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Is glandular formation of nitric oxide a prerequisite for muscarinic secretion of fructose in the guinea-pig seminal vesicle?

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Abstract The significance of nitric oxide (NO) formation in seminal secretion was studied in guinea-pig seminal vesicles. The nitric oxide synthase (NOS) activity was estimated and reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry was performed. Furthermore, cyclic guanosine 3,5-monophosphate (cGMP) concentration as well as fructose secretion from isolated vesicles was estimated. High Ca^{2+}-dependent NOS activity as well as prominent glandular NADPH-diaphorase staining was found in the secretory epithelium. The NOS inhibitors N^G-nitro L-arginine methyl ester (L-NAME) and N^G-nitro L-arginine (L-NNA) inhibited carbachol-induced fructose secretion but the D-isomer to L-NAME had no effect. When L-arginine was administered together with L-NAME, no inhibitory effect on the carbachol-induced fructose secretion could be seen. Nerve-induced fructose secretion was also inhibited by L-NAME. The NO donor glyceryl trinitrate (GTN) increased the fructose secretion. Carbachol or GTN did not increase cGMP levels, nor was fructose secretion inhibited by a guanylate cyclase inhibitor (ODQ). Our results suggests that glandular NO production is a prerequisite for muscarinic fructose secretion in the seminal vesicle via a cGMP-independent pathway.

Key words Cholinergic nerves · Glandular cells · Nitric oxide · Secretion · Seminal vesicle

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Introduction

Nitric oxide (NO) is a gaseous molecule involved in many different physiological processes. NO seems to act as a neurotransmitter causing smooth muscle relaxation by activating guanylate cyclase and increasing cyclic guanosine 3,5-monophosphate (cGMP) in the lower urinary tract in several species, including man [2] as well as in the upper urinary tract [10]. Furthermore, NO seems to be the final mediator of penile erection in man and other mammals [16]. NO is formed from the terminal guanidino group of L-arginine and oxygen by the enzyme NO synthase (NOS) with equimolar production of L-citrulline. NOS is a reduced form nicotinamide-adenine dinucleotide phosphate (NADPH)-diaphorase (NADPH-d) [22] and exists in different isoforms: constitutive Ca^{2+}-dependent and inducible Ca^{2+}-independent [12].

Ca^{2+}-dependent NOS activity has been found both in the human upper and lower urinary tract and also in the male reproductive organs, where the highest activity was found in the seminal vesicle although the activity in vas deferens, prostate and corpus cavernosum is also high [7]. Furthermore, we have demonstrated NADPH-d activity in secretory cells from essentially every part of the male reproductive tract of human and guinea-pig [18]. The aim of this study was to obtain further details of the localization of NOS and to define the possible function of glandular NOS and NO formation in the seminal vesicle. Furthermore, the possible involvement of the guanylate cyclase/cGMP pathway was also studied. Neuronal regulation of exocrine secretion in the male genital tract has always been difficult to study, but we have recently introduced a technique where the isolated everted seminal vesicle from guinea-pig serves as a "model organ" with fructose as a marker of secretion [15]. Thus, the glandular cells have a secretomotor innervation by short sympathetic cholinergic neurones with a preganglionic supply via the hypogastric nerve. The innervated excitatory receptors are muscarinic,

whereas adrenoceptors are inhibitory and either humoral and glandular (α_1 and β_2) or prejunctional (α_2). In this report carbachol or postganglionic nerve stimulation was used to excite the glandular muscarinic receptors. A preliminary account of the results has been presented [17].

Materials and methods

Male guinea-pigs weighing 570–1300 g were used. Ten days before the experiment they were injected with 125 mg testosterone enantate in 0.5 ml castor oil, and 25 mg testosterone propionate in 0.5 ml sesame oil, i.m. in the groin. At the experiment the animals were killed by stunning and bleeding and the seminal vesicles were dissected free and removed. The experiments followed the principles of laboratory animal care of the NIH and were approved by the regional board for animal care experimentation (acts no 216/92 and 126/96).

