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Regulation of Epithelial Na^+ Permeability by Protein Kinase C is Tissue Specific

M.L. Chalfant[3], J.M. Civan[1], K. Peterson-Yantorno[1], D.R. DiBona[5], T.G. O'Brien[4], M.M. Civan[1,2,3]

[1]Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104-6085
[2]Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6085
[3]Graduate Group in Bioengineering, University of Pennsylvania, Philadelphia, PA 19104-6085
[4]Lankenau Medical Research Center, Wynnewood, PA
[5]Marine Biomedical and Environmental Sciences, Medical University of South Carolina, Charleston, SC

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Abstract. Protein kinase C (PKC) is a major regulator of a broad range of cellular functions. Activation of PKC has been reported to stimulate Na^+ transport across frog skin epithelium by increasing the apical Na^+ permeability. This positive natriferic response has not been observed with other epithelial preparations, and could reflect the specific experimental conditions of different laboratories, or species or organ specificity of the response to PKC.

In the present study, measurements were conducted with skins and urinary bladders from the same animals of two different species. The PKC activator TPA uniformly increased the transepithelial Na^+ transport (measured as amiloride-sensitive short-circuit current, I_{SC}, across skins from *Rana temporaria* and *Bufo marinus,* and inhibited I_{SC} across bladders from the same animals. Inhibitors of PKC (staurosporine, H-7 and chelerythrine) partially blocked the TPA-induced stimulation of I_{SC} across frog skin. The specificity of the PKC response by amphibian skin could have reflected an induction of moulting, similar to that observed with aldosterone. However, light micrographs of paired areas of frog skin revealed no evidence of the putative moulting. Separation of *stratum corneum* from the underlying *stratum granulosum* could be detected following application of aldosterone.

We conclude that the effect of PKC on epithelial Na^+ channels is organ, and not species specific. The stimulation of Na^+ permeability in amphibian skin does not arise from sloughing of the *stratum corneum.* These observations are consistent with the hypothesis that the natriferic action arises from the calcium-independent isozyme of PKC previously detected in frog skin.

Correspondence to: M.M. Civan

Key words: Frog skin — Toad skin — Frog urinary bladder — Toad urinary bladder — Aldosterone — Moulting — PKC isozymes

Introduction

Protein kinase C (PKC) is a ubiquitous phospholipid-activated kinase which regulates many cellular functions (Nishizuka, 1986) and likely mediates the tumor promotion of phorbol esters (Nishizuka, 1984). In part, regulation by PKC is mediated by stimulation (Berger, Travis & Welsh, 1993; Tohse et al., 1990; Yazawa & Kameyama, 1990) and inhibition (Honore et al., 1991; Payet & Depuis, 1992) of ion channels. PKC consists of at least ten isozymes which have been cloned and sequenced (Nishizuka, 1992). The conventional (cPKC) forms of PKC (PKC-α, -β_I, -β_{II}, and -γ) have four conserved regions and are activated by Ca^{2+}, diacylglycerols and phorbol esters. The nonconventional (nPKC) forms (PKC-δ, -ϵ, -η, and -θ) lack the C_2 sequence and are Ca^{2+}-independent, albeit responsive to diacylglycerols and phorbol esters (Kiley et al., 1995; Nishizuka, 1988; Osada et al., 1990; Osada et al., 1992; Parker et al., 1989). The atypical (aPKC) forms (PKC -ζ and -λ) have one cysteine-rich zinc finger-like motif and are unresponsive to Ca^{2+}, diacylglycerols and phorbol esters; the physiologic signal which activates the aPKC isozymes is unknown (Nishizuka, 1992). The isozymes also differ in substrate specificity, kinetics, and sensitivity to activators and proteases (Parker et al., 1989; Huang et al., 1991).

Considerable activity has been focused on the possible functional significance of the diversity of PKC isozymes (Nishizuka, 1988; Parker et al., 1989; Kiley et al., 1992), particularly since some of these isozymes appear to localize to different cellular compartments (Kiley &

Parker, 1995). Because of the fundamental difference in Ca^{2+} dependence, only cPKC isozymes are translocated to the membranes of antigen-stimulated, basophilic RBL-2H3 cells in the absence of external Ca^{2+} (Ozawa et al., 1993). Because of the differing substrate specificities, only the cPKC isozymes, and not the nPKC isozymes, activate c-Raf protein kinase (Sözeri et al., 1992). Other work has led to the suggestions that one or another isozyme may play a special role in normal cell-cycle progression (Watanabe et al., 1992), epithelial differentiation (Osada et al., 1993), and in the vascular complications of diabetes (Inoguchi et al., 1992). However, no target protein has been documented to respond to different PKC isozymes in qualitatively different ways within the same cell, and the functional importance of the isozyme diversity remains unclear.

