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EFFECT OF SANGUINARINE, A BENZOPHENANTHRIDINE ALKALOID, ON FROG SKIN POTENTIAL DIFFERENCE AND SHORT CIRCUIT CURRENT

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Summary

Sanguinarine, a benzophenanthridine alkaloid, causes an initial stimulation of frog skin short circuit current I_{sc} when present in the mucosal bathing medium at 10^{-4} M. The stimulation is accompanied by an increase in spontaneous potential difference (PD) and increase in D.C. resistance. No effects are seen with sanguinarine in the serosal bathing medium. The inital stimulation is followed by a decrease in I_{sc} and PD, but a continued increase in resistance. In skins whose initial spontaneous PD is high, no initial stimulation in I_{sc} and PD is seen; however, clamping these skins to a lower potential does not alter their initial inhibitory response to sanguinarine. Likewise, clamping the lower potential skins to higher potential does not alter their initial stimulatory response. Sanguinarine seems to be acting on the permeability barriers at the outer surface of the frog skin.

Introduction

Comparisons of effects of inhibitors or activators of Na^+ transport in whole cells or tissues with changes in the ($Na^+ + K^+$)-ATPase have yielded considerable information on the mechanisms of ion transport and the relationship of ion transport to enzymatic activities. The most important of these inhibitors have been the cardiac glycosides which have been known to be inhibitors of Na^+ transport and have been used to characterize the ($Na^+ + K^+$)-ATPase as a specific enzymatic activity directly involved in active Na^+ transport [1,2]. On the other hand, ADH and amiloride, respectively, are activators and inhib-

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itors of Na^+ transport in frog skin. Their effects have been ascribed to changes in access of Na^+ to the pump sites by changes in passive permeability at the outer barrier, rather than the pump itself [3].

Sanguinarine is a benzophenanthridine alkaloid which inhibits ($Na^+ + K^+$)-ATPase from guinea pig brain [4]. They suggest that it inhibits at a site different from ouabain, oligomycin and harmaline. Moore [5] has reported that sanguinarine inhibits the membrane-bound ATPase from frog skeletal muscle. We report here that sanguinarine, apparently an inhibitor of ($Na^+ + K^+$)-ATPase, stimulates parameters which reflect increased Na^+ transport.

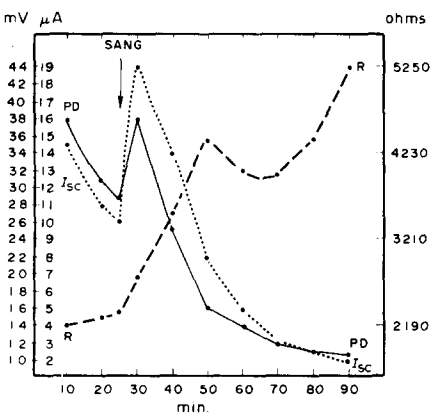
Materials and Methods

Grass frogs, *Rana pipiens*, were purchased from a biological supply company and used in the spring and summer season. The ventral abdominal skin was mounted as a flat sheet (1 cm diameter) between two Lucite chambers and potential difference (PD) and short circuit current (I_{sc}) measurements were made according to the technique of Ussing and Zehran [6]. Amphibian Ringer's solution buffered at pH 7.4 with 50 mM imidazole was used. The solutions in each chamber (11 ml each) were circulated and oxygenated by bubbling with air. Transepithelial resistance was measured in two ways: by calculating the ratio PD/I_{sc} in each experiment and by increasing the external current during I_{sc} measurement so that the mucosal surface was driven 20 mV positive; the current necessary to drive the mucosal surface from 0 potential to +20 mV was used to calculate the resistance. In these experiments, these two methods give identical results.

After mounting the tissue between the chamber halves, several minutes were allowed for equilibration. Following stabilization of PD, sanguinarine sulfate (Pfaltz and Bauer, Inc., Flushing, New York) (water solution) was added in a volume of 110 μl to make the appropriate concentration on either the serosal or mucosal side. PD was monitored constantly and I_{sc} was applied at 5 or 10 min intervals.

Results

Addition of sanguinarine to the mucosal bathing solution results in two separate responses. In those skins with low spontaneous potential differences, sanguinarine elicits an immediate stimulation of both potential difference and short circuit current with little change in calculated resistance, as shown in Fig. 1. for a typical experiment. The stimulation usually peaks in 3—10 min and lasts from 15 to 40 min. Eventually the spontaneous PD and I_{sc} are markedly inhibited and the calculated resistance increases 2—4-fold. Changing the bathing medium with several volumes of fresh buffer does not result in reversal of these effects. Addition of sanguinarine to the serosal bathing medium at comparable concentrations has no effect. The response of the PD and I_{sc} is dose related with very little effect at 10^{-5} M sanguinarine and near maximal effects at 10^{-4} M. The results of these experiments in skins of low initial PD are summarized in Table I. It can also be seen that at the highest concentration of sanguinarine, there is a small, but apparently signifi-



