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Ca^{2+}-Independent Form of Protein Kinase C May Regulate Na^+ Transport across Frog Skin

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Summary. Activators of protein kinase C (PKC) stimulate Na^+ transport (J_{Na}) across frog skin. We have examined the effect of Ca^{2+} on PKC stimulation of J_{Na}. Both the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and the diacyl-glycerol *sn*-1,2-dioctanoylglycerol (DiC_8) were used as PKC activators. Blocking Ca^{2+} entry into the cytosol (either from external or internal stores) reduced the subsequent natriferic effect of the PKC activators. This negative interaction did not simply reflect saturation of activation of the apical Na^+ channels, since the stimulations produced by blocking Ca^{2+} entry and adding cyclic AMP were simply additive.

The Ca^{2+} dependence of the natriferic effect could have reflected either a direct action of cytosolic Ca^{2+} on PKC or an indirect action on the final receptor site (the Na^+ channel). To distinguish between these possibilities, the TPA- and phospholipid-dependent kinase activity of broken-cell preparations was assayed. The kinase activity was not stimulated by physiological levels of Ca^{2+}, and in fact was inhibited at millimolar concentrations of Ca^{2+}.

We conclude that the effects of Ca^{2+} on the natriferic response to PKC activators are indirect. Reducing cytosolic uptake of Ca^{2+} may have stimulated Na^+ transport by a chemical modification of the apical channels observed in other tight epithelia. The usual stimulation of Na^+ transport produced by PKC activators in frog skin may reflect the operation of a nonconventional form of PKC. This enzyme is Ca^{2+} independent and seems related to the *n*PKC or PKCε observed in other systems.

Key Words frog skin · PKC · *n*PKC · Na^+ transport · Ca^{2+} · Cd^{2+} · Co^{2+}

Introduction

Transcellular Na^+ absorption by tight epithelia proceeds by apical entry from the external or mucosal medium into the cell and by basolateral extrusion from the cell into the serosal or interstitial fluid (Koefoed-Johnsen & Ussing, 1958). Over time frames of hours or more, transepithelial transport can be regulated by altering the number (Geering et al., 1982) and possibly the kinetics (Collins, Pon & Sen, 1987) of basolateral Na^+,K^+ exchange pumps. Over briefer time domains, regulation is effected by modifying Na^+ entry (Macknight, DiBona & Leaf, 1980; Civan, 1983; Garty & Benos, 1988). Entry can be speeded by increasing either the electrical driving force or the apical Na^+ permeability (P^{ap}_{Na}). The permeability is a composite function, reflecting the number, fractional open time, and single-channel characteristics of the apical Na^+ channels.

Many factors may modify (P^{ap}_{Na}) (Civan, 1983). Of clearest physiologic significance is 3′,5′-cyclic AMP (cAMP), which is the principal second messenger for the natriferic effect of neurohypophyseal hormones (Orloff & Handler, 1962). cAMP may well act by stimulating a protein kinase A to directly phosphorylate gating sites at or near the Na^+ channels. Phosphorylation of membrane and cytoplasmic proteins has been observed following stimulation with neurohypophyseal hormones (Schwartz et al., 1974; Konieczkowski & Rudolph, 1985; Shimada, Mishina & Marumo, 1985). Thus far, efforts to document this model more directly have proved unsuccessful (Garty & Benos, 1988).

Intracellular Ca^{2+} activity (a^c_{Ca}) may also be an important physiologic regulator of transepithelial Na^+ transport (Grinstein & Erlij, 1978). Intracellular Ca^{2+} can activate a family of K^+ channels (Latorre & Miller, 1983; Petersen & Maruyama, 1984; Latorre, 1986; Latorre et al., 1989), thereby increasing the electrical force favoring apical Na^+ entry. However, the value of a^c_{Ca} activating a given K^+ channel varies over two orders of magnitude (Latorre et al., 1989). In addition, studies of vesicle suspensions have demonstrated that intracellular Ca^{2+} exerts an inhibition of apical Na^+ entry (Chase & Al-Awqati, 1983), which is pH dependent (Garty, Asher & Yeger, 1987). Finally, Ca^{2+} can interact less directly

with the Na^+ channels by triggering protein modifications and by altering arachidonic acid and cAMP metabolism (Garty & Benos, 1988).

Activators of protein kinase C (PKC) also have marked effects on Na^+ transport across frog skin (Civan et al., 1985, 1989; Civan, Petersen-Yantorno & O'Brien, 1987, 1988; Andersen, Bjerregaard & Nielsen, 1987; Mauro, O'Brien & Civan, 1987). PKC stimulates Na^+ transport across frog skin epithelium by as much as 40–60% (Civan et al., 1989). The natriferic action appears to reflect an increase in P^{ap}_{Na} (Civan et al., 1987, 1989).

In order to explore the possible physiologic and pathophysiologic importance of the PKC modulation of Na^+ transport across frog skin, we have been studying the interactions among PKC, cAMP and a^c_{Ca}. We have previously reported that PKC activation inhibits the subsequent natriferic response to cAMP (Civan et al., 1985, 1987, 1988, 1989; Mauro et al., 1987). In the present study, we have found that reducing Ca^{2+} cytosolic entry from the extracellular fluid or from the endoplasmic reticulum reduces the PKC-triggered natriferic response. This effect of a^c_{Ca} is indirect, since the enzyme activated by TPA application to frog skin epithelium is a form of PKC, which is Ca^{2+} insensitive.

Materials and Methods

Transepithelial Measurements

Frogs (*Rana pipiens pipiens*) obtained from West Jersey Biological Supply (Wenonah, NJ) were doubly pithed. After removal, the abdominal skins were rinsed and bathed with a 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 and pH were 240 mOsm and 7.6, respectively.

Most of the skins were studied as full-thickness preparations, mounted between the two halves of a Lucite double chamber. Adjoining areas of 0.79 cm^2 were studied, permitting control and experimental measurements of each skin to be conducted 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. A dual-pen chart recorder continuously displayed the transepithelial current (I_T). The transepithelial conductance (g_T) was measured as the ratio of the current deflection to the 10-mV voltage step.

Three skins were studied as split-skin preparations. These isolated sheets were prepared by collagenase preincubation (Fisher, Erlij & Helman, 1980) and mounted serosal-side-up in the chamber described below.

Intracellular Measurements

Skins were mounted between the two halves of a Lucite chamber, mucosal surfaces up. As previously described (DeLong & Civan, 1984), exposed areas of 1.9 cm^2 were separately perfused on their mucosal and serosal sides, and the effluents discarded. Impalements were performed across the apical membranes of the cells. Micropipettes were prepared from omega-dot capillary glass tubing (0.75-mm inner and 1.5-mm outer diameter; Glass Co. of America, Millville, NJ) and filled with 0.5 M KCl to minimize saline release into the cells during the recordings. Minimal criteria for acceptability of impalements are discussed elsewhere (Civan et al., 1983; DeLong & Civan, 1983). The present measurements of intracellular potential (ψ^{mc}, cell positive with respect to mucosa) were obtained during impalements with the micropipette tips in place for ≥ 15 min. ψ^{mc} and I_T were continuously recorded with a dual-pen recorder and a storage oscilloscope, while clamping ψ^{ms} alternately to 0 mV for 7.7 sec and to 20 mV for 4.3 sec. The baseline pulsing was periodically interrupted to impose a sequence of voltage pulses across the skin. Each train comprised 12 pairs of alternately hyperpolarizing and depolarizing pulses, the magnitude increasing by 20 mV in successive steps (DeLong & Civan, 1984; Leibowich, DeLong & Civan, 1988). Data were acquired with pulse durations of 16 or 32 msec and interpulse intervals of 240–640 msec, but the choice of pulse duration is not critical (Civan et al., 1989). The final eight data points (for ψ^{mc}, ψ^{ms} and I_T) were sampled at each voltage step over a period of 0.96 msec. The resulting mean values were used in the subsequent data analysis [similar to that of Fuchs, Larsen and Lindemann (1977)]. The apical Na^+ current (I^{ap}_{Na}) was taken as the amiloride-sensitive transepithelial current at a given transepithelial potential. The I^{ap}_{Na}-ψ^{mc} relationship was fit with the constant field equation (Goldman, 1943) by nonlinear least-squares analysis. The apical Na^+ permeability (P^{ap}_{Na}) and the intracellular Na^+ concentration (a^c_{Na}) were estimated from the values chosen to obtain these best fits.

