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REVERSAL OF COPPER-INDUCED INHIBITION OF VASOPRESSIN RESPONSIVENESS BY REDUCING AGENTS

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Copper inhibits the hydro-osmotic response to vasopressin in the urinary bladder of *Bufo marinus* at a site proximal to cyclic AMP production. This effect is not reversed by washing in Cu^{2+}-free Ringer's solution but is overcome by serosal addition of reducing agents, suggesting that vasopressin responsiveness in this tissue is modulated by either the redox potential or the sulfhydryl content of the serosal membrane.

Copper has been shown to inhibit both the hydro-osmotic and natriferic responses to neurohypophyseal hormones in anuran epithelial membranes when added to the serosal bathing solution. Parisi and Piccinni [1] demonstrated that copper inhibited the hydro-osmotic response to oxytocin in toad bladder and that the inhibitory effect could not be reversed by extensive washing in copper-free Ringer's solution. They also demonstrated that copper inhibited the hydro-osmotic response to theophylline but not to exogenous cyclic AMP suggesting that the inhibitory effect was at or proximal to cyclic AMP generation. Ferreira [2] demonstrated that serosal copper inhibited the natriferic response to oxytocin in frog skin, whereas mucosal copper addition has been shown by numerous investigators to increase sodium transport in this tissue [2–7]. Ferreira [8] also demonstrated that the mucosal effect can be mimicked by the sulfhydryl oxidizing agent 5,5′-dithiobis(2-nitrobenzoic acid) and can be reversed by the sulfhydryl reducing agent, dithiothreitol. The latter observations suggest that the epithilial membrane effects of copper might be mediated through oxidation of membrane sulfhydryls.

We have recently demonstrated that dithiothreitol, at higher concentrations than used in the above studies, can inhibit the hydro-osmotic water flow induced by vasopressin in the urinary bladder of the toad, *Bufo marinus*, and that these effects could be partially reversed by oxidizing agents [9]. We were therefore interested in determining if the inhibitory effects of copper on vasopressin stimulated water flow could also be reversed by reducing agents.

We first studied the effect of serosal addition of copper on the hydro-osmotic response to vasopressin in the urinary bladder of *B. marinus*. Studies were conducted by the gravimetric method of Bentley [10] with paired hemibladders incubated in toad Ringer's of the following composition (mM): NaCl, 114.4; KCl, 3.5; NaH_2PO_4, 5.0; $NaHCO_3$, 10; $CaCl_2$, 1.0, dextrose, 5.0; (250 mosM). Bladders were filled with 8.0 ml Ringer's diluted 1:5 with deionized water (50 mosM) and aerated by constant bubbling with room air through a glass port (pH 8.4–8.5).

After equilibration, copper was added to the bath for 20 min prior to stimulation with either

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vasopressin, theophylline or cyclic AMP. Serosal addition of 0.1 mM $CuSO_4$ had no effect on basal osmotic water flow (not shown) but inhibited the osmotic water response to exogenous vasopressin (10 mU/ml) and theophylline (10 mM) by 91.5 ± 0.74% and 97.9 ± 1.9%, respectively (Table I). The response to exogenous cyclic AMP was unaffected by addition of Cu^{2+}. Washing the Cu^{2+}-treated hemibladders in fresh Ringer's solution failed to restore the response to vasopressin (vidi infra) confirming the observations noted above.

Next, in order to determine if the inhibitory effect of Cu^{2+} on vasopressin responsiveness might be due to its oxidant effects, as has been noted for its 'mucosal' stimulatory effect on the short circuit current in toad skin by Ferreira [8], we investigated the ability of reducing agents to reverse the inhibitory effect. Bladders were exposed to 0.1 mM $CuSO_4$ for 20 min and then washed and resuspended in Cu^{2+}-free Ringer's solution. Vasopressin was then added simultaneously with 0.1 mM of either dithiothreitol, glutathione, cysteine, or ascorbic acid as indicated by the format in Fig. 1. This concentration of these agents had minimal effects on vasopressin responsiveness on their own as indicated in Table II. Addition of each of these agents to Cu^{2+}-treated, washed bladders resulted in a further increase in the response compared to washing alone (Table III).

