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Diacylglycerols Stimulate Short-Circuit Current across Frog Skin by Increasing Apical Na^+ Permeability

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Summary. The phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) stimulates baseline Na^+ transport across frog skin epithelium and partially inhibits the natriferic response to vasopressin. The effects are produced largely or solely when TPA is added to the mucosal surface of the tissue. Although TPA activates protein kinase C, it has other effects, as well. Thus, the biochemical basis for the effects and the ionic events involved have been unclear. Furthermore, the physiologic implications have been obscure because of the sidedness of TPA's actions.

We now report that two synthetic diacylglycerols (DAG) replicate the stimulatory and inhibitory effects of TPA on frog skin. DAG is the physiologic activator of PKC. In this tissue, it produces half-maximal stimulation at a concentration of $\leqq 19\ \mu M$. In contrast to TPA, DAG is about equally effective from either tissue surface.

In a series of eight experiments, DAG was found to depolarize the apical membrane. Diacylglycerol also increases the paracellular conductance of frog skins bathed with mucosal Cl^- Ringer's solution. The latter effect can be minimized by replacing NO_3^- for Cl^- in the mucosal solution. Under these conditions, combined intracellular and transepithelial measurements indicated that DAG increased both the apical Na^+ permeability and intracellular Na^+ concentration. These results are qualitatively similar to the effects of cyclic 3′,5′-AMP on this tissue, suggesting that activation of PKC by DAG causes phosphorylation of the same or nearby gating sites phosphorylated by cAMP.

We propose that apical Na^+ entry is regulated in part by activation of PKC, and that insulin may be a physiologic trigger of this activation.

Key Words phorbol esters · TPA · protein kinase C · insulin · Na^+ transport · intracellular potential · intracellular Na^+ concentration · current-voltage relationship

Introduction

One of the most effective and well-characterized tumor promoters is the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (Diamond, O' Brien & Baird, 1980; Dicker & Rozengurt, 1981). TPA has been found both to activate protein kinase C (PKC) (Castagna et al., 1982) and to affect a number of transport processes in a range of biological cells (summarized by Mauro, O'Brien & Civan, 1987). These observations have suggested that the modulation of these transfer mechanisms may be mediated by PKC-dependent phosphorylations and that the observed changes in cellular activities may be triggered by the ionic translocations produced by TPA.

In order to pursue this hypothesis, we have initiated a study of the effects of TPA on the ionic, biochemical and biological properties of frog skin. This preparation has been chosen because: (i) the epithelium has been particularly well-characterized, (ii) net transepithelial Na^+ transport can be measured precisely and instantaneously as the short-circuit current (I_{sc}) (Ussing & Zerahn, 1951), and (iii) the membrane properties and intracellular ionic composition can be studied by several biophysical techniques not readily applied to other experimental models, such as toad urinary bladder (Civan & Garty, 1987).

We have already reported that TPA stimulates baseline Na^+ transport across frog skin and partially inhibits the subsequent natriferic response to vasopressin (Civan et al., 1985). TPA can exert different effects in other systems; for example, Yanase and Handler (1986) have reported inhibition of amiloride-sensitive current and stimulation of an amiloride-insensitive current across A6 cultured epithelial cells. In frog skin, the TPA-induced effects are elicited largely or entirely from the mucosal side of both whole-skin and split-skin preparations (Civan et al., 1985; Mauro et al., 1987). The stimulation of I_{sc} does not reflect nonspecific effects of TPA since the K_m is $\simeq 3$ nM (Mauro et al., 1987). Blockage of leukotriene production, but not of prostaglandin formation, from arachidonic acid partially inhibits the natriferic response to TPA (Civan et al., 1985; Mauro et al., 1987).

Despite the information already available concerning TPA stimulation of Na^+ transport across

frog skin, a number of fundamental points have been unclear. First, the biochemical basis for the stimulation has been uncertain. Although PKC might have mediated the effect, TPA is known to have multiple additional effects; for example, leukotrienes may also play a role (Mauro et al., 1987). Second, particularly in view of the sidedness of the response, it has been unclear whether the mechanisms triggered by TPA in vitro are also triggered by physiologic stimuli in vivo. Third, the ionic basis for the stimulated transport has been unknown.

In the present work, we have examined the effects of synthetic diacylglycerols (DAG), agents well documented to activate PKC (Kaibuchi, Takai & Nishizuka, 1981). The results of our transepithelial and intracellular electrophysiologic measurements suggest that DAG can replicate the transport effects of TPA, that these effects can be elicited on both mucosal and serosal surfaces, and that DAG appears to act by increasing the Na^+ permeability (P_{Na}^{ap}) of the apical plasma membrane. Based on these findings, we suggest possible physiologic implications for the TPA-induced stimulation of Na^+ transport across frog skin.

Materials and Methods

Transepithelial Measurements

Abdominal skins were removed from doubly-pithed frogs (*Rana pipiens pipiens*, West Jersey Biological Supply, Wenonah, NJ). The tissues were rinsed and bathed with a standard Ringer's solution (Civan et al., 1983; Mauro et al., 1987) containing (in mM): 120.0 Na^+, 3.5 K^+, 1.0 Ca^{2+}, 118.0 Cl^-, 2.5 HCO_3^-, and 10.0 HEPES (N-2-hydroxyethyl piperazine N'-2-ethanesulfonic acid; half in the basic and half in the acidic form). The pH was adjusted to about 7.6 U, and the osmolality was 240 mOsm.

Full-thickness skins were mounted between the two halves of a Lucite double chamber. Adjoining areas of 0.79 cm^2 were exposed, permitting control and experimental areas of each skin to be studied simultaneously. The transepithelial potential (ψ^{ms}, serosa positive with respect to mucosa) was clamped at 0 mV except for 5-sec intervals during which ψ^{ms} was increased to 10 mV. The transepithelial current (I_T) was continuously measured with a dual-pen chart recorder. The transepithelial slope conductance (g_T) was calculated as the ratio of the current deflection to the 10-mV voltage step.

Intracellular Measurements

Full-thickness skins were mounted mucosal side up between the two halves of a Lucite-chamber (DeLong & Civan, 1978), exposing 1.9 cm^2 for study. The mucosal and serosal surfaces were separately superfused with solutions. Cells were impaled across their apical membranes using single-barrelled micropipettes drawn from omega-dot capillary glass tubing (1.5 mm outer and 0.75 mm inner diameter; Glass Co. of America, Millville, NJ). The micropipettes were filled with 0.5 M KCl solution in order to reduce the rate of release of saline into the intracellular fluids during the impalements. Minimal criteria for acceptability of penetrations have been discussed elsewhere (Civan et al., 1983; DeLong & Civan, 1983). All of the intracellular measurements included in the present study were obtained during impalements which were stable for at least several minutes. Most of the data were collected during periods when the intracellular potential (ψ^{mc}, cell positive with respect to mucosa) was stable over periods of 30 min or more. The apical fractional resistance (f^{ap}), defined as the resistance of the apical membrane divided by the total transcellular resistance, was measured as the ratio of the change in apical membrane potential to that imposed across the entire epithelium while applying voltage pulses.

