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EFFECT OF A LIPID EXTRACT OF FROG SKIN ON SHORT-CIRCUIT CURRENT AND SODIUM TRANSPORT OF ISOLATED FROG SKIN

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SUMMARY

Lipid extracts obtained from frog skin and shown by thin-layer chromatography to contain materials with R_F values of cholesterol, cholesteryl linoleate, lecithin, palmitic acid, tripalmitin, and a number of methylated fatty acids stimulated short-circuit current and net sodium isotope flux of isolated frog skin. In contrast to the delayed response to adrenal steroids, the rise in short-circuit current was almost immediate. Change in net sodium flux was due entirely to rise in sodium influx. Sodium outflux was not stimulated, as it is by catecholamines. Treatment with the extract rendered the skins almost completely refractory to a second dose of extract but did not impair responsiveness to acetylcholine. Similarly, treatment of the skins with acetylcholine rendered them insensitive to a second dose of acetylcholine but not to the extract. Atropine failed to block the stimulatory action of the extract. These observations suggest that the extracts contained an agent or agents which are part of the natural sodium transport mechanism, which are not unaltered adrenal steroids, catecholamine, nor acetylcholine and whose actions are not mediated by these substances.

INTRODUCTION

In a preliminary communication[1] we described extracts of frog skin which stimulated short-circuit current (s.c.c.) of isolated frog skin. The present article reports more detailed experience with these extracts.

METHOD

A 16-channel, automatic, short-circuiting and recording apparatus was used to measure and record s.c.c.[2]. The frogs were female *Rana pipiens* (E. H. Steinhilber, Oshkosh, Wisc.). Frogs which were obviously ill or were members of a shipment with a high mortality rate within a few days of arrival in the laboratory were rejected. Until used, each frog was kept separately and unfed at about 75°F in a 2-cm layer of distilled water in an autoclaved Mason jar. The water was changed daily. Before

Abbreviation: s.c.c., short-circuit current.

being skinned, the frogs were decapitated and pithed. Each frog provided two symmetrical pieces of abdominal skin (skin halves). After being inserted in the recording apparatus with circulating aerated bathing medium[2], the skin halves were allowed to stabilize in the short-circuited condition for 3 or 4 h before any test substances were added to the reservoirs. The experiments were conducted at room temperature.

The lipid extracts of frog skin were obtained from an 80 % acetone extract of ground tissue. This was shaken with an equal volume of petroleum ether overnight. The supernatent was then decanted and evaporated, and the residue was dissolved in absolute ethanol. Cooling of this solution to about 0° resulted in formation of a white precipitate. After centrifugation and separation, the supernatent was evaporated to dryness under N_2 to yield the lipid extract. The extract was examined for activity with respect to s.c.c. and sodium isotope flux of isolated frog skin; it also was subjected to thin-layer chromatography on silica gel, petroleum ether–ether–glacial acetic acid (85:15:1, by vol.) being used as the solvent system.

Lipid extract was dissolved in absolute ethanol so that 25 μl solution, when added to the medium bathing the inside of the frog skins, yielded a concentration of 10 μg ml bathing medium. Twenty-five μl ethanol were used as control. In some experiments acetylcholine or atropine sulphate was added to the 'inside' medium to yield a concentration of 2 μg/ml. Preliminary experiments had shown these doses to be in excess of amounts needed to produce maximum responses. Isotope experiments were performed with ^{22}Na as a tracer when Na^+ influx or outflux was measured alone. ^{22}Na and ^{24}Na were used simultaneously* when net flux was being determined[3]. Samples of radioactive bathing media were counted in plastic vials in an automatic well counter, a γ spectrometer being used to count ^{24}Na. The ^{22}Na was counted after the ^{24}Na had been allowed to decay for about 2 weeks. The combination of spectrometer setting and decay of ^{24}Na ($t_{1/2}$, 15 h) permitted separation of the radioactive contribution of each isotope in the bathing media.

RESULTS

Lipid extract stimulated s.c.c., which reached a maximum within 30 min and returned to a steady level, usually slightly above the prestimulus steady level, within 1 h (Fig. 1). Fig. 2 illustrates responses to lipid extract of sodium isotope transport, entered to show correspondence between sodium influx and s.c.c. Increases in s.c.c. and sodium isotope influx are approximately the same (see also Table I). They are not, however, synchronous. There are delays as a result of diffusion of isotope through the skin[4]. Secondly, measurements of s.c.c. are momentary whereas measurements of isotope flux are cumulative. Thus, the two will appear out of phase to an extent determined in part by the frequency of measurements. Fig. 3 and Table I also show that changes in sodium isotope flux induced by lipid extract reflect changes in influx; outflux appears to be little affected by the extract.

