# Effects of Muscarine Agonists and Antagonists on Vasopressin-Stimulated Water Transport in the Amphibian Bladder

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The effects of $M_1$ and $M_2$ cholinoceptors on stimulated water transport in the urinary bladder of the common frog *Rana temporaria L.* are described. In the presence of pirenzepine, a selective $M_1$ cholinoceptor antagonist, carbachol stimulated water transport. Activation of $M_2$ cholinoceptors by oxotremorine in concentrations of 0.5-5.0 μM inhibited water transport, whereas their activation by this compound in higher concentrations (10-100 μM) stimulated it. The use of the phospholipase C inhibitor neomycin (0.5 mM) and the calmodulin inhibitor W-7 (1 mM) indicated that activation of $M_2$ cholinoceptors switches on phospholipid-$Ca^{2+}$-calmodulin-dependent mechanisms.

**Key Words:** *amphibian bladder; arginine vasopressin; $M_1$ and $M_2$ cholinoceptors; oxotremorine*

Information on how cholinergic substances influence antidiuretic hormone(vasopressin)-stimulated water transport is very limited. In a study using toad bladders, carbachol in concentrations of 0.1 to 1.0 mM increased the basal water flow but inhibited the water transport stimulated by arginine vasopressin (AVP) [3]; the latter effect was attributed to activation of muscarine receptors and increased entry of calcium into epithelial cells. We, for our part, have found that acetylcholine in high concentrations (e.g., 1 mM) inhibits stimulated water transport across the wall of the frog bladder, and this effect is not abolished either by atropine or by calcium blockers, but, paradoxically, is inhibited by acetylcholinesterase inhibitors. Hence the most plausible explanation for this effect of acetylcholine may be its interaction with acetylcholinesterase which, as shown in our studies [1,2], is present in the bladder epithelium of the frog *Rana temporaria L.* Recently, phosphoinositides have been found to play a role in the inhibition of AVP-stimulated water transport by carbachol [13]. A study with cultured renal tubule cells revealed that the suppression of AVP-stimulated water transport by muscarine agonists is associated with activation of $M_3$ cholinoceptors [9]. However, the type of muscarine receptors in amphibian bladders (classic objects for studying the intracellular mechanisms of action of antidiuretic hormone) has not been identified and it remains unknown how these receptors interact with the intracellular mediators of this hormone. Accordingly, the purpose of our present study was to determine the type of M cholinoceptors in the bladder epithelium and how they are coupled with phospholipase C and adenylate cyclase, as this coupling largely determines the biochemical specificity of muscarine receptor subtypes.

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## MATERIALS AND METHODS

The study was carried out during autumn and winter on bladders of common frogs (*Rana temporaria*

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*L.*). The bladders were dissected out as described by Bently [5] from frogs with disintegrated brain and spinal cord and incubated at room temperature in an aerated Ringer's solution of the following composition (mM): 112 $Na^+$, 113 $Cl^-$, 5.7 $K^+$, 0.89 $Ca^{2+}$, 0.6 $H_2PO_4^{2-}$, and 2.4 $HPO_4^{2-}$ (pH 7.3, 220 mosmol). The magnitude of osmotic water flows was estimated gravimetrically, at a 176 mosmol/liter concentration gradient of osmotically active substances between the incubation medium and the bladder contents, and expressed in μl per $cm^2$ of bladder wall over a 60-min incubation period. Cholinergic substances were added to one half of the bladder after a 60-min control period at 30 min before an agent stimulating the osmotic flow was added to that half. Antagonists were added to the medium 15 min before the addition of agonists. The other half of all bladders was used as the control. The osmotic water flow was stimulated with arginine vasopressin ([$Arg^8$]-vasopressin; Sigma). Carbachol (Sigma) was used as a nonselective ligand of M cholinoceptors and oxotremorine (Aldrich) as a selective agonist of $M_2$ receptors. Selective antagonists of $M_1$, $M_2$, and $M_3$ cholinoceptors were, respectively, pirenzepine, AF-DX 116, and 4-DAMP (all from RBI). Neomycin and W-7 (both from Sigma), respectively, were used as phospholipase C and calmodulin inhibitors. The results were analyzed with Student's *t* test.

