Sidedness of the inhibitory effects of diamide on Na and water transport in amphibian skin[1]

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Summary. Diamide, a thiol-oxidizing agent, blocks cAMP-mediated stimulation of Na and water transport when added to the outer solution bathing frog skin. No inhibition was found with diamide in the inner solution. These effects may be due to a perturbation of the thiol-disulfide status of specific components of the outer membrane of the epithelium.

In 1964 Bentley[3] reported that exogenous glutathione inhibits Na and water transport in frog bladder. On the other hand, extensive work by Kosower et al.[4-7] has shown that diamide, a thiol-oxidizing agent with a high affinity for glutathione, perturbs the thiol-disulfide status of a variety of cell systems, in particular the ratio between reduced (GSH) and oxidized (GSSG) glutathione. These findings prompted the study of diamide in amphibian skin as a step towards defining a possible role of the endogenous GSH/GSSG ratio of epithelial cells on Na and water transport[8].

Materials and methods. The ventral skin of 2 amphibians, frogs *Rana ridibunda* and toads *Bufo bufo*, was used. Net Na transport was measured by means of the short circuit current (SCC) technique[9]. Net water flow (J_{H_2O}) was continuously monitored with either of 2 different volumetric techniques. The 1st one, based on an optical signal, has been described in detail[10]. The 2nd technique, recently developed, is fully automated. A capacitance signal triggers the movement of a carrier that follows the displacement of the meniscus inside a horizontal pipette connected to a chamber holding the skin. The carrier has 2 metal pieces which are placed around the pipette. The position of the meniscus in between them originates the capacitance signal. The latter is fed into an electronic device that drives the carrier and calculates the average J_{H_2O} ($\mu l \cdot min^{-1} \cdot cm^{-2}$) during a preset time interval varying from 0.1 to 30 min. The J_{H_2O} values thus obtained were recorded on a 600 Tarkan W+W instrument. All chemicals were reagent grade. The hormones used were: vasopressin (Pitressin, Park-Davis), oxytocin (Syntocinon, Sandoz), D-L norepinephrine (Fluka). Diamide and dithiothreitol were purchased from Calbiochem. All results were expressed as mean±SEM, and the p-values obtained by means of Student's t-test for paired data.

Results and discussion. Diamide per se altered Na and water transport in resting skins. The effects were somewhat variable, however, and ranged from moderate stimulation to inhibition, depending on the concentration of the drug (0.1-1.0 mM) and on the side of the skin exposed to diamide. These results will be dealt with in another publication; in this report we will focus our attention on the interference of diamide with the hormonal stimulation of net Na and water fluxes across the skin.

A striking feature emerges from the results summarized in tables 1 and 2: the sidedness of the effects of diamide on the stimulation of SCC. At 0.5 mM, diamide added to the external solution bathing the skin, produced a 77% inhibition of the natriferic response to a supramaximal concentration of oxytocin (table 1). In contrast, addition of the drug to the inner solution did not significantly disturb the hormonal effect (table 2). At a higher concentration (1 mM), the 'outer' effect of diamide was even more marked (94% inhibition of oxytocin), while, in the inner side, diamide induced only a 20% inhibition of the hormone which appeared to be accounted for by a competitive stimulation of SCC by diamide itself. In fact, if the increments in SCC induced sequentially by diamide and by oxytocin were lumped together, no significant difference was found between the ΔSCC values of experimental and control tissues (table 2). This asymmetry of the diamide action was not specific for oxytocin. As shown in the tables, a similar pattern of results was found when frog skin was challenged with other natriferic agents, such as isoproterenol and theophylline. Finally, a normal response to oxytocin in skins exposed to 'external' diamide could be elicited when 1 mM dithiothreitol, a classic thiol-reducing agent, was present in the outer Ringer solution (table 1).

At this point the question arose whether diamide had a similar interaction with other transport processes stimulat-

Table 1. Effect of 'external' diamide on natriferic responses in frog skin

Experimental protocol	N	ΔSCC ($\mu A \cdot cm^{-2}$) (mean±SEM)	p
OT (50 mU/ml)	28	36.7±3.86	<0.001
D (0.5 mM)+OT		8.4±1.69	
OT (50 mU/ml)	5	24.4±1.89	<0.001
D (1 mM)+OT		1.4±1.37	
Iso (1 µM)	22	33.4±3.76	<0.001
D (0.5 mM)+Iso		9.3±1.81	
Theo (10 mM)	13	21.6±3.42	<0.01
D (0.5 mM)+Theo		6.8±1.92	
Theo (10 mM)	15	30.8±4.29	<0.001
D (1 mM)+Theo		16.7±3.80	
cAMP (5 mM)	7	16.1±3.39	<0.01
D (1 mM)+cAMP		11.8±3.46	
D (1 mM)+OT	9	1.2±0.57	<0.001
DTT (1 mM)+D+OT		27.1±3.77	

Increments in SCC induced by several natriferic agents in control skins (1st row) and in paired skins pre-exposed to diamide added to the external Ringer solution (2nd row). OT, oxytocin; Iso, isoproterenol; Theo, theophylline; D, diamide; DTT, dithiothreitol; N, number of paired studies.