NOS activity

The seminal vesicles were everted, rinsed in Ringer solution (4 °C) and blotted. Half of the seminal vesicle of one side was separated for determination of NOS activity in "whole organ". In the rest of the seminal vesicles the secretory linings were separated from the compact muscular coat by scraping with the blunt side of a fine scalpel. Finally, possible remnants of the secretory mucosa were rubbed off the muscle coat with a cotton swab. (The efficiency of the separation was verified by light microscopy on Bouin-fixed material stained with trichomatic stain.) Tissue specimens ("whole organ" 110–330 mg, secretory linings 110–330 mg, muscle coat 220–330 mg) were immediately frozen in liquid nitrogen after preparation and stored at −70 °C. To determine the NOS activity the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline was studied as previously described [7].

Histochemistry

The nitroblue NADPH-based technique [21] was used for demonstration of NADPH-d positive structures. The thiocholine method according to Karnowsky and Roots [11] was used for demonstration of acetylcholinesterase (AChE) positive nerves. The details have been described [18].

Fructose secretion study

The method and the calculations has been outlined in detail previously [15]. In short: the seminal vesicles were everted and placed in two different 25-ml organ baths containing Tyrode solution, kept at 36°C and aerated with 5% CO_2 and 95% O_2. After immersion for 85 min and five changes of Tyrode solution, each seminal vesicle was used for four sampling periods of 10 min during which the organ was bathed in glucose free Tyrode solution which was then collected for analysis of fructose. Between each collecting period there was a 20-min resting period in Tyrode solution with glucose. The first, second and fourth sampling periods were used for determination of the basal secretion level and exposure to drug or nerve stimulation was performed during the third period. The fructose content of the collected fluid was analysed and fructose secretion was calculated and expressed as the percentage change from resting secretion level [15]. Carbachol (550 µM) or stimulation of the distal part of the hypogastric nerve was used to induce the secretion of fructose. The secretory response to carbachol is blocked by scopolamine but not by hexamethonium, mecamylamine, tetrodotoxin or destruction of the neurones innervating the secretory cells and hence muscarinic [15]. When nerve stimulation was performed, platinum electrodes (2 mm between the rings) were placed around the hypogastric nerve at a distance of about 5 mm from the seminal vesicle, i.e. the stimulation was postganglionic [15]. The electrodes were immersed in the bath fluid. Voltage was 25, stimulus duration was 0.5 ms and pulse train 10 s every 30 s at 10 Hz. Square wave pulses were delivered from a Grass S44 stimulator (Grass CO, Quincy, Mass.). When NOS inhibitors were added, the inhibitor was administered to one of the two seminal vesicles from the same animal, 20 min before the collecting period and at the start of the test period, while the other vesicle was used as control. We also compared the effect of carbachol with the NO donor glyceryl trinitrate (GTN) and a combination of carbachol and GTN. The guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) was solved in dimethylsulfoxide (DMSO). Similar amounts of solvent was added to the controls.

Cyclic GMP content

Secretory linings were separated from the seminal vesicles at end of the test period in the organ bath (see above) and were immediately frozen. The tissue was homogenized in ice-cold 50 mM TRIS buffer and incubated for 20 min in buffer containing 50 mM TRIS, 2 mM DL-dithiothreitol, 10 mM $MgCl_2$, 5 mM guanosine triphosphate (GTP) and 20 µM isobutylmethylxantine (IBMX), after which it was heated to 100°C for 15 min. The homogenate was centrifuged at 10 000 g for 30 min at 4°C, and the soluble fraction was used for the measurement of cGMP by a specific radioimmunoassay using a commercially available kit (Amersham, UK).

Statistics

Values are given as means ± SEM. Student's *t*-test for unpaired observations was used to analyse the differences in NOS activity and cGMP content. Student's *t*-test for paired variables was used to compare the fructose secretion in the seminal vesicles given NOS inhibitors versus the controls and guanylate cyclase inhibitor, respectively. Student's *t*-test for paired variables was also used to compare the effect of GTN, carbachol and carbachol/GTN with the basal secretion. Values of $P < 0.05$ were considered statistically significant.

