some forms are being sold in parts of Europe today to treat a variety of ailments such as diabetes, inflammation of nose or throat membranes, or lung diseases, and in other parts of the world, eg. India and South America, they have been popular as treatments for liver diseases and for a number of other purposes. *Evernia vulpina* (*Letharia vulpina*) has been used as a poison for wolves (Hale 1983; Fink 1910).

In the last three decades several lichen-forming fungi have been investigated for the pharmacological activity of their primary and secondary metabolites. Some of these compounds appear to have useful effects including antibiotic activity (Varita 1973), antiviral and antitumor activities (Fukuoka et al. 1968; Hirabayashi et al. 1989), and anti-inflammatory effects (Otsuka et al. 1972; Shibuya et al. 1983). The possibility that more activities of these compounds will be discovered and utilized is likely, and may become an important part of the bio-pharmaceutical industry (Yamamoto et al. 1993).

Soderberg, in the early 1950’s performed a series of investigations to determine the *in vivo* effects of usnic and vulpinic acids on anesthetized cats. He showed that usnic acid increased respiration rate and amplitude, produced a transient fall in blood pressure, and increased body temperature. A dose-dependent relationship between usnic acid and a rise in blood glucose level was also observed. At concentrations above 10 mg/kg, lethality occurred and muscular rigor was observed almost immediately after death. These effects were similar to those produced by dinitrophenols (Soderberg 1953). The use of
vulpinic acid in cats resulted in a similar hyperventilation and hypotension, but it had an opposite effect on blood glucose regulation. In some experiments vulpinic acid normalized blood-sugar levels in depancreatized cats (Soderberg 1954).

A review of published literature over the past thirty years reveals that little basic research has been performed on the pharmacological and physiological effects of lichen compounds on mammalian systems or on the mechanisms of these effects. This study is the first known investigation of the effects of lichen products on the electrical properties of ventricular muscle cells.

The purpose of this experiment was twofold: 1.) To investigate the effects of several lichen extracts and purified lichen compounds on electrophysiological parameters of rat papillary muscle in vitro, and 2.) To determine ionic mechanisms of the effects related to a purified lichen metabolite (vulpinic acid) utilizing whole-cell patch-clamp techniques; primarily the effects on potassium and calcium currents were examined in isolated, single myocytes from rat heart.

MATERIALS AND METHODS

**Extract preparation.** Extracts from 6 lichen species—*Letharia vulpina* (L.) Hue,*Cladina subtenuis* (des Abb.) Hale & Culb., *Umbilicaria americana* Poelt & T. Nash, *Xanthoparmelia cumberlandia* (Gyelnik) Hale, *Parmotrema austrosinense* (Zahlbr.) Hale, and *Rimelia reticulata* (Taylor) Hale & Fletcher—were prepared as follows: 0.5 g of each of the lichens was dispensed into separate test tubes and crushed with a pestle, followed by the addition of 7.5 mL of acetone to each tube. The tubes were shaken vigorously and then stoppered and allowed to stand overnight (approximately 16 hours). By the end
of this time all insoluble thallus material had settled to the bottom, and the acetone was decanted into a clean tube. The tubes were then left unstoppered and the acetone was allowed to evaporate in a fume hood. The dissolved material was deposited on the side of the tube as the acetone evaporated (approx. 72 hours). 7.5 mL of 100% ethanol was then added to each tube and shaken vigorously to redissolve the extracted material. For the experiments, the extracts were diluted to 0.1% in Tyrodes solution. All lichens used for this study were kindly supplied by Dr. Robert Egan, Dept. of Biology, University of Nebraska at Omaha. A listing of abbreviations used in the thesis is included in Table I.

Pure samples of vulpinic acid (VA), usnic acid (UA), stictic acid (SA), and atranorin (At) were obtained from Sigma Chemical Company. Stock solutions of 2 mM in 100% ethanol were prepared, and working concentration for all experiments were 2 μM of the purified compounds. The control solution for all experiments was 0.1% ethanol in normal Tyrodes solution. The composition of this solution was (in mM): NaCl, 127.9; NaH₂PO₄, 0.9; NaHCO₃, 20.0; glucose, 5.5; MgSO₄, 0.5; KCl, 4.0; and CaCl₂, 2.5; pH 7.4.