Table 2. Effect of 'internal' diamide on natriferic responses in frog skin

Experimental protocol	N	ΔSCC ($\mu A \cdot cm^{-2}$) (mean±SEM)	p
OT (50 mU/ml)	10	46.4±4.06	NS
D (0.5 mM)+OT		39.9±3.97	
OT (50 mU/ml)	17	29.2±3.80	<0.01
D (1 mM)+OT		23.3±3.10	
		(29.1±3.58)*	NS
Iso (1 µM)	11	33.5±5.46	NS
D (0.5 mM)+Iso		32.9±4.82	
Iso (1 µM)	7	22.4±3.70	NS
D (0.5 mM)+Iso		20.4±4.14	
Theo (10 mM)	25	27.4±3.05	<0.05
D (0.5 mM)+Theo		23.4±2.62	
		(29.1±3.00)*	NS

Experimental design, symbols and abbreviations as in table 1. NS, non significant. * The values of this 3rd row correspond to the sum of SCC increments sequentially induced by D and by OT or Theo. They are not significantly different from control values (1st row).

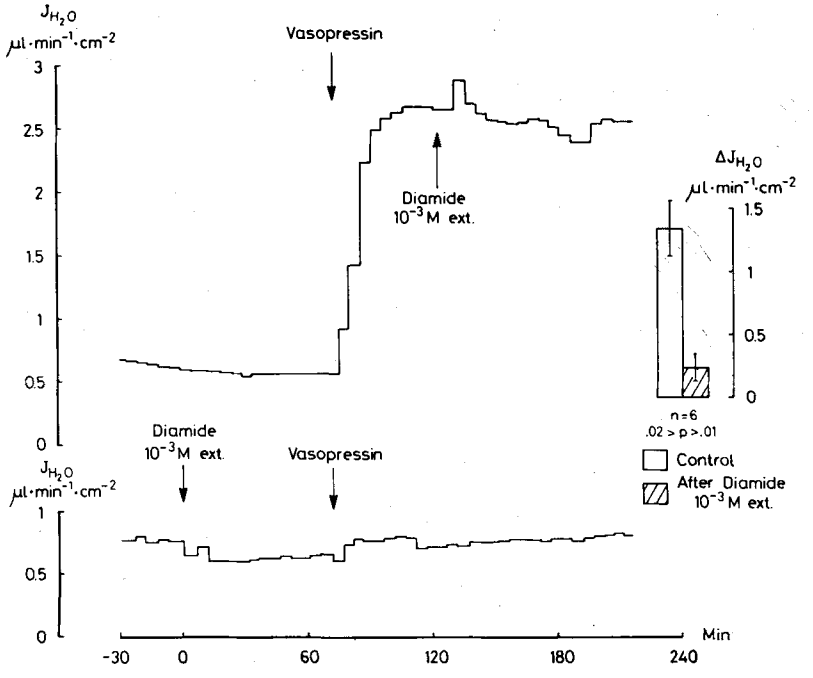

Fig. 1. Block of the hydrosmotic effect of vasopressin (100 mU/ml) in toad skins exposed to diamide added to the external solution. The example shows the time-course of the variations of J_{H_2O} in 2 paired skins, followed with the 2nd technique described in the methods. At the right side, comparison of the increments in J_{H_2O} induced by vasopressin with and without pre-exposure of the skins to diamide. Osmotic gradient: Ringer solution diluted 10 times on the external side; normal Ringer solution on the internal side.

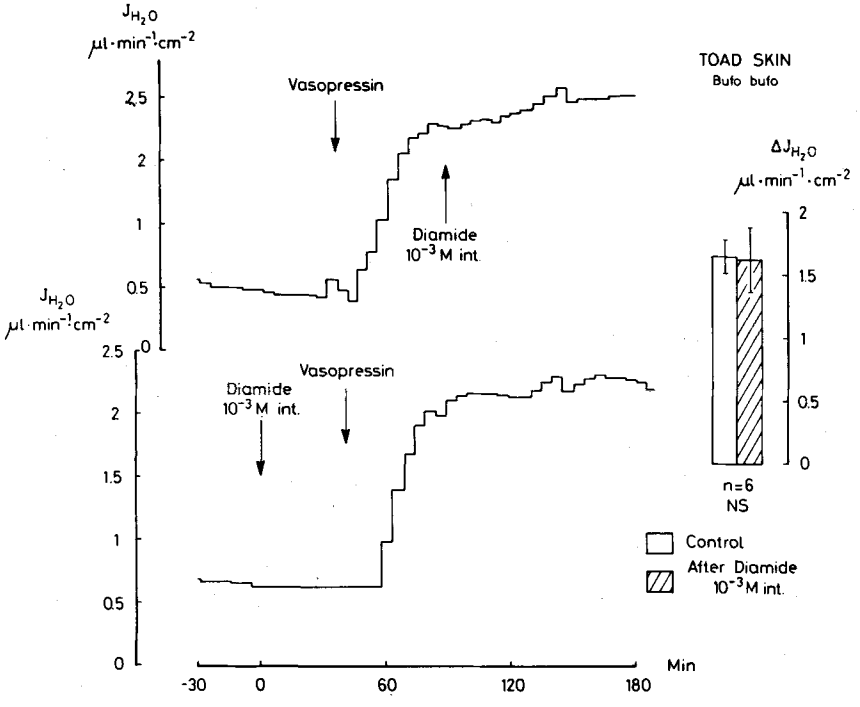

Fig. 2. Normal hydrosmotic responses to vasopressin in toad skins exposed to diamide added to the internal solution. For other details see legend of figure 1.