Under many experimental conditions, Na^+ absorption by frog skin (from apical to basolateral solution) can be monitored instantaneously and precisely by measuring the short-circuit current [I_{SC}, the current necessary to voltage clamp the epithelium to zero (Ussing & Zerahn, 1951; Civan, 1983)]. Activation of PKC with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulates I_{SC} across frog skin by as much as 40–60% (Civan et al., 1985). Only later (after ≈ 1 hr), is the stimulation succeeded by a reduction in baseline Na^+ transport (Civan et al., 1989). The PKC-dependent stimulation has since been documented and further analyzed both electrophysiologically (Civan et al., 1987, 1988, 1989, 1991; Mauro, O'Brien & Civan, 1987) and by measurements of $^{22}Na^+$ flux across frog skin (Andersen, Bjerregaard & Nielsen, 1990). The increase in I_{SC} reflects an increase in the apical Na^+ permeability (Civan et al., 1987).

In contrast to the response of frog skin, PKC activation has been reported to have little effect (Schlondorff & Levine, 1985; Rorsman, Arkhammar & Berggren, 1986) or actually depress (Yanase & Handler, 1986; Hays, Baum & Kokko, 1987; Mohrmann, Cantiello & Ausiello, 1987; Ling & Eaton, 1989; Satoh & Endou, 1990; Graham et al., 1992) Na^+ channel activity in several other epithelial preparations. These differences could reflect a number of factors. In the present study, we examine whether the difference in PKC responsivity reflects species or tissue specificity, and whether changes in the unique structure of skin, the *stratum corneum,* might mediate the natriferic effect of PKC.

Materials and Methods

Electrophysiologic Measurements

Frogs (*Rana pipiens* and *Rana catesbeiana*) and toads (*Bufo marinus*) were obtained from West Jersey Biological Supply (Wenonah, NJ) and sacrificed by double pithing. Abdominal skins and urinary bladders were excised and mounted between the two halves of a Lucite double chamber and bathed with an amphibian Ringer's solution containing (in mM): 120.0 Na^+, 3.5 K^+, 1.0 Ca^{2+}, 118.0 Cl^-, 2.5 HCO_3^-, and 10.0 N-2-hydroxyethyl piperazine N′-2-ethanesulfonic acid (HEPES; half in the basic and half in the acidic form). The osmolality was 240 mOsm and the pH 7.6. Adjoining areas of 0.79 cm^2 were studied, permitting control and experimental measurements of each skin or bladder to be conducted simultaneously. The transepithelial potential V_{ms} (serosa positive to mucosa) was clamped at 0 mV, except for 5-sec intervals during which V_{ms} was increased to 10 mV. The transepithelial conductance g_T was measured as the ratio of the current deflection to the 10-mV voltage step. A dual-pen chart recorder continuously displayed the transepithelial currents.

Morphologic Studies

Frog skins were fixed by simultaneous addition of suitable volumes of 50% (w/v) glutaraldehyde to the mucosal and serosal solutions to attain a final concentration of 1%. After 20–30 min, the tissue areas studied were excised and transferred to 1% glutaraldehyde in phosphate buffer. Samples were post-fixed in osmium tetroxide, embedded in epoxy, and sectioned for light microscopy. Examination of the sections was initially performed by D.R.D. without prior knowledge of the experimental protocol to avoid possible bias in interpretation.

Chemicals

Chemicals were reagent grade. D-aldosterone and sn-1,2-dioctanoylglycerol (DiC_8) were obtained from the Sigma Chemical (St. Louis, MO), and 12-O-tetradecanoylphorbol 13-acetate (TPA) from Chemsyn Science Laboratories (Lenaxa, KS). Vasopressin was purchased (as 8-arginine vasopressin) from Calbiochem (San Diego, CA). The PKC inhibitors 1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine (H-7) (Hidaka et al., 1984), staurosporine (Tamaoki, 1991) and chelerythrine (Herbert et al., 1990) were obtained from Sigma (St. Louis, MO), Biomol (Plymouth Meeting, PA), and LC Services (Woburn, MA), respectively. Amiloride was a gracious gift from Dr. George M. Fanelli, Jr. (Merck Institute for Therapeutic Research, West Point, PA).

Data Reduction

Values are presented as the means ± 1 SE. The number of experiments is indicated by the symbol *N*. The probability (*P*) of the null hypothesis has been calculated using Student's *t*-test.

Results

Tissue Specificity of TPA-induced Natriferic Effect

As illustrated by Fig. 1, 160 nM TPA stimulated the short-circuit current across the abdominal skin of the toad, while inhibiting I_{SC} across the urinary bladder from the same animal. The results obtained from a paired series of five experiments are summarized in Table 1. TPA increased I_{SC} by 7.9 ± 2.4 μA across the skins and reduced I_{SC} by 10.7 ± 1.8 μA across the bladders. The damped oscillations triggered by TPA in Fig. 1*A* were observed with all five toad urinary bladders studied, but these oscillations were not observed with frog bladders (Fig. 2*B*).