The apical fractional resistance is defined as the ratio of the apical membrane resistance to the total transcellular resistance. Its value (f^{ap}_{SC}) in the short-circuited state was estimated as the ratio of the change in ψ^{mc} to that in ψ^{ms}. The calculation was performed by linear least-squares analysis of the ψ^{mc}-ψ^{ms} data points obtained during the application of the trains of voltage pulses over the range in ψ^{ms} from −40 to 40 mV. The transepithelial conductance was analogously calculated as the ratio of the change in transepithelial current to that in transepithelial potential.

Preparations of Epithelial Extracts

Skins were placed in a beaker containing either ice-cold deionized water or Ringer's solution and then transferred to beakers containing deionized water at 55°C for 15 sec. Immediately thereafter, the tissues were placed in a beaker containing ice-cold deionized water. Subsequently, the skins were individually placed on a chilled glass plate and the epidermis scraped off with a razor blade. The complete separation of all epidermal cell layers from dermis by this technique was verified by examining fixed skin sections (stained with hematoxylin and eosin) under light microscopy. Epidermal sheets from 2–4 skins were pooled in a chilled Corning glass centrifuge tube together with 1.5 ml of buffer A solution, containing: 10 mM Tris(hydroxymethyl)-aminoethane (Tris), 3 mM $MgCl_2$, 1 mM ethyleneglycol-bis-(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonylfluoride (PMSF) and 10 μM leupeptin, at pH 7.5. Tissues were homogenized with two 20-sec bursts of a Polytron® homogenizer. Sucrose was then added to 0.25 M, and the homogenate was centrifuged for 1 hr at 100,000 × g at 4°C. The pellet was resuspended in buffer A plus 1% Triton X-100 and gently mixed for 1 hr at 4°C. After centrifugation as before, the supernatant

was saved as the membrane fraction. Both supernatants were run on small (approximately 25 mm) diethylaminoethyl-(DEAE)-cellulose columns at 4°C. Columns were equilibrated with buffer B solution, containing: 20 mM Tris, 1 mM DTT (dithiothreitol), 100 μM PMSF, and 1 μM leupeptin, at pH 7.5. The columns were subsequently washed with four column volumes of buffer B, after which the kinase activity was eluted by means of a stepwise gradient of 0–300 mM NaCl in buffer B.

Kinase Assay

Silanized glass tubes (13 × 100 mm) were used, and the enzyme assay was conducted at 30°C in a 20 mM Tris-10 mM $MgCl_2$ buffer at pH 7.4. Where indicated, the following reagents were also added: 50 μg/ml phosphatidylserine (PS), 1 mM $CaCl_2$, 1 mM EGTA, 200 μg/ml histone, and 0.125 μg/ml 12-O-tetradecanoylphorbol 13-acetate (TPA). Sufficient deionized water was added to bring the volume to 140 μl. Fifty μl of the fraction to be measured were added to the tube and preincubated at 30°C for 5 min. To start the assay, 10 μl of a ^{32}P-ATP solution ($\simeq 2 \times 10^4$ cpm/pmol) was added to a final concentration of 20 μM, the tube was vortexed, and the tube was then placed in a water bath at 30°C. After 10 min, 50 μl of the assay mixture were pipetted onto a piece ($\simeq$20 × 20 mm) of Whatman P81 ion-exchange chromatography paper. Filters were washed with occasional stirring for 10 min four times, in 400 ml of deionized water. After the final wash, filters were washed with acetone, allowed to air dry, placed in scintillation vials containing 5 ml of scintillation fluid, and counted.

Chemicals

PMSF, leupeptin, and PS were purchased from Sigma Chemical (St. Louis, MO). TPA was obtained from Chemsyn Science Laboratories (Lenexa, KS). *sn*-1,2-dioctanoylglycerol (DiC_8) was purchased from Avanti Polar Lipids (Birmingham, AL). 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) and vasopressin (as 8-arginine vasopressin) were obtained from Calbiochem (San Diego, CA). Porcine insulin, donated by Eli Lilly in crystalline form (lot 615-2H2-300), was dissolved in 5 mM HCl. Amiloride was generously provided by Dr. George M. Fanelli, Jr. (Merck Institute for Therapeutic Research, West Point, PA).

Data Reduction

Unless otherwise indicated, the results are presented as means ±SE. The probabilities (P) of the null hypotheses have been calculated with Student's t test.

Results

Approaches for Altering Cytosolic Activity of Ca^{2+}

Activation by TPA or DAG markedly increases the affinity of PKC for Ca^{2+} (Nishizuka, 1986). Therefore, increasing the cytosolic Ca^{2+} activity in frog skin epithelium above its baseline value [215 ± 39 nM (Kelepouris, Agus & Civan, 1985)] is unlikely to affect PKC stimulation of Na^+ transport. On the other hand, large reductions in c^a_{Ca} might possibly limit the ability of PKC to phosphorylate critical modulating sites (Kojima, Kojima & Rasmussen, 1985; Sawamura, 1985; Rose, Yada & Loewenstein, 1986; Pershadsingh, Gale & McDonald, 1987). We have attempted to reduce cytosolic Ca^{2+} activity in two ways: by decreasing the rate of Ca^{2+} entry from the external media (with Co^{2+} or Cd^{2-}), and by lowering the rate of Ca^{2+} release from the endoplasmic reticulum (with TMB-8).

The divalent cations Co^{2+} and Cd^{2+} block Ca^{2+} entry through the T-, L- and N-type Ca^{2+} channels (Miller, 1987). These cations have been reported to affect Na^+ transport across tight epithelia (Borghgraef, Stymans & van Driessche, 1971; Banks, 1974; Hillyard & Gonick, 1976; Hayashi, Takada & Arita, 1977; Takada & Hayashi, 1978, 1980; Takada, 1985; Goncharevskaya, Monin & Natochin, 1986; Natochin, Goncharevskaya & Monin, 1986; Verbost et al., 1987*a*, 1988; Verbost, Senden & van Os, 1987*b*).

Effects on Baseline I_{SC} of Reducing Cell Uptake of Ca^{2+} with Co^{2+} or Cd^{2+}

In a series of six experiments, the administration of 1 mM Co^{2+} to the external media produced an immediate increase in the short-circuit current (I_{SC}) of 15 ± 4 $\mu A \cdot cm^{-2}$, while the I_{SC} of the control area fell by 1.3 $\mu A \cdot cm^{-2}$ (Table 1, Fig. 1). The major increase in the experimental short-circuit peaked 2.1 ± 0.2 min after addition of the Co^{2+}, although a more sustained, further slight increase was sometimes observed. A stimulation of comparable magnitude and similar time course was noted after adding 2 mM Cd^{2+} to the media bathing the experimental areas of four additional skins (Table 1). Averaging the results for the 10 experiments and correcting for the time-dependent changes in I_{SC} across the control, the divalent cations increased short-circuit current by 17 ± 3 $\mu A \cdot cm^{-2}$ (a stimulation of $\simeq$43%).