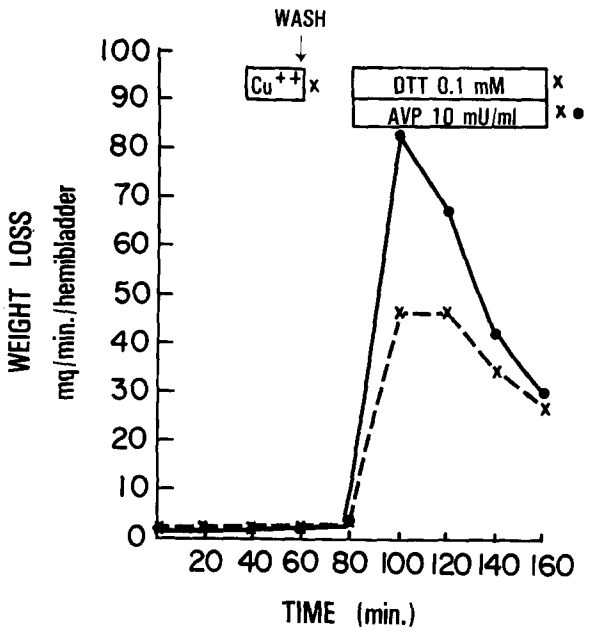

Fig. 1. Bladders were incubated with (×) or without (○) $CuSO_4$ (0.1 mM) for 20 min, washed in Cu^{2+}-free Ringer's and stimulated with vasopressin (10 mU/ml) alone (○), or plus dithiothreitol (DTT) (0.1 mM), and weight loss recorded every 20 min. The same format was used to study the ability of each reducing agent to reverse the inhibition due to Cu^{2+} (Table III) ($n=9$ pair).

TABLE I

EFFECT OF $CuSO_4$ (0.1 mM) ON OSMOTIC WATER FLOW

1.0 mM $CuSO_4$ was added to serosal bath of experimental hemi-bladders for 20 min before the addition of vasopressin, theophylline or cyclic AMP. The incremental water flow in response to these agents is compared in control and experimental hemi-bladders over 80 min. Bladders were incubated in 20 ml bicarbonate Ringer's (pH 8.4; 250 mosM) and filled with 8 ml Ringer's diluted 1 : 5 (50 mosM). Results are expressed as means ± S.E.

	N	mg/min/hemibladder	
		Control	Cu^{2+}
Vasopressin (10 mU/ml)	4	50.5 ± 4.8	8.5 ± 1.5 [a]
Theophylline (10 mM)	5	25.8 ± 7.1	0.5 ± 0.4 [a]
Cyclic AMP (10 mM)	4	4.9 ± 1.3	5.8 ± 1.9

[a] $P = < 0.05$.

TABLE II

EFFECT OF REDUCING AGENTS ON THE OSMOTIC RESPONSE TO VASOPRESSIN

Control and experimental hemi-bladders were exposed to vasopressin (10 mU/ml) alone, or vasopressin plus reducing agent (0.1 mM) administered simultaneously, and incremental water flow measured over 80 min. Although cysteine produced a slight, but significant, inhibition of the response to vasopressin there were no major effects of these agents on vasopressin response. Results are expressed as means ± S.E.

	N	mg/min/hemibladder	
		Control	Experimental
Dithiothreitol	5	45.1 ± 4.7	41.4 ± 3.2
Glutathione	4	55.8 ± 9.9	58.6 ± 3.0
Cysteine	5	49.7 ± 2.8	42.7 ± 3.4 [a]
L-Ascorbic acid	4	55.1 ± 3.2	56.1 ± 4.4

[a] $P < 0.05$.

Although the effect of ascorbic acid in reversing the inhibitory effect of Cu^{2+} was significantly less than that of any of the sulfhydryl compounds used, there were no differences noted between the abilities of the three sulfhydryl compounds used to reverse this effect at equimolar concentrations. We did not calculate the redox potential of the resultant solutions at pH 8.4–8.5 but might have expected an order of reversibility of dithiothreitol > gluthathione > cysteine > ascorbic acid, based on the known characteristics of these compounds at alkine pH [11–13]. Although we cannot speculate on why this was not observed, it should be noted that the ability to reverse the inhibitory effects of Cu^{2+} appears to depend not solely upon the presence of -SH groups but rather on the reducing potential of these agents as ascorbic acid was effective in this regard. The relative reducing potentials for dithiothreitol and ascorbic acid at pH similar to that used here are reported at −0.36 and −0.012, respectively [12,13], suggesting that their effect on Cu^{2+} might be related to their reducing capacity. We could find no reported data on the redox potentials of either glutathione or cysteine at this pH but would expect them to lie approximately midway between that of ascorbate and dithiothreitol.

To further characterize the ability of reducing agents to reverse the inhibitory effects of Cu^{2+} on vasopressin induced osmotic water flow, the effect of varying concentrations of dithiothreitol (0.001–0.1 mM) was studied (Higher concentrations were avoided as we have previously demonstrated that concentrations of dithiothreitol above 0.1 mM inhibit the hydro-osmotic response to vasopressin [9].). These studies demonstrate (Fig. 2) that the ability of dithiothreitol to reverse the inhibitory effect of Cu^{2+} are biphasic with a maximum effect between 0.01 and 0.05 mM under the conditions of this study. This suggests either several independent effects of this agent or that the optimum number of membrane-SH groups or redox state for the Cu^{2+} effect is quite narrow.