Both ψ^{mc} and I_T were continuously displayed on a storage oscilloscope and a dual-pen recorder. The intracellular measurements were conducted while ψ^{ms} was clamped alternately to 0 mV for 7.7 sec and to 20 mV for 4.3 sec. When appropriate, this baseline voltage pulsing was interrupted and a train of voltage pulses applied across the epithelium. The pattern consisted of 12 pairs of alternately hyperpolarizing and depolarizing pulses, the magnitude increasing by 20 mV in successive steps. A modification (S. Leibowich, J. DeLong, & M.M. Civan, *in preparation*) of our initial program (DeLong & Civan, 1984) was used, permitting variation of both the pulse duration and interpulse interval over a broad range of values. The present data were collected with pulse durations of 16 or 32 msec and interpulse intervals of 240-1,280 msec. At the end of each voltage step, ψ^{mc}, ψ^{ms} and I_T were sequentially sampled eight times over a period of 0.96 msec. The mean values of these eight data points were used in the subsequent data analysis, using the approach of Fuchs, Larsen and Lindemann (1977). The apical Na^+ current (I_{Na}) was taken as the amiloride-sensitive current at a given transepithelial voltage. With the experimental approach used in the present study, plots of I_{Na} as a function of ψ^{mc} can be fit by the Goldman equation, frequently over a range of several hundred millivolts (DeLong & Civan, 1984; Schoen & Erlij, 1985; S. Leibowich, J. DeLong, & M.M. Civan, *in preparation*). From the fit, both the apical Na^+ permeability (P_{Na}^{ap}) and the intracellular Na^+ concentration (c_{Na}^c) can be estimated. All data are reported as the means ±SE. The probability (P) of the null hypothesis has been calculated by application of Student's t test.

Chemicals

The initial sample of 1-oleoyl-2-acetylglycerol (OAG) was a generous gift from Dr. Enrique Rozengurt and James Sinnett-Smith. Later samples of both OAG and *sn*-1,2-dioctanoylglycerol (DIC) were purchased from Avanti Polar Lipids (Birmingham, AL). Both OAG and DIC were prepared as follows: The chloroform in which the compounds were received from the supplier was evaporated with a stream of nitrogen and the compounds redissolved in dimethylsulfoxide (DMSO) just before use; the concentration of DMSO applied to the tissue was always <0.3% (vol/vol). In order to reduce adsorption of the diacylglycerols onto glass and plastic surfaces, the solvent Ringer's solution always contained 1 mg · cm^{-3} of albumin (Fraction V, Sigma Chemical Co., St. Louis, MO) whenever these highly hydrophobic agents were applied to the tissues. The baseline measurements were conducted in the presence of the same concentration of albumin.

The amiloride was kindly provided by Dr. George M. Fanelli, Jr. (Merck Institute for Therapeutic Research, West

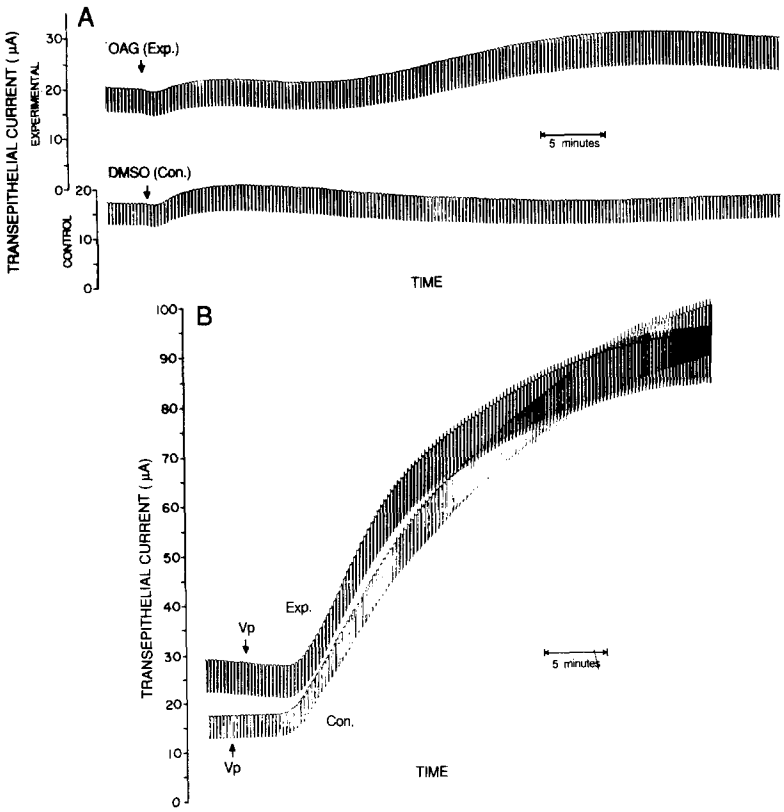

Fig. 1. Effects of OAG (1-oleoyl-2-acetylglycerol) on transepithelial current across frog skin. The upper envelope of each trace is the short-circuit current (I_{sc}). The lower envelope is the transepithelial current required to clamp the transepithelial potential (ψ^{ms}) at 10 mV. Thus, the transepithelial conductance (g_T) is proportional to the displacement of the two leaves of each trace. The tissue was bathed with a standard Cl^- Ringer's solution in an Ussing double-chamber. (*A*) Addition of the DMSO solvent produced a transient increase in I_{sc}. However, the introduction of 375 μM OAG into the mucosal and serosal solutions bathing the experimental area also produced a much larger and more sustained stimulation of I_{sc} and an increase in g_T. (*B*) Both the experimental and control portions of the skin subsequently displayed large responses of I_{sc} and g_T to the application of 100 mU · cm^{-3} serosal vasopressin. However, the OAG-pretreated area displayed a reduced percentage stimulation of I_{sc}. This can be appreciated by noting that just prior to adding vasopressin, the baseline value of I_{sc} was larger on the OAG-pretreated side (*Exp*), while it was smaller than that of the control (*Con*) hemiskin at the time of the peak response

Point, PA), and was stored in the form of concentrated aqueous solutions at 4°C. Vasopressin was purchased as 8-arginine vasopressin (Calbiochem, San Diego, CA), dissolved in deionized water and stored frozen.

