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

Richard C. Witt

THE EFFECTS OF SELECTED LICHEN EXTRACTS AND PURIFIED COMPOUNDS ON THE RAT HEART

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Master of Arts

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Richard C. Witt

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

THESIS ACCEPTANCE

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Committee

Name	Department/School
[illegible]	UNMC
A. T. Norman Walter	Biology

Chairperson R S Egan, Biology

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ABSTRACT

Extracts from Letharia vulpina, 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.*

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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 seperate 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; NaH_2PO_4, 0.9; $NaHCO_3$, 20.0; glucose, 5.5; $MgSO_4$, 0.5; KCl, 4.0; and $CaCl_2$, 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).

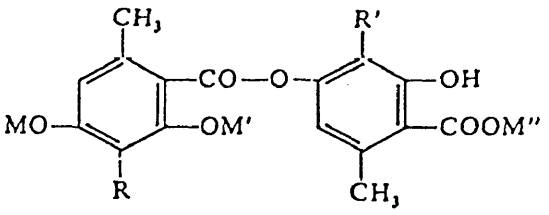
Electrophysiology. Papillary muscles were isolated from the hearts of euthanized laboratory rats (*Ratus norvegicus,* Sprague Dawley) and pinned to the floor of a tissue bath perfused with oxygenated (95% O_2, 5% CO_2) Tyrodes

VA= Vulpinic Acid
UA= Usnic Acid
SA= Stictic Acid
At= Atranorin
RMP= Resting Membrane Potential
Amp= Action Potential Amplitude
APD= Action Potential Duration
APD_{50}= Action Potential Duration at 50% of Repolarization
APD_{90}= Action Potential Duration at 90% of Repolarization
I_{to}= Transient Outward Potassium Current
I_{Ca}= L-Type Calcium Current
I_{SS}= Steady State Current
I_K=Delayed Rectifier Potassium Current
$I_{Na/Ca}$= Sodium/Calcium Exchange
K_{ATP}= ATP-Sensitive Potassium Current
4-AP= 4-Aminopyridine
$CoCl_2$= Cobalt Chloride
V_m= Membrane Voltage
V_{max}= Maximum Rate of Rise of the Action Potential Upstroke

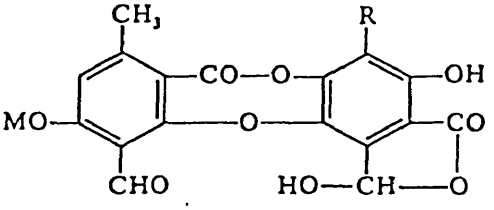
TABLE I. Abbreviations used in the thesis.

SPECIES	MAJOR METABOLITES
Letharia vulpina	**vulpinic acid**
Cladina subtenuis	**usnic acid & fumarprotocetraric acid**
Umbilicaria americana	**gyrophoric acid**
Rimelia reticulata	**atranorin & lecanoric acid**
Parmotrema austrosinense	**atranorin & salazinic acid**
Xanthoparmelia cumberlandia	**stictic acid, usnic acid, & norstictic acid**

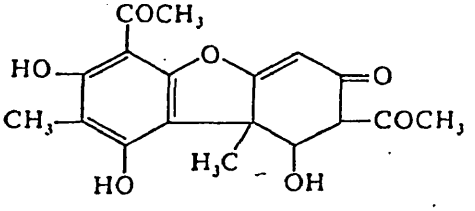
TABLE II. Metabolites detected in each extract by thin layer chromatography.



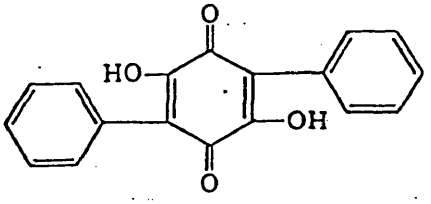
Atranorin (MW= 374.3)



Stictic Acid (MW= 386.3)



Usnic Acid (MW= 344.3)



Vulpinic Acid (MW=322.3)

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 Ca^{2+}). 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 Ca^{2+}. 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

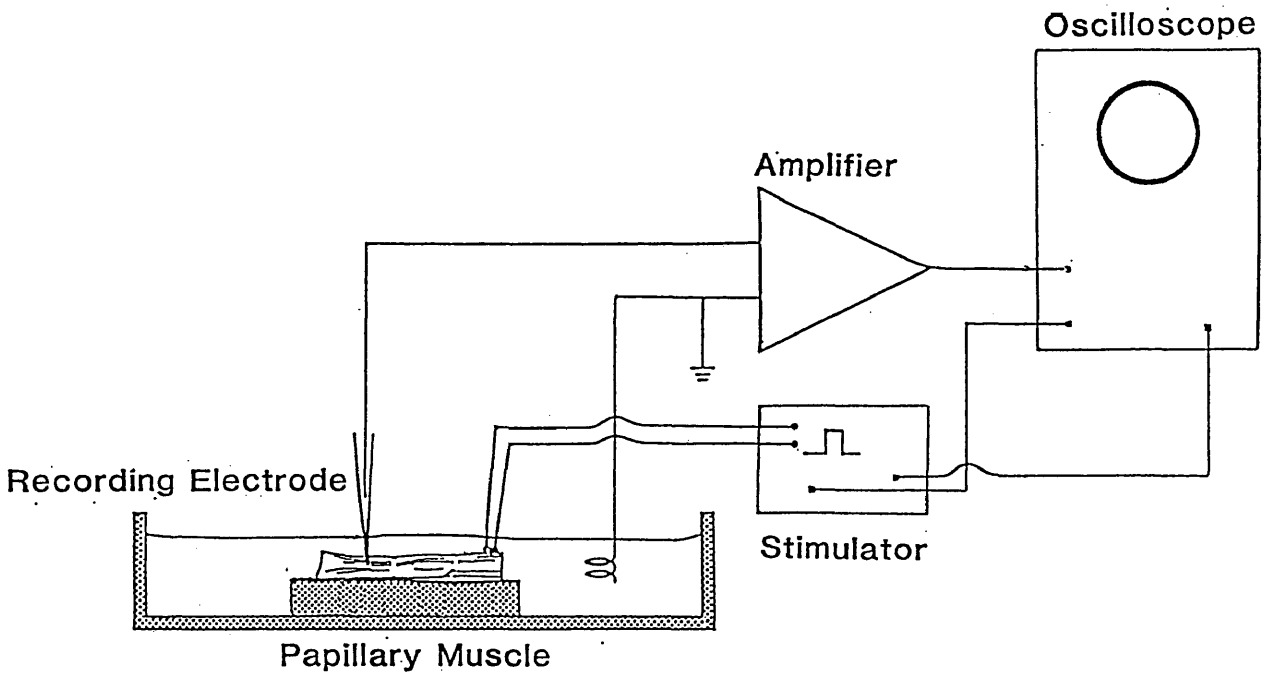


FIGURE 2. A schematic of the experimental set-up for recording action potentials from rat papillary muscle.