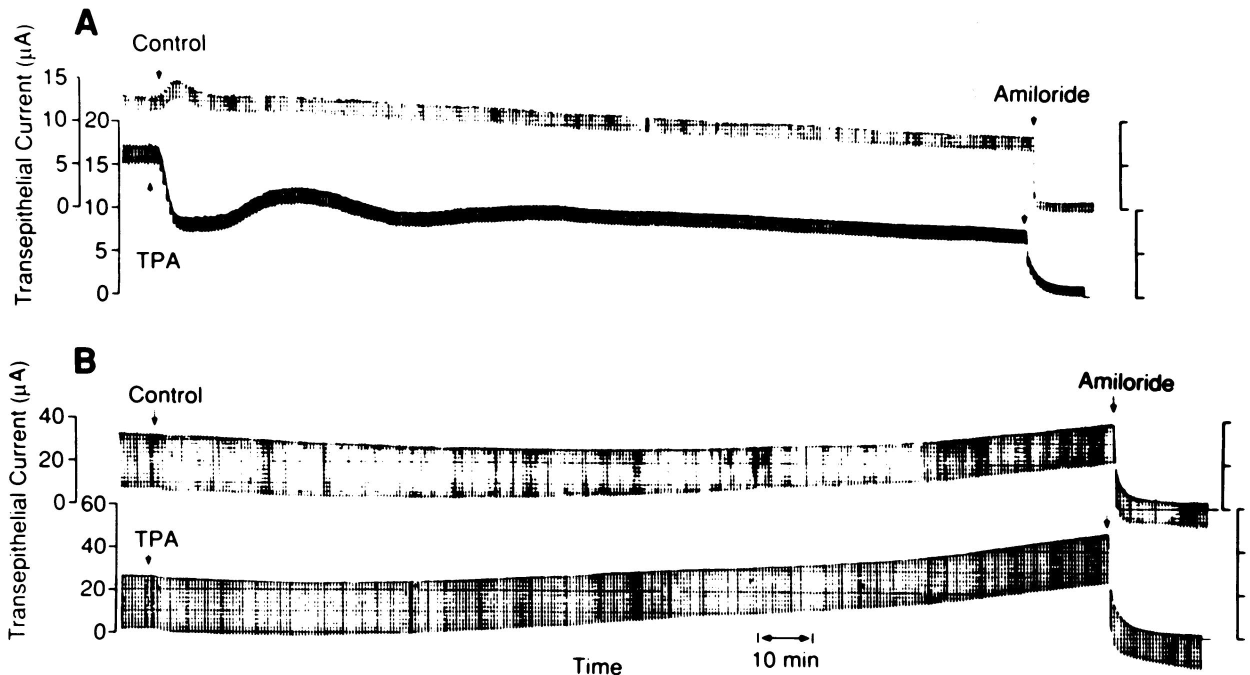

Fig. 1. Effects of PKC activation on currents across the urinary bladder (*A*) and abdominal skin (*B*) from the same toad (*Bufo marinus*). The upper and lower envelopes of each trace are the currents at 0 mV (short-circuit current, I_{SC}) and 10 mV (serosa positive to mucosa), respectively. At the arrows, 160 nM TPA in ethanol was added to the media bathing the experimental tissue area and ethanol alone was added to the solutions bathing the control area. At the conclusion of each experiment, blocking Na^+ channels with 100 μM amiloride abolished I_{SC}. TPA markedly inhibited I_{SC} across the bladder and stimulated I_{SC} across the toad skin by 22.0 μA, 2.7 times more than the increase (of 8.6 μA) associated with ethanol alone.

Table 1. Effects of sequential addition of TPA and vasopressin to skins and urinary bladders from the same toads

Tissue	Condition	Baseline I_{SC} (μA)	Change (μA) in I_{SC} following: 160 nM TPA
Skin:	Con	31.8 ± 2.9	3.3 ± 2.9 ($P > 0.3$)‡
	Exp	28.1 ± 1.8	11.2 ± 3.2 ($P < 0.05$)
	Exp − Con	−3.8 ± 4.4 ($P > 0.4$)	7.9 ± 2.4 ($P < 0.05$)
Bladder:	Con	20.4 ± 6.2	−0.4 ± 0.9 ($P > 0.6$)
	Exp	26.1 ± 4.7	−11.1 ± 1.2 ($P < 0.01$)
	Exp − Con	5.6 ± 2.7 ($P > 0.1$)	−10.7 ± 1.8 ($P < 0.01$)

‡ *P* is the probability of the null hypothesis. The data were obtained from measurements of the abdominal skins and urinary bladders excised from the same 5 toads. The addition of TPA produced a mean percentage increase of 34.1 ± 10.2% in I_{SC} across the skins after 116 ± 21 min. The response of the bladders was much faster; a mean percentage decrease of 47.9 ± 9.3% in I_{SC} was noted after 12 ± 2 min.

Differential effects were also noted following addition of 160 nM TPA to skins and urinary bladders taken from the same animals of another species, the bullfrog *Rana catesbeiana* (Fig. 2). In a series of seven paired experiments, TPA increased I_{SC} across the skins by 17.2 ± 6.4 μA, and reduced I_{SC} by 15.5 ± 2.8 μA across the bladders (Table 2). The negative responses to TPA of the I_{SC} across both frog and toad bladders were consistently faster than the positive responses of I_{SC} across the corresponding skins (Figs. 1–2, Tables 1–2).We conclude that the natriferic response to TPA is tissue specific, and not species specific, at least among amphibia.