**Thin-layer chromatography.** Thin-layer chromatography (Culberson 1972) was performed on all purified compound solutions to verify their presence and on all extracts to ascertain the existence of the desired metabolites. Results of the procedure are shown in Table II. Chemical structures of the purified compounds are shown in Figure 1 (from Hale 1983).

**Electrophysiology.** Papillary muscles were isolated from the hearts of euthanized laboratory rats (*Ratus norvegicus*, Sprague Dawley) and pinned to the floor of a tissue bath perfused with oxygenated (95% O₂, 5% CO₂) Tyrodes
TABLE I. Abbreviations used in the thesis.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA</td>
<td>Vulpinic Acid</td>
</tr>
<tr>
<td>UA</td>
<td>Usnic Acid</td>
</tr>
<tr>
<td>SA</td>
<td>Stictic Acid</td>
</tr>
<tr>
<td>At</td>
<td>Atranorin</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting Membrane Potential</td>
</tr>
<tr>
<td>Amp</td>
<td>Action Potential Amplitude</td>
</tr>
<tr>
<td>APD</td>
<td>Action Potential Duration</td>
</tr>
<tr>
<td>APD$_{50}$</td>
<td>Action Potential Duration at 50% of Repolarization</td>
</tr>
<tr>
<td>APD$_{90}$</td>
<td>Action Potential Duration at 90% of Repolarization</td>
</tr>
</tbody>
</table>

TABLE II. Metabolites detected in each extract by thin layer chromatography.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>MAJOR METABOLITES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Letharia vulpina</td>
<td>vulpinic acid</td>
</tr>
<tr>
<td>Cladina subtenuis</td>
<td>usnic acid &amp; fumarprotocetraric acid</td>
</tr>
<tr>
<td>Umbilicaria americana</td>
<td>gyrophoric acid</td>
</tr>
<tr>
<td>Rimelia reticulata</td>
<td>atranorin &amp; lecanoric acid</td>
</tr>
<tr>
<td>Parmotrema austrosinense</td>
<td>atranorin &amp; salazinic acid</td>
</tr>
<tr>
<td>Xanthoparmelia cumberlandia</td>
<td>stictic acid, usnic acid, &amp; norstictic acid</td>
</tr>
</tbody>
</table>
FIGURE 1. Chemical structures and molecular weights of purified lichen compounds used in the experiment.
solution warmed to 37% C. Isolated tissues were paced with bipolar electrodes (Frederick Haer, P6i stimulator) at a frequency of 1 Hz with 3-5 msec duration pulses. Transmembrane potentials were recorded (World Precision Instruments Model KS 700) with microelectrodes (DC resistance 10-20 Mohms), displayed on an oscilloscope (Tektronix Model 5111A), and photographed with a Polaroid camera. After equilibration of the tissue in Tyrodes solution for at least one hour, the control solution (0.1% ethanol in Tyrodes) was allowed to superfuse the tissue for at least 15 minutes, followed by the extract (0.1% of ethanol-resolubolized extract) or purified compound (0.1% of the ethanol solution to give a final concentration of 2 μM) for 10-15 minutes before being washed out with 0.1% ethanol control solution. A photograph of the action potential trace on the oscilloscope was made immediately before beginning wash-out. A schematic diagram of the experimental set-up is shown in Figure 2 (modified from Jalife and Moe 1979).

Isolation of cardiac myocytes. Excised rat hearts were mounted on a modified Langendorff perfusion system, and digested using collagenase Type 2 (Worthington Biochemical) as previously described (Rozanski and Witt 1994, 1995). Once dissociated, cells are placed in a nominal calcium Tyrodes solution (~100 μM Ca2+). Calcium is added back to the cells in an incremental manner by increasing calcium concentration in each of 3 additional washes with Tyrodes, ie. the first wash has approximately 0.5 mM, the second wash has approximately 0.9 mM and the third wash contains approximately 1.4 mM Ca2+. Cells are cultured in Dulbecco’s modified Eagle’s medium plus Ham’s F-12 (1:1) after the third wash and are incubated at 37% C until used on the same day of isolation. Aliquots of myocytes were transferred to the cell chamber
FIGURE 2. A schematic of the experimental set-up for recording action potentials from rat papillary muscle.
mounted on the stage of an inverted microscope and superfused at a rate of 1-2 mL/min with modified Tyrodes solution containing 0.5 mM CdCl₂ to block residual Ca²⁺ channels and ethanol at the same concentration as that used with the VA solution (for controls) or VA solution itself (for experiments). The modified Tyrodes solution contained (in mM): NaCl, 130; KCl, 4.0; MgCl₂, 1.2; CaCl₂, 1.8; n-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5.0; glucose, 18; pH 7.4 adjusted with NaOH.