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 $CdCl_2$ to block residual Ca^{2+} 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, 138; KCl, 4.0; $MgCl_2$, 1.2; $CaCl_2$, 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; $MgCl_2$, 3.0; HEPES, 10; Na_2-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 (C_m) 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 (I_{to}) 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

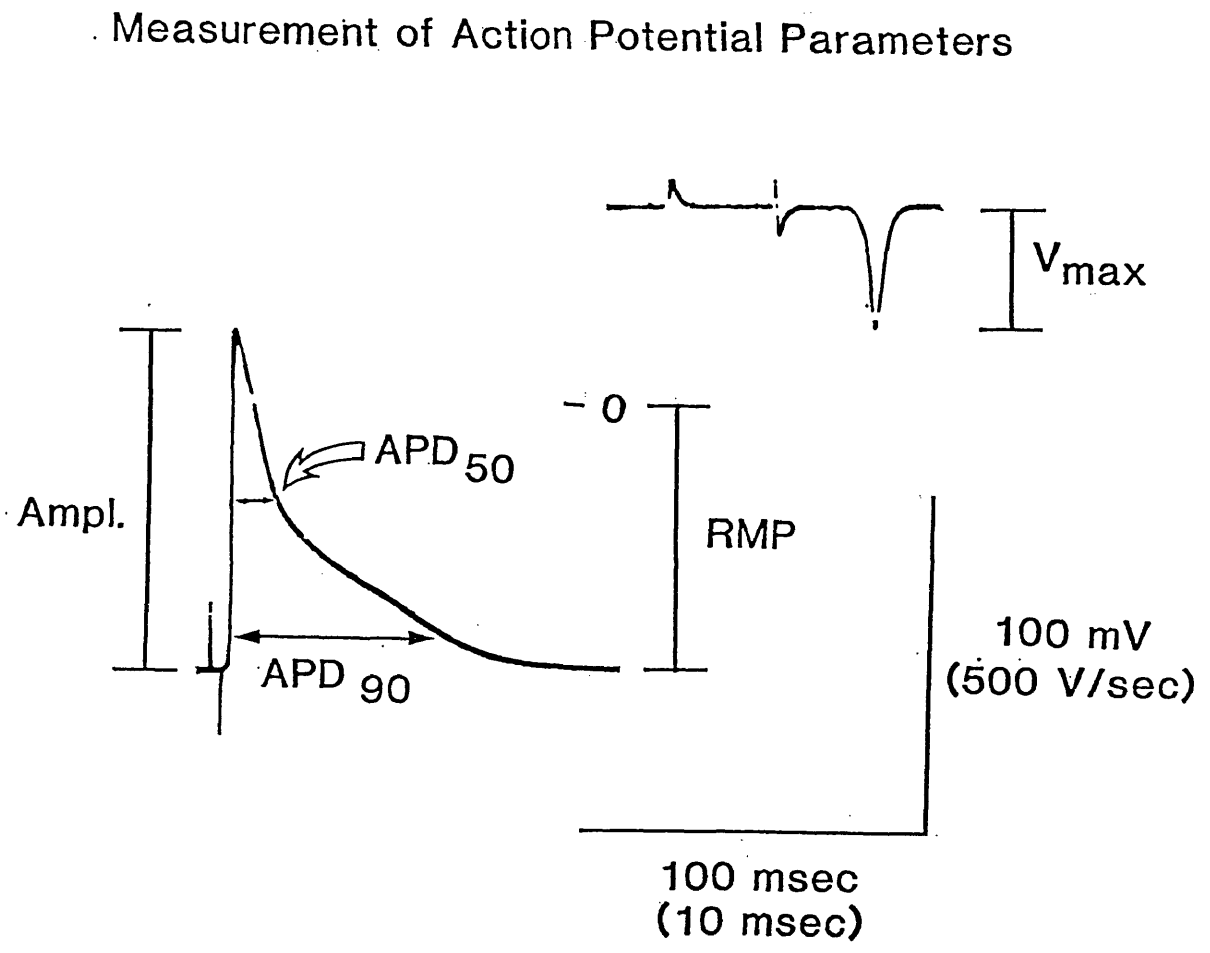


FIGURE 3. Illustration of parameters measured for rat action potentials. Action potential calibrations are n mV and msec and values in parentheses are for V_{max}. Abbreviations are explained in the text.

potential amplitude (Amp.), action potential duration at 50% and 90% of repolarization (APD_{50} and APD_{90}), and maximum rate of rise of the action potential upstroke (V_{max}), as measured from the ouput 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_{max}, APD_{50}, or APD_{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_{max} (excluding *Letharia vulpina*) were minimal, but changes in APD_{50} and APD_{90} were more marked. Four of the extracts caused shortening, with *Letharia vulpina* eliciting the greatest shortening, while *Rimelia reticulata* and *Umbilicaria americana* caused prolongation (since both *Rimelia reticulata* and *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 *Letharia* which did not usually wash out and was eventually toxic to the cell. The *Letharia* extract caused significant shortening of both APD_{50} and APD_{90} ($p<0.01$) and the *Cladina* extract caused

