ACTIVATION OF Na/H EXCHANGE BY PHORBOL ESTERS IN RED CELLS OF THE DOGFISH SHARK (SQUALUS ACANTHIAS)

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Na/H exchange has been detected in a variety of cells where it is implicated in regulation of cell volume and pH. In human fibroblasts, activation of this antiporter also precedes stimulation of cell proliferation by growth factors (Moolenaar et al., Nature 304: 645-648, 1983). This effect is mimicked by exogenous diacylglycerol—a product of the receptor–mediated turnover of inositol phospholipids—or by tumor–promoting phorbol esters (Moolenaar et al., Nature 312: 371-374, 1984). The final common pathway for these effects is protein kinase C. Phorbol esters, which act as structural analogs of diacylglycerol, bind to protein kinase C and render it irreversibly active. In this report, we describe experiments in which a phorbol ester, 4–β–phorbol 12–myristate 13–acetate (PMA), activated an external Na–dependent, amiloride–sensitive proton efflux from red cells of the dogfish shark, Squalus acanthias. We also attempted to determine the natural physiological activator of this putative Na/H exchange.

Spiny dogfish (Squalus acanthias) were tonically immobilized by dorsal inversion in a trough containing fresh sea water. Handled in this way, they were able to maintain normal gill perfusion during the procedure. Blood drawn into a heparinized syringe from the caudal vein was processed as previously described (Payne and Evans, Bull. MDIBL 27: 67-69, 1988) except for the preincubation protocol. In this instance, washed red cells were preincubated for 3 hours at 12°C in the following medium (mM): NaCl, 270; KCl, 6; $CaCl_2$, 5; $MgCl_2$, 3; Na_2SO_4, 0.5; NaH_2PO_4, 1; urea, 350; TMAO, 72; glucose, 5; tetramethylammonium (TMA)–TES (pH= 7.8 @ 12°C), 15.

All experiments were carried out at 12 ± 0.1°C in a jacketed beaker perfused from a Haake refrigerated bath and circulator (A-80). Net proton efflux was assayed by monitoring external pH (pH_o) changes induced by addition of substances to an unbuffered suspension of cells (7.5% hematocrit) in a medium of the following composition (mM): NaCl, 270; KCl 6; $CaCl_2$, 5; $MgSO_4$, 2.5; urea, 350; TMAO, 72; glucose, 5; sucrose, 21. The cell suspension was stirred with a small magnetic follower, and pH_o was monitored by a combination electrode connected to a Radiometer pH meter (PHM 84). The results were recorded on a Radiometer servograph (REC 61).

All stock solutions of the phorbol ester (1.62×10^{-3}M), as well as amiloride analogs (10^{-2}M) and 3–isobutyl–1–methylxanthine (IBMX, 0.5M), were made up in DMSO. Forskolin was prepared as a stock solution (10^{-2}M) in ethanol. Stock solutions of the remaining substances were prepared in the unbuffered medium described above.

Addition of PMA to a suspension of DIDS–treated (10^{-4}M) dogfish red cells (7.5% hematocrit) stimulated proton efflux in a dose-dependent manner. The 50% effective dose was 5×10^{-8}M with the maximal effect at 10^{-7}M. To maximize net proton efflux, the cells were pretreated with DIDS, thus preventing equilibration of acid-base equivalents across the plasma membrane via capnophorin (the "band 3" anion exchange protein), which has been shown to be present in these cells (Payne and Evans, Bull. MDIBL 27: 67-69, 1988). It is interesting that the delay in onset of proton efflux after addition of PMA was 2–3 minutes, similar to the delay reported at 37°C for PMA–stimulated lymphocytes (Grinstein et al., Proc. Nat. Acad. Sci. USA 82: 1429-1433, 1985).

Addition of amiloride (10^{-3}M) or 5–dimethylamiloride (10^{-4}M) inhibited PMA-stimulated proton efflux, as did replacement of external NaCl with TMA–Cl . Subsequent re-introduction of isotonic NaCl (final concentration: 100 mM) caused a prompt reappearance of amiloride–sensitive proton efflux. Although amiloride at these doses is known to inhibit protein kinase C (Besterman et al., J. Biol. Chem. 260: 1155-1159, 1985), the effect of the dimethyl analog supports the conclusion that the diuretics are acting directly on Na/H exchange. Thus, dimethylamiloride, which is just as potent against Na/H exchange as amiloride, has little effect at 10^{-4}M on the kinase.