Recording techniques. Ionic currents were recorded using the whole-cell configuration of the patch-clamp technique. Borosilicate glass capillaries were pulled (Sutter Instruments, Model P-87) to produce an internal tip diameter of 1-2 μm and filled with pipette solution containing (in mM): KCl, 135; MgCl₂, 3.0; HEPES, 10; Na₂-ATP, 3.0; EGTA, 10; Na-GTP, 0.5, pH 7.2 adjusted with KOH. Filled pipettes with resistances of 2-4 MΩ were coupled to a patch-clamp amplifier (Axopatch 1C, Axon Instruments) and after creation of a gigaohm seal, the membrane within the pipette was ruptured. Series resistance was compensated at this time and whole-cell capacitance (Cₘ) derived as the area under the capacitative transient divided by the amplitude (-5 mV) of an applied test pulse. A computer program (pClamp, Axon Instruments) controlled command potentials and acquired current signals which were filtered at 2 kHz using a four-pole low-pass Besel filter. Currents were sampled at 4 kHz by a 12-bit resolution analog-digital-converter (Tecmar Labmaster) and stored on the hard disk of a 486 computer. All experiments were performed at room temperature (22-24% C).

The transient outward K⁺ current (Iₒ) was evoked in each cell by 500 ms depolarizing pulses to test potentials between -40 and +60 mV (0.2 Hz). The
holding potential in these experiments was -80 mV and a 100 ms prepulse was applied to -60 mV to inactivate the fast Na+ current. At each test pulse the amplitude of $I_{to}$ was measured as the difference between peak outward current and the current level at the end of the depolarizing clamp pulse, referred to in this experiment as the steady state. The steady state current ($I_{ss}$) was measured from the same current trace as the difference from the current level at the end of the clamp pulse and 0 current level. Data were normalized as current densities by dividing measured current amplitude by whole-cell capacitance (pA/pF).

The L-type Ca$^{2+}$ current ($I_{Ca}$) was also measured in some cells by 300 ms depolarizing pulses from a holding potential of -40 mV. Currents were monitored continuously by repetitive (0.1 Hz) test pulses to 0 mV, and at regular intervals current-voltage (I-V) data were recorded by varying the test potential in 10 mV steps (0.2 Hz) over a range of -30 to +60 mV. For each test pulse the amplitude of $I_{Ca}$ was measured as the difference between the peak inward and the current level at the end of the depolarizing clamp pulse. Data for $I_{Ca}$ were also normalized by dividing the measured current amplitude by whole cell capacitance.

*Patch-clamp experimental protocols.* After seal formation on each cell, the modified Tyrodes containing CdCl$_2$ and ethanol was allowed to perfuse the cell for 15 minutes with recordings made every 5 minutes. The solution was then switched to one containing the VA and after a 1-2 minute wash-in, recordings were made every five minutes to observe effects.

*Measurements.* Measurements were made from transmembrane recordings as follows (see Figure 3): resting membrane potential (RMP), action
Measurement of Action Potential Parameters

FIGURE 3. Illustration of parameters measured for rat action potentials. Action potential calibrations are n mV and msec and values in parentheses are for $V_{\text{max}}$. Abbreviations are explained in the text.
potential amplitude (Amp.), action potential duration at 50% and 90% of repolarization (APD\textsubscript{50} and APD\textsubscript{90}), and maximum rate of rise of the action potential upstroke (V\textsubscript{max}), as measured from the output of an electronic differentiator (WPI). Statistical analyses were done with a commercial computer program using the paired T-test comparison for control and extract or metabolite data.