The observations reported here are similar to those of Parisi and Piccini [1] and, in addition, demonstrate that the inhibitory effects of Cu^{2+} on vasopressin-stimulated osmotic water flow can be reversed by reducing agents whether or not the reducing agent contains SH groups. The effect of ascorbic acid was approx. 50% of that obtained with the sulfhydryl compounds. That differential effects of the sulfhydryl compounds on the reversibility of the Cu^{2+} effect were not observed may have been due to the conditions chosen for the

TABLE III

EFFECT OF WASHING AND REDUCING AGENTS ON THE INHIBITORY EFFECT OF 0.1 mM $CuSO_4$ ON VASOPRESSIN-INDUCED OSMOTIC WATER FLOW

Experimental hemi-bladders were incubated with $CuSO_4$ (0.1 mM) for 20 min, then exposed to vasopressin (10 mU/ml) directly or after washing in Cu^{2+}-free Ringer's alone, or after washing plus addition of the specified reducing agent at a final concentration of 0.1 mM. Washing slightly reversed the inhibitory effect of Cu^{2+} and addition of reducing agents further reversed this effect. Results are expressed as means ± S.E.

	N	mg/min/hemibladder		% Response [c]
		Control	Experimental	
Cu^{2+}	4	50.5 ± 4.8	4.2 ± 0.1	8.5 ± 0.7
Cu^{2+} + washing	12	54.9 ± 2.3	13.0 ± 2.5 [a]	23.8 ± 3.7
+ dithiothreitol	9	52.9 ± 2.8	37.6 ± 3.3 [b]	70.2 ± 3.9
+ glutathione	7	56.2 ± 1.7	38.7 ± 3.7 [b]	69 ± 6.5
+ cysteine	8	59.3 ± 3.9	41.1 ± 2.7 [b]	69.8 ± 3.8
+ L-ascorbic acid	5	43.8 ± 3.1	17.6 ± 2.0 [b]	40.9 ± 6.0

[a] $P < 0.05$ compared to Cu^{2+}.

[b] $P < 0.05$ compared to Cu^{2+} + washing.

[c] Response = (Δ Expt./Δ Control) × 100.

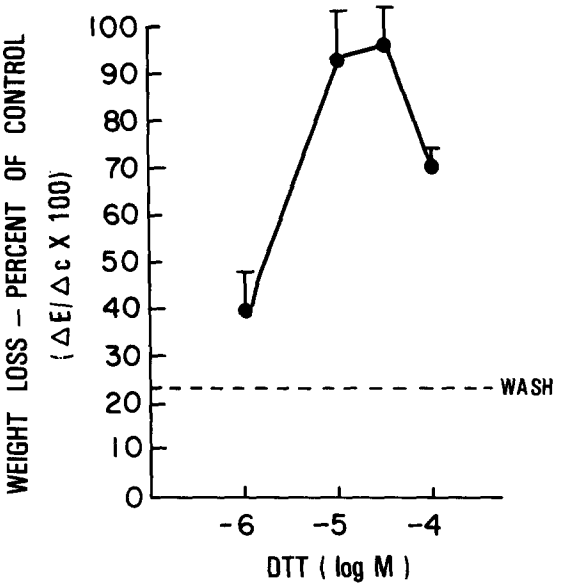

Fig. 2. Bladders exposed to 0.1 mM $CuSO_4$ for 20 min were washed in Cu^{2+}-free Ringer's and then exposed to 10 mU/ml vasopressin plus varying concentrations of dithiothreitol (DTT). DTT produced complete reversal of the Cu^{2+} induced inhibition of vasopressin at concentrations of 0.01–0.05 mM. Dashed line represents the mean response of bladders exposed to vasopressin after Cu^{2+} had been washed out and no reducing agent added (see Table III) ($n=23$ pair).

study. As can be seen in Fig. 2, the maximum effect of dithiothreitol at restoring the response to vasopressin was observed at 0.01–0.05 mM. Had we chosen a concentration at or below this for the comparative study, or performed similar titrations with the other agents, a clearer relation to reducing potential might have occurred.

We believe that these data are most consistent with Cu^{2+} acting as a membrane oxidant to inhibit the effect of vasopressin. The inhibitory effect could result from a direct effect on altering membrane SH groups or through the reduction of Cu^{2+} to Cu^{+} by membrane SH groups with subsequent auto-oxidation of Cu^{+} to generate Cu^{2+} and O_2. Evidence for such an effect of Cu^{2+} on red cell membranes has been presented by Kumar et al. [14]. We have no evidence to distinguish between these mechanisms.

In previous studies we demonstrated that higher concentrations of dithiothreitol than used here could inhibit the hydro-osmotic, natriferic, adenylate cyclase response to vasopressin and that these effects could be partially reversed by oxidizing agents [9]. Evidence exists for similar inhibitory effects with other reducing agents [15,16]. Those observations together with the current findings would suggest that the hydro-osmotic and natriferic responses to vasopressin are dependent upon an optimum state of serosal membrane oxidation or reduction, changes in either direction tending to reduce the responsiveness. It is possible that several sites may be involved in this response. Recent observations suggest that the opiate receptor may be similarly affected [17].

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