curs from a cytoplasmic transmitter store rather than from a vesicle-bound store. In contrast, the extra release of transmitters induced by stimulation with K^+ (4.0 nmole of [^{14}C]AHCh and 0.5 nmole of ACh) had a ratio of 8.1 ± 0.8 (Table 1), close to that of the P_3 fraction and clearly different from that of the S_3 fraction; this result strongly suggests that evoked release originates from vesicle stores and not from cytoplasmic stores. Either omission of Ca^{2+} or elevation of Mg^{2+} reduced the evoked release of [^{14}C]AHCh, but not of ACh (Table 2). Neither treatment affected the spontaneous release of [^{14}C]AHCh [2.7 ± 0.1 versus 2.4 ± 0.1 nmole/g per 5 minutes (N = 8), and 2.2 ± 0.3 versus 1.6 ± 0.2 nmole/g per 5 minutes (N = 13), respectively] or of ACh (data not shown), a result which also suggests that the spontaneous release of cholinergic transmitter occurs from a different subcellular pool than the evoked release.

Treatment of brain tissue in lithium–high potassium Krebs solution, as compared to treatment of brain tissue in normal Krebs solution, increases the ratio of [^{14}C]AHCh to ACh markedly in the P_3 (0.46 to 7.3) and increases this ratio to a similar extent (0.28 to 8.1) when transmitter release is stimulated by K^+, without appreciably increasing the ratio in the S_3 (0.26 to 0.44). This result provides additional evidence that the K^+-induced release of cholinergic transmitter occurs from the vesicle-bound fraction.

We used a lithium–high potassium Krebs treatment of mouse brain tissue and homocholine to distinguish the transmitter content of the cytoplasmic and vesicle-bound fractions before release. This treatment selectively lowers the ACh content of the vesicle-bound fraction, possibly by depolarizing the tissue and releasing vesicle-bound ACh while simultaneously blocking the transport of extracellular precursor essential for refilling this fraction with acetylated product. Subsequent incubation of the treated tissue in normal Krebs with either [^{14}C]choline or [^{14}C]homocholine augments accumulation of the precursors by the P_3 fraction, independently of the S_3 fraction (*7*). The precursors may then be acetylated by choline *O*-acetyltransferase (E.C. 2.3.1.6) associated with this fraction (*9*, *10*) to achieve a ratio of [^{14}C]ACh to ACh or of [^{14}C]AHCh to ACh that is higher in the P_3 fraction than in the S_3 fraction (*7*). Homocholine is similar to choline in most respects [transport, acetylation by intact tissue, and release as an acetylated product (*11*, *12*)], but differs from choline in one very important respect. It is not acetylated by soluble choline acetyltransferase (*9*, *12*, *13*), believed to exist in the cytoplasm (*14*). Therefore, the high ratio of [^{14}C]AHCh to ACh achieved in the P_3 fraction during repletion may be due to its exclusive acetylation by membrane-bound choline acetyltransferase. The relatively low ratio of [^{14}C]AHCh to ACh that is simultaneously achieved in the S_3 fraction may be due to the inability of soluble choline acetyltransferase in the cytoplasm to acetylate homocholine.

This study confirms the predictions made by others (*5*) that the spontaneous release of cholinergic transmitter occurs from the cytoplasm independently of extracellular Ca^{2+}, whereas the evoked release of cholinergic transmitter occurs from the vesicle-bound fraction by a Ca^{2+}-dependent process.

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Oral Contraceptives, Lanosterol, and Platelet Hyperactivity in Rat

Abstract. *Lanosterol, a cholesterol precursor that increases considerably in the platelets of rats treated with oral contraceptives, was incubated with either platelet-rich plasma or washed platelet suspension. After 2 minutes there was a remarkable dose-related increase in platelet activity. This platelet hyperactivity as measured by clotting time and platelet aggregation could not be reproduced by cholesterol or ethinylestradiol.*

An increase in the activity of several plasma factors has been noted in women taking oral contraceptives (*1*) but, apparently, the mechanism of this enhanced activity has not been elucidated. It is even a matter of controversy whether the level of these plasma factors has clinical significance (*2*). Our group has shown (*3*, *4*) that the hypercoagulability resulting from oral contraceptives might depend essentially on an increase in the clotting activity of platelets.