RESULTS

Effects of lichen extracts and purified compounds. Rat papillary muscles were superfused initially with ethanol at various concentrations ranging from 0.01% to 0.2% in Tyrodes solution for 15 minutes to determine if the solvent had direct effects on electrophysiological properties. Ethanol had little effect on RMP, amplitude, V\textsubscript{max}, APD\textsubscript{50}, or APD\textsubscript{90} at any of the concentrations tested (APD data shown in Figure 4), and ethanol at 0.1\% was the control used throughout the experiment. Figure 5 summarizes the effects of different extracts on ventricular tissue for all measured parameters. These data illustrate that the effects on RMP, Amp., and V\textsubscript{max} (excluding \textit{Letharia vulpina}) were minimal, but changes in APD\textsubscript{50} and APD\textsubscript{90} were more marked. Four of the extracts caused shortening, with \textit{Letharia vulpina} eliciting the greatest shortening, while \textit{Rimelia reticulata} and \textit{Umbilicaria americana} caused prolongation (since both \textit{Rimelia reticulata} and \textit{Parmotrema austrosinense} contained atranorin, an average of the two were used for the value recorded in Figure 8). The effects of all extracts were reversible except for that from \textit{Letharia} which did not usually wash out and was eventually toxic to the cell. The \textit{Letharia} extract caused significant shortening of both APD\textsubscript{50} and APD\textsubscript{90} (p<0.01) and the \textit{Cladina} extract caused
FIGURE 4. Summary of the effects of lichen extracts on each measured parameter. Abbreviations are: C=Cladina; L=Letharia, P=Parmotrema; R=Rimelia; U=Umbilicaria; X=Xanthoparmelia. *=p<0.05, **=p<0.01. n=5 for all extracts except Letharia (n=6).
FIGURE 5. The effects of ethanol at various concentrations on $\text{APD}_{50}$ and $\text{APD}_{90}$. 
significant shortening of both APD's as well (p<0.05). For all extracts and purified compounds the time of onset of effect was usually 4--8 minutes.

The purified compounds caused similar effects as the extracts, as shown in Figure 6. RMP, Amp., and $V_{\text{max}}$ were not significantly altered while APD$_{50}$ and APD$_{90}$ were more profoundly changed. Two of the purified metabolites caused significant shortening of APD, with VA having the greatest effects on APD$_{50}$ and APD$_{90}$ (62.2% and 52.7% of control, respectively; p<0.01), while UA also produced large APD$_{50}$ and APD$_{90}$ shortening (65.0% and 65.1% respectively; p<0.05). Figure 7 shows superimposed action potentials recorded from the same cell before and after superfusion with VA.

Relationship of purified compounds and extracts. A positive, qualitative relationship between purified compounds and lichen extracts was found as shown in Figure 8. An exact concentration of VA contained in the extract from *Letharia vulpina* could not be determined, but spotting densities on chromatography plates revealed a concentration of VA in the extracts probably greater than 2 μM. The APD$_{90}$ used for this comparison was the best correlative parameter, but APD$_{50}$ also showed a positive relationship between purified compounds and extracts (Appendix i). A comparison of $V_{\text{max}}$ data also showed a similar trend, while the other parameters (RMP and Amp.) did not show a relationship between the two groups of muscles studied (Appendix ii).

Effects of blocking agents. Although the focus of the present study was to examine the overall effects of lichen metabolites, some experiments were performed with blocking agents to explore possible ionic mechanisms. The use of 4-aminopyridine (4-AP), a blocker of $I_{\text{to}}$ (Castle & Slawsky 1992), did not
FIGURE 6. Effects of purified compounds on each measured parameter.

Abbreviations are: UA=usnic acid; VA=vulpinic acid; At=atrnorin; SA=stictic acid. * = p<0.05, ** = p<0.01. n=5 for at and UA; n=6 for VA and SA.
FIGURE 7. Superimposed action potentials with 2 μM VA and control (0.1% ethanol). Calibration value in parentheses is for $V_{\text{max}}$.

FIGURE 8. Comparison of changes in $\text{APD}_{90}$ in response to extracts and purified compounds. Abbreviations are the same as for previous figures, except P=$Parasotrema/Rimella$. 
prevent further shortening of APD\textsubscript{50} when VA was subsequently applied (Figure 9A). With 1 mM 4-AP, there was no reduction of the effect whereas at 2 and 5 mM 4-AP, action potential duration was initially prolonged but the usual effect of VA was eventually observed after a delay of about 10 minutes.