Two observations indicate that the skin glands played little or no role in expressing the effects of Co^{2+} and Cd^{2+} on I_{SC}. First, as described below, intracellular electrophysiologic measurements in the whole-skin preparation documented parallel transport effects within the epithelial syncytium and across the entire preparation. Second, the effect of 0.3–1.0 mM Cd^{2+} was also studied with a series of three split-skin preparations. Isolation of the epithelium from the underlying dermis minimizes the contribution of the skin glands. In contrast to studies with whole-thickness skin, Ca^{2+} (Ziyadeh et al., 1985) and Cl^- (Thompson & Mills, 1983) transport through the glands are not detected. In studying these delicate sheets of isolated epithelia, it was experimentally desirable to add Cd^{2+} solely to the serosal surface. [Cd^{2+} or Co^{2+} (Table 2) stimulated

Table 1. Effects of Co^{2+} or Cd^{2+} on baseline and response of I_{SC} to TPA

Agent	Parameter	Experimental	Control	Experimental–Control	P
Co^{2+}	Baseline I_{SC} ($\mu A \cdot cm^{-2}$)	43 ± 9 (6)	45 ± 8 (6)	−2 ± (4) (6)	>0.6
	Response to Co^{2+} ($\mu A \cdot cm^{-2}$)	15 ± 4 (6)	−1.3 ± 0.4 (6)	16 ± 4 (6)	<0.01
	Response to TPA ($\mu A \cdot cm^{-2}$)	12 ± 7 (4)	20 ± 4 (4)	−8 ± 3 (4)	>0.05
	% Inhibition of TPA effect	—	—	49 ± 15% (4)	<0.05
	Baseline g_T ($mS \cdot cm^{-2}$)	2.2 ± 0.6 (6)	2.2 ± 0.5 (6)	0.0 ± 0.2 (6)	>0.8
	Final g_T in amiloride ($mS \cdot cm^{-2}$)	0.8 ± 0.3 (6)	1.2 ± 0.3 (6)	−0.4 ± 0.1 (6)	<0.05
Cd^{2+}	Baseline I_{SC} ($\mu A \cdot cm^{-2}$)	33 ± 6 (4)	33 ± 4 (4)	0 ± 4 (4)	>0.95
	Response to Cd^{2+} ($\mu A \cdot cm^{-2}$)	17 ± 4 (4)	0.0 ± 0.5 (4)	17 ± 4 (4)	<0.02
	Response to TPA ($\mu A \cdot cm^{-2}$)	11.0 ± 0.5 (4)	21 ± 2 (4)	−10 ± 2 (4)	<0.05
	% Inhibition of TPA effect	—	—	44 ± 9% (4)	<0.02
	Baseline g_T ($mS \cdot cm^{-2}$)	1.9 ± 0.2 (4)	1.8 ± 0.1 (4)	0.0 ± 0.1 (4)	>0.7
	Final g_T in amiloride ($mS \cdot cm^{-2}$)	0.6 ± 0.1 (4)	1.3 ± 0.3 (4)	−0.7 ± 0.2 (4)	<0.05
Co^{2+}	Baseline I_{SC} ($\mu A \cdot cm^{-2}$)	39 ± 6 (10)	40 ± 5 (10)	−1 ± 3 (10)	>0.6
or	Response to Co^{2+}/Cd^{2+} ($\mu A \cdot cm^{-2}$)	16 ± 3 (10)	−0.8 ± 0.4(10)	17 ± 3 (10)	<0.001
Cd^{2+}	Response to TPA ($\mu A \cdot cm^{-2}$)	11 ± 3 (8)	21 ± 2 (8)	−9 ± 2 (8)	<0.01
	% Inhibition of TPA effect	—	—	47 ± 8% (8)	<0.001
	Baseline g_T ($mS \cdot cm^{-2}$)	2.1 ± 0.4(10)	2.1 ± 0.3(10)	0.0 ± 0.1 (10)	>0.7
	Final g_T in amiloride ($mS \cdot cm^{-2}$)	0.7 ± 0.2(10)	1.2 ± 0.2(10)	−0.5 ± 0.1 (10)	<0.01

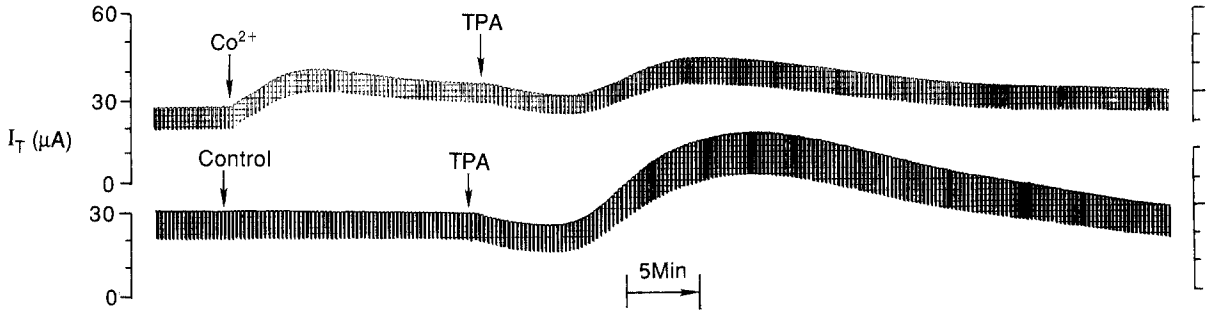

Fig. 1. Effect of Co^{2+} on baseline value and response of transepithelial current to TPA. The two traces are the transepithelial currents measured across the contiguous experimental and control areas of frog skin. The upper envelope of each trace is the short-circuit current, and the deflections are the responses to periodic applications of 10-mV voltage pulses. After equilibration, 1 mM Co^{2+} was added to the solutions bathing both surfaces of the experimental area, while equal volumes of solvent were added to the reservoirs of the control area. The Co^{2+} promptly increased the short-circuit current by ≃48%. In this experiment, I_{SC} thereafter declined slowly with time. In other experiments, I_{SC} could also either increase slowly with time or remain stable. Both tissue areas responded to the subsequent addition of 160 nM mucosal TPA. However, the pretreatment with Co^{2+} inhibited the magnitude of the current stimulation by 65%. At the conclusion of the experiment (*not included*), 100 μM mucosal amiloride reduced I_{SC} across the experimental and control areas to 0.3 and 0.7 μA, respectively

Table 2. Sidedness of effects of Co^{2-} on baseline value and response of I_{SC} to TPA

Parameter	I_{SC} ($\mu A \cdot cm^{-2}$)			
	Mucosal Co^{2+}	Serosal Co^{2+}	Difference	Probability
Baseline	38.3 ± 4.5	38.4 ± 4.9	−0.1 ± 1.7	>0.9
Changes after:				
Co^{2+}	4.8 ± 1.1	10.9 ± 4.1	−6.1 ± 3.9	>0.2
TPA	20.8 ± 5.6	15.1 ± 6.2	5.6 ± 5.9	>0.4
Final I_{SC} in amiloride	2.7 ± 0.6	3.5 ± 0.4	−0.8 ± 0.9	>0.4

Under baseline conditions, four skins were bathed with standard Cl^--containing Ringer's solutions on both surfaces. Co^{2+} was then added to a final concentration of 1 mM to either the mucosal or serosal reservoir. The short-circuit current was stimulated by Co^{2-} applied to either surface. However, the response to mucosal Co^{2+} was 55 ± 13% of that to serosal Co^{2+} ($P < 0.025$). The subsequent responses to addition of 160 nM mucosal TPA were comparable.