There are also conflicting reports concerning the effect of oral contraceptives

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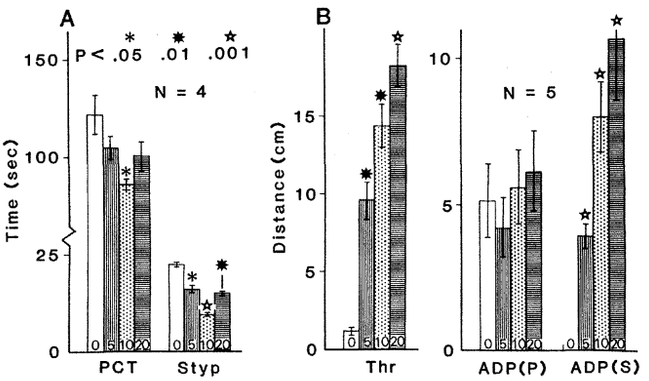

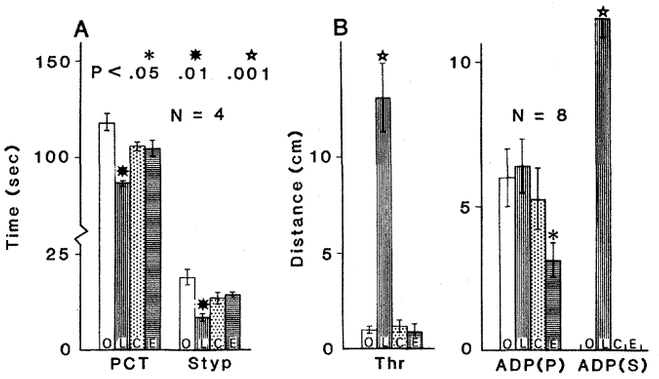

Fig. 1 (left). Effects of lanosterol on (A) coagulation and (B) platelet aggregation. (A) Platelet-rich plasma (0.1 ml) was mixed with saline (0.95 percent, 0.1 ml) and incubated for 10 minutes at 37°C. In the last 2 minutes of incubation, 4 μl of ethyl alcohol alone or containing lanosterol at the concentrations shown (5, 10, or 20 μg) was added. The test was then started by adding 0.1 ml of 0.04*M* $CaCl_2$; *Styp*, Stypven time, a test similar to the plasma clotting time (*PCT*) except that the $CaCl_2$ solution (0.025*M*) contained Russell's viper venom (1/100,000). (B) A washed platelet suspension (0.5 ml, in Tyrode's solution containing Mg^{2+} and gelatin, *p*H 7.4) was incubated for 2 minutes at 37°C with either alcohol alone or alcohol containing lanosterol, as above. The aggregating agents thrombin (*Thr*) or ADP were then added in complete Tyrode's solution. The amount of thrombin (bovine, Sigma) used was 0.17 unit (NIH) per milliliter of platelet suspension. The ADP was a sodium salt (Sigma) having a final concentration in the platelet suspension of $0.8 \times 10^{-6}M$. Primary aggregation is shown by (*P*) and secondary aggregation by (*S*); *N* is the number of experiments; the results are shown as means ± standard error. Fig. 2 (right). The effects on coagulation and aggregation of lanosterol (*L*), cholesterol (*C*), and ethinylestradiol (*E*) diluted at the same concentration (10 μg) in ethyl alcohol. The experiments were performed as in Fig. 1.

on platelet aggregation (*5*). In female rats we found that a marked platelet hyperaggregability could be induced in 4 days by the administration of an oral contraceptive (*6*). This hyperaggregability did not seem to be due to changes in the fatty acid composition of the platelet phospholipids (*6*), although we observed a marked increase in the platelet lipid synthesis of contraceptive-treated rats (*7*). We also found that the substances most actively synthesized in the platelets of rats receiving the contraceptive (or the estrogen alone) were lanosterol and dihydrolanosterol (*8*).

Our purpose in the present study was to determine, by incubating these substances in vitro with platelet-rich plasma (PRP) or washed platelets, whether these cholesterol precursors might induce the hypercoagulability and hyperaggregability observed in female rats treated by an oral contraceptive.