The use of 3 mM cobalt chloride (CoCl\textsubscript{2}), a blocker of Ca\textsuperscript{2+} channels, however, did prevent further shortening of the APD\textsubscript{50} and reduced the shortening of APD\textsubscript{90} when VA was applied (Figure 9B). This would indicate that Ca\textsuperscript{2+} channels were being influenced by the extracts and purified compounds.

Pretreatment of the tissue with 10\textsuperscript{-7} M Bay K 8644, a Ca\textsuperscript{2+} channel agonist which influences gating characteristics, caused a slight increase in APD\textsubscript{50} and APD\textsubscript{90} as expected. Application of VA in the presence of Bay K 8644 however, caused nearly identical shortening of APD\textsubscript{50} as was observed with VA alone (Figure 9C). Possible modification or antagonism of the gating characteristics or activation of these channels was evidenced when the tissue was treated with VA, washed out, then followed by application of SA. By itself, SA has little effect on APD\textsubscript{50} and APD\textsubscript{90} (105.6 % and 106.3% of control, respectively). When the SA followed VA, however, APD\textsubscript{50} and APD\textsubscript{90} were reduced to 36.7% and 40.0% of control, respectively.

When glibenclamide, a blocker of ATP-sensitive K+ channels, was used in this study in the presence of VA, APD\textsubscript{50} was not shortened, but in fact was slightly prolonged, and no shortening of APD\textsubscript{90} was observed (Fig 9D).

*Effects of VA on isolated myocytes.* Because VA had the greatest effects on APD in the tissue phase of the experiment, VA was chosen for use during patch-clamp investigations. Single, dissociated rat myocytes were exposed to
FIGURE 9. Superimposed action potentials from the same cell with various channel blocking agents with and without VA. Arrows in all panels point to control action potential in the presence of blocker alone; the other trace is with blocker and VA. 9A: 5 mM 4-aminopyridine; 9B: 3 mM cobalt chloride; 9C: 10^{-7} M Bay K 8644; 9D: 1 \mu M glibenclamide.
varying concentrations of VA (0.2-2.0 μM) for purposes of exploring a dose-
response relationship on ionic mechanisms. At a membrane voltage (V_m) of 60
mV, at which maximum current is usually observed in this protocol, I_o was
decreased significantly as VA concentration increased (Figure 10A). At VA
concentrations above 1.2 μM, peak I_o current traces were usually higher than
was measurable with the instrumentation available. Also at 60 mV, I_S increased
proporionally and significantly, by over 900% when compared to
control (Figure 10B). Because current traces for I_S were much lower than for
I_o, measurement was possible for VA concentrations through 2.0 μM.

Data from the isolated tissues suggested that I_Ca may be inhibited by VA,
and thus a number of cells were exposed to VA to confirm or reject this possible
mechanism (n=7). The data from these experiments (Figure 11) showed that I_Ca
increased slightly at lower membrane voltages. The increase was not
significant however, and is also shown to be slightly less at higher V_m's.

Effects of blocking agents on isolated currents. As shown in other studies
(Castle and Slawsky 1992), 1 mM 4-AP nearly completely blocked control I_o
(Figure 12A). Yet, when VA with 1 mM 4-AP was added to the cell, no similar
block of I_o was observed, and significant increase in I_o was observed (Figure
13A). Representative current traces illustrating this effect are shown in Figure
12. 4-AP completely blocked I_o and reduced I_S in the control cell (middle
panel), but didn't block the outward currents when VA was present (lower
panel). This coincides with what was seen in Figure 13B, where I_S was
similarly blocked with 4-AP alone, yet with VA and 4-AP, no such block was
observed, in fact there was very little difference either with or without 4-AP in the
FIGURE 10. VA dose-response curves for $I_{lo}$ (10A) and $I_{SS}$ (10B). * = p < 0.05, ** = p < 0.01.
FIGURE 11. Current-voltage relationship for $I_{Ca}$ before and after treatment with 1 μM VA.
FIGURE 12. Current traces of a control cell with and without 4-AP, and of the same cell with VA and 4-AP. The inset in the first panel shows voltage protocol used.
FIGURE 13. Effect of 4-AP on $I_{t0}$ in cells with and without VA (13A), and on $I_{ss}$ (13B).
presence of VA (Figure 13B).