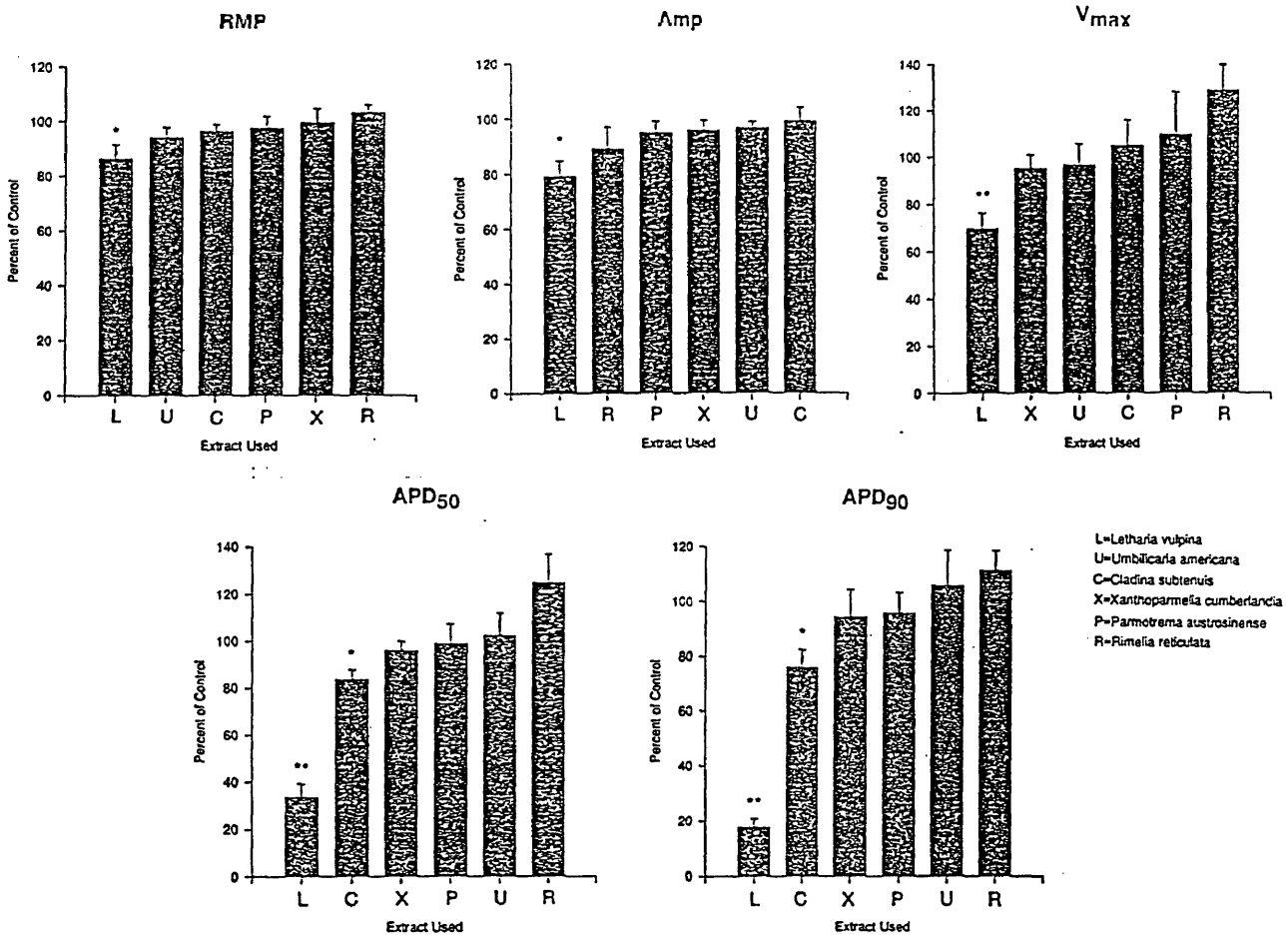


FIGURE 4. Summary of the effects of lichen extracts on each measured parameter. Abbreviations are: C=*Cladina*; L=*Letharia*, P=*Parmotrema*; R=*Rimelia;* U=*Umbilicaria*; X=*Xanthoparmelia* . *=p<0.05, **=p<0.01. n=5 for all extracts except *Letharia* (n=6).

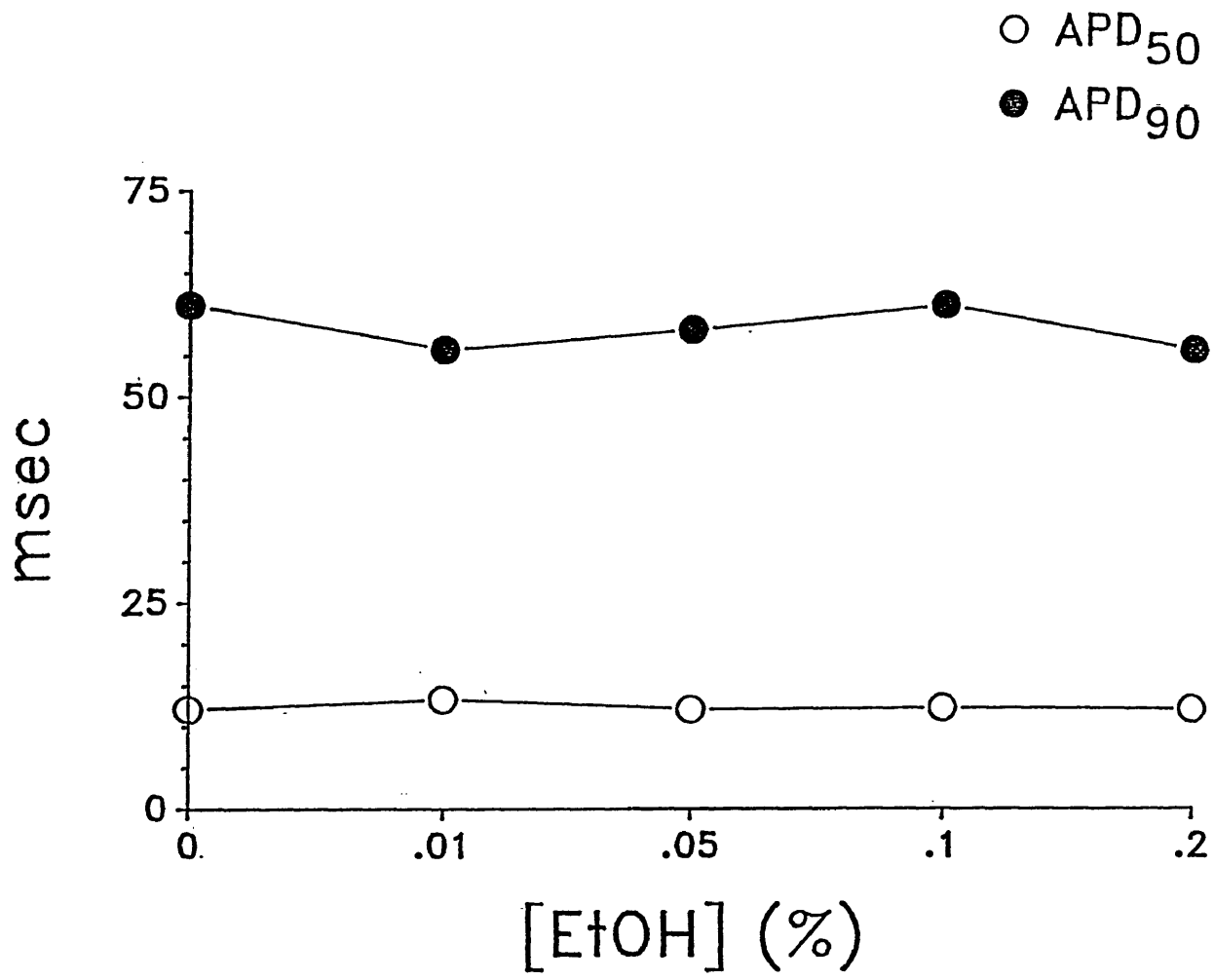


FIGURE 5. The effects of ethanol at various concentrations on APD_{50} and APD_{90}.

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_{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_{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_{to} (Castle & Slawsky 1992), did not