Results

Effects of Diacylglycerols

Figure 1*A* presents representative results obtained by adding 375 μM OAG to the mucosal and serosal media bathing an experimental hemiskin (*Exp.*) and equal volumes of DMSO solvent to the two surfaces of the control area (*Con*) of the same preparation. The short-circuit current is given by the upper envelope of each trace of transepithelial current; the conductance is proportional to the deflection in current associated with each voltage pulse. Even at the low concentrations (<0.3%) used in the present study, DMSO often produced transient increases in short-circuit current (I_{sc}) and/or transepithelial conductance (g_T) (Fig. 1*A*). Superimposed on these transient effects, the addition of 375 μM OAG to the mucosal and serosal media characteristically caused larger, more sustained stimulations of I_{sc} and g_T. The responses of the experimental trace are representative of the effects characteristically observed. Averaging the results from a series of six such experiments, OAG increased short-circuit current by 40 ± 6% and conductance by 17 ± 5% (Table 1).

Following the peak stimulation by OAG, the subsequent natriferic response to vasopressin was blunted. This effect can be appreciated from Fig. 1*B*. Before the addition of supramaximal doses of hormone, the value of I_{sc} for the control was less than that for the experimental hemiskin. This inequality was reversed at the time of the peak natriferic response. The natriferic response was also partially inhibited by OAG pretreatment in each of the additional four experiments so conducted (Table 1).

The stimulation of baseline parameters and the partial inhibition of vasopressin's effect were not limited to OAG. The diacylglycerol DIC exerted similar effects, both qualitatively and quantitatively (Table 1). Averaging the pooled results of six experiments conducted with OAG and five experiments performed with DIC, diacylglycerol was found to increase I_{sc} and g_T by 42 ± 4% and 27 ± 6%, respectively. The diacylglycerols also reduced the subsequent effect of vasopressin on short-circuit current by approximately half (Table 1). These data estab-

Table 1. Effects of diacylglycerols (DAG) on baseline values of short-circuit current and transepithelial conductance, and on the subsequent responses to vasopressin

A. Effects on short-circuit current

DAG	Parameter	Baseline ($\mu A \cdot cm^{-2}$)			% Changes after DAG			% Changes after vasopressin		
		E	*C*	*E-C*	*E*	*C*	*E-C*	*E*	*C*	*E-C*
OAG	Mean	40	41	0	34	−6	40[d]	141	300	−159[c]
	± SE	±6	±7	±2	±6	±4	±6	±42	±50	±30
	(*N*)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(5)	(5)
DIC	Mean	43	40	3	36	−8	44[c]	72	151	−79
	± SE	±6	±8	±2	±8	±2	±6	±24	±28	±31
	(*N*)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
OAG	Mean	42	40	1	35	−7	42[d]	106	225	−119[d]
or	± SE	±4	±5	±2	±5	±2	±4	±25	±37	±24
DIC	(*N*)	(11)	(11)	(11)	(11)	(11)	(11)	(10)	(10)	(10)

B. Effects on Transepithelial conductance

DAG	Parameter	Baseline ($mS \cdot cm^{-2}$)			% Changes after DAG			% Changes after vasopressin		
		E	*C*	*E-C*	*E*	*C*	*E-C*	*E*	*C*	*E-C*
OAG	Mean	1.3	1.5	−0.1	21	4	17[b]	28	55	−27[a]
	± SE	±0.2	±0.2	±0.1	±8	±5	±5	±8	±14	±8
	(*N*)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(5)	(5)
DIC	Mean	1.6	1.6	0.0	42	2	39[a]	20	75	−54[a]
	± SE	±0.4	±0.4	±0.1	±11	±4	±11	±21	±36	±17
	(*N*)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
OAG	Mean	1.5	1.5	0.0	30	3	27[c]	24	65	−41[c]
or	± SE	±0.2	±0.2	±0.1	±7	±3	±6	±11	±19	±10
DIC	(*N*)	(11)	(11)	(11)	(11)	(11)	(11)	(10)	(10)	(10)

In each case, one of two diacylglycerols was applied to both the mucosal and serosal solutions bathing the experimental tissue area (*E*). OAG (1-oleoyl-2-acetylglycerol) was added to final concentrations of 250 μM in the first experiment, and of 375 μM in the remaining five experiments of the series. DIC (sn-1,2-dioctanoylglycerol) was applied at a concentration of 450 μM in the first experiment and at 375 μM in the remaining four experiments of the series. In each case, an equal volume of solvent [dimethylsulfoxide (DMSO)] was added to the control tissue area (C); the final DMSO concentration was 0.14-0.29% (vol/vol). At the time of the peak response of short-circuit current to the OAG or DIC, vasopressin was added to a final serosal concentration of 100 $mU \cdot cm^{-3}$ to both the experimental and control areas. The entries under *% Changes after Vasopressin* have been calculated with respect to the values of short-circuit current and conductance at the time of addition of hormone, i.e. close to the time of peak response to the diacylglycerol. *N* is the number of both the experiments and skins studied in each series. Since the results obtained with the OAG and DIC were similar, the values calculated from analyzing the combined data are also presented in the third rows of Table 1*A* and *B*, The columns headed *E-C* present the results of paired analyses of each series. The statistically significant values of (*E-C*) have been identified in this and subsequent tables by the superscripts: ([a]) the probability (*P*) of the null hypothesis is < 0.05, ([b]) $P < 0.02$, ([c]) $P < 0.01$, and ([d]) $P < 0.001$.

lish that the stimulatory and inhibitory effects of TPA on frog skin can be reproduced by the addition of diacylglycerols, documented activators of protein kinase C.

Dose-Response Relationship

Because of the nonpolar nature of the diacylglycerols, their true concentrations in solution did not necessarily match their nominal concentrations in the aqueous media. In the absence of albumin, no effect of diacylglycerol was consistently noted, presumably because of adsorption of DAG onto glass and plastic surfaces. Even with albumin present, there was appreciable tissue variation in the quantitative responses to the diacylgylcerols. Most of the data reported in the present study were obtained with 188-375 μM diacylglycerol.

An approximate estimate of the DAG concentration required for half-maximal stimulation was obtained from a series of five experiments. DIC was added to the mucosal and serosal media of each experimental hemiskin over a concentration range of 4-75 μM, while equal volumes of DMSO were added to the control hemiskin. After a peak response to the lower dose was noted, a supramaximal concentration (375 μM) of DIC was added to the media bathing the control hemiskin. In the experiment conducted at a DIC concentration of 4 μM, no

Table 2. Dose-response relationship for DIC-induced stimulation of short-circuit current

Exp.	Baseline ($\mu A \cdot cm^{-2}$)			Low	% Changes after DIC			% Changes after VP		
	E	*C*	*E-C*	[DIC] (μM)	Low [DIC]	High [DIC]	% Effect at low dose	After low [DIC]	After high [DIC]	Diff
A	38	45	−8	19	17	30	58	399	295	104
B	38	32	6	38	27	55	50	97	8	89
C	40	32	8	75	45	35	130	105	98	8
D	17	15	2	75	92	153	60	289	60	229
Mean	33	31	2			68		223	90	74
± SE	±5	±6	±7			±29		±74	±39	±19