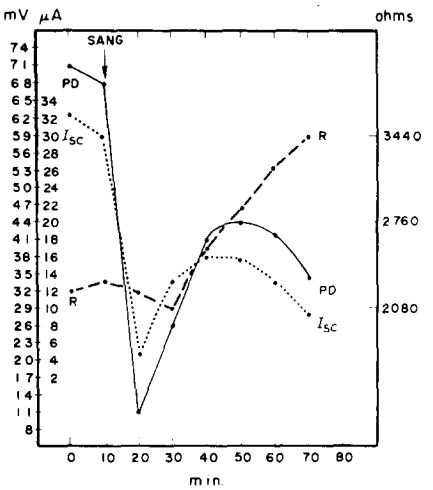


Fig. 1. Response of frog skin PD, I_{sc} and resistance (R) to sanguinarine. Low spontaneous PD (38 mV). Sanguinarine (SANG), 10^{-4} M final concentration, was added to mucosal bathing medium as indicated.

Fig. 2. Response of frog skin PD, I_{sc} and resistance to sanguinarine. High spontaneous PD (71 mV). Sanguinarine, 10^{-4} M final concentration, was added to mucosal bathing medium as indicated.

cant, increase in resistance at 10 min as well as a large increase in PD and I_{sc}.

In those skins with an initial PD which is high the response to sanguinarine is inhibitory, as can be seen in a typical experiment shown in Fig. 2. After

TABLE I

EFFECT OF SANGUINARINE ON FROG SKIN POTENTIAL, SHORT CIRCUIT CURRENT AND RESISTANCE

The figures are given in percent of initial or control values (before addition of sanguinarine). Number of experiments (n) given in parentheses. Resistance is calculated as from current necessary to drive the skin +20 mV (outside positive).

Treatment	% PD after 5 min	P	% I_{sc} after 5 min	P	% Resistance 10 min	P
$1 \cdot 10^{-4}$ mucosal *	180.57 ± 33.44 (n = 14)	<0.001	162.55 ± 28.92 (n = 11)	<0.001	132.17 ± 38.66 (n = 12)	<0.1
$1 \cdot 10^{-4}$ serosal	102.38 ± 7.52 (n = 16)		98.64 ± 10.41 (n = 11)		107.64 ± 15.57 (n = 11)	
$5 \cdot 10^{-5}$ mucosal **	166.43 ± 33.98	<0.01	177.00 ± 33.68	<0.01	119.60 ± 17.12	<0.01
$5 \cdot 10^{-5}$ serosal	102.25 ± 14.06 (n = 4)		104.00 ± 19.71 (n = 4)		93.75 ± 5.74 (n = 4)	
$1 \cdot 10^{-5}$ mucosal	114.14 ± 13.98 (n = 7)	n.s.	106.43 ± 12.20 (n = 7)	n.s.	106.93 ± 9.87 (n = 7)	n.s.
$1 \cdot 10^{-5}$ serosal	104.75 ± 9.64 (n = 4)		104.75 ± 31.00 (n = 4)		101.81 ± 13.18 (n = 4)	

* Initial potential difference <50 mV.
** Initial potential difference <38 mV.
n.s., not significant.

TABLE II

RELATIONSHIP OF INITIAL SPONTANEOUS PD TO RESPONSE TO SANGUINARINE

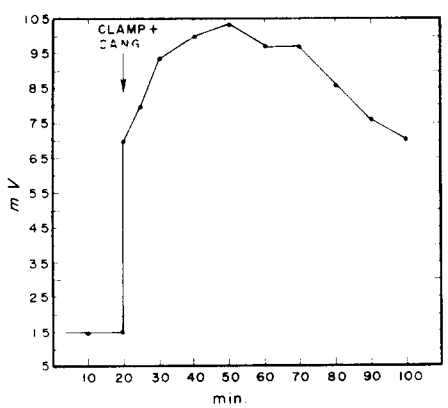
The figures are given as percent of initial or control values (before addition of sanguinarine).

Concentration	Initial potential difference	% PD after 5 min	P	% I_{sc} after 5 min	P'
	<50 mV	180.57 ± 32.44 (n = 14)		162.55 ± 28.92 (n = 11)	
$1 \cdot 10^{-4}$ mucosal			<0.001		<0.001
	50 mV and over	77.83 ± 30.62 (n = 6)		66.40 ± 29.16 (n = 5)	
	<38 mV	166.42 ± 33.98 (n = 7)		177.03 ± 33.68 (n = 7)	
$5 \cdot 10^{-5}$ mucosal			<0.01		<0.01
	38 mV and over	101.33 ± 11.66 (n = 3)		96.69 ± 9.02 (n = 3)	

the addition of sanguinarine to the mucosal bathing medium, this PD and I_{sc} begin to decline sharply with very litttle change in skin resistance; however, after 20—40 min, the PD and I_{sc} rebound and decline again at the same time as resistance is rising. Even in skins with high initial potential, there is usually a very short (1—2 min) small increase in PD before the decline, but after 5 min of sanguinarine, a decrease is always seen.