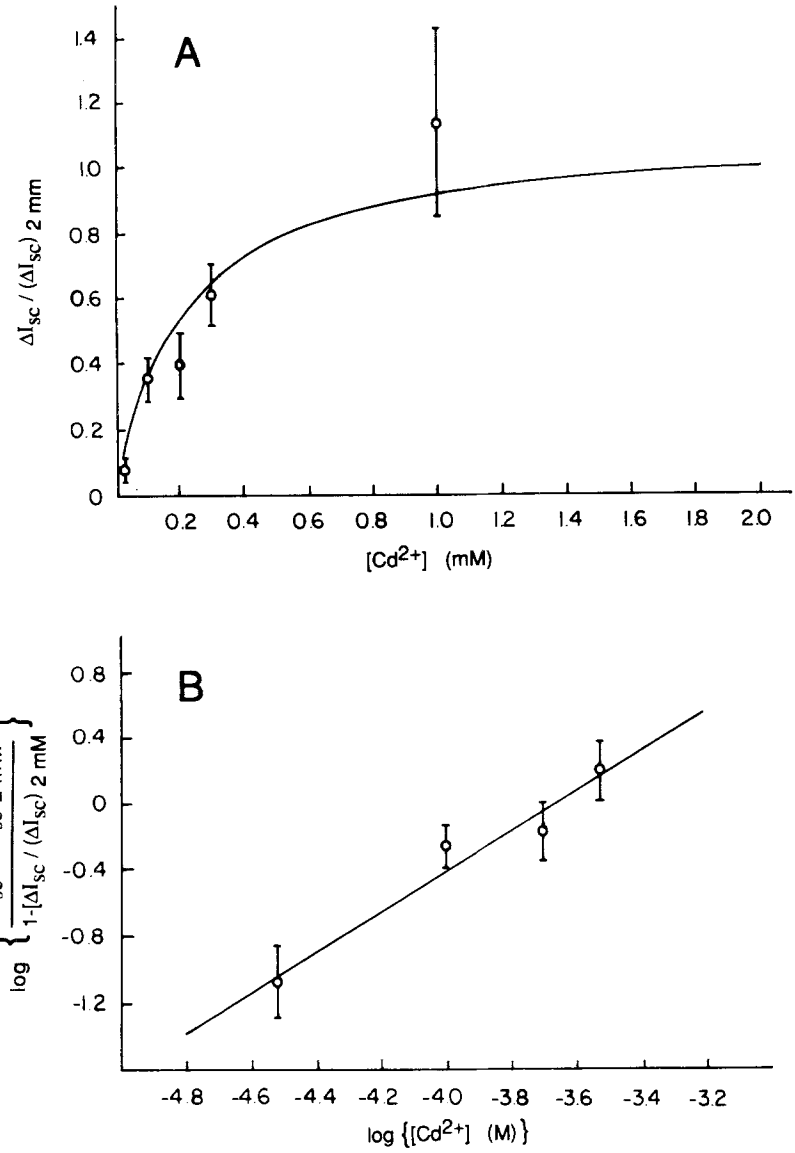

Fig. 2. Dose-response relationship of Cd^{2+} on short-circuit current. (*A*) Woolf-Augustinsson-Hofstee plot with the $[Cd^{2+}]$ for half-maximal stimulation taken to be 211 μM. (*B*) Data presentation in the form of a Hill plot. The slope is 1.2 ± 0.2, not significantly different from one

I_{SC} when added to either surface of whole skins, although the response to mucosal addition was somewhat smaller.] Serosal Cd^{2+} also increased the baseline I_{SC} across the split skins by 2.2 ± 0.4 $\mu A \cdot cm^{-2}$ ($P < 0.05$), a stimulation of 55 ± 20%.

The dose-response relationship between stimulation of I_{SC} and $[Cd^{2+}]$ was examined with a series of five frog skins, over a range of Cd^{2+} concentrations from 10 μM to 2 mM. The slope of the Hill plot (presented in Fig. 2*B*) is indistinguishable from one (1.2 ± 0.2), so that the data are consistent with a stoichiometry of 1 Cd^{2+} to 1 Ca^{2+} channel. From a Lineweaver-Burk reciprocal plot, the half-maximal stimulation was achieved at a Cd^{2+} concentration ($K_{0.5}$) of 211 ± 68 μM. This value has been used to generate the solid curve of Fig. 2*A*, using the form of a Woolf-Augustinsson-Hofstee plot (Segel, 1976). The estimated value of $K_{0.5}$ is consistent with Cd^{2+} inhibition of T-type Ca^{2+} channels, but is an order of magnitude larger than that characterizing the blockage of N- or L-type Ca^{2+} channels (Miller, 1987). This observation is consistent with data obtained with 5 μM verapamil in an additional series of six experiments. Verapamil is expected to inhibit L-, but not N- or T-type, channels. Verapamil was noted to stimulate I_{SC} by only 2.2 ± 0.4 μA (≃6%). We conclude that the effects of the Ca^{2+} channel blockers on I_{SC} across frog skin are consistent with a primary action of Cd^{2+} and Co^{2+} on T-type channels. This primary effect likely reduces a^c_{Ca}, leading to a secondary increase in apical Na^+ permeability.

In the present study, transepithelial Na^+ transport was monitored as I_{SC}. This approach is valid

for frog skin and toad urinary bladder under a variety of experimental conditions (Civan, 1983) and was further substantiated in the present study. Exposure of an experimental area to 100 μM mucosal amiloride (blocking the apical Na^+ channels) abolished I_{SC} and entirely prevented the stimulation in current observed upon adding 100 μM Cd^{2+} to the adjoining control area.

In principle, the stimulation of Na^+ transport could reflect one or more primary actions of Cd^{2+} and Co^{2+} to: (*i*) increase the apical Na^+ permeability, (*ii*) increase the electrical driving force favoring apical entry into the cell (by increasing the basolateral K^+ conductance), or (*iii*) stimulate the Na,K exchange pumps (by increasing the number of functioning units, increasing the rate of cycling, or increasing the affinity of the intracellular site for binding Na^+). Cd^{2+} does not directly stimulate Na,K-activated ATPase (Takada & Hayashi, 1978). However, blockage of Ca^{2+} entry could relieve the intracellular Ca^{2+} inhibition thought to be exerted through calnaktin (Yingst, 1988). To resolve this issue, we conducted combined intracellular and transepithelial electrophysiologic measurements. Insofar as Cd^{2+} is thought to reduce the apical Cl^- conductance of frog skin (Hayashi et al., 1977), these experiments were performed with skins bathed with the standard Ringer's solution on their serosal surfaces, but with an equimolar replacement of NO_3^- for Cl^- on their mucosal surfaces. This approach has been empirically used in studies of frog skin to reduce the baseline, non-Na^+ conductance (Nagel, Garcia-Diaz & Essig, 1983; Civan et al., 1987; Leibowich et al., 1988).

In a series of 11 experiments conducted with eight skins, 0.3–2.0 mM mucosal Cd^{2+} increased I_{SC} by 4.7 ± 1.0 $\mu A \cdot cm^{-2}$, constituting a stimulation of 38 ± 10% ($P < 0.01$). As illustrated by Fig. 3, a parallel membrane depolarization was noted in each experiment. The mean value of ψ^{mc} increased algebraically by 9 ± 2 mV ($P < 0.001$) from a baseline value of −64 ± 4 mV. The subsequent addition of 20–100 μM mucosal amiloride uniformly hyperpolarized the membrane. In the seven experiments where the peak amiloride effect was measured, the membrane potential fell to −100 ± 2 mV.

The observation of a Cd^{2+}-induced depolarization is inconsistent with a single primary action either to increase the basolateral K^+ permeability or to stimulate the Na,K exchange pumps. In either case, a hyperpolarization would have been expected, contrary to observation. By exclusion, possibility (*i*) above was indicated. This conclusion was more directly supported by the results of five of the experiments, in which technically satisfactory measurements of P_{Na}^{ap} were obtained. The data, summarized in Table 3, indicate that Cd^{2+} does increase the apical Na^+ permeability. In the series of Table 3, the stimulation of I_{SC} produced by 0.3–2.0 mM mucosal Cd^{2+} was small (2.2 ± 0.4 $\mu A \cdot cm^{-2}$). However, an increase in P_{Na}^{ap} was noted in each of the five experiments. The mean elevation of apical Na^+ permeability was $(1.4 \pm 0.3) \times 10^{-7}$ cm · sec^{-1}, statistically significant at the 0.02 probability level.