Following a baseline period, the experimental hemiskin was initially exposed to a low dose of DIC (19-75 μM) on both surfaces, while the control hemiskin was treated with equal volumes of DMSO. After the peak stimulation of I_{sc}, the control tissue was treated with the high dose of DIC (375 μM). The ratio ($\times 100\%$) of the effect at the low to that at the high dose is entered for each tissue in the column headed *% Effect at low dose*; a value of 100% would indicate that the response at the lower dose was just as effective as that produced by the higher concentration of DIC. After a maximal response to the high concentration of DIC was observed, vasopressin (VP, 100 $mU \cdot cm^{-3}$) was added to both serosal media.

stimulation of I_{sc} was observed. The results obtained with the other four experiments over the range 19-75 μM are entered in Table 2. Over this concentration range, the stimulation produced by the low dose of DIC was no less than half the maximal response noted with 375 μM. Even at a concentration of 19 μM, the DIC-elicited stimulation was 58% of the maximal effect. The response to vasopressin was also partially inhibited by the low doses of DIC in each of the four experiments.

Thus, the results of these five experiments indicate that half-maximal stimulation could be produced at a DIC concentration $\leqq 19$ μM and > 4 μM.

Sidedness of Effects

Despite the similarity of the transport effects of diacylglycerols and phorbol esters on frog skin, the sidedness of the effects is distinctly different. TPA stimulates short-circuit current across this tissue largely or only when added from the mucosal side (Civan et al., 1985; Mauro et al., 1987). In contrast, the diacylglycerols act from either surface of frog skin. This point is illustrated by Fig. 2. OAG was sequentially and separately added to the serosal and mucosal media of the two hemiskins of a single preparation; at the same time, DMSO solvent was administered to the adjoining tissue area. In each case, OAG stimulated baseline transport, whether added to mucosal or serosal surface. The results of eight such mucosal and serosal additions to five preparations are summarized in Table 3. In an additional series of four frog skins, similar results were obtained when DIC was separately added to the mucosal and serosal surfaces of the same tissue (Table 3).

The results of averaging the 12 mucosal and 12 serosal additions of the diacylglycerols are also presented in Table 3. The separate applications of mucosal and serosal DAG increased short-circuit current by 25 ± 6% and by 25 ± 5%, respectively. Thus, the diacylglycerols were comparably effective from either tissue surface in stimulating I_{sc}.

Intracellular Measurements

The sidedness of the responses to phorbol esters has suggested that TPA stimulates Na^+ transport across frog skin at an apical site of action (Civan et al., 1985; Mauro et al., 1987). The site of action was less apparent in the present context, since the diacylglycerols were equally effective in stimulating short-circuit current when added to the apical or basolateral membranes. This point was examined by a series of intracellular impalements.

In a preliminary series of three experiments, DIC was administered at a concentration of 188 or 375 μM either to the mucosal or to both tissue surfaces. At the season of year in which the intracellular measurements were conducted (late Fall–early Winter), DIC stimulated I_{sc} to a lesser extent than in late Summer–early Fall. However, in each of the experiments, DIC clearly increased I_{sc} and g_T, and depolarized the apical membrane. Averaging these initial results, the changes (Δ) produced in these parameters were: $\Delta I_{sc} = 0.8 \pm 0.3$ $\mu A \cdot cm^{-2}$, $\Delta g_T = 0.022 \pm 0.004$ $mS \cdot cm^{-2}$, and $\Delta \psi^{mc} = 4 \pm 1$ mV.

The observation that DIC depolarized the intracellular potential while stimulating the short-circuit current suggested that diacylglycerols exert a cAMP-like effect, increasing the apical Na^+ permeability. This possibility was next examined in a se-

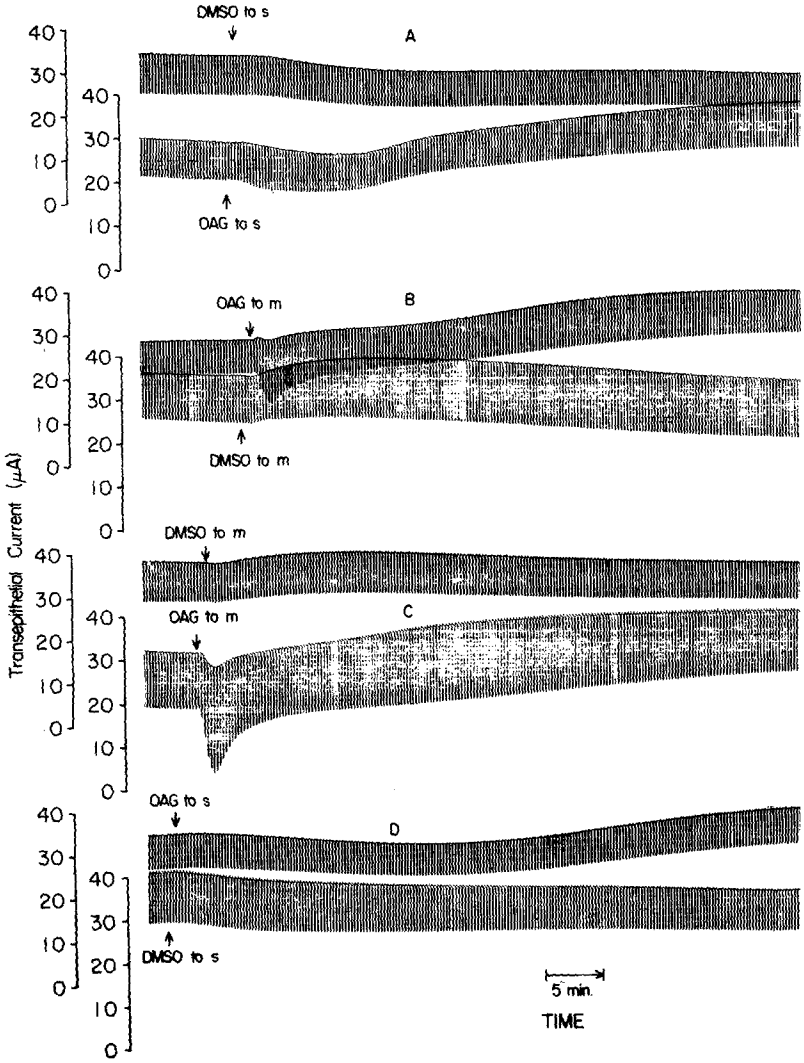

Fig. 2. Sidedness of effect of OAG on short-circuit current and transepithelial conductance. During the course of a single experiment, 375 μM diacylglycerol was sequentially added to: (*A*) the serosal surface of the "experimental" hemiskin, (*B*) the mucosal medium bathing the "control" hemiskin, (*C*) the mucosal reservoir of the "experimental" area, and (*D*) the serosal surface of the "control" tissue. In each case, identical volumes of pure DMSO solvent were added to the corresponding surface of the adjoining hemiskin