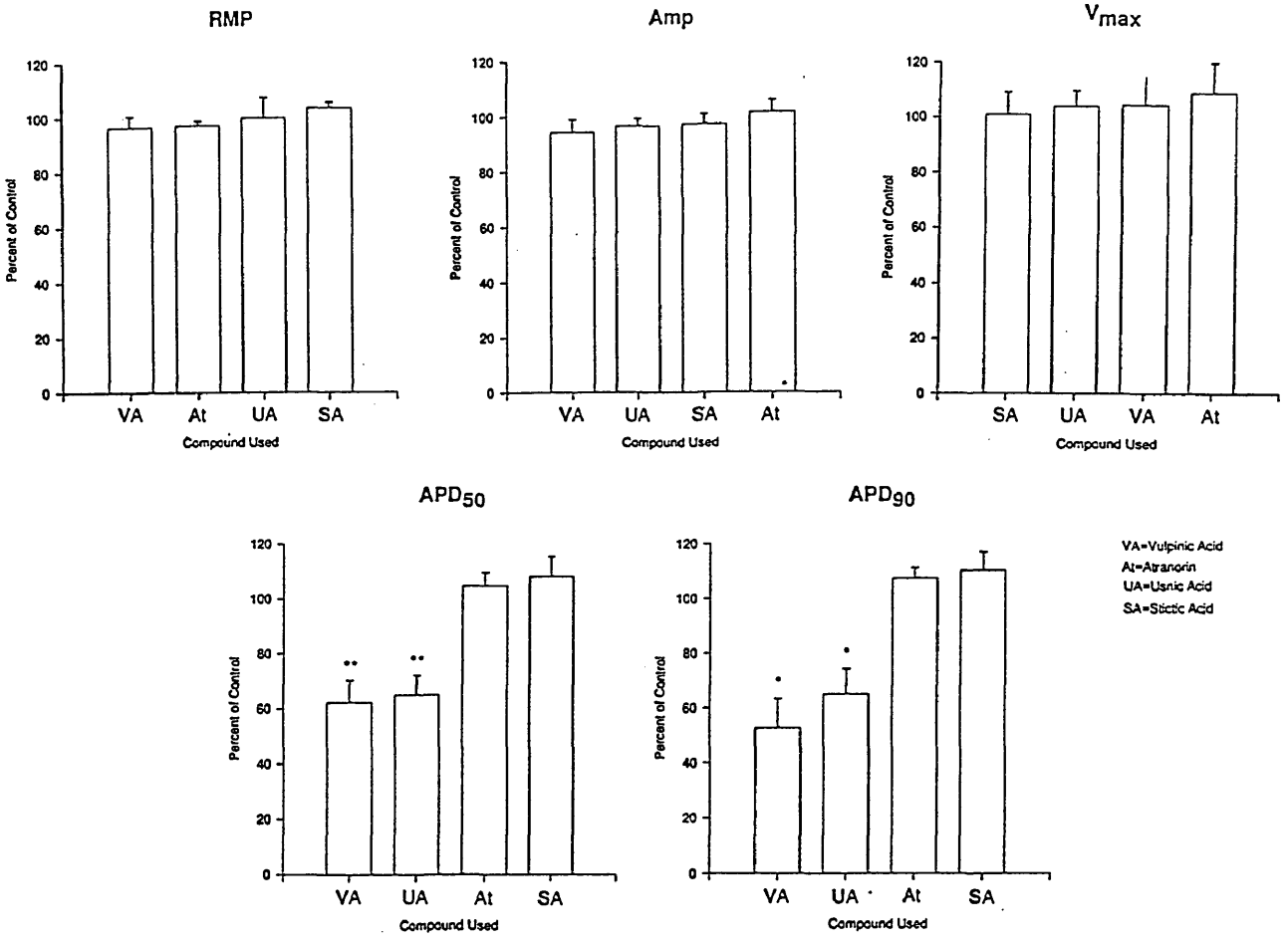


FIGURE 6. Effects of purified compounds on each measured parameter. Abbreviations are: UA=usnic acid; VA=vulpinic acid; At=atranorin; SA=stictic acid. *=p<0.05, **=p<0.01. n=5 for at and UA; n=6 for VA and SA.

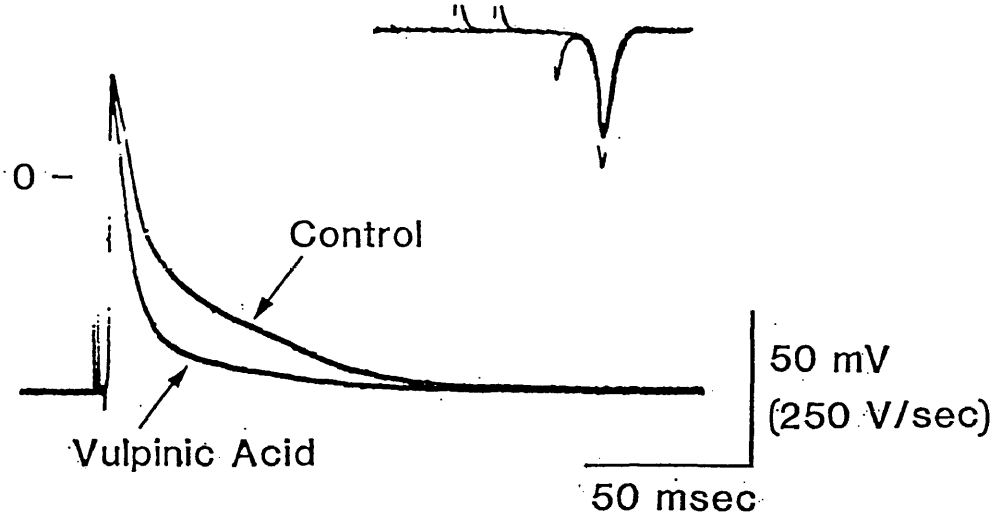


FIGURE 7. Superimposed action potentials with 2 μM VA and control (0.1% ethanol). Calibration value in parentheses is for V_{max}.

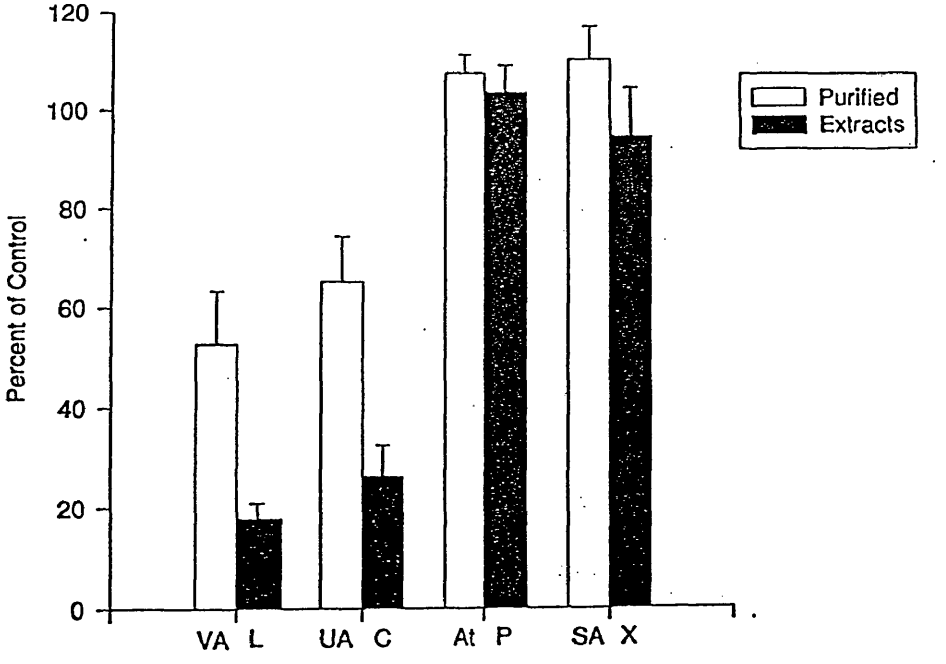


FIGURE 8. Comparison of changes in APD_{90} in response to extracts and purified compounds. Abbreviations are the same as for previous figures, except P=*Parmotrema/Rimelia*.

prevent further shortening of APD_{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 ($CoCl_2$), a blocker of Ca^{2+} channels, however, did prevent further shortening of the APD_{50} and reduced the shortening of APD_{90} when VA was applied (Figure 9B). This would indicate that Ca^{2+} channels were being influenced by the extracts and purified compounds.