Interaction of TPA and Vasopressin

We have previously noted that activation of PKC with either phorbol esters or diacylglycerols stimulates baseline I_{SC}, but also inhibits the subsequent characteristic

Table 2. Effects of sequential addition of TPA and vasopressin to skins and urinary bladders from the same bullfrogs

Tissue	Condition	Baseline I_{SC} (μA)	Change (μA) in I_{SC} following:	
			160 nM TPA	100 mU Vasopressin
Skin:	Con	58.1 ± 9.2	−0.2 ± 1.2 ($P > 0.8$)	39.5 ± 10.2 ($P < 0.01$)
	Exp	56.9 ± 21.5	17.0 ± 6.4 ($P < 0.05$)	−6.3 ± 5.8 ($P > 0.3$)
	Exp − Con	−1.2 ± 5.0 ($P > 0.8$)	17.2 ± 6.4 ($P < 0.05$)	−45.8 ± 11.1 ($P < 0.01$)
Bladder:	Con	17.1 ± 3.6	−0.2 ± 1.1 ($P > 0.8$)	16.0 ± 3.7 ($P < 0.01$)
	Exp	21.6 ± 3.2	−15.7 ± 2.6 ($P < 0.001$)	4.4 ± 1.9 ($P > 0.05$)
	Exp − Con	4.6 ± 2.9 ($P > 0.1$)	−15.5 ± 2.8 ($P < 0.005$)	−11.6 ± 4.2 ($P < 0.05$)

The data were obtained from measurements of the abdominal skins and urinary bladders excised from the same 7 bullfrogs. The addition of TPA produced a mean percentage increase of 42.8 ± 8.2% in I_{SC} across the skins after 43 ± 6 min. TPA reduced I_{SC} across the bladders by 74.2 ± 3.3% after 23 ± 2 min. Vasopressin was applied to the basolateral surface of the skins and bladders after preincubation with/without TPA for 64 ± 5 min and 65 ± 4 min, respectively. The peak hormonal stimulation was noted 37 ± 8 min later by the skins and 22 ± 3 min later by the bladders.

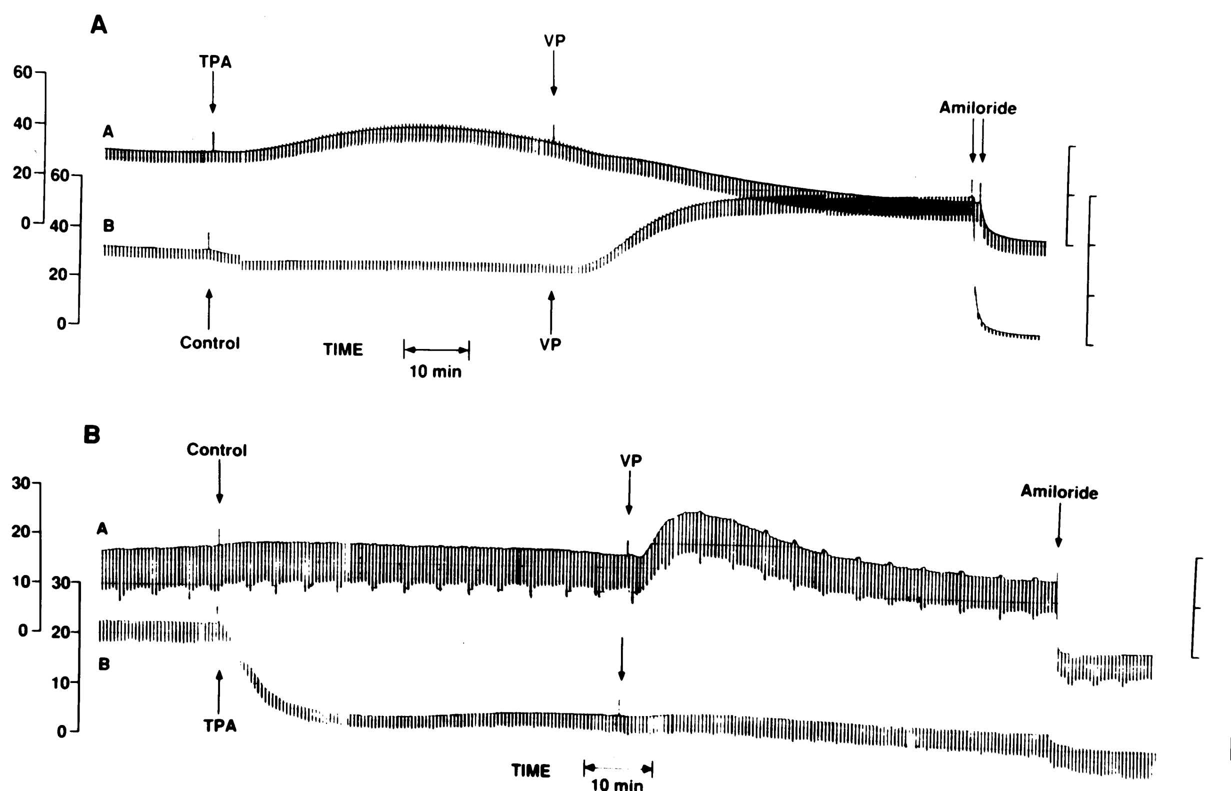

Fig. 2. Effects of PKC activation on currents across the skin (*A*) and urinary bladder (*B*) from the same frog (*Rana catesbeiana*). TPA stimulated I_{SC} across the frog skin and inhibited I_{SC} across the frog bladder.