Table 3. Sidedness of effect of diacylglycerols (DAG) on short-circuit current

DAG	Parameter	Initial baseline ($\mu A \cdot cm^{-2}$)			% Changes after DAG		
		E	*C*	*E-C*	Muc	Ser	Muc & Ser
OAG	Mean	39	41	−2	29	20	12
	± SE	±7	±9	±2	±7	±6	—
	(*N*)	(5)	(5)	(5)	(8)	(8)	(1)
DIC	Mean	26	23	2	19	35	48
	± SE	±6	±5	±1	±10	±10	±23
	(*N*)	(4)	(4)	(4)	(4)	(4)	(3)
OAG or DIC	Mean	33	33	0	25	25	39
	± SE	±5	±6	±2	±6	±5	±19
	(*N*)	(9)	(9)	(9)	(12)	(12)	(4)

The first three rows of entries (OAG) are results obtained with a series of five skins treated by adding 375 μM OAG separately to the mucosal (Muc) and serosal (Ser) media 1-2 times; in one experiment, OAG was also added simultaneously to the mucosal and serosal reservoirs. The second three rows of values were measured in another series of experiments, where 375 μM DIC was applied to three skins and 187.5 μM DIC was applied to a fourth tissue. In the latter series, DIC was applied once separately to mucosa and serosa. In both series, equal volumes of DMSO solvent were always added to the adjoining area serving as control. The final three rows of entries are the results calculated from analyzing the combined results of the two preceding series of experiments. The hemiskins identified as "experimental" (*E*) were the tissue areas first to receive either OAG or DIC, while the "control" (*C*) hemiskins were the first tissue areas to be exposed to DMSO solvent alone.

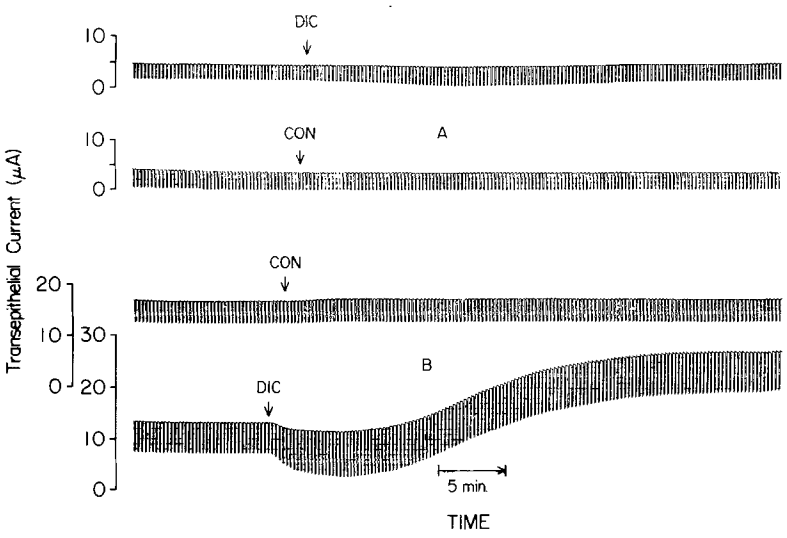

Fig. 3. Effect of *sn*-1,2,-dioctanoylglycerol (DIC) on I_{sc} and g_T of tissues bathed with mucosal NO_3^-. (*A*) The experimental (upper) and control (lower) traces were obtained with a single skin perfused with 20 μM amiloride and NO_3^- (rather than Cl^-) in the mucosal medium. The serosal medium was a standard Cl^- Ringer's solution. At the arrows, 188 μM DIC was added to the mucosal and serosal media bathing the experimental area, while identical volumes of DMSO solvent were added to the reservoirs of the control hemiskin. In the presence of amiloride and mucosal NO_3^-, the DIC had little effect, increasing g_T slightly. (*B*) The media were subsequently replaced, fresh NO_3^- Ringer's solution bathing the mucosal surfaces and Cl^- Ringer's solution bathing the serosal surfaces. At the arrows, addition of 188 μM DIC now stimulated I_{sc} by approximately 100% and increased g_T more than three times as much in the absence of amiloride as in its presence (panel *A*). Both panels demonstrate that the transient effects of DMSO noted with mucosal Cl^- solution (Figs. 1-2) were almost completely abolished by replacing mucosal Cl^- with NO_3^-, whether or not amiloride was present

ries of four experiments conducted in the presence of 20 μM mucosal amiloride. In each of three experiments conducted with standard Ringer's solution bathing the tissue surfaces, mucosal and serosal addition of 188 μM DIC had little or no effect on short-circuit current, but clearly increased the transepithelial conductance. For these three skins, ΔI_{sc} was 0.0 ± 0.2 $\mu A \cdot cm^{-2}$ and Δg_T was 0.6 ± 0.2 mS $\cdot$ cm^{-2}. These data supported the concept that DIC was stimulating short-circuit current by increasing Na^+ movement through the apical amiloride-sensitive channels, but also indicated that DIC was increasing the conductance of the paracellular junctions.

In order to estimate intracellular Na^+ concentration and apical Na^+ permeability by the electrophysiologic approach described under Materials and Methods, it is necessary to minimize any changes in paracellular conductance. This can be achieved by bathing the mucosal surface of frog skins with nitrate, rather than chloride (Nagel, Garcia-Diaz & Essig, 1983). In a series of three experiments conducted with 20 μM amiloride and nitrate quantitatively replacing chloride in the mucosal solution, 188 μM DIC had very little effect on transepithelial transport. ΔI_{sc} was -0.4 ± 0.2 $\mu A \cdot cm^{-2}$ and Δg_T was 0.04 ± 0.04 mS $\cdot$ cm^{-2}. Two of these experiments were performed during parallel applications of DIC bathed with mucosal nitrate and with mucosal Cl^- on the two adjoining hemiskins of the same preparation. The change in g_T in the presence of NO_3^- was only 0-2.5% of the increase in g_T observed with chloride Ringer's solution. The results of the third experiment of the series are presented in Fig. 3. In this case, DIC was first added to an amiloride-treated skin, the solutions were replaced, and DIC was added once again in the absence of amiloride. In both cases, the mucosal medium was nitrate Ringer's solution. This tissue displayed an unusually large conductance response (0.09 mS) to DIC in the presence of mucosal amiloride and nitrate; the conductance of the other two skins increased by only 0.00-0.01 mS. However, even in this preparation, the DIC-induced increase in g_T was more than threefold larger in the absence of amiloride than in its presence. Thus, replacement of mucosal Cl^- by NO_3^- proved effective in minimizing changes in the paracellular conductance produced by DIC. Figure 3 also demonstrates that the transient effects exerted by DMSO in the presence of mucosal Cl^- (Figs. 1-2) were almost entirely absent when nitrate replaced the chloride. Therefore, the electrophysiologic measurements of c_{Na}^c and P_{Na}^{ap} entered in Table 4 were all obtained in the presence of mucosal nitrate Ringer's solution.