Pretreatment of the tissue with 10^{-7} M Bay K 8644, a Ca^{2+} channel agonist which influences gating characteristics, caused a slight increase in APD_{50} and APD_{90} as expected. Application of VA in the presence of Bay K 8644 however, caused nearly identical shortening of APD_{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_{50} and APD_{90} (105.6 % and 106.3% of control, respectively). When the SA followed VA, however, APD_{50} and APD_{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_{50} was not shortened, but in fact was slightly prolonged, and no shortening of APD_{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

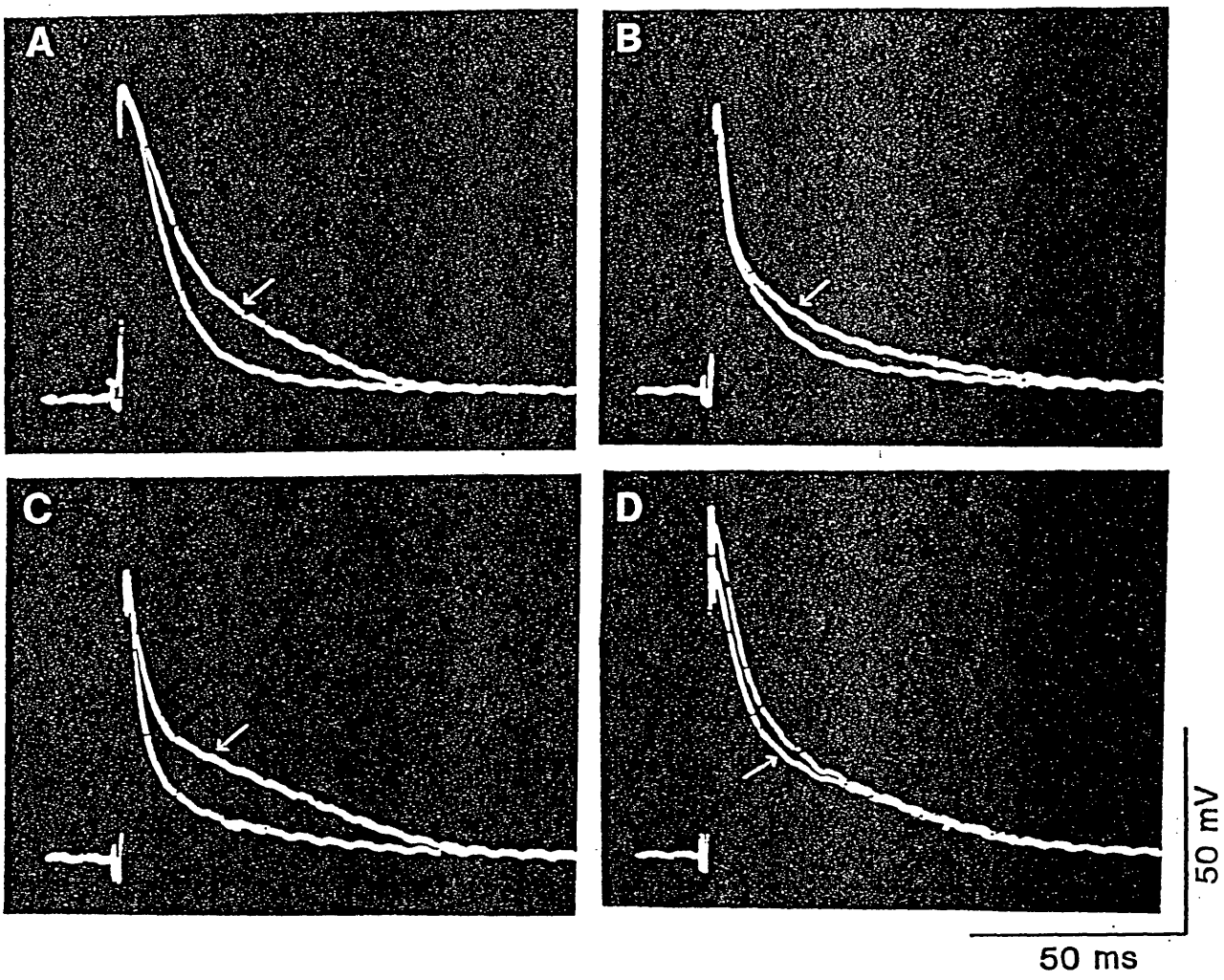


FIGURE 9. Superimposed action potentials from the same cell with various channel blocking agents with and without VA. Arrows in all panels point to control action potential in the presence of blocker alone; the other trace is with blocker and VA. 9A: 5 mM 4-aminopyridine; 9B: 3 mM cobalt chloride; 9C: 10^{-7} M Bay K 8644; 9D: 1 μM 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_{to} was decreased significantly as VA concentration increased (Figure 10A). At VA concentrations above 1.2 μM, peak I_{to} current traces were usually higher than was measurable with the instrumentation available. Also at 60 mV, I_{SS} increased proportionally and significantly, by over 900% when compared to control (Figure 10B). Because current traces for I_{SS} were much lower than for I_{to}, 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_{to} (Figure 12A). Yet, when VA with 1 mM 4-AP was added to the cell, no similar block of I_{to} was observed, and significant increase in I_{to} was observed (Figure 13A). Representative current traces illustrating this effect are shown in Figure 12. 4-AP completely blocked I_{to} and reduced I_{SS} 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_{SS} 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

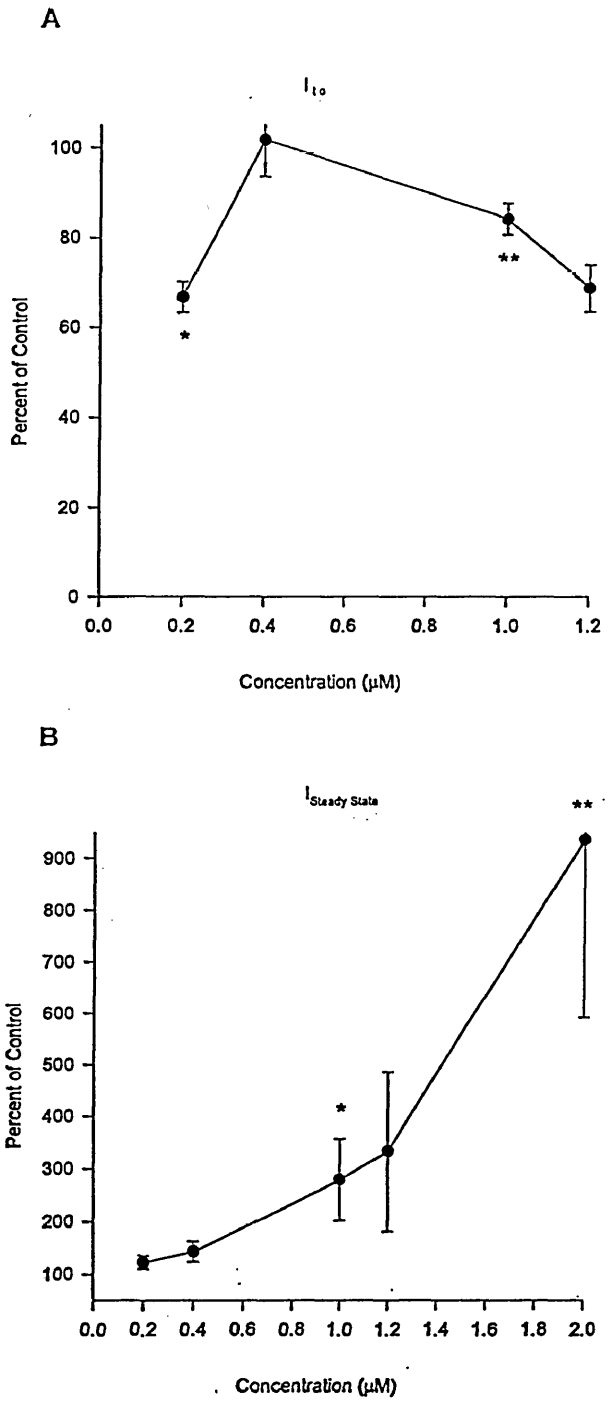


FIGURE 10. VA dose- response curves for I_{to} (10A) and I_{SS} (10B). *=p<0.05, **=p<0.01.