Effects of Co^{2+} or Cd^{2+} on the Natriferic Effect of TPA

In the 10 experiments of Table 1, 160 nM mucosal TPA was added to the control and experimental areas after pretreatment of the experimental areas with either 1 mM Co^{2+} or 2 mM Cd^{2+}. In 9 of the 10 experiments, the divalent ion reduced the subsequent response to TPA. In one experiment, TPA produced a large stimulation of I_{SC}, whether or not the area had been pretreated with 1 mM Co^{2+}. In order to facilitate quantification of the effect of Co^{2+} and Cd^{2+} on the subsequent response to TPA, data were averaged only from those eight skins whose control areas displayed a clearly measurable stimulation of I_{SC} (≥4 μA). Within this framework, the I_{SC} of the eight control areas increased by 21 ± 2 $\mu A \cdot cm^{-2}$ (a stimulation of ≈52%). The pretreatment with Co^{2+} or Cd^{2+} reduced the response of the experimental areas to TPA by 47 ± 8% ($P < 0.001$).

One possible interpretation of this observation was that the less than additive response of I_{SC} to divalent cation and TPA simply reflected a saturation phenomenon. Since both Co^{2+} or Ca^{2+} and TPA likely stimulate I_{SC} by increasing P_{Na}^{ap}, no further responese in I_{SC} would be expected after maximally increasing apical Na^+ permeability. However, the data of Table 4 suggest that this interpretation is unlikely. cAMP is also thought to increase Na^+ transport across frog skin and toad bladder by increasing P_{Na}^{ap}. However, in eight experiments, the stimulation in I_{SC} produced by 1 mM serosal cAMP under control conditions (14 ± 2 $\mu A \cdot cm^{-2}$) was not significantly different from that produced after pretreatment with 1–2 mM Cd^{2+} (11 ± 4 $\mu A \cdot cm^{-2}$). Likewise, 1–2 mM external Cd^{2+} stimulated I_{SC} by similar amounts whether cAMP was absent (13 ± 2 $\mu A \cdot cm^{-2}$) or present (12 ± 2 $\mu A \cdot cm^{-2}$) in the serosal reservoir. Therefore, the data of Table 1 do not reflect a simple saturation phenomenon. Rather, these data suggest that the stimulations produced by pretreatment with Cd^{2+} or Co^{2+} and addition of TPA are less than additive in a more specific way.

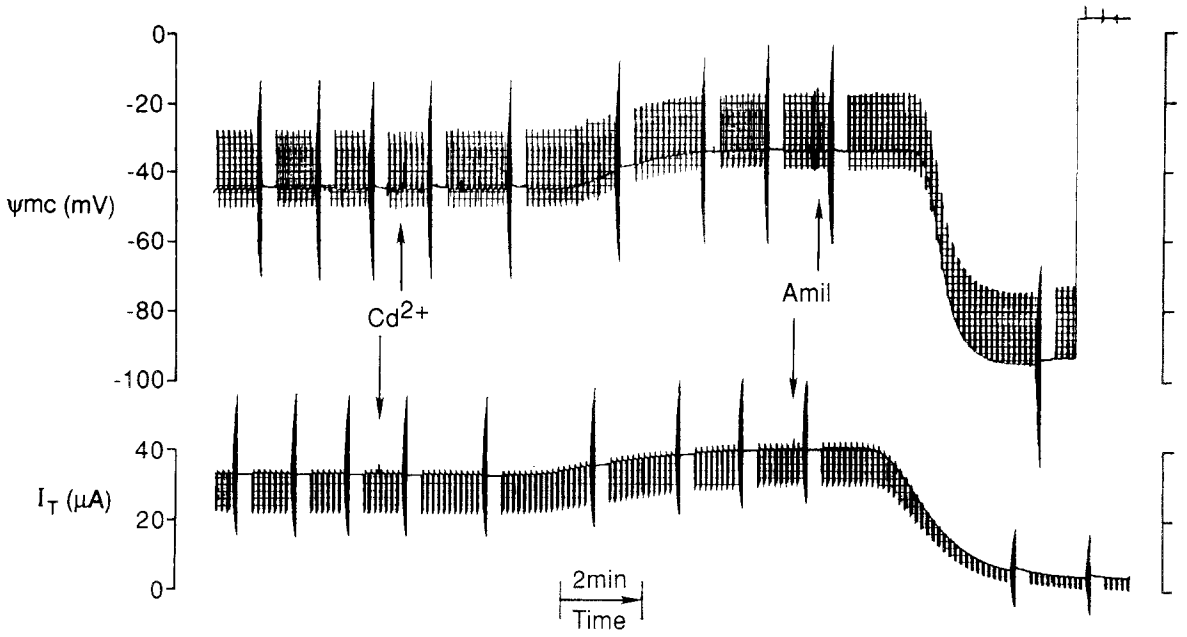

Fig. 3. Effects of Cd^{2+} on intracellular and transepithelial electrophysiologic parameters. The upper and lower traces are the apical membrane potential (ψ^{mc}) and transepithelial current (I_T), respectively. The thin dark triangles are the responses of ψ^{mc} and I_T to the imposition of trains of brief voltage pulses across the skin. Perfusion with 2 mM mucosal Cd^{2+} (indicated as "Cd^{2+}"), increased I_{SC} by 6.0 μA (3.2 $\mu A \cdot cm^{-2}$) and depolarized ψ^{mc} by 11 mV. At the conclusion of the experiment, 100 μM mucosal amiloride was added, inhibiting I_{SC} and hypolarizing ψ^{mc} to -95 mV

Table 3. Effects of mucosal Cd^{2+} on intracellular and transepithelial parameters

Parameter	Before Cd^{2+}	After Cd^{2+}	Difference	Probability
I_{SC} ($\mu A \cdot cm^{-2}$)	27.1 ± 4.6	29.3 ± 4.6	2.2 ± 0.4	<0.01
ψ^{mc} (mV)	−52 ± 2	−47 ± 3	5 ± 2	<0.05
g_T ($\mu S \cdot cm^{-2}$)	435 ± 64	435 ± 63	0 ± 14	>0.8
f^{ap}_{SC}	0.902 ± 0.009	0.894 ± 0.011	−0.008 ± 0.007	>0.3
P^{ap}_{Na} (10^{-7} cm · sec^{-1})	8.4 ± 1.2	9.7 ± 1.4	1.4 ± 0.3	<0.02
c^c_{Na} (mM)	29 ± 9	21 ± 3	−8 ± 8	>0.3

Means ± SE for measurements taken during five experiments conducted with four skins. Cd^{2+} was added to the mucosal surface at a concentration of 2 mM in four experiments and 300 μM in one experiment. After adding 100 μM mucosal amiloride, the transepithelial conductance (g_T) fell to 111 ± 27 $\mu S \cdot cm^{-2}$.

Effects of Reducing Intracellular Mobilization of Ca^{2+}

The foregoing data indicate that blocking Ca^{2+} channels is associated with a reduction of the natriferic action of TPA. We have also tried to decrease a^c_{Ca} by inhibiting the release of Ca^{2+} from the endoplasmic reticulum (Streb et al., 1984). Mobilization of intracellular Ca^{2+} has been blocked in other preparations with TMB-8 (Chiou & Malagodi, 1975; Mix, Dinerstein & Villereal, 1984; Sawamura, 1985; Yada, Rose & Loewenstein, 1985; Rose et al., 1986). In these experiments, we have used only a 70-μM concentration. As discussed by Rose et al. (1986), at significantly higher concentrations TMB-8 may have a direct inhibitory effect on PKC (Kojima et al., 1985; Sawamura, 1985). At this concentration, TMB-8 had no significant effect on baseline I_{SC}, when added to the external solutions bathing the experimental areas of five skins (Table 5). However, the TMB-8 did inhibit the subsequent stimulation of current associated with activation of PKC by DiC_8. Addition of 290 μM DiC_8 to the external media stimulated I_{SC} by 18 ± 6 $\mu A \cdot cm^{-2}$ across the control areas, but by only 8 ± 5 $\mu A \cdot cm^{-2}$ across the areas pretreated with TMB-8. Thus, application of an