Figure 4 presents the unprocessed data recorded during Exp. 2 of Table 4. A cell was impaled while monitoring the output voltage of the exploring micropipette and the transepithelial current (I_T). After a baseline period of observation, trains of volt-

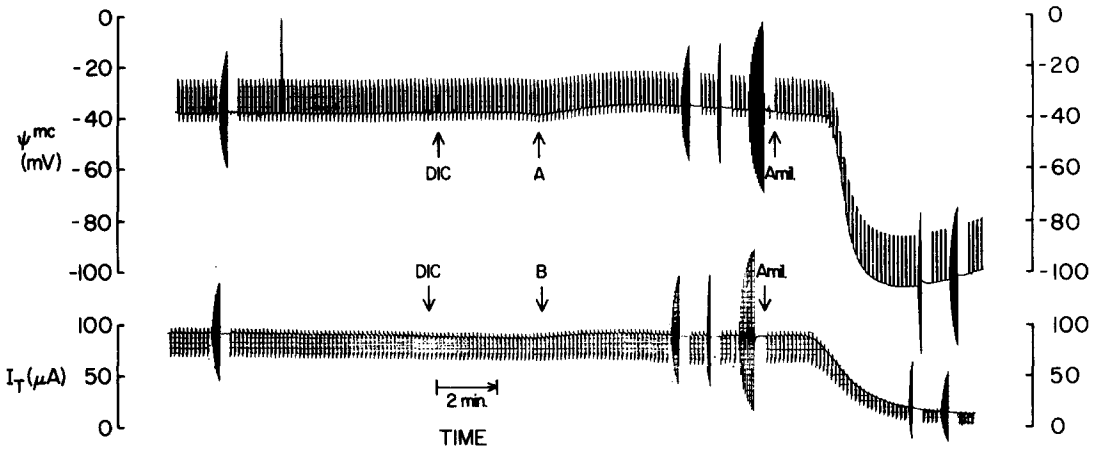

Fig. 4. Effects of DIC on apical membrane potential, short-circuit current and transepithelial conductance. At periodic intervals, the baseline pattern of voltage pulsing was interrupted, and trains of pulses (appearing as filled triangles) applied across the tissue. At the arrows, 188 μM DIC was applied to the mucosal and serosal surfaces; the separation between the two arrows reflect the displacement of the two pens of the paper chart recorder. ψ^{mc} began to depolarize at arrow *A*, while both I_{sc} and g_T began to increase shortly before arrow *B*. The fall in membrane potential did not reflect partial slippage of the micropipette tip out of the cell since the subsequent addition of 20 μM mucosal amiloride (*Amil*) produced a characteristic hyperpolarization to more than -100 mV

Table 4. Effects of DIC on intracellular, apical membrane and transepithelial parameters of tissues bathed with mucosal nitrate

Exp.	I_{sc} ($\mu A \cdot cm^{-2}$)		g_T mS $\cdot$ cm^{-2})		ψ^{mc} (mV)		No. of analyses		P^{ap}_{Na} (10^{-7} $cm^3 \cdot sec^{-1}$)		c^c_{Na} (mM)	
	BL	Δ	BL	Δ	BL	Δ	BL	DIC	BL	Δ	BL	Δ
1	8	2.0	0.30	0.053	−65	4.1	5	8	3.9	2.7 ±0.1[d]	4.3	33.6 ±0.9[d]
2	46	2.6	0.58	0.079	−38	2.5	3	3	33.2	7 ±2[a]	14	26 ±3[d]
3	28	2.4	0.47	0.053	−50	4.0	4	4	14.2	1.8 ±0.2[d]	3.6	4 ±1[b]
4	68	4.2	0.78	0.053	−43	2.4	3	9	46.6	2.8 ±0.5[d]	19.4	10.2 ±0.7[d]
5	40	4.5	0.47	0.026	−49	6.7	3	5	25.3	4.0 ±0.5[d]	36.6	−1 ±1
Mean ± SE	38 ±10	3.1 ±0.5[c]	0.52 ±0.08	0.053 ±0.008[c]	−49 ±5	3.9 ±0.8[c]			25 ±7	3.7 ±0.9[b]	16 ±6	14 ±7

After completing baseline measurements (BL), 188 μM DIC was added to the mucosal and serosal surfaces of each skin. The number of analyses is the number of times that pulse trains were applied and analyzed in estimating P^{ap}_{Na} and c^c_{Na} during the baseline (BL) and experimental (DIC) periods. Statistically significant differences have been identified by the letter superscripts defined in Table 1.

age pulses were applied across the tissue. One such train is included in the beginning of the upper (ψ^{mc}) and lower (I_T) traces, appearing as filled triangles at the time resolution of the paper chart record. The vertical line appearing 2 min later on the upper trace is an artifact caused by passing a pulse of current through the micropipette in order to monitor the micropipette resistance periodically. At the time of the arrows identified by *DIC* 188 μM diacylglycerol was added to the mucosal and serosal reservoirs; the appearance of additional noise on the ψ^{mc} trace was caused by the investigator placing his hands in the Faraday cage in order to change solutions. After a lag period of 3 min, changes can be detected in the upper (*A*) and lower (*B*) traces. The lag period reflects both a contribution of approximately 2 min from the flow rate and dead space of the tubing in this experiment, and the true response time of the

tissue following arrival of DIC at the skin surface. Although the magnitudes of the changes are small, the increase in I_{sc} and g_T and the membrane depolarization (upward displacement of ψ^{mc}) are clearly detectable. The experiment was concluded by applying 20 μM amiloride to the mucosal surface, producing a marked inhibition of I_{sc}, reduction of g_T, and hyperpolarization of the apical membrane to well over 100 mV (cell negative to mucosal solution). As can be appreciated by the Figure, additional trains of voltage pulses were applied at the peak DIC effect and after amiloride was added.

Figure 5 presents reduced data from the same experiment (Exp. 2 of Table 4, Fig. 4). The transcellular Na^+ current (I_{Na}) is plotted as a function of apical membrane potential. The data symbolized by the rhomboids were derived from measurements before adding DIC. The points presented as closed circles were calculated from measurements obtained at the peak response of I_{sc}. Thereafter, 20 μM amiloride was added to measure the amiloride-insensitive transepithelial current as a function of transepithelial voltage. The amiloride was then washed out with fresh Ringer's solution. The data points displayed as open circles were calculated from a train of voltage pulses applied after a new steady state had been reached. The solid lines are Goldman fits to the data. From these fits, P_{Na}^{ap} is estimated to have been 31.7×10^{-7} cm^3 · sec^{-1} before the DIC, 43.7×10^{-7} cm^3 · sec^{-1} during the stimulation by DIC, and 24.9×10^{-7} cm^3 · sec^{-1} after reversing the DIC effect. Similarly, the intracellular Na^+ concentration (c_{Na}^c) is estimated to have been 16.8, 44.5 and 18.1 mM before, during and after the application of DIC, respectively.