## RESULTS

In the absence of pirenzepine, a selective exogenous $M_1$ receptor antagonist, carbachol (0.1 mM) acted on the AVP (0.3 μM)-stimulated water transport in a random fashion; it could either activate or inhibit this transport, but typically it had little or no effect on it (Fig. 1, *a*, *1*). In the presence of pirenzepine (0.1 μM), carbachol invariably increased the water flow (Fig. 1, *a*, *2*).

To find out to what extent the enhancement of AVP-stimulated water transport was due to the dominance of muscarine receptors other than $M_1$, we measured the effects of oxotremorine, which displays preferential affinity for $M_2$ receptors in a number of biological objects [7]. As depicted in Fig. 2, oxotremorine inhibited water transport in concentrations of 0.5 to 5 μM and enhanced it in concentrations of 10 to 100 μM. These opposite effects were both blocked by the $M_2$ antagonist AF-DX 116 [10], which confirms that the effects were associated with activation of $M_2$ cholinoceptors. However, since the selectivity of oxotremorine and AF-DX 116 is not absolute [6], we also tested oxotremorine for its effects on water transport in the presence of the $M_1$ and $M_3$ cholinoceptor antagonists pirenzepine and 4-DAMP and found that both were ineffective.

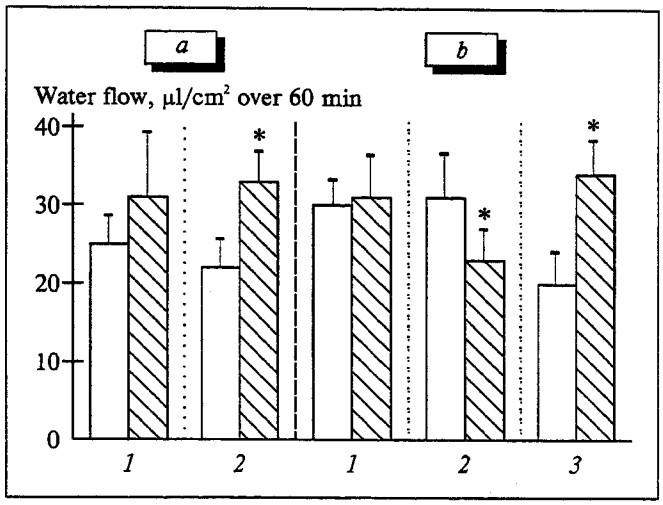


**Fig. 1.** Effects of carbachol (100 μM, *a*) and physostigmine (1 μM, *b*) on AVP−stimulated water transport across the frog bladder wall. *White bars*: control samples; *black bars*: test samples (with added carbachol in *a* and with added physostigmine in *b*). *1*) AVP; *2*) AVP+pirenzepine; *3*) AVP+AF−DX 116. $^*p<0.05$; $n=4$.

Which of the two oxotremorine effects (inhibitory or stimulatory) on water transport across the bladder wall reflects the physiological role of $M_2$ receptors in the bladder epithelium? To answer this question, we sought to find out how physostigmine, an acetylcholinesterase inhibitor, would influence the intrinsic cholinergic system of the bladder. Physostigmine (1 μM), like carbachol, had variable effects on the AVP-stimulated water transport but, unlike carbachol, inhibited rather than enhanced it in the presence of pirenzepine. Preincubation of bladders with AF-DX 116, however, led to stimulation of the water transport by phys-