natriferic response to vasopressin (Civan et al., 1985). As illustrated by Fig. 2, 100 mU/ml vasopressin strongly stimulated the short-circuit current across the control areas of skin and bladder. In each case, however, TPA largely blocked the later response to vasopressin. This observation was characteristic of the entire series of experiments (Table 2). Whether the TPA exerted a natriferic effect which was positive (across skin) or negative (across bladder), the action of vasopressin was subsequently blunted.

Effects of PKC Inhibitors on the Response to TPA

As considered in the Discussion, considerable evidence supports the concept that the positive natriferic action

Table 3A. Effects of sequential addition of staurosporine and TPA to frog skins

Condition	Baseline I_{SC} (μA)	Change (μA) in I_{SC} following:	
		300 nM Staurosporine	160 nM TPA
Con	38.8 ± 6.6	−0.4 ± 0.6 ($P > 0.5$)	15.6 ± 2.6 ($P < 0.001$)
Exp	36.5 ± 4.7	5.9 ± 1.0 ($P < 0.005$)	8.4 ± 1.6 ($P < 0.005$)
Exp − Con	−2.3 ± 2.9 ($P > 0.4$)	6.2 ± 1.2 ($P < 0.005$)	−7.2 ± 2.4 ($P < 0.025$)

The data were derived from 7 skins from *R. pipiens* which were initially preincubated with/without staurosporine for 44 ± 1 min, and then exposed to TPA for 47 ± 12 min. The control areas displayed a peak increase in short-circuit current of 46.5 ± 9.8% following the phorbol ester.

exerted by TPA on amphibian skin is mediated by protein kinase C. However, no information has yet been presented concerning the effects of PKC inhibitors on the TPA-triggered response of frog skin. This point was addressed by applying the nonspecific PKC inhibitors staurosporine (Tamaoki, 1991) and H-7 (Hidaka et al., 1984), and the more selective PKC inhibitor chelerythrine (Herbert et al., 1990) (Tables 3*A*–*C*) to skins excised from the frog *Rana pipiens.* In each case, addition of the PKC inhibitor increased the baseline short-circuit current by 5–8 μA. This is consistent with the observation that long-term activation of PKC (after ~ 1 hr) leads to inhibition of Na^+ transport, following the initial phase of Na^+-channel activation (Civan et al., 1989). Each of the PKC inhibitors also significantly reduced the response to the subsequent addition of TPA. It will be noted that the final currents following the sequential addition of first staurosporine or H-7 and secondly TPA were actually not very different for the control and experimental areas (Tables 3*A* and *B*). However, this was certainly not the case with the more specific inhibitor chelerythrine (Table 3*C*), where the final control currents (exposed only to TPA) were twice as large as the final experimental currents (sequentially exposed to inhibitor and then TPA). We conclude that the data support the concept that the response to the phorbol ester was indeed mediated by PKC activation.

TPA is also known to stimulate prostaglandin formation and release in some preparations (Ohuchi & Levine, 1978). The nonselective inhibitor ETYA blocks all three (lipoxygenase, cycloxygenase and epoxygenase) pathways of arachidonic acid metabolism (Downing et al., 1970). However, in contrast to the above results observed with PKC inhibitors, 50 μM ETYA had no significant effect either on the baseline I_{SC} or on the response to TPA 41–75 min after adding the TPA. In a series of three sets of control and experimental areas of skins from the frog *Rana pipiens,* 160 nM TPA increased I_{SC} by 9.4 ± 2.0 μA ($P < 0.01$), a stimulation of 27.0 ± 5.3%. The paired difference in TPA-stimulated I_{SC} was −0.8 ± 3.6 μA ($P > 0.8$). These results are consistent with reports that more selective inhibition of prostaglandin synthesis with indomethacin does not block the positive natriferic action of TPA (Civan et al., 1985; Mauro et al., 1987).

Morphology of Frog Skin Epithelium following Exposure to TPA

TPA in high concentration (1 μM) has been found to markedly stimulate endo- and exocytosis (Masur, Sapirstein & Rivero, 1985; Masur & Massardo, 1987). We considered the possibility that the unique positive natriferic response to TPA of amphibian skin might reflect a specific action of phorbol ester on the structure of this epithelium. In particular, the positive natriferic response of aldosterone has indeed been associated with the induction of moulting of the amphibian skin (Nielsen, 1969). This possibility was examined by comparing photomicrographs of adjoining areas of control and TPA-treated tissues taken from the same skins.