Drugs

The following drugs were used: acetylthiocholine iodide, aprotinin, carbamylcholine chloride (carbachol), DMSO, Dowex AF 50W-

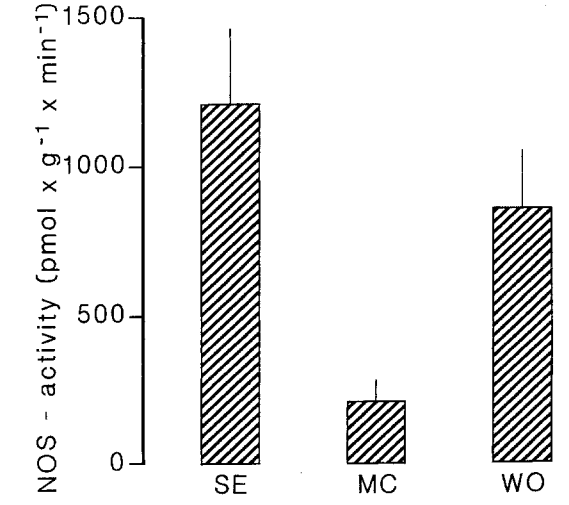

Fig. 1 Calcium-dependent nitric oxide synthase (*NOS*) activity in the guinea-pig seminal vesicle as measured by citrulline formation (pmol × min^{-1} × g^{-1}). Both secretory epithelium (*SE*) and whole organ (*WO*) showed significantly higher citrulline formation compared with the muscular coat (*MC*). $P < 0.01$, mean ± SEM, $n = 5$ in all columns