When 100 nM glibenclamide was present with VA (Figure 14), \( I_{SS} \) was significantly and completely blocked when compared to the cells with VA alone. Control cells with glibenclamide had higher \( I_{SS} \) than cells with VA and glibenclamide at all voltages tested.

![Graph showing the effect of glibenclamide on \( I_{SS} \) in the presence of VA and control cells.](image)

**FIGURE 14.** Effect of glibenclamide on \( I_{SS} \) in the presence of VA and on control cells. *\( p<0.05 \).
Secondary metabolites from lichens may have significant effects on the electrical behavior of cardiac cells of mammalian hearts. Compounds from various lichen metabolic pathways were tested on rat ventricular muscle and isolated cardiac myocytes. Some pathways represented were the shikimic acid pathway (vulpinic acid) and the acetate-polymalonate pathway (p-depsides and depsidones such as atranorin and stictic acid respectively, and the dibenzofuran derivative usnic acid), as well as additional compounds from acetone-solubilized extractions. The parameters affected the most by these compounds were the action potential duration at 50% and 90% of repolarization, which were both shortened significantly with VA and UA and extracts containing VA from *Letharia vulpina* and UA from *Cladina subtenuis*.

The heart's plasma membrane contains channels that influence the repolarization and the duration of the action potential (Katz 1992). The pertinent channels in the rat are unique in that they can be divided into an early phase with brief duration and a later phase which has a longer duration (Nobe et al. 1990). A decrease in APD$_{50}$ (the early phase), suggests an effect on $I_{to}$—an outward K+ channel. $I_{to}$ significantly contributes to the repolarization phase of the action potential and is voltage sensitive (-40 to +60 mV); if it is large (as in rat myocytes), it leads to short repolarization times. Parallel to this and operating more or less simultaneously is an opposing inward current carried by Ca$^{2+}$ ($I_{Ca}$). The Ca$^{2+}$ current determines APD and refractory period, and also is an important determinant of the plateau phase, although in rat myocytes it is thought to have a lesser influence than in some other species, eg. guinea pig.
The effects on APD$_{90}$ (the later phase) are usually attributed to an inward current arising from electrogenic Na$^+$/Ca$^{2+}$ exchange ($I_{Na/Ca}$) which is itself secondarily activated by $I_{Ca}$ and the subsequent release of Ca$^{2+}$ from the sarcoplasmic reticulum (Nobe et al. 1990) which initiates cardiac contraction. When ryanodine, a blocker of Ca$^{2+}$ release from sarcoplasmic reticulum, is present, APD$_{90}$ is shortened similarly to that seen in this study (Nobe et al. 1990). Also independent of this is an outward K$^+$ channel, called the delayed rectifier ($I_K$) or the potassium steady state current ($I_{SS}$), which is responsible for repolarization of the cardiac cell back to the resting membrane potential (Apkon & Nerbonne, 1991). $I_K$ is activated at voltages positive to -45 mV and inactivates slowly. As $I_K$ may contribute to $I_{SS}$, in this experiment $I_{SS}$ will be used, in synonymy with $I_K$. Since both APD$_{50}$ and APD$_{90}$ are shortened significantly, these data suggest that either $I_{to}$ is enhanced and/or $I_{Ca}$ is decreased. Further, it may be proposed that $I_K$ was increased or the Na$^+$/Ca$^{2+}$ exchange decreased due to block of Ca$^{2+}$ release from sarcoplasmic reticulum.

The effects of blockers may indicate some of the possible mechanisms responsible for the effects of purified compounds and extracts. The fact that 4-AP did not prevent further shortening of APD$_{50}$ when VA was applied, suggests that $I_{to}$ is not a likely target of these compounds. The prevention of further shortening of APD$_{50}$ and reduced shortening of APD$_{90}$ when CoCl$_2$ is used with VA indicates that $I_{Ca}$ is influenced by the extracts and purified compounds. It is possible VA may itself be a blocker of the Ca$^{2+}$ channel and act similarly to cobalt chloride, which by itself led to a marked shortening of action potential duration. These data also suggest that $I_{Ca}$ plays an important role in
determining the duration of the rat action potential at 50% and 90% of repolarization.