A summary of the comparison between high and low potential skins is compiled in Table II at two different concentrations of sanguinarine. For 10^{-4} M concentration, the transition between initial stimulation and inhibition was about 50 mV and for $5 \cdot 10^{-5}$ M sanguinarine the crossover was approximately 38 mV.

The relationship between skin potential and effect of sanguinarine was explored by voltage clamping the skins. Fig. 3 is a typical experiment in which



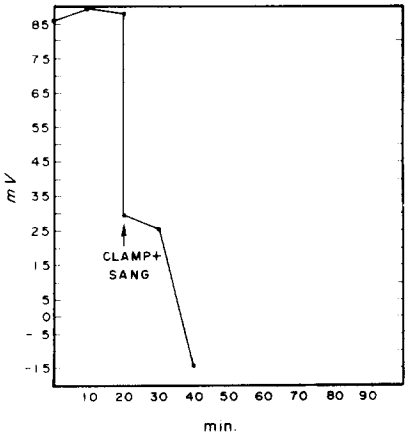


Fig. 3. Effect of sanguinarine on spontaneous low potential frog skin clamped at high PD to sanguinarine. Sanguinarine, final concentration 10^{-4} M, was added to mucosal bathing medium 1 min after application of clamp.

Fig. 4. Effect of sanguinarine on spontaneous high potential frog skin clamped at low PD. Sanguinarine, final concentration 10^{-4} M, was added to mucosal bathing medium 1 min after application of clamp.

TABLE III

EFFECT OF POTENTIAL CLAMPING ON THE RESPONSE TO SANGUINARINE

Low spontaneous potential skins were clamped to 70 mV and high spontaneous potential skins were clamped to 30 mV and subsequently sanguinarine was added to outside solution at 10^{-4} M. Effect of sanguinarine was measured at 5 min after addition and expressed as percent of clamped potential.

	Initial PD	Clamped PD	10^{-4} M Sanguinarine (% clamped PD at 5 min)
Low spontaneous potential	6	70	166
	14	70	134
	19	70	117
	20	70	160
	25	70	123
	30	70	117
Mean ± S.D. (n = 6)	19.00 ± 8.39		136.17 ± 21.78
High spontaneous potential	70	30	34
	74	30	67
	88	30	−157
	88	30	−171
Mean ± S.D. (n = 4)	80 ± 9.38		−82 ± 109

a skin with an initially low PD is clamped to a high PD and is treated with sanguinarine on the mucosal surface. In spite of being clamped at the high potential, the skin responded as if it were still at a low potential, i.e., by increase in PD. Likewise, in Fig. 4, a high potential skin is clamped to low potential and sanguinarine added to mucosal medium. The skin responds as if it were at high potential with an immediate and steep decrease in PD. A summary of a series of such experiments is presented in Table III. Clamping low potential skins to high potentials did not alter the stimulating response to sanguinarine and clamping of high potential skins to low potentials did not alter the inhibitory response to sanguinarine.

Discussion

The stimulation of PD and I_{sc} by sanguinarine in low potential frog skins is interpreted as a stimulation of Na^+ transport. This finding is unexpected because of the inhibitory effects of sanguinarine on ($Na^+ + K^+$)-ATPase from frog skeletal muscle [5] and a mammalian preparation [4]. Also perplexing is the fact that sanguinarine inhibits PD and I_{sc} if the initial PD is relatively high.

Using the Koefoed-Johnson and Ussing [7] two-step transfer model for Na^+ from the mucosal to the serosal side of frog skin, Larsen [8] notes that this active transport could be regulated at any one of three points: at the outward-facing surface by regulating passive Na^+ movement, by regulating the supply of ATP, or by regulating the $Na^+ + K^+$ pump itself on the inward-facing surface.

Considering these in reverse order, it seems that, if we identify the $Na^+ + K^+$ pump with ($Na^+ + K^+$)ATPase, sanguinarine should inhibit at this step. The supply of ATP available to the transport mechanism also be inhibited by sanguinarine since it is reported to inhibit oxidative phosphorylation in mitochon-

dria [9]. This leaves only the step involving movement of Na^+ from the mucosal side into the cell as a possible site for stimulation.