For each experiment, PRP (*9*) from three to four fasted female rats (Sprague-Dawley, 250 to 300 g) was pooled. The plasma clotting time (PCT) and the Stypven time were measured as before (*4*), except that 4 μl of either ethyl alcohol alone or of alcohol containing lanosterol (Sigma), cholesterol (Calbiochem), or ethinylestradiol (ICN Nutritional Biochemicals) was added to the diluted PRP (0.1 ml of PRP plus 0.1 ml of 0.95 percent NaCl) in the last 2 minutes of the incubation period at 37°C.

Aggregation tests were performed on platelets washed as in previous studies (*8*) except that we replaced the gelatin by albumin in the Tyrode's solution as described by other investigators (*10*). The alcohol alone (4 μl), or containing the above-mentioned agents, was added 2 minutes before initiating aggregation by thrombin or adenosine diphosphate (ADP) diluted in complete Tyrode's solution.

Addition of three different concentrations of lanosterol to PRP induced a marked shortening of both the PCT and the Stypven time, the maximum effect being obtained with 10 μg (100 μg per milliliter of PRP) of lanosterol (Fig. 1).

Lanosterol had a significantly greater effect on thrombin aggregation, the greatest effect being obtained with the highest concentration (20 μg corresponding to 40 μg per milliliter of platelet suspension).

With ADP-induced primary aggregation, there was no significant increase in the platelet response. However, a secondary response to ADP could be observed, the extent of which also appeared to depend on the concentration of lanosterol (Fig. 1).

Figure 2 shows that at the concentrations used (10 μg), only lanosterol significantly increased the reactivity of platelets in the two series of tests. By contrast, ethinylestradiol significantly reduced the response of platelets to ADP.

Consequently, it seems that lanosterol (alone or combined with dihydrolanosterol since it was a contaminant of the lanosterol) might be responsible for the marked increase in the activity of platelet functions observed in female rats treated with an oral contraceptive (*6*, *7*). After stimulation with ADP, lanosterol can even induce rat platelets to exhibit secondary aggregation, a phenomenon observed with human platelets but never before with rat platelets, at least under our conditions. We also noted that aspirin inhibited the lanosterol-induced secondary aggregation.

The acceleration of PCT in the current study suggests our treatment modified the platelet clotting activity, an effect previously observed under many circumstances (*3*, *4*, *11*). A similar shortening of the Stypven time lends weight to the postulation that lanosterol acts primarily at the level of platelets to accelerate clotting.

An increase in the ratio of cholesterol to phospholipid in the platelet membrane after a 5-hour period of incubation with cholesterol has been shown to be associated with hyperaggregability (*12*). Under our conditions, after 2 minutes of incubation, only lanosterol was able to induce hyperaggregability in rat platelets. It has also been found, in artificial membranes, that the permeability of vesicles containing lanosterol is greater than that of vesicles containing cholesterol (*13*). Thus, it seems feasible that lanosterol might be much more potent than cholesterol in stimulating the platelet membrane activities responsible for both coagulation and aggregation.

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Somatostatin: Occurrence in Urinary Bladder Epithelium and Renal Tubules of the Toad, *Bufo marinus*

Abstract. *Immunohistochemical techniques were used to detect immunoreactive somatostatin-like material in toad urinary bladder epithelium and in kidney distal tubules and collecting ducts. This material has immunological and chromatographic properties identical to those of synthetic cyclic somatostatin. The occurrence of somatostatin-like material in antidiuretic hormone-sensitive portions of the renal urinary system suggests a local regulatory or paracrine role for somatostatin.*

The recent demonstration that somatostatin inhibits the effects of vasopressin on water transport in toad urinary bladder (*1*) and that this tissue contains relatively large amounts of immunoreactive somatostatin led Forrest and his colleagues to propose that this peptide is an intrinsic regulator of epithelial transport in toad bladder (*1a*). Somatostatin, a tetradecapeptide originally identified as a hypothalamic hypophysiotropic hormone that inhibits secretion of growth hormone (*2*), is widely distributed in the extrahypothalamic central nervous system, the peripheral nervous system, various tissues of the gastroenteropancreatic system (*3*, *4*), and thyroid tissue (*5*), but has not been consistently measured in rat kidney (*3*, *4*, *6*). The dual neuronal and gastrointestinal distribution of somatostatin has been demonstrated in all vertebrate classes (*7*).