The data in Table II supplement those in Table I and data published elsewhere[1,2,5] with regard to the stimulatory action of lipid extract and acetylcholine on s.c.c. In addition, these tables show that treatment with one substance makes most

* ^{24}Na was obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn. The ^{22}Na was obtained from Abbott Laboratories, North Chicago, Ill. Assays were performed by suppliers. The purity of the isotopes was checked by us with a γ spectrometer (Tracerlab Inc., Waltham, Mass.).

skins insensitive to a second treatment with the same substance but not to the other substance. Atropine had no significant effect on the stimulatory action of lipid extract (Table III). This in contrast to the complete block it imposes on acetylcholine in the same system[2]. Thin-layer chromatography (Fig. 4) showed that lipid extract contained materials with R_F values of lecithin, cholesterol, palmitic acid, tripalmitin, several methylated fatty acids, and cholesteryl linoleate. We have been unsuccessful so far in localizing activity in any of these fractions with certainty.

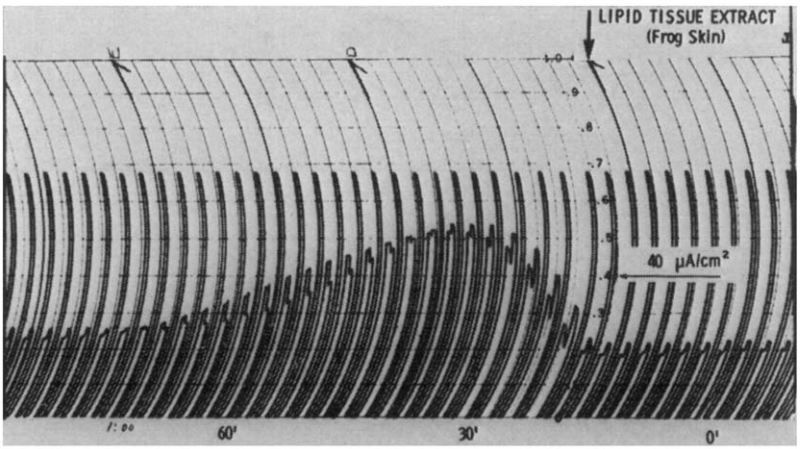

Fig. 1. Graphic record (Graphic ammeter (Model AW) Esterline–Angus Co., Indianapolis, Ind.) of typical response of s.c.c. of isolated frog skin to lipid extract of frog skin (lipid extract). Tall peaks are calibration marks; double peak marks are sequential readings from left and right skin halves of a single skin.

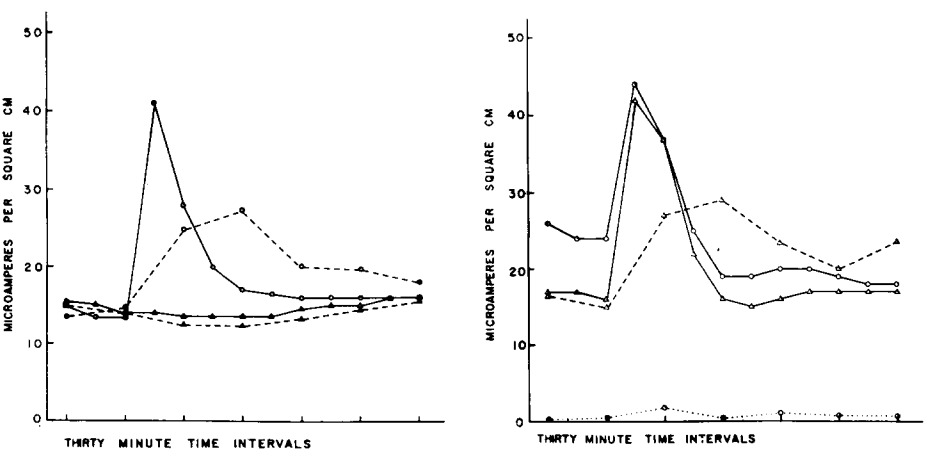

Fig. 2. The figure represents an experiment with one frog skin divided into halves. One-half (represented by o) was treated with lipid extract at 30 min. The other half (represented by △) was untreated. s.c.c. is represented by unbroken lines, and sodium influx by broken lines.