Table 4. Interaction of Cd^{2+} and cAMP on I_{SC}

Parameter	I_{SC} ($\mu A \cdot cm^{-2}$)			
	Cd^{2+} 1st	cAMP 1st	Difference	Probability
Baseline (5)	30.2 ± 6.3	30.4 ± 4.2	−0.2 ± 2.7	>0.9
Changes after:				
Cd^2	12.7 ± 2.5	12.3 ± 1.9	0.3 ± 2.0	>0.8
cAMP	11.2 ± 3.6	14.3 ± 2.3	−3.1 ± 2.4	>0.2
Final I_{SC} in amiloride (4)	5.4 ± 1.1	6.0 ± 1.0	−0.5 ± 1.3	>0.6

The "experimental" and "control" areas of eight skins were exposed to both 1–2 mM external Cd^{2+} and to 1 mM serosal cAMP. The areas identified as "Cd^{2+} 1st" were first treated with Cd^{2+}, while Ringer solvent was simply added to the contiguous areas identified as "cAMP 1st." After a peak response to Cd^{2+} was observed, cAMP was simultaneously added to the serosal solutions bathing both areas of skin. Finally, Cd^{2+} was added to the mucosal and serosal solutions bathing the "cAMP 1st" areas of skin. In order to document that the concentration of Cd^{2+} chosen was close to a saturating dose, an additional 1–2 mM Cd^{2+} was added to the external media bathing the "Cd^{2+} 1st areas." This second dose of Cd^{2+} produced no detectable effect in three of the preparations and stimulated I_{SC} by a mean value of only 0.5 ± 0.1 $\mu A \cdot cm^{-2}$ (≈4% of the initial response). Similar responses were noted to Cd^{2+}, whether or not cAMP was present, and to cAMP, whether or not Cd^{2+} was present.

Table 5. Effect of TMB-8 on baseline value and response of I_{SC} to DiC_8

Parameter	I_{SC} ($\mu A \cdot cm^{-2}$)			
	Experimental	Control	Difference	Probability
Baseline (5)	37.8 ± 10.4	39.6 ± 9.0	−1.7 ± 2.0	>0.4
Changes after:				
TMB-8 to experimental (5)	−6.2 ± 2.7	−2.7 ± 2.2	−3.5 ± 1.9	>0.1
DiC_8 (4)	8.2 ± 4.6	18.1 ± 5.9	−9.8 ± 2.3	<0.05
Final I_{SC} in amiloride (4)	4.7 ± 0.6	4.6 ± 0.8	0.1 ± 0.7	>0.8

Under baseline conditions, five skins were bathed with standard Cl^--containing Ringer's solution on both their mucosal and serosal surfaces. The mucosal solution was thereafter replaced with a Cl^--free, NO_3^- Ringer's solution before adding 70 μM TMB-8 to the mucosal and serosal solutions bathing the experimental area and ethanol solvent to the solutions bathing the adjoining control area of each skin. The experimental and control areas were subsequently exposed to the sequential additions of 290 μM external DiC_8, 100 mU · ml^{-1} serosal vasopressin, and 100 μM mucosal amiloride. In all five experiments, TMB-8 pretreatment qualitatively reduced the subsequent response to DiC_8. However, one skin displayed an unusually small response to DiC_8 even across the control area; as indicated by the numbers of skins in parentheses, that experiment was excluded from the data averaging. The probabilities (P) of the null hypotheses are presented in the final column. Pretreatment with TMB-8 inhibited the natriferic response to DiC_8 by 60 ±14% ($P < 0.05$).

agent reported to reduce Ca^{2+} mobilization from intracellular stores inhibited the Na^+ stimulation associated with PKC activation by 60 ± 15% ($P < 0.05$).

Civan et al. (1988) have suggested that insulin may serve as a physiologic trigger for PKC activation, and as a regulator of Na^+ reabsorption by frog skin. If so, we would expect that pretreatment with TMB-8 should also reduce the natriferic response to insulin. This prediction was tested in a series of five experiments (Table 6). Without prior exposure to 70 μM TMB-8, insulin increased I_{SC} by 8 ± 2 $\mu A \cdot cm^{-2}$. After exposure to mucosal and serosal TMB-8, the experimental areas displayed an increase of only 2 ± 1 $\mu A \cdot cm^{-2}$. Thus, TMB-8 lowered the characteristic hormonal effect by 76 ± 10% ($P < 0.01$).

Analysis of Kinase Activity

The electrophysiologic data have suggested that a reduction in a^c_{Ca}, produced by blocking either Ca^{2+} entry or mobilization of Ca^{2+} stores, reduces the natriferic responses to PKC activators and to a puta-

Table 6. Effect of TMB-8 on subsequent natriferic response to insulin

Parameter	I_{SC} ($\mu A \cdot cm^{-2}$)			
	Experimental	Control	Difference	Probability
Baseline	58.6 ± 1.9	58.2 ± 3.4	0.4 ± 3.8	>0.9
Changes after:				
TMB-8 (to experimental)	−3.7 ± 4.4	−7.7 ± 1.0	4.1 ± 3.8	>0.3
Insulin	1.9 ± 1.4	8.2 ± 2.5	−6.3 ± 1.8	<0.02
Final I_{SC} in amiloride	6.7 ± 0.8	6.5 ± 1.5	0.3 ± 0.9	>0.4

Under baseline conditions, five skins were bathed with standard Cl^--containing Ringer's solutions on both their mucosal and serosal surfaces. The mucosal solution was thereafter replaced with a Cl^--free, NO_3^- Ringer's solution before adding 70 μM TMB-8 to the mucosal and serosal solutions bathing the experimental area and ethanol solvent to the solutions bathing the adjoining control area of each skin. The experimental and control areas were subsequently exposed to the sequential additions of 10 mU · ml^{-1} serosal insulin, 100 mU · ml^{-1} serosal vasopressin, and 100 μM mucosal amiloride. The probabilities (P) of the null hypotheses are presented in the final column. Pretreatment with TMB-8 inhibited insulin's natriferic response by 76 ± 10% ($P < 0.01$).

tive physiologic trigger of PKC activation in frog skin. However, the foregoing data did not permit identification of the precise step at which a^c_{Ca} modulates the action of PKC on Na^+ transport. One possibility was that reducing cytosolic Ca^{2+} inhibited PKC activation. An alternative possibility was that reduction in a^c_{Ca} altered a number of other parameters, permissive or required for the natriferic expression of activated PKC. To resolve this issue, TPA- and phospholipid-sensitive kinase activity was measured in extracts of frog skin epithelium.

Chromatography of cytosolic or membrane extracts on DEAE-cellulose resulted in the appearance of a TPA-stimulated kinase activity in the 0.1 M NaCl fraction (cytosol, Fig. 4, top) or the 0.2 and 0.3 M NaCl fractions (membrane, Fig. 4, bottom). Further characterization of this kinase activity (Fig. 5) indicated that the TPA-stimulated kinase did not require Ca^{2+}, and in fact, enzyme activity was often reduced in the presence of added Ca^{2+}. This lack of requirement for added Ca^{2+} was consistently observed in each of 12 frog epidermal preparations examined and included both cytosolic and membrane extracts (*data not shown*). When a range of Ca^{2+} concentrations was tested, the results shown in Fig. 6 were obtained. Using 10 mM EGTA to buffer free Ca^{2+} to the indicated concentrations, a biphasic effect was observed: at low Ca^{2+} concentrations (10^{-9}–10^{-8} M), little change in kinase activity occurred, but at intermediate Ca^{2+} concentrations (10^{-7}–10^{-5} M), a strong inhibitory effect was noted. It is at these free-Ca^{2+} concentrations (in the presence of 10 mM EGTA) that the concentration of the Ca^{2+}-EGTA complex is significant (i.e., in the μM range). However, if EGTA is omitted and the Ca^{2+} concentrations made by serial dilution of a concentrated stock,