The movement at this first barrier has been considered recently [10,11]. This does not seem to be an energy-requiring step, but is often considered to be a facilitated transport involving a carrier [12]; the possibility of saturable pores should not be ruled out, however [13]. Benos et al. [12] utilized the common inhibitor amiloride and analogues to show a specific, but reversible, inhibition of Na^+ passage across this outer barrier. If amiloride decreases the PD and I_{sc} by inhibiting the passage of Na^+ into the cell, then sanguinarine might stimulate PD and I_{sc} by increasing the passage of Na^+ into the cell. The entrance of Na^+ into the cell could be accompanied by sanguinarine entrance whereupon the internal sanguinarine would now inhibit oxidative phosphorylation and the $(Na^+ + K^+)$-ATPase directly. Our experiments generally show such an inhibition of PD and I_{sc} following the stimulation. Similar behavior including initial stimulation followed by inhibition was observed for procaine [14] and *N*-dodecyl-pyridine-2-aldoxime [15]. These authors have considered the primary effect of the quartenary nitrogen containing compounds to be an increase of Na^+ permeability at the outer barrier. The subsequent inhibition was considered to be a separate effect on the active transport mechanism, perhaps by the unchanged form of the nitrogen (undissociated base). No voltage-dependent behavior was reported by these authors, although some seasonal variation was evident and lack or response to the quartenary nitrogen compounds could be corrected by administration of estradiol (female) or testosterone (male) [15].

If the skin has a high PD initially, this may be interpreted as a high Na^+ transport activity due to initial easy access through the outer barrier to the pump. Since the outer barrier is already readily permeated by Na^+, sanguinarine may enter directly the internal compartments of the cell and inhibit the transport activity. However, clamping high potential skins to low levels and low potential skins to high levels did not change the response of PD and I_{sc} based on initial spontaneous PD. Therefore, the initial potential difference is an indicator of response to sanguinarine, but the PD does not itself determine the response since clamping the PD did not alter the sanguinarine effect. Thus, the voltage-dependent changes in outer barrier transport [16] are probably not important in the response of the frog skin to sanguinarine.

Thus, sanguinarine may facilitate the Na^+ reaching the pump unless the Na^+ already has good access; in this case sanguinarine may enter the cells with the Na^+ and inhibit the transport, by either inhibiting the energy production in mitochondria and/or the $(Na^+ + K^+)$-ATPase at the serosal membrane.

A similar relationship between sodium entry at the mucosal membrane and pump activity has been noted for sodium transport in the isolated rabbit colon [17]. Thus, replacement of Cl^- by impermeant anions could only stimulate transcolonic sodium transport when the potential and short circuit current were low. When anion replacement was made at high potential and short circuit current, no stimulation was seen. In the rabbit colon, the anion replacement was shown to decrease Na resistance at the mucosal surface. The increase in active Na transport on anion replacement was, therefore, assumed to be due to removal of a rate-limiting Na^+ permeability barrier at the mucosal surface

and at high Na^+ transport rates, the permeability barrier was already at its minimum and anion replacement could lower it no further. These authors have suggested a negative feedback mechanism between pump activity at the serosal surface and sodium permeability at the mucosal surface so that "limitation of Na pump activity inhibits the ease of Na entry".

The resistance measurements bear no relationship to the proposed increase on Na^+ permeability due to sanguinarine. Thus, as the PD and I_{sc} are increasing presumably because of increase in Na^+ permeability at the outer barrier, the overall skin resistance is also increasing. If the paracellular pathways are the major contributors to this resistance, sanguinarine has two effects on permeability, i.e., an increase in outer barrier Na^+ permeability for transcellular transport of Na^+ and a decrease in ionic permeability in the paracellular pathways. Since the paracellular pathways predominate in the d.c. resistance, the observed change is to give decreased overall ionic permeability.

An alternative explanation can be proposed if assuming, as before, that the stimulation is due to an increase in outer barrier permeability and that the inhibition is a consequence of further reaction of sanguinarine with this outer barrier which leads to decreases in permeability to sodium. This might explain the increase in transepithelial resistance after prolonged exposure. In this view, sanguinarine does not enter the cell for its action, but reacts with the outer surface only.

It must be concluded, however, that sanguinarine effect on transepithelial ion transport in frog skin is not easily explainable as inhibition of the (Na^+ + K^+)-ATPase alone. At least one site of action is at the outer or mucosal barrier where sanguinarine probably increases membrane permeability for transcellular transport of Na^+. In addition, there appears to be an opposite effect on paracellular ion transport. The physiological state of the frog skin as reflected by the spontaneous PD is a determinant of whether the initial response to sanguinarine will be stimulatory or inhibitory.

Sanguinarine and its closely related benzophenanthridine compounds [18] have a broad range of activity in membrane systems. Thus, membrane functions such as oxidative phosphorylation, photophosphorylation [9], and (Na^+ + K^+)-ATPase [4], ouabain binding and excitability in myocardial membranes [19], as well as effects on frog skin noted in this report, are evidences of this activity toward various membranes. These compounds may prove useful as probes for further study of membrane structure and function.

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