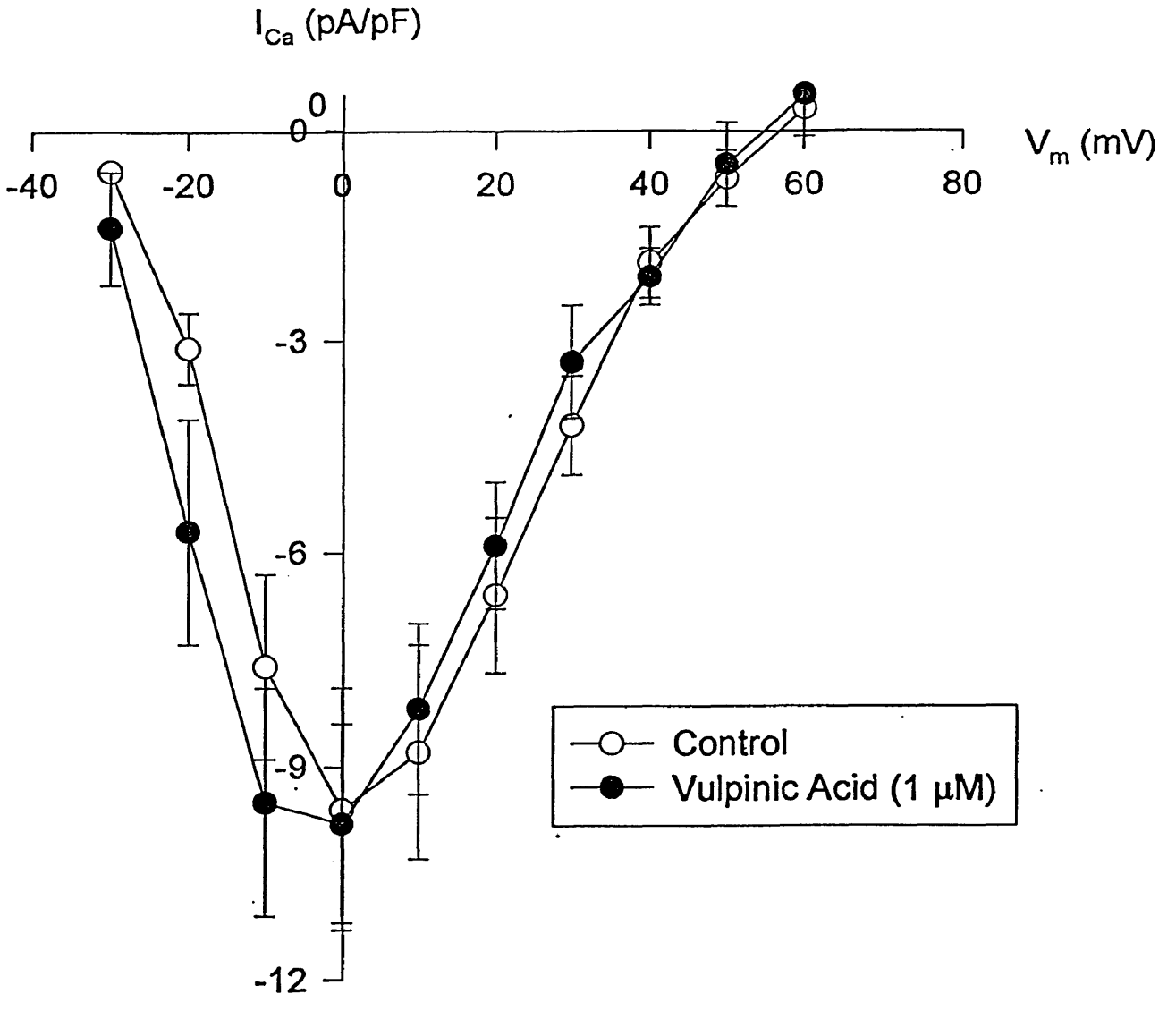


FIGURE 11. Current-voltage relationship for I_{Ca} before and after treatment with 1 μM VA.

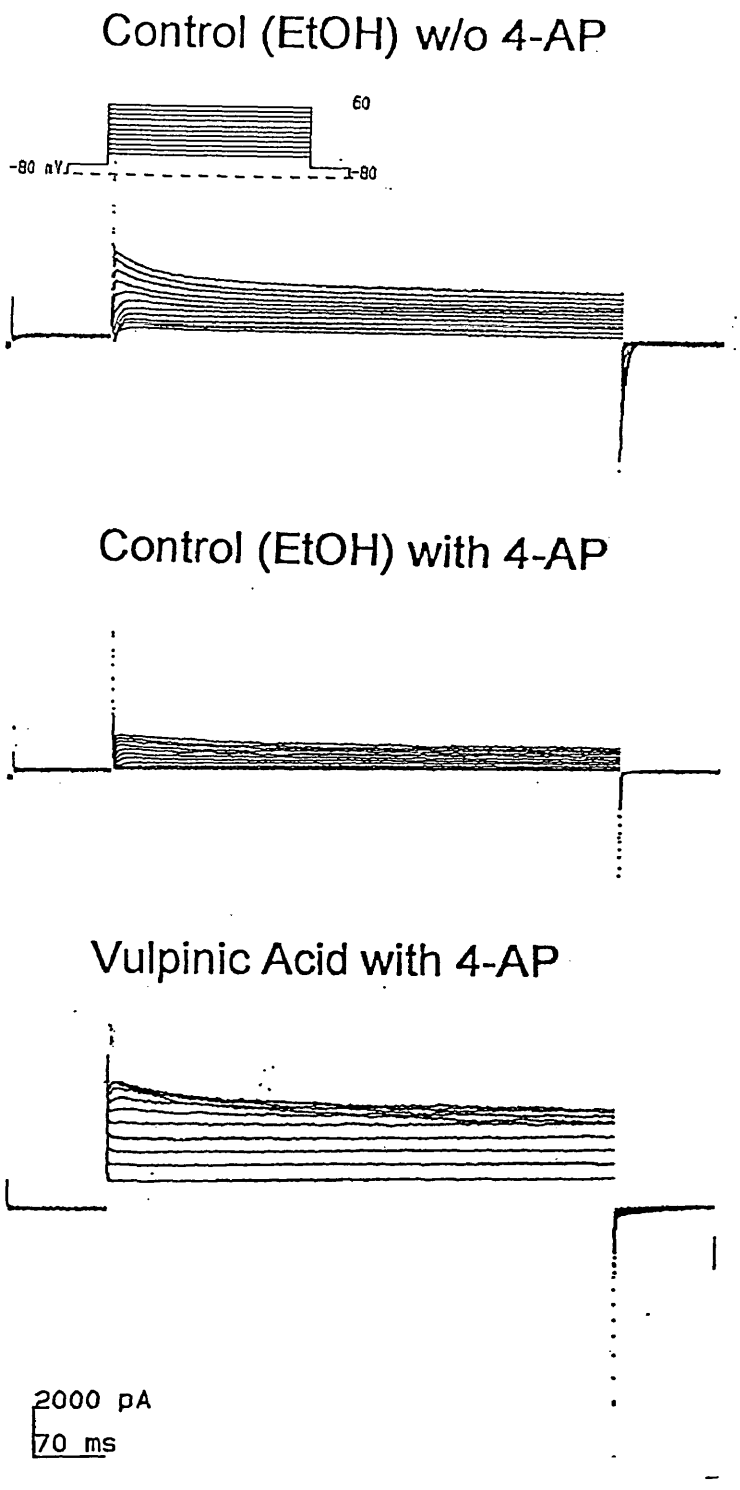


FIGURE 12. Current traces of a control cell with and without 4-AP, and of the same cell with VA and 4-AP. The inset in the first panel shows voltage protocol used.