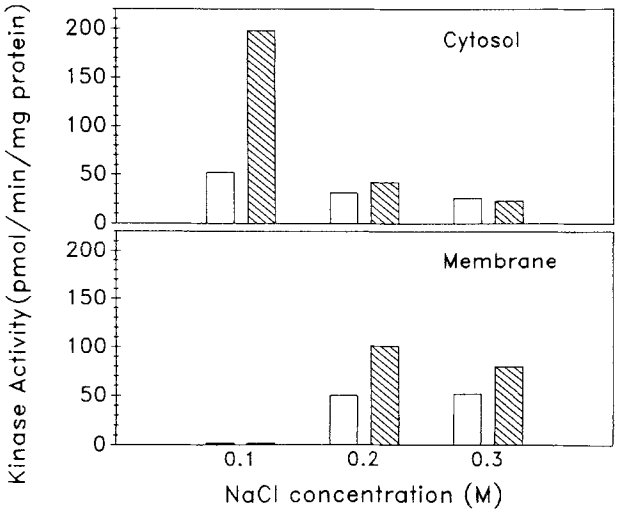

Fig. 4. Partial purification of kinase activity from cytosol and membrane fractions. An epithelial homogenate was fractionated into cytosolic and membrane fractions, as described in Materials and Methods. Each fraction was then applied to a small DEAE-cellulose column, and kinase activity eluted stepwise with increasing NaCl concentrations. Column fractions were assayed in the presence of phosphatidylserine (open bars) or phosphatidylserine plus TPA (hatched bars). The experiment shown is representative of three independent preparations

no strong inhibitory effect of Ca^{2+} was observed at nominal concentrations in the range of 10^{-7}–10^{-5} M. Our tentative interpretation of these data is that the Ca^{2+}-EGTA complex is a moderately potent inhibitor of this kinase. Ca^{2+} itself at mM concentrations is also somewhat inhibitory. Under no experimental conditions was a stimulatory effect of free Ca^{2+} on this kinase activity observed.

The frog epidermal kinase activity has an appar-

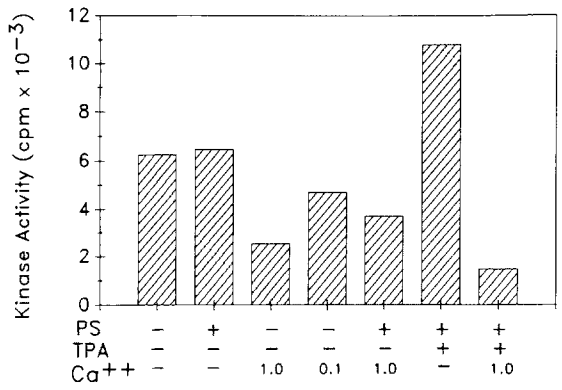

Fig. 5. Characteristics of frog epidermal kinase activity. The cytosolic enzyme partially purified by DEAE-cellulose chromatography was assayed in the presence of PS (50 μg/ml), TPA (200 nM) or Ca^{2+} (free ionic concentrations in mM), as indicated. Similar results were seen with the enzyme from the membrane fraction

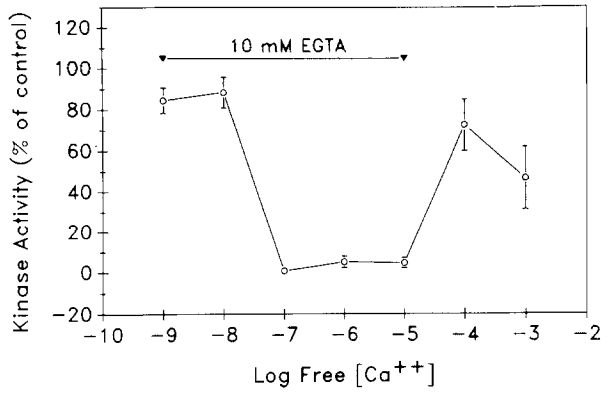

Fig. 6. Dependence of kinase activity on free-Ca^{2-} concentration. Kinase activity partially purified on DEAE-cellulose was incubated with the indicated concentrations of free Ca^{2+} in the presence of phosphatidylserine (50 $\mu g \cdot ml^{-1}$) and TPA (0.16 μM). Suitable amounts of $CaCl_2$ were added to 10 mM EGTA in order to vary the free $[Ca^{2+}]$ over the range 10^{-9} to 10^{-5} M, whereas dilutions of a 100-mM solution of $CaCl_2$ were used to fix the free $[Ca^{2+}]$ at 10^{-4} and 10^{-3} M. The means (± SE) of three separate experiments are displayed and expressed as percentages of the activity in the absence of Ca^{2+} or EGTA

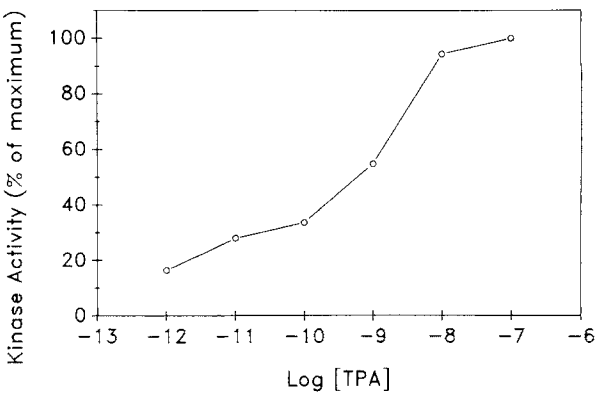

Fig. 7. Dependence of kinase activity on TPA concentration. Kinase activity was measured in the presence of phosphatidylserine (PS) and the indicated concentrations of TPA. Results shown are from a representative experiment out of a total of three

Table 7. Phosphorylation of different substrates by frog epidermal kinase

Substrate	Kinase activity (nmol P_i incorporated/min/mg protein)	
	+PS	+PS/TPA
Histone	11.1	18.6
RRRRYGSRRRRRRY[a]	135.0	181.0
[CGG]PLSRTLSVSS[b]	21.3	33.6
[CGG]URKRTLRRL[c]	17.1	31.8

A frog epidermal cytosolic extract was assayed for kinase activity in the usual manner except different substrates were employed, each at 20 μM. Ca^{2+} was omitted from the enzyme assay, and EGTA was present at 0.25 mM. Amino acid residues within brackets are not present in the native proteins from which these peptides were derived but were added for reasons unrelated to their use as kinase substrates.

[a]Residues 52–65 of galline (Ferrari et al., 1985).

[b]Residues 1–10 of glycogen synthetase (House, Wettenhall & Kemp, 1987).

[c]Residues 650–658 of the epidermal growth factor receptor (Hunter, Ling & Cooper, 1984).

ently high affinity for TPA. A representative TPA concentration-response curve is illustrated in Figure 7. Half-maximal activation of kinase activity occurred at approximately 1 nM. This value is consistent both with the K_m for the transport response of intact frog skin epithelium [≈3 nM (Mauro et al., 1987)] and with results obtained with protein kinase C isozymes (Nishizuka, 1986).

The question of whether the predominant TPA-stimulated kinase activity in frog epidermis is a member of the protein kinase C family was addressed by the use of synthetic peptide substrates. As shown in Table 7, each of the synthetic peptides (whose sequences are derived from the phosphorylation sites of known PKC substrates) are excellent substrates for frog epidermal kinase. At an equivalent concentration (20 μM), RRRRYGSRRRRRRY was notably phosphorylated to a greater extent than the standard substrate, histone IIIs. These results suggest, but do not prove, that the TPA-sensitive kinase is a member of the PKC family of kinases.

Discussion

PKC Stimulation of Na^+ Transport and Interaction with cAMP

Activators of protein kinase C markedly stimulate the short-circuit current (I_{SC}) across frog skin (Civan et al., 1985, 1987, 1988, 1989; Andersen et al., 1987; Mauro et al., 1987). Under many experimental conditions, I_{SC} is a reliable index of net Na^+ transport across frog skin and the urinary bladder of the toad (Civan, 1983). This point has been documented for the PKC-stimulated current, as well, by measurement of isotopic Na^+ flux before and after TPA administration (Andersen et al., 1987). Electrophysiological measurements have suggested that PKC activation stimulates Na^+ transport largely by increasing the apical Na^+ permeability (P^{ap}_{Na}) of frog skin epithelium (Civan et al., 1987, 1989). Insulin may serve as the physiological trigger of the PKC-stimulated Na^+ transport in this tissue (Civan et al., 1988, 1989).