The results observed when Bay K 8644 was used in the presence of VA indicates that these compounds may modify or antagonize the gating characteristics or activation of Ca\(^{2+}\) channels, perhaps related to voltage sensing. When tissues were treated with VA, washed out, then followed by application of SA, a large shortening of both APD\(_{50}\) and APD\(_{90}\) was seen, thus providing evidence that these compounds may cause sensitization or modification of Ca\(^{2+}\) channels.

The possibility exists that these compounds may act as metabolic inhibitors. As stated above, the work of Soderberg (1953) suggests that these products may have effects similar to dinitrophenols. The fact that the *Letharia* extract was often not reversible and eventually toxic would support this possibility. A recent paper by Ogbaghebriel and Shrier (1994) utilized 2,4-dinitrophenol and cyanide, both inhibitors of oxidative phosphorylation, to abolish \(I_{\text{to}}\). They also discovered that another K+ channel controlled by intracellular ATP \((K_{\text{ATP}})\) is induced in rabbit cardiomyocytes in the presence of these metabolic inhibitors, resulting in a shortening of APD. When intracellular ATP concentration is reduced below normal levels in the presence of cyanide, action potential shortening also occurs due to activation of \(K_{\text{ATP}}\) (Nichols et al. 1991). During blockade of these channels by sulfonylurea agents like glibenclamide or tolbutamide, \(I_{\text{to}}\) is partially restored and APD is prolonged (Faivre & Findlay 1989; Ogbaghebriel and Shrier 1994). Other compounds, such as pinacidil, nicorandil, or cromakalin, have been shown to activate these currents directly (Arena & Kass 1989), or by regulation of GTP-binding proteins
(G proteins) (Kirsch et al. 1990). The possibility of pinacidil-like activities is attractive because these compounds may be medically useful as antihypertensive agents and vasodilators. The effect seen in these experiments when glibenclamide was used would suggest that lichen metabolites may activate the ATP-sensitive K+ currents either directly or by lowering intracellular ATP.

Analysis of patch clamp data confirms and further illuminates some of the previously discussed mechanisms. That $I_{to}$ is not involved is suggested by the tissue electrophysiology data when the effect of VA was not blocked by 4-AP and further supported by patch-clamp data which shows that $I_{to}$ is significantly decreased as VA concentration is increased (Figure 10A). When 4-AP is used with controls, as shown in Figures 12A and 13 (middle panel), $I_{to}$ is blocked nearly completely, showing the expected effectiveness of 4-AP as a blocker of $I_{to}$. The appearance of an increase in $I_{to}$ when VA is added (Figure 12) relates to current overlap, ie. some other outward K+ current must be activated at these same membrane voltages. Figure 13 (lower panel) illustrates that even with 4-AP, VA caused large currents to develop.

Figure 13 (middle panel) shows that $I_{SS}$ is also largely blocked by 4-AP, yet Fig. 12B shows a large $I_{SS}$ produced by VA even in the presence of 4-AP and which is concentration related (Figure 10B). This overlapping current then, obscures the true $I_{to}$ and $I_{SS}$, making them appear that they are increasing when in fact they are not.

The actual current being activated by VA, however, can be blocked by glibenclamide, as illustrated in Figure 14. When $K_{ATP}$ is blocked by glibenclamide, $I_{SS}$ is reduced significantly. Thus, VA may activate $K_{ATP}$ either directly or
by reducing intracellular ATP. This is supported by the tissue electrophysiology phase, which showed that the ADP was restored to normal or even longer duration by the addition of glibenclamide (Figure 9D).