The tissues were fixed after displaying typical responses to TPA. In one experiment, the addition of 160 nM TPA to both the mucosal and serosal solutions stimulated baseline current by 64% and inhibited the subsequent response to 100 mU/ml serosal vasopressin by 76%. In a second experiment (Figs. 3*B*–*C* and *E*–*F*), 160 nM TPA was added solely to the mucosal bath, increasing I_{SC} by 30% and inhibiting the subsequent response to vasopressin by 66%. The later addition of 100 μM amiloride abolished the short-circuit current. Thorough scanning of the sections revealed no detectable difference in the structure of the TPA-treated tissue at low (Figs. 3*B* and *E*) or high (Fig. 3*C* and *F*) magnification. Specifically, no evidence of epidermal sloughing could be detected.

It was possible that the putative TPA-induced moulting was too subtle to be detected by our experimental approach. This possibility was addressed by comparing aldosterone-treated and control areas from the same skin (Fig. 3*A* and *D*). Hormone was added at 7×10^{-7} M to the mucosal and serosal surfaces of the experimental area, and an equal volume of DMSO solvent was added to the corresponding surfaces of the control area. After 50 min of incubation, the tissue was removed and the

Table 3B. Effects of sequential addition of H-7 and TPA to frog skins

Condition	Baseline I_{SC} (μA)	Change (μA) in I_{SC} following:	
		25 μM H-7	160 nM TPA
Con	40.0 ± 4.1	−1.3 ± 0.9 ($P > 0.2$)	17.1 ± 6.5 ($P > 0.05$)
Exp	39.0 ± 4.4	3.3 ± 1.1 ($P < 0.05$)	11.8 ± 5.7 ($P > 0.1$)
Exp − Con	−1.0 ± 0.5 ($P > 0.05$)	4.6 ± 1.0 ($P < 0.02$)	−5.2 ± 1.0 ($P < 0.01$)

The data were derived from 5 skins from *R. pipiens* which were initially preincubated with/without H-7 for 37 ± 3 min, and then exposed to TPA for 32 ± 6 min. The control areas displayed a peak increase in short-circuit current of 41.8 ± 13.0% following the phorbol ester.

Table 3C. Effects of sequential addition of chelerythrine and TPA to frog skins

Condition	Baseline I_{SC} (μA)	Change (μA) in I_{SC} following:	
		10–30 μM Chelerythrine	30–160 nM TPA
Con	59.2 ± 11.1	−2.8 ± 1.1 ($P < 0.05$)	24.7 ± 4.5 ($P < 0.001$)
Exp	57.8 ± 7.8	4.9 ± 2.0 ($P < 0.05$)	8.3 ± 6.3 ($P > 0.2$)
Exp − Con	−1.4 ± 3.9 ($P > 0.7$)	7.7 ± 2.4 ($P < 0.02$)	−16.4 ± 6.8 ($P < 0.05$)

The data were derived from 8 skins from *R. pipiens* which were initially preincubated with/without chelerythrine for 72 ± 4 min, and then exposed to TPA for 51 ± 5 min. The control areas displayed a peak increase in short-circuit current of 60.1 ± 13.4% following the phorbol ester.

mucosal surfaces of both areas were gently rubbed with a cotton applicator (Nielsen, 1969). This approach is necessary to uncover the initial stimulatory natriferic action of aldosterone (but not of TPA) on amphibian skin. The tissue was remounted, and the short-circuit current monitored for another 50 min before applying 100 μM amiloride to the mucosal solutions to verify that I_{SC} was amiloride-blockable. Relative to the paired control, aldosterone increased I_{SC} by 56%. These electrophysiologic changes were accompanied by clearly detectable areas of separation of the *stratum corneum* from the underlying remaining skin (Fig. 3*A* and *D*).

We conclude that TPA stimulates Na^+ transport across frog skin by a direct or indirect action on the plasma membranes, and not by triggering moulting or any other dramatic change in tissue architecture at the light-micrographic level.

Discussion

The phorbol ester TPA produces an initial large increase in the amiloride-inhibitable short-circuit current across frog skin of some 20–60%, followed by an inhibition (Fig. 2). This initial positive natriferic effect is highly reproducible (Civan et al., 1987, 1988, 1989, 1991; Mauro et al., 1987) and is accompanied by an increase in the mucosal-to-serosal flux of radioactive Na^+ (Andersen et al., 1990). However, the strongly stimulatory action appears to be unique among the epithelial preparations thus far treated with TPA and other activators of protein kinase C (Schlondorff & Levine, 1985; Yanase & Handler, 1986; Rorsman, Arkhammar & Berggren, 1986; Hays, Baum & Kokko, 1987; Mohrmann, Cantiello & Ausiello, 1987; Ling & Eaton, 1989; Satoh & Endou, 1990; Graham et al., 1992). The uniqueness of this observation necessarily raises the questions whether the effect of TPA is species specific and whether the positive natriferic response is mediated by PKC activation. The present data bear on both issues.