In the gastrointestinal tract and pancreas, somatostatin-secreting cells have been found adjacent to other types of secretory cells whose function is influenced by somatostatin. This observation led to the postulation that such somatostatin-secreting cells outside the brain exert a local, "paracrine," effect on neighboring cells (*8*). In this study we demonstrated that somatostatin is present in the layer of toad urinary bladder epithelium that is responsive to antidiuretic hormone. Further, because somatostatin influences water resorption in mammalian kidney (*9*), we measured somatostatin in kidney by immunoassay and demonstrated its presence immunohistochemically in collecting ducts and distal tubules (but not in proximal tubules or glomeruli). The presence of this peptide modulator in renal tissue, when considered together with evidence that somatostatin impairs the hydroosmotic response to antidiuretic hormone in toad urinary bladder (*1*) and inhibits the response to this hormone in the mammalian kidney in vivo (*9*), suggests that somatostatin-like material may have a paracrine role in the renal-urinary tract.

Urinary bladders and kidneys were quickly excised from decapitated *Bufo marinus* (*10*), rinsed in 200 m*M* NaCl, and frozen on dry ice for immunofluorescence processing or fixed for 2 to 4 hours in 10 percent neutral buffered formaldehyde for peroxidase-antiperoxidase (PAP) immunohistochemistry (*11-13*). Acetic acid extracts of tissue homogenates were lyophilized and reextracted with acetone-petroleum ether (*14*). Procedures for Biogel P4 chromatography, protein determination, and somatostatin radioimmunoassay have been described (*15*).

Immunoreactive somatostatin was readily demonstrated by immunofluorescence in the epithelial and subepithelial layer of urinary bladder (Fig. 1a). Concentrations of antibody to somatostatin ranged from 1:50 to 1:200. Fluorescence was most intense in the epithelial layers, where it was present in virtually all cells, and was less intense in the subepithelial layer. Immunoreactivity was eliminated by preabsorption of the antiserum with synthetic somatostatin. In the kidney, a distinct pattern of immunoreactive somatostatin was observed in many tubules (Fig. 1b). As in the bladder, immunofluorescence was eliminated by preabsorption with synthetic somatostatin.

Immunohistochemical preparations with the PAP technique confirmed that immunoreactive somatostatin was distributed in the urinary bladder epithelium. In the kidney, positive reaction product was concentrated in most collecting ducts and many distal tubules (Fig. 1c). Positive staining was observed at antibody to somatostatin titers between 1:200 and 1:1000, but staining was negative at an antibody dilution of

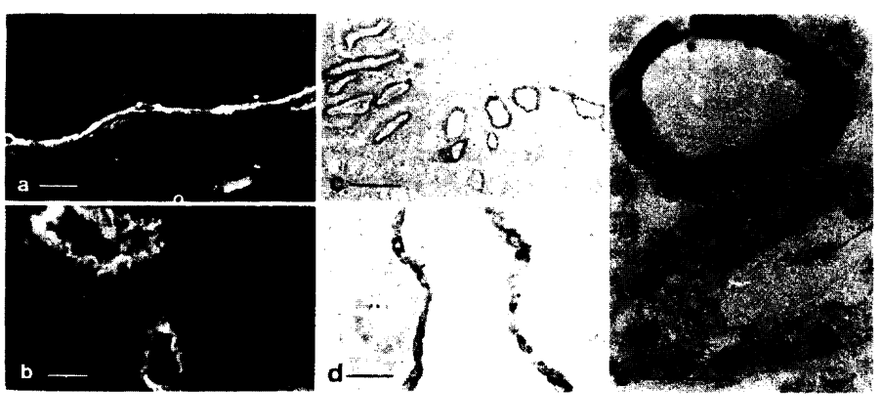

Fig. 1. (a and b) Fluorescence micrographs of toad urinary bladder (a) showing immunoreactive somatostatin in the epithelial layer and of toad kidney (b) showing somatostatin in two tubules, with many nonfluorescing tubules. (c, d, and e) Peroxidase-antiperoxidase visualization of somatostatin in kidney distal tubules and collecting ducts, with many somatostatin-negative proximal tubules; (d) shows somatostation-positive collecting duct and somatostatin-negative proximal tubule (left of duct) and glomerulus (right of duct); and (e) shows a tubule with dense cytoplasmic immunoreactivity (upper part of photo) in contrast to a complete absence of immunoreactivity in the proximal tubule (lower part of photo). Scale bars: 25 μm in (a), (b), (d), and (e); 100 μm in (c).

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