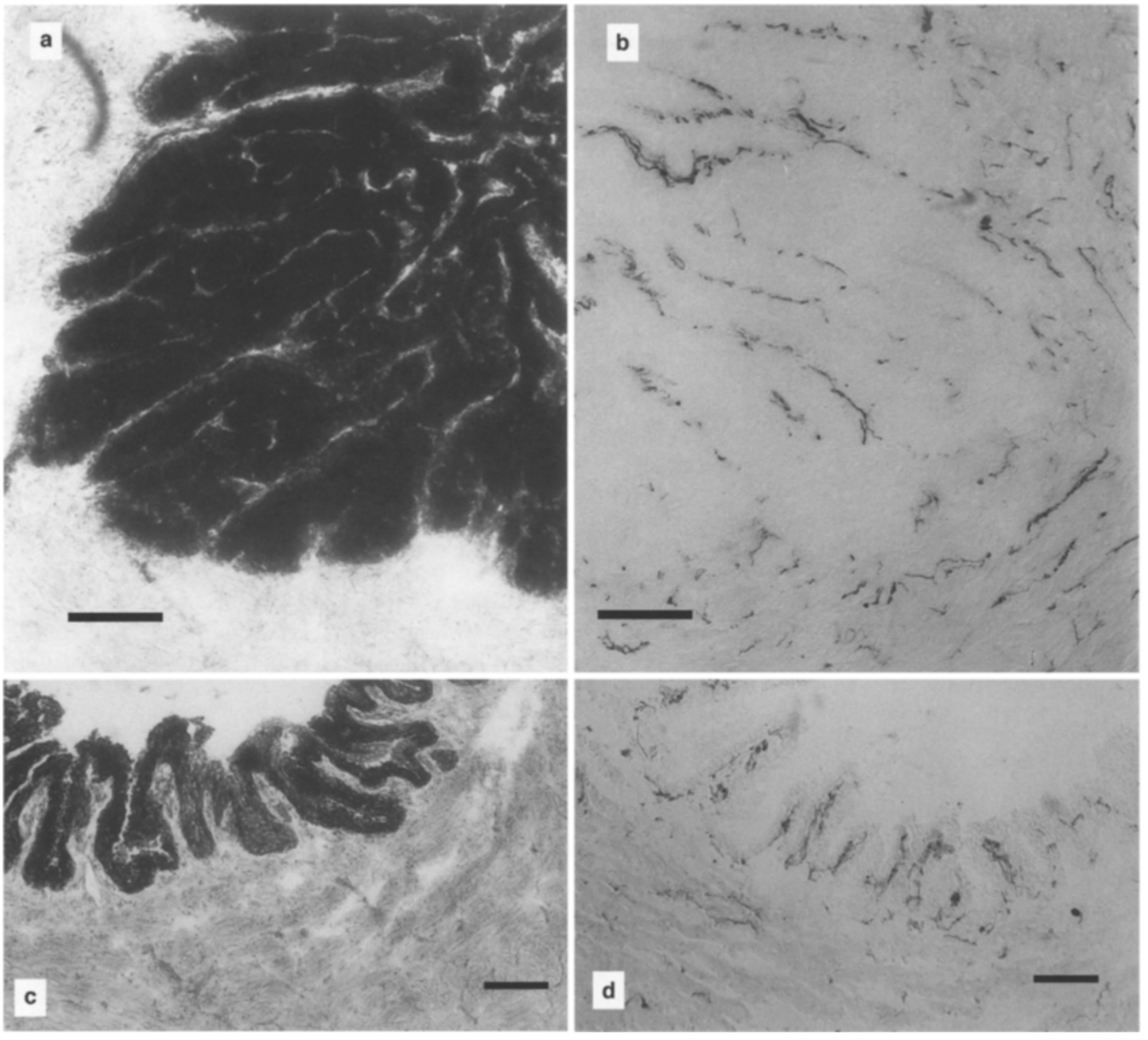

Fig. 2a–d Reduced form nicotinamide-adenine dinucleotide phosphate-diaphorase (NADPH-d) and acetylcholinesterase (AChE) stainings of the guinea-pig seminal vesicle. **a** NADPH-d staining is prominent in the secretory cells of the mucosal folds. Section from base of the seminal vesicle. **b** AChE staining of a reversed parallel section from the same tissue block. Darkly stained nerve fibres are present beneath the secretory cells in the folds of the mucosa. Fibres are also seen in the muscle layer. **c** Strongly NADPH-d positive glandular cells are still present in the neck of the seminal vesicle although the folds are much smaller. **d** AChE positive fibres are seen in the narrow folds of a reversed parallel section. **a** and **b** are unfixed sections, **c** and **d** are sections fixed in formaldehyde before colouring. Scale bars represent 100 μM

X8, DL-dithiothreitol, guanosine triphosphate, IBMX, isoompa, L-arginine, L-citrulline, L-valine, N^G-nitro L-arginine methyl ester (L-NAME), N^G-nitro L-arginine (L-NNA), NADPH, nitroblue, phenylmethylsulfonyl fluoride, Triton-X-100, TRIS buffer, trypsin inhibitor (all Sigma Chemical Co., St Louis, Mo.); N^G-nitro D-arginine methyl ester (D-NAME, Bachem, Bubendorf, Switzerland); GTN, nitroglycerin (Tika, Lund, Sweden); copper sulfate, formaldehyde, hydrochloric acid, potassium ferricyanide, resorcinol (Merck, Darmstadt, Germany); L-[U-^{14}C]arginine (150 000 dpm, Amersham); leupeptin (Peninsula Labs, USA); N^G-monomethyl-L-arginine (L-NMMA, Wellcome Research Laboratories, UK); Testoviron-Depot (Schering); ODQ, (Tocris Cookson, Bristol, UK); and testosterone propionate in sesame oil from the pharmacy of Karolinska Hospital.

Results

NOS activity

The NOS activity in the seminal vesicle was Ca^{2+}-dependent and was almost 6 times higher in the secretory epithelium than in the smooth muscular coat (Fig. 1). Both secretory epithelium and whole organ showed

significantly ($P < 0.01$) higher citrulline formation than the smooth muscle. Relative figures were 5.6 : 4 : 1 respectively. No Ca^{2+}-independent NOS activity could be found under the present experimental conditions.

Histochemistry

The secretory cells showed prominent staining for NADPH-d (Fig. 2a,c). Vascular endothelium was also stained and in about 10% of the sections a few nerves beneath the secretory cells and in the smooth muscle were also stained. Beneath the secretory cells and in the folds of the secretory mucosa strong staining of AChE positive terminals was evident (Fig. 2b,d). Such fibres were also present in the lamina muscularis. In a few sections AChE co-localized with NADPH-d in some nerves [18].