In other experiments, we found that PMA (5×10^{-7}M) also caused a marked increase in net Na uptake in ouabain-treated (10^{-4}M) cells incubated in a buffered medium (TMA-TES, pH=7.8 @

12°C). This effect was completely inhibited by 5–dimethyl amiloride (10^{-4}M). PMA stimulation also caused a net Cl uptake, which can be explained as an exchange (in the absence of DIDS) of intracellular base equivalents for Cl (via capnophorin) following alkalinization of the cytosol due to exchange of internal protons for external Na via Na/H exchange. These observations, therefore, lend further support to the conclusion that net proton efflux in the presence of phorbol ester is due to activation of Na/H exchange.

Stimulation of proton efflux by phorbol esters was specific for the 4–ß–phorbol analog. As shown in Table 1, neither of the 4–α–phorbol analogs were effective even at relatively high concentrations. The 4–α–phorbol analogs are also ineffective activators of protein kinase C, thus providing useful negative controls demonstrating that the 4–ß–phorbol response is not a non-specific effect of phorbol itself.

The adrenoceptors that activate Na/H exchange in teleost red cells are of the $ß_1$–type (Tetens et al., J. exp. Biol. 134: 267-280, 1986). We tested two agonists of this class, isoproterenol and norepinephrine, and found them to be inactive (Table 1). Neither forskolin nor dibutyryl cAMP (supplemented with the phosphodiesterase inhibitor IBMX) had any effect either, further confirming the absence of a cAMP–mediated pathway in these cells. Since neither of the agonists with α_1 activity, phenylephrine and norepinephrine, were effective, we conclude that these cells also lack an α_1 response, which is interesting since α_1 receptors have been shown to activate inositol phospholipid turnover and protein kinase C in many cells (Berridge, Biochem. J. 220: 345-360, 1984). In all these studies, the observation of a negative response to any agent was followed by subsequent addition of PMA (5×10^{-7}M) which always elicited an extrusion of protons.

In summary, we have presented evidence for the presence of Na/H exchange in dogfish red cells under protein kinase C control, but unaffected by agonists for receptor-mediated regulation. In a recent abstract, Borgese et al. (Proc. of the European Assoc. for Red Blood Cell Research, Cadarache, France, May 2-6, 1988) reported that Na/H exchange in trout red cells can be activated by both the catecholamine–cAMP and phorbol ester pathways. This does not appear to be the case in dogfish red cells which appear to lack a hormonally controlled system. In the trout cell, adrenergic control of this response is thought to play a crucial role in maintenance of cell pH and hemoglobin-oxygen affinity during respiratory stress (Fievet et al., Am. J. Physiol. 252: R269-R275, 1987). Dogfish red cells must therefore have other methods of coping with these exigencies. An interesting suggestion recently put forward by Primmett et al. (J. exp. Biol. 122: 139-148, 1986) draws attention to the reduction in plasma pH due to lactic acidosis that occurs during burst activity in salmonids. As a consequence of the Bohr and Root effects, a detrimental decrease in hemoglobin–oxygen binding might be expected. This is prevented by an offsetting increase in red cell pH due to the β–adrenergically mediated Na/H exchange. Thus, a catecholamine response in the red cell may be of importance only in those organisms that possess a Root effect hemoglobin. Significantly, Squalus hemoglobin does not show a Root shift (Wells and Weber, J. exp. Biol. 103: 95-108, 1983).

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TABLE 1

Agents Tested for Activation of Proton Efflux
from Red Cells of the Spiny Dogfish
(Squalus acanthias)

Agent	Concentration moles / l	Maximum Rate of Acidification $\Delta(pH_o)$/min*	
Phorbol Ester Analogs			
4–ß–Phorbol 12–Myristate 13–Acetate	10^{-7}	0.030 ± 0.003	(3)
4–α–Phorbol 12–Myristate 13–Acetate	10^{-6}	0	(3)
4–α–Phorbol 12,13–Didecanoate	10^{-6}	0	(2)
α and ß Receptor Agonists			
Isoproterenol ($ß_1 + ß_2$)	10^{-4}	0	(3)
Norepinephrine ($ß_1 + \alpha_1$)	10^{-4}	0	(2)
Phenylephrine (α_1)	10^{-4}	0	(4)
Forskolin	10^{-5}	0	(3)
Dibutyryl cAMP (+IBMX 10^{-3}M)	2×10^{-3}	0	(1)

All cells pretreated with DIDS (10^{-4} M)
* Mean ± 1 S.D. (n)