ed by hormonal agents. The answer is given in figures 1 and 2 and concerns the hydrosmotic effect of vasopressin on toad skin. In the experiment depicted in figure 1, 'external' diamide induced an almost total block of the stimulation of J_{H_2O}. Such inhibition was reproducible, highly significant and did not occur if J_{H_2O} was already stimulated by pre-exposure to vasopressin (figure 1). In contrast, no difference in hydrosmotic response was found in control skins and in paired skins pre-exposed to 1 mM diamide added to the internal solution (figure 2).

The conspicuous sidedness of the diamide action raises a host of interesting questions. 2 main points must be discussed: a) the effects of other SH-reagents on Na and water transport; b) the mechanism of action of diamide in other cell systems.

Handler and Orloff[11] reported that 1 mM cysteine also blocked vasopressin- and theophylline-induced stimulation of Na and water flow in toad bladder. Several features, however, were clearly distinct from those of diamide, namely: 1. cysteine was effective on the serosal but not on the mucosal side of toad bladder; 2. cysteine did not block supramaximal concentrations of vasopressin, but markedly inhibited the effect of high concentrations (10 mM) of theophylline. Work by Frenkel et al.[12] with several sulfhy-

dryl reagents, showed that SH groups of the apical membrane of toad bladder are involved in the regulation of both basal and stimulated Na transport, but no data on water transport were reported by these authors. Conversely, Bentley[3] found that both GSH and GSSG blocked the hydrosmotic effect of oxytocin in frog bladder, but the natriferic effect of the hormone was not investigated in the presence of glutathione.

A survey of the literature concerning the action of diamide in other cell systems, shows that a perturbation of the thiol-disulfide status has been the mechanism usually invoked to explain the multiple effects induced by this agent[4-7,13-16]. Although specificity of diamide as an intracellular oxidant of glutathione has been challenged[17,18], it is widely accepted that the shift of the GSH/GSSG ratio towards oxidized glutathione is a prominent feature of diamide action. Moreover, several recent reports suggest that the perturbation of GSH homeostasis by diamide can affect microfilaments[19], intracellular Ca^{++} levels[20,21] and the assembly of microtubules[14,15,22]. A newly described effect, however, deserves particular attention here: the specific inhibition by diamide of protein kinases[23,24] which are cAMP-dependent[25]. Such an action may provide the most important clue for the understanding of the block of oxytocin, isoproterenol and theophylline reported in this work. It would imply, however, that diamide blocks the hormonal stimulus-effect coupling at a step beyond the generation of cAMP. To test this hypothesis, we examined the effect of 'external' diamide (1 mM) on exogenous cAMP and found a moderate (26%), although significant, inhibition of its natriferic action (table 1).

Diamide appears to be a unique chemical probe to study the role of intracellular and/or membrane SH-groups on a variety of transport processes. The mechanism(s) underlying the multiplicity and the sidedness of its effects in asymmetric cells are still unknown. Inhibition of kinase activity[23-25] and/or cross-linking of membrane proteins[26] are appealing lines of research deserving further investigation.

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Occurrence of an endocrine centre in the gonad of a land slug *Laevicaulis alte* (Gastropoda: Pulmonata)

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Summary. An endocrine centre consisting of club-shaped cells has been found in the gonad of a slug for the first time. It is situated in the right half of the gonad near the periphery.

Garnault[2] and Chalaux[3] noted that in snails castrated by infection, the reproductive tract becomes attenuated. Laviolette[4] confirmed this and considerably extended these results by experimental studies using various limacid and arionid slugs. The conclusion from these experiments is that the maturation of the albumen gland and the common duct is controlled by a hormone from the gonad. In *Ariolimax columbianus*, the production of tentacle spermatogenic hormone appears to be controlled by a hormone releases from the gonad[5]. But no cytological evidence for any endocrine cells has so far been found in the gonad of these gastropods. Therefore it is of interest that we have located the position of the endocrine centre in the gonad of a land slug *Laevicaulis alte*.

The slugs *L. alte* are available in great abundance in Nanjundapuram area in the vicinity of the Coimbatore city during the monsoon season. 'Susa' fixative was injected into the freshly collected large slugs to kill them and then the reproductive system was dissected out. The gonad was isolated and left in fresh fixative for 6-12 h, according to Runham and Laryea[6], and serial sections at 5-8 µm prepared by paraffin method. The sections were stained in Gomori's chrome-haematoxylin-phloxin and paraldehyde fuchsin according to modifications of Halmi and Dawson[7].