Organ bath experiments

There was a significant increase in fructose secretion after application of carbachol (550 μM) (Fig. 3a–d). The NOS inhibitors L-NAME (100 μM) and L-NNA (50 μM) inhibited the carbachol-induced fructose secretion (Fig. 3a,d). Simultaneous addition of high concentration (2 mM) of the NOS substrate L-arginine blocked the effect of L-NAME (Fig. 3b). Furthermore, the D-isomer analogue to L-NAME, D-NAME, had no significant effect on carbachol-induced fructose secretion (Fig. 3c). Hypogastric nerve stimulation caused a marked increase in fructose secretion (Fig. 3e). After application of L-NAME (100 μM), the secretory response was blocked (Fig. 3e). There was no significant change in basal secretion after application of the NOS inhibitors.

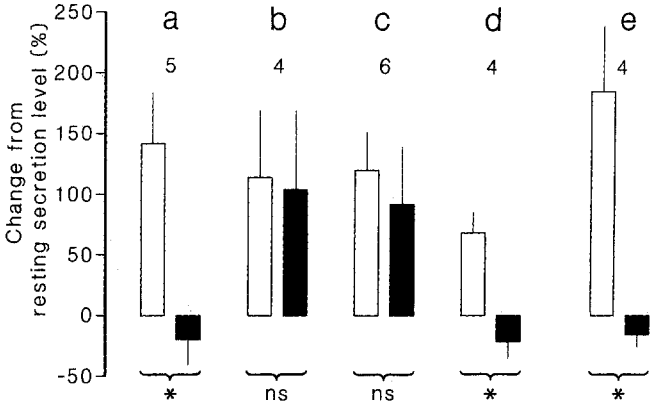

Fig. 3 Fructose secretion from the guinea-pig seminal vesicle. **a** □ carbachol, 550 μM, ■ carbachol, 550 μM + N^G-nitro L-arginine methyl ester (L-NAME), 100 μM; **b** □ carbachol, 550 μM, ■ carbachol, 550 μM + (L-NAME), 100 μM + L-arginine, 2 mM; **c** □ carbachol, 550 μM, ■ carbachol, 550 μM, + N^G-nitro D-arginine methyl ester (D-NAME), 100 μM; **d** □ carbachol, 550 μM, ■ carbachol, 550 μM + N^G-nitro L-arginine (L-NNA), 50 μM; **e** □ nerve stimulation, ■ nerve stimulation + L-NAME, 100 μM.
* $P < 0.05$, *ns* not significant. Values are means ± SEM. Number of experiments is given above columns

Administration of the NO donor GTN (10 μM) caused a significant increase of fructose secretion compared with the resting secretion level (40% ± 9%, $n = 6$, $P < 0{,}01$). A combination of carbachol and GTN also caused an increase in fructose secretion but no synergistic effect between the drugs could be seen (72% ± 17%, $n = 6$; carbachol only, 104% ± 31%, $n = 7$).

No increase in cGMP levels were noted after stimulation by carbachol (550 μM) or GTN (10 μM), (21.8 ± 1.6 pmol/g, $n = 6$ and 19.1 ± 2.3 pmol/g, $n = 6$, respectively, compared with controls, 20.8 ± 2.7 pmol/g, $n = 6$). Neither did ODQ (1 μM) reduce the secretory response to carbachol. However, the solvent DMSO in itself reduced the response (carbachol + DMSO 43.4% ± 14.6%, $n = 5$; carbachol + DMSO + ODQ 43.2% ± 15.4%, $n = 5$).

Discussion

The high Ca^{2+}-dependent NOS activity in the secretory linings of the seminal vesicle corresponds well with the pronounced NADPH-d staining of the glandular cells. Apparently most of the NOS activity of the seminal vesicle is confined to these cells. The comparatively low Ca^{2+}-dependent NOS activity that was found in the smooth muscle layer correlates well to the presence of NADPH-d positive nerves and vascular endothelium in this layer. The network of AChE positive fibres observed beneath the glandular cells is as described previously [1].