In order to gain further information concerning the mechanisms by which PKC activation increases P^{ap}_{Na}, we have been studying the interactions among PKC, cAMP and intracellular Ca^{2+} activity in frog skin. Pretreatment with PKC activators clearly inhibits the subsequent responsiveness to vasopressin, whose hormonal action is mediated by cAMP (Orloff & Handler, 1962). The effects of PKC cannot be explained solely by actions on the catalytic unit or the G-proteins regulating adenylate cyclase, since PKC activation also markedly inhibits the natriferic response of frog skin to cAMP, itself (Civan et al., 1985). Whether the interaction between PKC and cAMP is at the level of the Na^+ channel (Civan et al., 1985) or at some more proximal step is unclear.

Effects of Altering a^c_{Ca} on PKC-stimulated Na^+ Transport

In the present work, we have begun to examine the interrelationship between intracellular Ca^{2+} and PKC by using two techniques to lower a^c_{Ca}: (*i*) blocking calcium entry with external Co^{2+} or Cd^{2+}, and (*ii*) blocking Ca^{2+} mobilization from intracellular stores with TMB-8. This approach (using two complementary perturbations to lower a^c_{Ca}) was chosen since actions of inhibitors are rarely unique. In particular, Co^{2+} and Cd^{2+} may not only block Ca^{2+} channels, but also inhibit Ca^{2+}-activated ATPase (Verbost et al., 1987*a*, 1988), and Na^+,K^+-activated ATPase (Rifkin, 1965; Takada & Hayashi, 1978). However, several observations indicate that the predominant effect of these divalent ions in frog skin is to inhibit Ca^{2+} uptake: (*i*) removal of mucosal Ca^{2+} produces a rapid, reversible stimulation of Na^+ transport [monitored as short-circuit current (I_{SC}) across frog skin (Curran & Gill, 1962; Hillyard & Gonick, 1976)], (*ii*) addition of Cd^{2+} or Co^{2+} stimulates I_{SC} (Borghgraef et al., 1971; Banks, 1974; Hayashi et al., 1977; Takada & Hayashi, 1980, 1981; Takada, 1985; Goncharevskaya et al., 1986; Natochin et al., 1986), and (*iii*) prior removal of mucosal Ca^{2+} with EDTA increases I_{SC}, but abolishes the characteristic stimulatory response to subsequently added Cd^{2+} (Hillyard & Gonick, 1976). It is likely that the reduced a^c_{Ca} secondarily affects plasma membrane channels for Na^+ (Grinstein & Erlij, 1978), Cl^- (Hayashi et al., 1977) and K^+ (Latorre et al., 1989). In the present study, Co^{2+} or Cd^{2+} increased the baseline values of apical Na^+ permeability (Table 3) and I_{SC} (Table 1). The $K_{0.5}$ for the latter effect was $\simeq$200 μM (Fig. 2), consistent with a primary action to block T-type Ca^{2+} channels (Miller, 1987).

Similarly, TMB-8 may not only inhibit mobilization of Ca^{2+} from the endoplasmic reticulum (Streb et al., 1984), but also (in high concentration) may directly inhibit PKC (Kojima et al., 1985; Sawamura, 1985). To minimize this possibility, we have followed the approach of Rose et al. (1986) in not exceeding a bath concentration of 70 μM.

Both manipulations designed to reduce a^c_{Ca} had the same qualitative effect, reducing the PKC-stimulated Na^+ transport by approximately 50% (Tables 1 and 5). Furthermore, preincubation with TMB-8 also markedly reduced the natriferic effect of insulin (Table 6). This observation is consistent with the hypothesis that insulin stimulates Na^+ transport across frog skin by triggering activation of PKC (Civan et al., 1988, 1989). The electrophysiologic observations of the present study are also consistent with biochemical results reported by Pershadsingh et al. (1987). The latter investigators reduced a^c_{Ca} by preloading rat adipocytes with the calcium buffer quin2. This procedure reduced the stimulation of D-glucose uptake characteristically produced by insulin and by TPA.

The foregoing results suggested that intracellular Ca^{2+} interacts with PKC in modifying Na^+ transport across frog skin. However, it was unclear whether that interaction: (*i*) directly reflected the documented Ca^{2+} dependence of conventional forms of PKC (Nishizuka, 1986), or (*ii*) arose from later nonindependent events (such as chemical modification of the same target site, the Na^+ channel). In order to distinguish between these possibilities,

kinase activity has been assayed in broken-cell preparations from the epithelium. The results of Figs. 4–8 have strongly suggested that the latter possibility is correct, since altering the free-Ca^{2+} concentration had no direct stimulatory effect on the measured kinase activity (*discussed below*). The precise nature of the indirect negative interaction between a^a_{Ca} and PKC cannot be as yet identified. Possibly it arises from a negatively cooperative effect exerted on the Ca^{2+}- and temperature-dependent modulating site reported for Na^+ channels in toad urinary bladder (Garty & Asher, 1985), rat colon (Bridges et al., 1988) and rat cortical collecting tubules (Palmer & Frindt, 1987*b*). At least in those tight epithelia, intracellular Ca^{2+} inactivates apical Na^+ channels by a mechanism apparently distinct from direct blocking of the channel (Garty & Benos, 1988). Whether or not activation of an isozyme of PKC can reactivate the calcium-modified channel remains to be explored.

Kinase Activity

The properties of frog skin PKC(s) have not been described previously. Somewhat surprisingly, the predominant protein kinase C-like activity present in frog skin differs in at least one important respect from the major mammalian PKC isoforms, termed α, β_I, β_{II} and γ (Ono et al., 1987). Partially purified frog skin cytosolic extracts (eluted from an anion exchange column) exhibit a protein kinase activity that is phosphatidyl serine-dependent, activated by phorbol esters at nM concentrations, but does not require Ca^{2+} for activity. At high, nonphysiological concentrations of Ca^{2+}, frog epidermal kinase activity is actually inhibited (Fig. 6). Interestingly, the Ca^{2+}-EGTA complex appears to be a moderately potent inhibitor of this kinase; we are not aware that this observation has been reported for conventional PKC isoforms (α, β_I, β_{II}, γ). Further evidence that this kinase is a member of the PKC family is provided by the use of different substrates. Each of three peptides whose sequences were derived from the phosphorylation site of known PKC substrates proved to be better substrates for the frog epidermal kinase than the standard PKC substrate, histone IIIs. The above properties of this kinase, especially its lack of requirement for Ca^{2+}, suggest that the amphibian equivalent of *n*PKC (Ohno et al., 1988) or PKCε (Ono et al., 1988) was being measured in these assays. Further characterization by isozyme-specific immunological and nucleic acid probes should allow us to confirm this conclusion.

Significance of Phospholipid- and TPA-Sensitive Kinase in Frog Skin

The epithelium of frog skin has served as a model system for transport across tight epithelial tissues for over 140 years (Matteucci & Cima, 1845; Du Bois Reymond, 1848). During this period, ion and water transport across frog skin have been studied by a wide range of biophysical and biochemical techniques. However, in its responsiveness to activators of PKC, frog skin has proven to be different from several other tight epithelia. TPA produces a prompt, marked stimulation of Na^+ transport across frog skin which can last >1 hr (Civan et al., 1985, 1987, 1988, 1989; Anderson et al., 1987; Mauro et al., 1987). A secondary phase of inhibition is observed after 1–2 hrs (Civan et al., 1989). Only the inhibitory phase has been noted with A6 cells (Yanase & Handler, 1986), and intermediate effects have been reported for other epithelial cells (Rorsman, Arkhammar & Berggren, 1986; Schlondorff & Levine, 1986; Palmer & Frindt, 1987*a*). The results of the present study provide support for the concept (Civan et al., 1988) that the natriferic response of a given tissue to PKC activators may reflect the principal isozyme of the PKC family present.

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