Our studies of frog skin are conducted primarily with *Rana pipiens,* a species whose urinary bladders are too small for convenient transepithelial study. This problem was addressed by studying the urinary bladder of the bullfrog *Rana catesbeiana.* TPA produced a clear, prompt inhibition of short circuit current (Fig. 2*B,* Table 2), consistent with the inhibition noted in a number of other epithelial tissues (Yanase & Handler, 1986; Hays, Baum & Kokko, 1987; Mohrmann, Cantiello & Ausiello, 1987; Ling & Eaton, 1989; Satoh & Endou, 1990; Graham et al., 1992). Nevertheless, addition of the same concentration of TPA to skins excised from the same animals elicited the characteristic stimulation of I_{SC} observed across the skin of *Rana pipiens*. These observations were confirmed with tissues excised from another species, the toad *Bufo marinus*. Once again, TPA stimulated I_{SC} across the skin and inhibited I_{SC} across the urinary bladder. We conclude that the transepithelial response to TPA is organ specific, not species specific.

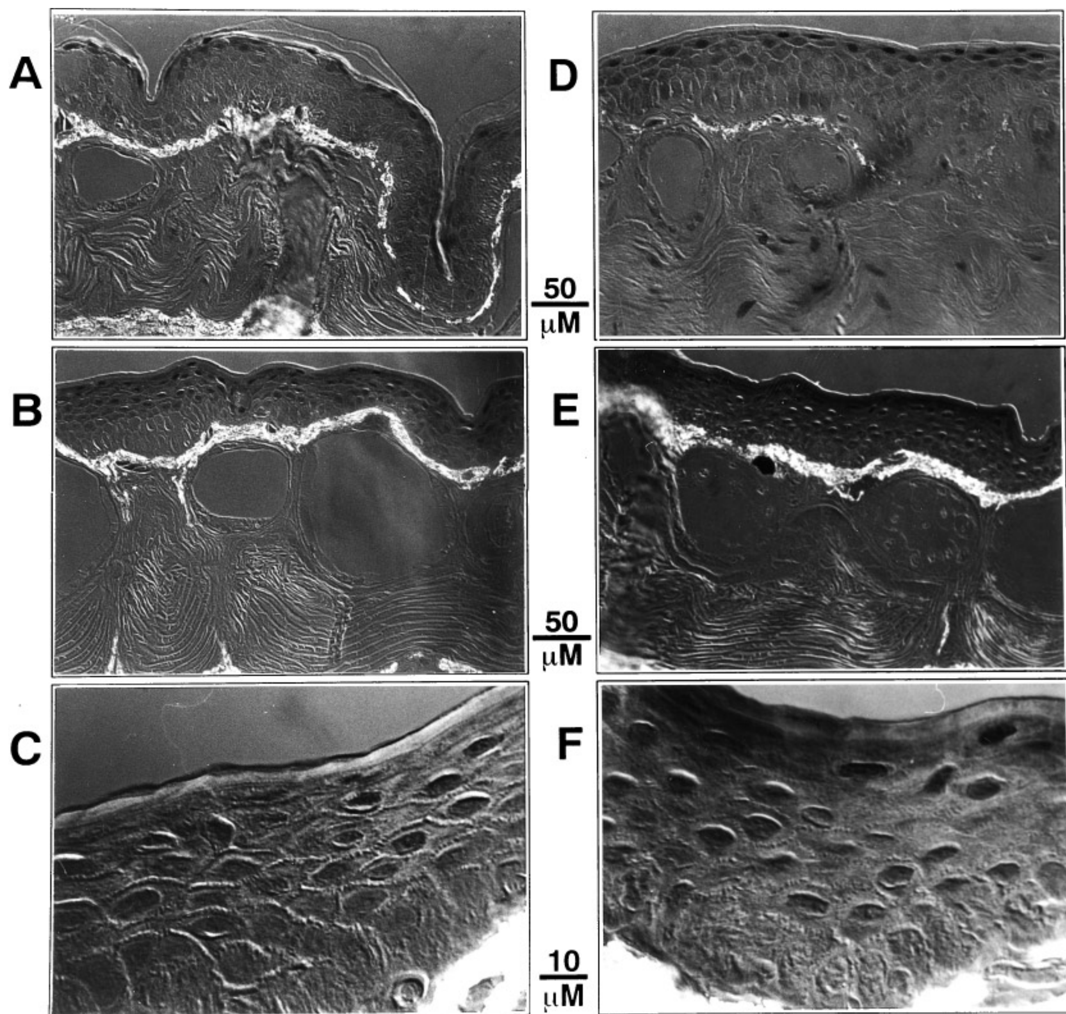

Fig. 3. Light photomicrographs of paired experimental (panels *A–C*) and control (panels *D–F*) areas of frog skin. (*A, D*): Aldosterone (7×10^{-7} M) was added to the mucosal and serosal surfaces of the experimental area, and an equal volume of DMSO solvent was added to the baths of the control area. The hormone both stimulated the short-circuit current and produced sloughing of the *stratum corneum* from the underlying remaining epidermis (*A*). (*B–C, E–F*): TPA (160 nM) was added to the mucosal surface of the experimental area, and an equal volume of ethanol solvent was added to the control mucosal bath. Subsequently, 100 mU/ml vasopressin was added to both serosal solutions and 100 μM amiloride was added to both mucosal solutions. Neither sloughing nor any other dramatic change could be detected at low (*B, E*) or high (*C, F*) magnification.