The main topic of this report is the functional significance of the NOS in the secretory cells and the possible role of glandular NO formation. Two tentative functions of NO formed in glandular cells are apparent and unstrained. NO might be a secretory product in the seminal plasma. Alternatively or additionally, formation of NO is a step in the glandular secretory process. Our results with specific NOS inhibitors focus on the latter possibility. Fructose, our marker of secretion, is the main free sugar of the human seminal plasma where it has been suggested to provide energy to the spermatozoa [13] and is produced in the seminal vesicle only. In this organ it is present in the luminal preformed secretion and in secretory granules of the glandular cells [3]. Muscarinic receptor stimulation releases fructose from glandular cells in the guinea-pig [15]. Carbachol in the concentration used raised the resting secretion by an average of 120%, which is in accordance with our previous result [15]. This secretory response seems to require the L-arginine : NO pathway. Thus it was effectively blocked by the specific NOS inhibitors L-NNA and L-NAME, whereas the stereoisomer D-NAME was without effect. Furthermore, high concentration of L-arginine added together with L-NAME prevented the inhibitory action of the analogue. It has

been claimed that L-NAME blocks muscarinic receptors [4]. We find it unlikely that such an effect of L-NAME played a substantial role in our results because it is ascribed to the ester modification at the carboxyl end of the L-arginine analogue and therefore is not counteracted by L-arginine [4]. Moreover, L-NNA, which lacks the molecule modification supposed to interact with muscarinic receptors blocked the muscarinic response, while D-NAME, which has the alkyl ester modification in the carboxyl terminal, but is isomerically reversed in the guanidino terminal, did not block the muscarinic response. In addition, doubts on the significance of the antimuscarinic action of L-NAME in the concentrations presently used have recently been presented by Cheng et al. [5].

L-NAME blocked the response to hypogastric nerve stimulation as it blocked the response to carbachol. That is, neurogenic activation of the excitatory receptors followed the pattern of exogenous activation of them. Our finding that an exogenous NO donor, GTN, also increased the fructose secretion confirms to the concept that NO is involved in the seminal vesicle secretion and also points at a possible function of the submucosal nitrergic nerves as an alternative source of NO. The lack of synergism between GTN and carbachol would be an expected finding if, as postulated above, carbachol raised the intracellular NO concentration. Then the concentration gradient would oppose diffusion of exogenously applied NO into the glandular cells. The detailed mechanism of the action of NO remains to be elucidated but neither carbachol nor GTN increased the level of cGMP in the seminal vesicle. Furthermore, the guanylate cyclase inhibitor ODQ [8] did not oppose the effect of carbachol. Thus, like the NO-induced inhibitory junction potential [6, 19] and the NO inhibition of cytochrome oxidase [14] the secretogogous action of NO in the seminal vesicle seems not to be mediated via the guanylate cyclase : cGMP pathway. Finally, the possibility that NO also is a constituent of seminal plasma should not be neglected. Thus the NO donor sodium nitroprusside has been claimed to improve the motility of human spermatozoa [9]. However, Weinberg et al. [20] claimed the opposite.

In conclusion, our study suggests that intracellular NO formation is a functional step in the secretory response to muscarinic stimulation of the glandular cells of the seminal vesicle as is schematically outlined in Fig. 4.

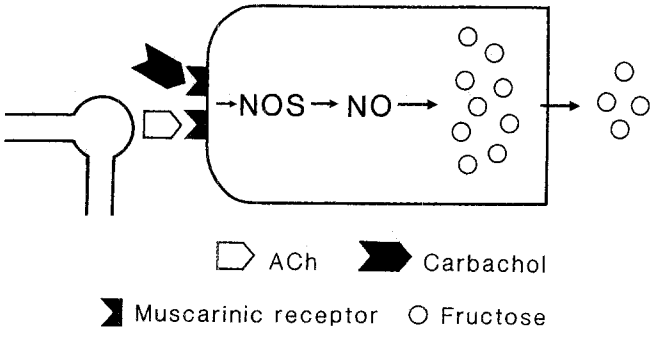

Fig. 4 Schematic drawing of the events leading to fructose secretion tentatively suggested by the results. Muscarinic receptors are activated by nervous release of acetylcholine (ACh) or exogenously administered carbachol. Receptor stimulation activates nitric oxide synthase (*NOS*). Nitric oxide (*NO*) is formed and stimulates secretion of stored fructose

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