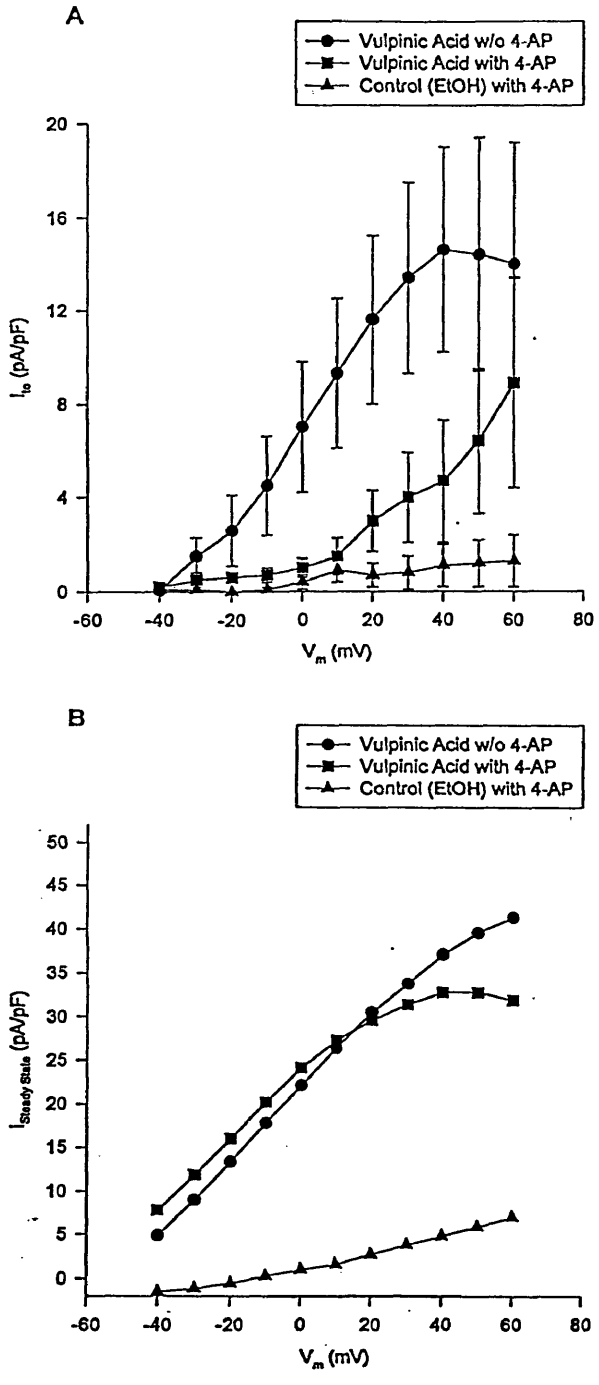


FIGURE 13. Effect of 4-AP on I_{to} in cells with and without VA (13A), and on I_{SS} (13B).

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.

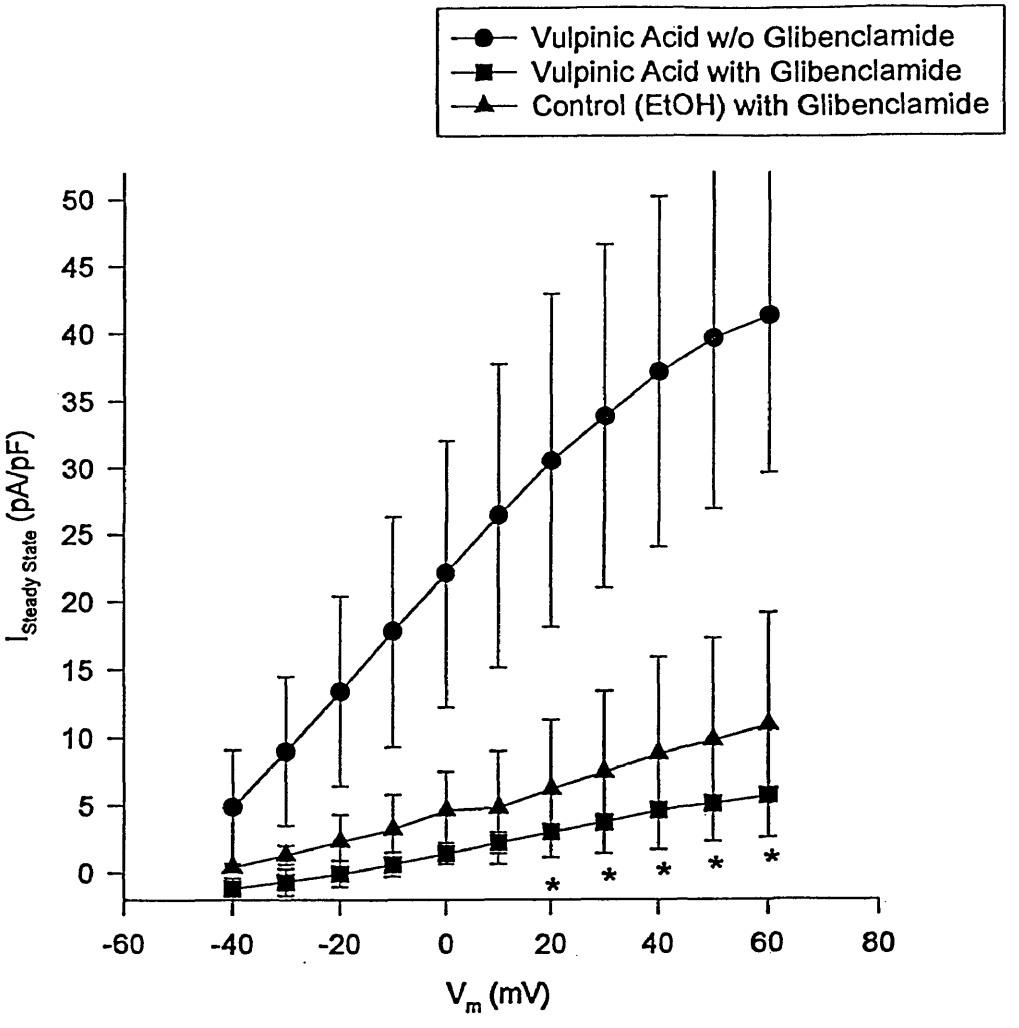


FIGURE 14. Effect of glibenclamide on I_{SS} in the presence of VA and on control cells. *=$p<0.05$.

DISCUSSION

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-solubolized 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+} exhange 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_{to}. They also discovered that another K^+ channel controlled by intracellular ATP (K_{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_{ATP} (Nichols et al. 1991). During blockade of these channels by sulfonylurea agents like glibenclamide or tolbutamide, I_{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_{Ca} may be influenced, and based on the effects of VA on APD one could surmise that I_{Ca} was possibly being decreased. The analysis of I_{Ca} patch-clamp data, however, suggests this is not the case (Figure 11). Rather, I_{Ca} was increased by VA, albeit not significantly. The hypothetical involvement of I_{Ca} was again probably caused by the huge influence upon K_{ATP} which would lead to similar effects one would normally see from decreased I_{Ca}. Since K_{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_{to}, I_K, and/or I_{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_{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^{2+} and K^{+} channels, has been studied and such informaiton 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^{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, synapatic activity, excitability, and particularly contraction in cardiac muscle are some functions at least partically dependent upon these channels. The potassium channels are also responsible for a number of actions. I_{to} 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_{max}).
3. Usnic acid and vulpinic acid cause significant shortening of APD_{50} and APD_{90}.
4. Transient outward current (I_{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_{to}.
6. An inward calcium current is consistently but not signficantly increased by vulpinic acid.
7. The steady state potassium current is reduced significantly by glibenclamide, suggesting it is an ATP-sensitive potassuim current (K_{ATP}), and may be activated either directly (eg. pinacidil) or by metabolic inhibition (eg. DNP or cyanide).