Not all effects of TPA are necessarily mediated by direct activation of PKC (Ohuchi & Levine, 1978), so that the positive natriferic response could possibly have reflected a PKC-independent action. However, many published observations are inconsistent with this possibility: (i) Two synthetic forms of diacylglycerol, the physiologic trigger of PKC, also stimulate I_{SC} across frog skin (Civan et al., 1987); (ii) Phorbol itself (inactive as a tumor promoter) has no effect on I_{SC} (Civan et al., 1985); (iii) The K_m of the TPA-induced stimulation of I_{SC} in frog skin is ~3 nM (Mauro et al., 1987), similar to that for PKC stimulation in broken-cell preparations (Nishizuka, 1984); (iv) TPA stimulates a phospholipid-dependent kinase in epithelial extracts from frog skin, with a substrate specificity similar to that of the PKC family of kinases (Civan et al., 1991); and (v) Using indomethacin to block the prostaglandin synthesis triggered by TPA does not block the TPA-induced stimulation of I_{SC} across frog skin (Civan et al., 1985; Mauro et al., 1987). Three of the present results provide complementary support of this reasoning. First, each of three inhibitors of PKC activity also inhibited the natriferic action of TPA (Table 3). Second, TPA blocked the natriferic effect of vasopressin (Table 2), whether its action on baseline I_{SC} was positive (across frog skin) or negative (across frog urinary bladder). The TPA-induced inhibition of vasopressin's hydro-osmotic effect on toad bladder has been reported to be PKC mediated (Schlondorff & Levine, 1985). If so, it is reasonable to presume that the block of the hormone's natriferic re-

sponse is also PKC mediated, both in frog skin and in frog bladder. Third, blocking all three pathways of arachidonic acid metabolism with ETYA did not block the natriferic action of TPA, further documenting that the stimulation of baseline I_{SC} is not mediated by a TPA-triggered increase in prostaglandin formation. Taken together with the earlier results summarized above, the evidence now appears overwhelming that the TPA-induced stimulation of Na^+ transport across frog skin is indeed mediated by activation of protein kinase C.

The preceding considerations establish that TPA's stimulation of Na^+ transport is not limited to a single genus, the frog, and is mediated by activation of the ubiquitous enzyme PKC. However, the possibility remained that TPA's natriferic action could have reflected a perturbation of the unique structure of the skin epithelium. In particular, Nielsen (1969) has demonstrated that the natriferic response of amphibian skin to the hormone aldosterone depends, in part, on the induction of moulting. The results of the current work unequivocally demonstrate that this is not the case for TPA. The epidermal sloughing triggered by aldosterone was verified (Fig. 3*A* and *D*), but no such sloughing was detected following exposure to sufficient TPA to stimulate Na^+ transport (Fig. 3*B* and *E,* 3*C* and *F*). Thus, the stimulation of Na^+ transport produced by PKC activation does not depend upon the unique structure of the epidermis, and is potentially of general relevance to nonamphibian preparations. This possibility has been recently supported by experiments conducted with *Xenopus* oocytes expressing the bovine α-subunit of the αβγ-heterotrimeric, amiloride-sensitive, low-conductance, highly selective Na^+ channel (Fuller et al., 1995). The cRNA injected was derived from bovine renal papillary collecting duct and the cDNA is homologous with human and rat α-subunits. Application of 100 nM TPA stimulated the expressed Na^+ current by 28–89%, mimicking the effect of PKC on the apical Na^+ channels of frog skin (Civan et al., 1987). It should be noted that although the Na^+ channels expressed were of mammalian origin, the forms of PKC stimulated by TPA (Fuller et al., 1995) were the endogenous amphibian isozymes.

In part, PKC regulates the voltage-gated Na^+ channel by directly phosphorylating two separate sites (Numann et al., 1994). In contrast, very little is known about the mechanisms by which PKC stimulates or inhibits voltage-insensitive, epithelial Na^+ channels. Consensus sites for phosphorylation by PKC are clearly displayed by the α-(Canessa, Merillat & Rossier, 1994; Renard et al., 1994; McDonald et al., 1994; Fuller et al., 1995) and the β- and γ-subunits (Canessa, Merillat & Rossier, 1994) of the heterotrimeric channel, but the functional consequences of such phosphorylations are unknown. Alternatively, PKC-induced phosphorylation of a G-protein might lead to down-regulation of Na^+ channels in epithelia displaying a negative natriferic response to PKC (Mohrmann, Cantiello & Ausiello, 1987). We have suggested that, whether the action of PKC on the Na^+ channel is direct or indirect, the isozyme specificity of the target cells may determine whether the natriferic response is stimulatory or inhibitory (Civan, Peterson-Yantorno & O'Brien, 1988), possibly by phosphorylating different members of the multiple phosphorylation consensus sites. Alternative interpretations are certainly possible. However, this hypothesis is consistent with the observations that toad bladder (whose I_{SC} is inhibited by PKC) displays Ca^{2+}-sensitive PKC activity (Schlondorff & Levine, 1985), whereas frog skin (whose I_{SC} is stimulated by PKC) displays predominantly Ca^{2+}-independent PKC activity (Civan et al., 1991).

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