Tissue evidence suggested that $I_{\text{Ca}}$ may be influenced, and based on the effects of VA on APD one could surmise that $I_{\text{Ca}}$ was possibly being decreased. The analysis of $I_{\text{Ca}}$ patch-clamp data, however, suggests this is not the case (Figure 11). Rather, $I_{\text{Ca}}$ was increased by VA, albeit not significantly. The hypothetical involvement of $I_{\text{Ca}}$ was again probably caused by the huge influence upon $K_{\text{ATP}}$ which would lead to similar effects one would normally see from decreased $I_{\text{Ca}}$. Since $K_{\text{ATP}}$ is not the one usually expected to be involved in development of these effects, a person usually begins developing hypotheses from the most usual and obvious causes, which in this case was $I_{\text{to}}$, $I_{K}$, and/or $I_{\text{Ca}}$.

The lichen species used to obtain the extracts, and the purified forms themselves, were chosen more or less randomly—primarily because of their availability or abundance. That two out of 4 purified lichen compounds caused significant shortening of the APD$_{50}$ and APD$_{90}$, and that one of those two had further significant effects on RMP, amplitude, and $V_{\text{max}}$, would indicate that certain lichen compounds effect a high degree of activity at the cellular level. This activity, eg. toxicity, may suggest a possible evolutionary role for these compounds, perhaps as pesticides or insecticides. Along with the known antibacterial properties some of these secondary metabolites possess, these additional activities may confer protection from consumption or invasion by small organisms. It is likely that many more of these compounds, which number at least 150 in several biochemical classes (Hale 1983), may have similar
dramatic effects at the cellular levels, and may have value as research tools, pharmacological application, or perhaps even medicinal uses.

The possible influence of lichen metabolites upon ion channels, particularly on Ca\textsuperscript{2+} and K\textsuperscript{+} channels, has been studied and such information may provide clues to some useful applications for these lichen compounds. The significance of these effects is best realized when one considers the role and importance that these channels play in many biological responses and physiological functions. The Ca\textsuperscript{2+} channel is an ubiquitous component of cellular function which is essential for many biological responses. It is found in nerve, muscle, and cardiac cells from *Paramecium* to humans. Secretions, synaptic activity, excitability, and particularly contraction in cardiac muscle are some functions at least partially dependent upon these channels. The potassium channels are also responsible for a number of actions. \( I_{lo} \) and \( I_K \) contribute to the repolarization phase of the cardiac action potential and determines repolarization of the action potential in squid axon, skeletal and heart muscle, and slow wave activity (smooth muscle). \( K_{ATP} \) is activated by metabolic inhibitors and is responsive to lowered intracellular ATP levels, hypoxia/anoxia, reduced intracellular pH, and lactate, and may protect against ischemia in ventricular muscle, skeletal muscle, and in pancreatic cells, may be sensitive to lowered glucose levels and link calcium and insulin secretion by maintaining depolarized voltages.

The following points summarize the effects of lichen metabolites on rat heart muscle tissue and isolated myocytes:

1. Extracts from *Letharia vulpina* and *Cladina subtenuis* cause significant shortening of action potential duration at 50 and 90\% of repolarization
(APD$_{50}$ and APD$_{90}$).

2. *Letharia vulpina* also causes significant modifications to resting membrane potential (RMP), amplitude (Ampl), and maximum rate of rise of the action potential upstroke ($V_{\text{max}}$).

3. Usnic acid and vulpinic acid cause significant shortening of APD$_{50}$ and APD$_{90}$.

4. Transient outward current ($I_{\text{to}}$) is decreased with increasing concentrations of vulpinic acid.

5. A significant increase in an outward steady state potassium current is elicited by vulpinic acid. The increased steady state current is not sensitive to 4-aminopyridine, suggesting it is not $I_{\text{to}}$.

6. An inward calcium current is consistently but not significantly increased by vulpinic acid.

7. The steady state potassium current is reduced significantly by glibenclamide, suggesting it is an ATP-sensitive potassium current ($K_{\text{ATP}}$), and may be activated either directly (eg. pinacidil) or by metabolic inhibition (eg. DNP or cyanide).


Fink, B. The Lichens of Minnesota. Contributions from the U. S. National Herbarium 14:1-269; 1910 (p. 35).


Rozanski, G. J.; Witt, R. C. Interleukin-1 enhances β-responsiveness of cardiac


A Comparison of the Effects of Extracts and Purified Compounds on APD$_{50}$ and $V_{\text{max}}$
A Comparison of the Effects of Extracts and Purified Compounds on Amp and RMP