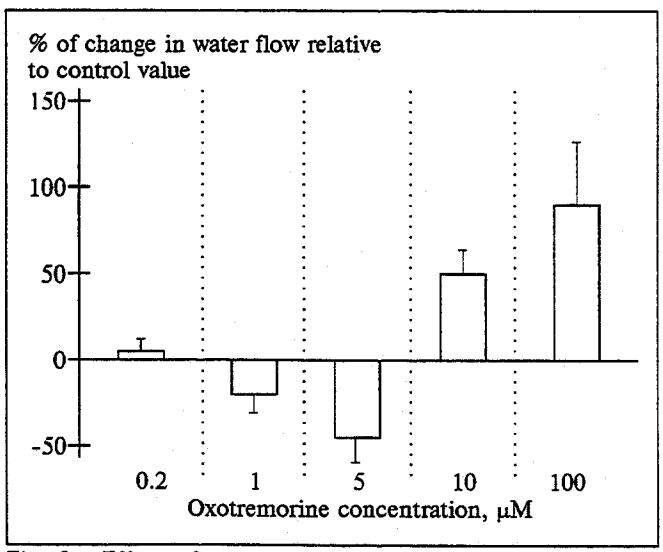


**Fig. 2.** Effect of oxotremorine on AVP−stimulated water transport across the frog bladder wall.

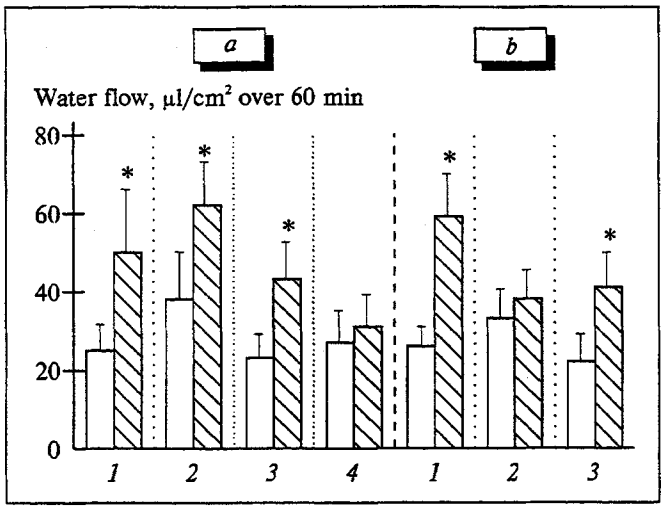


**Fig. 3.** Analysis of the activating action by oxotremorine (100 μM) on stimulated water transport across the frog bladder wall. *White bars*: control samples; *black bars*: test samples (with added oxotremorine). *a*: *1*) AVP; *2*) AVP+4−DAMP; *3*) AVP+pirenzepine; *4*) AVP+AF−DX 116. *b*): *1*) AVP; *2*) AVP+neomycin; *3*) AVP+W−7. *$p<0.05$; $n=4-6$.

ostigmine (Fig. 1, *b*, *3*). These findings indicate that $M_2$ cholinoceptors inhibit water flow across the bladder wall.

The inhibitory action of $M_2$ cholinoceptors has been associated with the inhibition of adenylate cyclase by the inhibitory G protein, with which these receptors are coupled [4]. However, these receptors may also be coupled with phospholipase C, whose activation stimulates phosphoinositide metabolism, and may be involved in raising $Ca^{2+}$ levels in the cells [12]. As a result, calmodulin and the calmodulin-dependent adenylate cyclase may be activated. The elevation of cAMP in the cell caused by stimulation of this (type 1) adenylate cyclase is abolished by W-7, a calmodulin inhibitor [8]. It has been shown that $M_2$ receptor activation resulting in stimulation of phosphoinositide metabolism can be induced by oxotremorine [11].

To determine the contributions of phospholipase C and calmodulin to the water transport-stimulating effect of oxotremorine, we used the phospholipase inhibitor neomycin (0.5 mM) and the calmodulin inhibitor W-7 (1 μM). Neomycin abolished this effect completely and W-7 decreased it by half. Hence the enhancement of the antidiuretic effect by oxotremorine can be associated, in part at least, with activation of the calmodulin-sensitive adenylate cyclase.

This study has provided evidence that the bladder epithelium of *R. temporaria* possesses $M_1$ and $M_2$ cholinoceptors via which the cholinergic system can exert complex effects, including activation and inhibition, on the chain of intracellular reactions initiated by antidiuretic hormone. The end result apparently depends both on the ratio of sensitivity thresholds between the M cholinoceptor subtypes and on how the receptors of different subtypes are coupled with adenylate cyclase and phospholipase C. Pinpointing the mechanisms responsible for the effects described above is one of the aims of our future research.

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