Table 4 summarizes the results obtained with all five skins of the series. As in the case of the three tissues initially studied with mucosal Cl^-, DIC depolarized the apical membrane (by 3.9 ± 0.8 mV). Averaging the results of all tissues bathed either with mucosal NO_3^- or Cl^-, the diacylglycerol depolarized ψ^{mc} by 4.0 ± 0.6 mV. Each entry of P_{Na}^{ap} and c_{Na}^c has been calculated by averaging the results of fits of at least three I_{Na}-ψ^{mc} relationships. In each of the five experiments of Table 4, DIC significantly increased P_{Na}^{ap} above its baseline value. Averaging the paired differences before and after DIC, ΔP_{Na}^{ap} was found to be $(3.7 \pm 0.9) \times 10^{-7}$ cm^3 · sec^{-1}.

DIC was also found to increase c_{Na}^c significantly in four of the five experiments. In three experiments, the increase was significant at the 0.001 probability level. In only one case (Exp. 5, Table 4) was c_{Na}^c unchanged. The basis for this single exception is unclear, although the baseline value of intracellular Na^+ concentration estimated for this tissue was much greater than that measured in the other four experiments (Table 4).

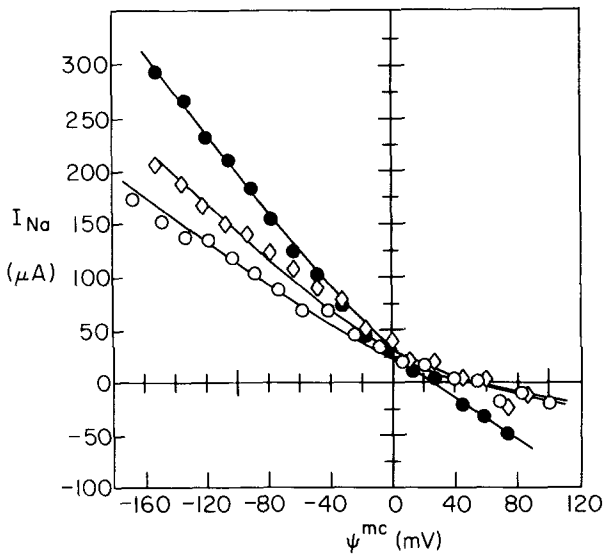

Fig. 5. Effect of DIC on relationship between transcellular Na^+ current (I_{Na}) and apical membrane potential (ψ^{mc}). The data were obtained from the same experiment as that of Fig. 4 (Exp. 2, Table 4). The data points represented by rhomboids were derived from initial control measurements; those symbolized by closed circles were based on measurements during mucosal and serosal stimulation with 188 μM DIC. After the peak response to DIC, 20 μM amiloride was added to the mucosal superfusate, and the transepithelial current-voltage relationship was redetermined. Thereafter, the tissue was perfused with fresh Ringer's solution free of DIC and amiloride. The points represented as open circles were based on results obtained during the second control period. All the data were collected during the application of voltage pulses 16 msec in duration, with interpulse intervals of 240 msec. The solid curves were constructed by fitting the data to the constant field equation (Goldman, 1943):

$$I_{Na} = -[(P_{Na}^{ap} F^2 \psi^{mc})/(RT)]\{c_{Na}^m - c_{Na}^c [e^{(\psi^{mc}F/(RT))}]\} \cdot \{1 - e^{(\psi^{mc}F/(RT))}\}^{-1}$$

where $\psi^{mc} \neq 0$, F and R are the Faraday and perfect gas constants, respectively, T is the absolute temperature, and c_{Na}^m is the mucosal Na^+ concentration. The three sets of values calculated for the apical Na^+ permeability (P_{Na}^{ap}, in 10^{-7} cm^3 · sec^{-1}) and for the intracellular Na^+ concentration (c_{Na}^c, in mM) were: 31.7 and 16.8 before adding DIC, 43.7 and 44.5 during DIC stimulation, and 24.9 and 18.1 after washing out the DIC

Discussion

The tumor promoter TPA stimulates Na^+ transport across frog skin and partially inhibits the natriferic effect of vasopressin (Civan et al., 1985; Mauro et al., 1987). In part, these effects may be mediated by stimulating leukotriene production (Mauro et al.,