Fig. 3. One frog skin was divided into halves, represented by o and △, respectively. Both halves were stimulated by lipid extract at 30 min. s.c.c. is represented by unbroken lines. Influx of sodium isotope is represented by the broken line and outflux by the dotted line.

TABLE I

SHORT-CIRCUIT CURRENT, RADIOACTIVE SODIUM INFLUX AND OUTFLUX OF 8 SKINS TREATED ON THE INNER SURFACE WITH LIPID EXTRACT

Measurements are in $\mu A/cm^2$ isolated frog skin.

Skin No.	*Pre-treatment*			*Maximum*			*Post-treatment*		
	s.c.c.	*Influx*	*Outflux*	*s.c.c.*	*Influx*	*Outflux*	*s.c.c.*	*Influx*	*Outflux*
1	16	16	0.5	41	29	1.0	17	20	1.0
2	19	20	0.5	51	42	2.0	22	35	1.5
3	20	22	1.0	32	29	1.0	21	21	1.0
4	20		2.0	30		2.5	23		2.0
5	20	18		30	26		20	19	
6	17	22		46	47		21	26	
7	13	14		41	27		16	20	
8	31	31		56	46		35	47	

TABLE II

EFFECT ON SHORT-CIRCUIT CURRENT OF SEQUENTIAL TREATMENT OF ISOLATED FROG SKIN WITH LIPID EXTRACT AND ACETYLCHOLINE AN HOUR APART

Expt. No.	*Treatments*	*Change in s.c.c.**
1 (16 skins)	Lipid extract	66 ± 31
	Lipid extract	6 ± 14
	Acetylcholine	32 ± 22
2 (16 skins)	Acetylcholine	39 ± 23
	Acetylcholine	−2 ± 8
	Lipid extract	43 ± 26

* Mean % ± S.D.

TABLE III

EFFECT OF ATROPINE ON RISE IN SHORT-CIRCUIT CURRENT PRODUCED BY LIPID EXTRACT

Values are given in $\mu A/cm^2$ frog skin (mean ± S.D.) 28 skins. The treatments with lipid extract were given at the same time; with atropine 1 h before.

Treatment	*Skin half A*		*Skin half B*	
	Initial	*Change*	*Initial*	*Change*
Atropine	17 ± 7	−1.0 ± 1.3		
Lipid extract	15.7 ± 6.4	4.0 ± 3.4		
Lipid extract			15.8 ± 6.2	4.3 ± 3.2

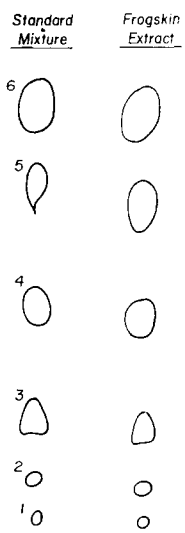

Fig. 4. Silica-gel thin-layer chromatograms of lipid extract of frog skin and a standard mixture of (1) lecithin; (2) cholesterol A.R.; (3) palmitic acid; (4) tripalmitin; (5) methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate; (6) cholesteryl linolenate. The solvent system was petroleum ether–ether–glacial acetic acid (85:15:1, by vol.).

DISCUSSION

A highly potent extract has been obtained from the skin of frogs which stimulates s.c.c. and sodium influx of isolated frog skin. It has little effect on outflux. The extract is a mixture of a number of lipids as judged by thin-layer chromatography. The method of preparation makes it unlikely that the extract has any major non-lipid constituents. The possibility, however, of the active material being non-lipid cannot be dismissed with certainty.

Our experience with isolated frog skin[6,7] and that of others[8] with toad bladders indicates that aldosterone, a major adrenal steroid of the frog[9,10], and other adrenal steroids stimulate short-circuit current and sodium flux only after a latent period of 2 h or more. The effect of our extract was almost immediate so that it is improbable that its active component is unaltered adrenal steroid. The fact that the extract does not stimulate sodium outflux would seem to exclude the possibility of its action being mediated in some way by catecholamine[2,3].

Previous studies showed that the stimulatory action on s.c.c. of acetylcholine is blocked by previous treatment of the skin with atropine[2]. The fact that atropine had no discerned effect on stimulation of s.c.c. by lipid extract and also the failure of previous treatment of the skins with acetylcholine itself to block the action of lipid extract make it unlikely that the effect of lipid extract on s.c.c. and presumably sodium isotope flux is mediated through raising the concentration of acetylcholine, at least extracellular acetylcholine[11].

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