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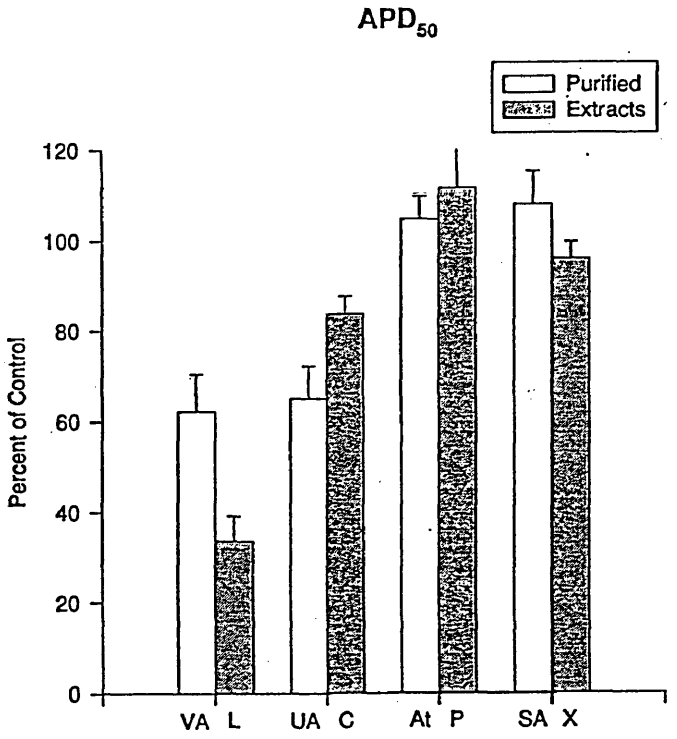
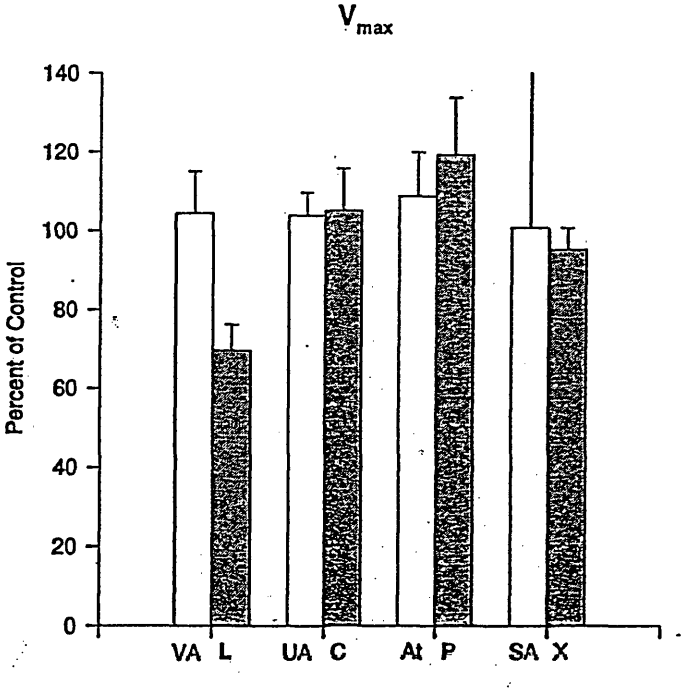
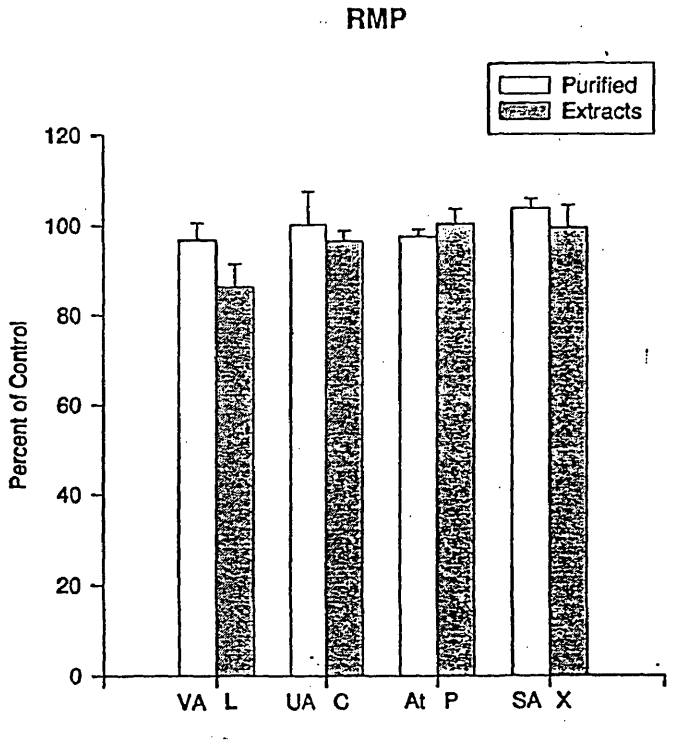
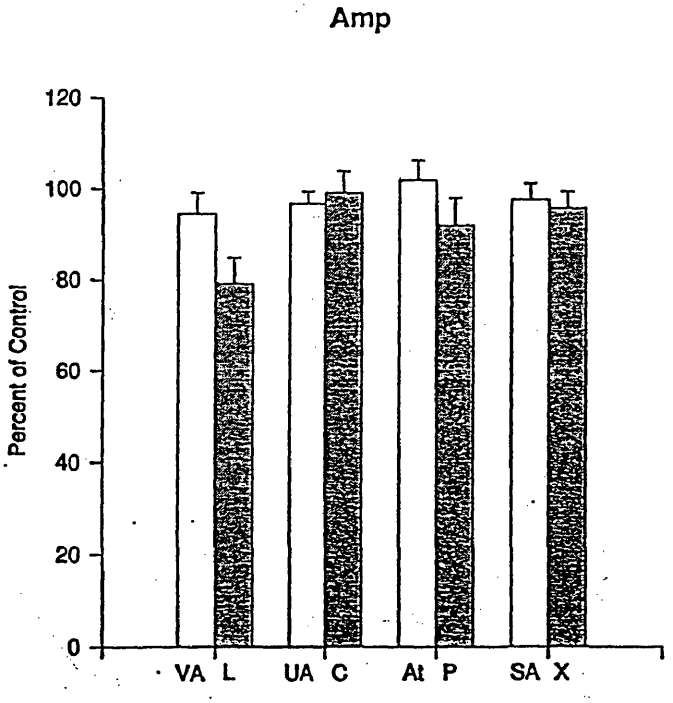




A Comparison of the Effects of Extracts and Purified Compounds on APD_{50} and V_{max}





A Comparison of the Effects of Extracts and Purified Compounds on Amp and RMP