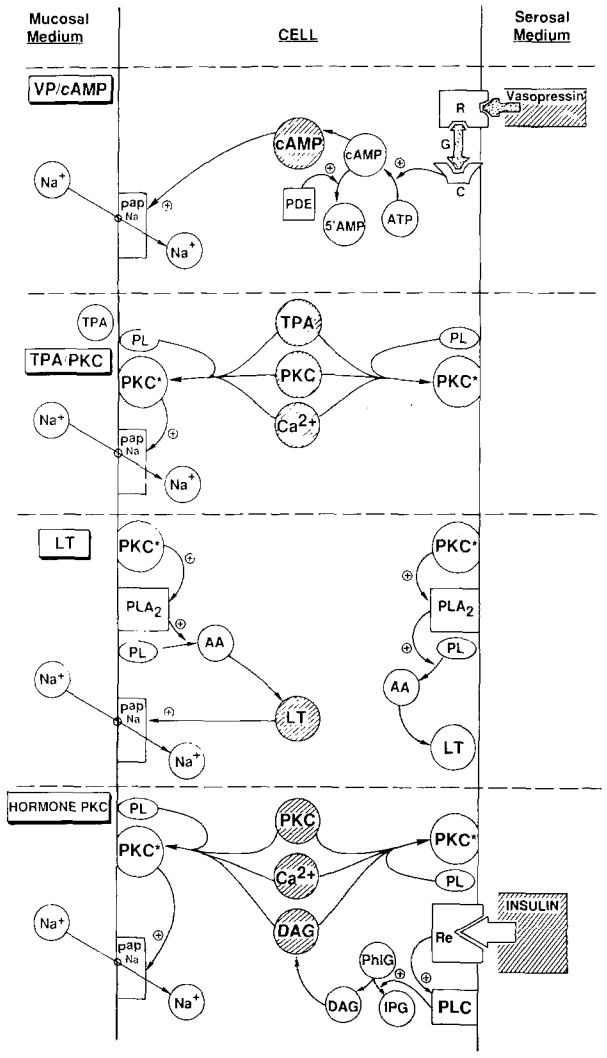

Fig. 6. Working hypothesis of interrelationships among biochemical factors regulating apical Na^+ permeability. The symbols represent: receptor and regulatory and catalytic subunits of adenyl cyclase (*R*, *G* and *C*, respectively); 12-O-tetradecanoylphorbol-13-acetate (*TPA*); inactive (*PKC*) and activated (*PKC**) forms of protein kinase C; membrane phospholipid (*PL*); leukotrienes (*LT*); phospholipase A_2 (PLA_2); arachidonic acid (*AA*); basolateral receptor for insulin (*Re*); phosphatidylinositol-glycan (*PhIG*); inositol-phosphate glycan (*IPG*); and diacylglycerol (*DAG*). The uppermost panel (*VP/cAMP*) outlines how vasopressin likely increases P_{Na}^{ap} by stimulating cAMP production, and thereby triggering phosphorylation of a site gating Na^+ entry. Panel *TPA PKC* suggests that mucosal addition of the phorbol ester could produce translocation of cytosolic PKC to either cell membrane. However, only the activated complex (*PKC**) in the vicinity of the apical Na^+ channel can phosphorylate and thereby activate the site gating Na^+ entry. Panel *LT* indicates that PKC-induced stimulation of leukotriene production can also play a role in regulating apical Na^+ entry (Mauro et al., 1987). The lowermost panel (*Hormone PKC*) suggests how basolateral binding of hormone may lead to activation of PKC, phosphorylation of an apical gating site, and finally activation of the apical Na^+ channel. For the reasons cited in the text, insulin may be one such physiologic trigger for PKC activation

1987) (Panel *LT*, Fig. 6). However, the current results now suggest that TPA acts on frog skin largely by activating protein kinase C (PKC). This conclusion is based on the observations that: (i) TPA has been documented to activate PKC in other cells (Castagna et al., 1982; Yamanishi et al., 1983); (ii) diacylglycerols are physiologic activators of PKC in other tissues (Kaibuchi et al., 1981); (iii) TPA has a half-maximal effect on frog skin at ≈3 nM, similar to the value reported for PKC activation in vitro (Castagna et al., 1982); and (iv) the present data document that the diacylglycerols OAG and DIC replicate both the stimulatory and inhibitory effects of TPA on transport across the frog skin (Table 1, Fig. 1).

The intracellular measurements of the current study (Table 4, Fig. 5) further suggest that the end effect of PKC activation may be similar to that produced by cAMP in this tissue (Panel *VP/cAMP*, Fig. 6). cAMP acts by increasing the apical Na^+ permeability of tight epithelia, thereby increasing transepithelial conductance, depolarizing the apical membrane and increasing the intracellular Na^+ concentration (Orloff & Handler, 1961, 1962; Civan, 1983; Kelepouris, Agus & Civan, 1985). All of these effects were also observed after PKC activation in this study. One effect not observed was the decrease in apical fractional resistance characteristically produced by cAMP. The inability to detect this anticipated change may have reflected the small size of the DIC-induced stimulation in the experiments of Table 4. Alternatively, the PKC activation may have exerted separate apical and basolateral effects, proportionately increasing the conductance of both membranes.

cAMP likely increases P_{Na}^{ap} by triggering a cascade of biochemical events (Cohen, 1982) resulting in the phosphorylation of a gating site at or near the apical Na^+ channel (Jard & Bastide, 1970; Kirchberger, Schwartz & Walter, 1972; Schwartz et al., 1974). The qualitative similarity of the electrophysiologic effects of cAMP and PKC activation suggests that PKC phosphorylates the same, or a nearby, site gating apical Na^+ entry.

Although it is likely that PKC can be activated by pharmacologic agents to modify the apical Na^+ channels, the physiologic importance of this observation has been unclear. In particular, phorbol esters have been found to stimulate Na^+ transport largely when added from the mucosal (or external) surface. Were PKC activated by agents added only from the external surface, it could scarcely be relevant to the physiologic regulation of transepithelial Na^+ transport. Such regulation must proceed in response to changes in hormonal levels on the contralateral serosal (inner or body) surface of the tissue.

The results of the present work document that PKC can actually be activated from either side of the tissue (Table 3, Fig. 2). Thus, the sidedness of the effects produced by TPA is specific to the phorbol ester and not to the enzyme it stimulates.

We suggest that the sidedness of the transport effects of TPA arises from the extremely nonpolar nature of the phorbol ester. Over relatively brief periods of time, phorbol esters are removed from solution by adsorption onto glass and plastic surfaces (Kreibich, Süss & Kinzel, 1974). It is likely that TPA is also adsorbed onto the underlying dermis and basolateral surfaces of the cells when added from the serosal side. Thus, only part of the serosal TPA likely reaches the cell cytoplasm, and succeeds in translocating inactive cytosolic PKC to activated membrane-bound PKC (PKC*) (Panel *TPA/PKC*, Fig. 6). Because of the limited depth of penetration of TPA, the PKC* it activates from the serosal side may also be restricted to the *strata germinativum* and *spinosum*. These sites are far removed from the apical plasma membrane of the outermost living layer of epidermis, the *stratum granulosum,* which likely constitutes the major site of Na^+ gating.

We further suggest that activation of PKC constitutes one mechanism for the physiologic regulation of apical Na^+ entry (Panel *Hormone PKC*, Fig. 6). In the model of Fig. 6, we propose that insulin is one of perhaps many activators of PKC in frog skin and other tight epithelial cells. This hypothesis is supported by many lines of indirect evidence: (i) insulin stimulates Na^+ transport across toad urinary bladder (Herrerra, 1965) and short-circuit current across frog skin (Herrerra, Whittembury & Planchart, 1963; Erlij & Schoen, 1981); (ii) this stimulation reflects not only a primary stimulation of the Na,K-exchange pump (Siegel & Civan, 1976), but an enhancement of P_{Na}^{ap} as well (Erlij & Schoen, 1981; Walker et al., 1984; Schoen & Erlij, 1985); (iii) insulin stimulates production of diacylglycerol (DAG) in cultured myocytes by increasing the rate of hydrolysis of a phosphatidylinositol-glycan (PhIG) to DAG and an inositol-phosphate glycan (IPG) (Saltiel et al., 1986); (iv) DAG can be translocated from one plasma membrane to intracellular membranes and presumably to the contralateral plasma membrane (Pagano & Longmuir, 1985); (v) DAG is a physiologic activator of PKC (Kaibuchi et al., 1981) which stimulates Na^+ transport across frog skin (Table 1, Fig. 1); and (vi) at least one Na^+ channel protein seems to be a substrate for PKC (Costa & Catterall, 1984). Not all published data are consistent with this formulation. For example, insulin does not produce translocation of PKC in adipocytes (Glynn et al., 1986). Whether this is a fundamental flaw in the model or simply reflects biological variance from tissue to tissue remains to be determined.

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