We also found that amorphous masses from the v. seminalis disappeared in regions where the apyrene sperm showed a churning motion, but remained in regions where apyrenes were not present. Thus, activated apyrene spermatozoa promoted digestion of this material which was probably necessary for sperm maturation. Vigorously flagellating apyrenes squeezed between individual eupyrenes which became partially loosened, though still largely remaining bound together. At 80 min after mating, dissociation of the bundle in the spermatophore, which had begun from the ends, was almost complete (fig. 2C). Thus, the individual eupyrene spermatozoa became separated from each other, acquired motility and began to flagellate (fig. 2D). However, they still moved with flagellar undulations of much longer wave length and lower frequency than those of apyrenes. Since addition of either 5 µl of the g. prostatica suspension (one gland homogenized in 50 µl silkworm Ringer) or a purified endopeptidase, trypsin from bovine pancreas (Type I, Sigma Chemical Co., St. Louis, USA) or endoproteinase Arg-C from g. submaxillaris of mouse (Boehringer Mannheim GmbH, Mannheim, West-Germany) to 5 µl of the seminal fluid (content of one v. seminalis diluted in 200 µl silkworm Ringer) had always a very similar effect in causing digestion of cysts and dissociation of bundles, the active factor in the prostatic secretion was presumably an endopeptidase. These in vitro experiments were repeated at least five times to confirm the results.

In the silkmoth, the separation of enzymes[15, 16, 18] from their substrates[8, 19] in the male reproductive glands must be an effective biological mechanism for the supply of fresh nucleated spermatozoa at fertilization, since both the enzymes and substrates remain intact before ejaculation, but react together after ejaculation, resulting in sperm maturation.

However, the high viscosity and heterogeneity of the contents of spermatophore[19] would retard these reactions. Therefore, apyrene spermatozoa must be activated in the spermatophore to stir the contents and promote dissociation of eupyrene bundles and separation of each individual eupyrene spermatozoon both mechanically and by biochemical reactions. The flagellating apyrenes also cause digestion of the soft plug, a proximal part of the spermatophore, resulting in opening to the ductus seminalis. These functions of apyrenes are similar to those of apyrenes in some molluscs[20, 21], which help in the migration of eupyrenes.

Recently, four possible functions of apyrene spermatozoa were suggested[3]. The possibility that they are a source of nutrition for eupyrenes[22] is unlikely, because they are present in such a small quantity. The other three possibilities; that they facilitate escape of eupyrenes from the testis[23, 24], that they promote eupyrene movement in females[25], and that they play a role in competition between rival sperms deposited by different males[3], are also unlikely.

The last possibility appears to be true in some Lepidoptera which can mate several times. In *Bombyx mori*, remating is often possible[26]. However, the first-inseminated spermatozoa are not always dominant over the later, reinseminated ones in fertilization[27]. In either case, apyrene spermatozoa do not have any relation to sperm competition.

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1 Toyama, K., Bull. Coll. Agr. Tokyo Imp. Univ. *2* (1894) 125.
2 Riemann, J. G., Thorson, B. J., and Ruud, R. L., J. Insect Physiol. *20* (1974) 195.
3 Silberglied, R. E., Shepherd, J. G., and Dickinson, J. L., Am. Nat. *123* (1984) 255.
4 Katsuno, S., Appl. Ent. Zool. *12* (1977) 241.
5 Kasuga, H., and Osanai, M., Zool. Sci. *1* (1984) 942.
6 Ômura, S., J. Fac. Agr. Hokkaido Imp. Univ. *40* (1938) 129.
7 Mann, T., in: Spermatophores. Development, Structure, Biochemical Attributes and Role in the Transfer of Spermatozoa, p. 234. Springer, Berlin 1984.
8 Kasuga, H., Osanai, M., and Aigaki, T., Zool. Sci. *2* (1985) 953.
9 Shepherd, J. G., J. Insect Physiol. *20* (1974) 2107.
10 Katsuno, S., Appl. Ent. Zool. *12* (1977) 352.
11 Katsuno, S., Appl. Ent. Zool. *12* (1977) 370.
12 Osanai, M., Aigaki, T., Kasuga, H., and Yonezawa, Y., Zool. Sci. *2* (1985) 904.
13 Osanai, M., Aigaki, T., Kasuga, H., and Yonezawa, Y., Insect Biochem, *16* (1986) 879–885.
14 Osanai, M., Aigaki, T., and Kasuga, H., Insect Biochem, *17* (1987) 71.
15 Aigaki, T., Kasuga, H., and Osanai, M., Insect Biochem. *17* (1987) 323.
16 Kasuga, H., Aigaki, T., and Osanai, M., Insect Biochem. *17* (1987) 317.
17 Kasuga, H., Osanai, M., Yonezawa, Y., and Aigaki, T., Dev. Growth Diff. *27* (1985) 514.
18 Aigaki, T., and Osanai, M., J. comp. Physiol. *B155* (1985) 653.
19 Kasuga, H., Osanai, M., and Aigaki, T., Dev. Growth Differ. *28*, suppl. (1984) 74.
20 Woodard, T. M., J. exp., Zool. *85* (1940) 103.
21 Bulnheim, H. P., Helgol. Wiss. Meeresunters. *18* (1968) 232.
22 Katsuno, S., Appl. Ent. Zool. *12* (1977) 142.
23 Holt, G. G., and North, D. T., J. Insect Physiol. *16* (1970) 2211.
24 Friedländer, M., and Gitay, H., J. Morph. *138* (1972) 121.
25 Riemann, J. G., and Gassner III, G., Ann. ent. Soc. Am. *66* (1973) 154.
26 Umeya, Y., and Ômura, S., Bull. Sericul. Exp. Sta. *13* (1950) 63.
27 Ômura, S., Jap. J. Genet. *15* (1939) 29.

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Evidence of a direct action of triiodothyronine (T_3) on the cell membrane of GH_3 cells: an electrophysiological approach

J. S. du Pont and J.-M. Israel

University of Amsterdam, Dept. of Zoology, Biologisch Centrum Gebouw 11, Kruislaan 320, NL-1098 SM Amsterdam (The Netherlands), and I.N.S.E.R.M. U. 176, Neurobiologie des compartements, Domaine de Carreire, Rue Camille Saint-Saens, F-33076-Bordeaux Cedex (France), 2 September 1986

Summary. Electrophysiological experiments demonstrate that triiodothyronine (T_3) exerts a direct effect on the membrane of a strain of cultured rat pituitary tumor cells, GH_3/B_6. These cells respond to pressure application of T_3 (2–5 nl, concentration $1 \cdot 10^{-10}$ M) with an increase in the membrane resistance (R_m) and a hyperpolarization. Spontaneously firing cells become silent.

Key words. Triiodothyronine; GH_3 cells; membrane potential; membrane resistance; action potentials.

Direct effects of T_3 (triiodothyronine) on the electrophysiological parameters of the cell membrane of pituitary cells have not yet been described, whereas those of TRH (thyrotropine-releasing hormone)[1-5] are well known. It is thought that T_3 acts at the nuclear level in a way similar to lipophilic steroid hormones, that it passes the cell membrane passively and that its effects become manifest only after a considerable time delay[6,7]. However the effects of T_3 in vivo on the pituitary are relatively rapid[8,9] and it has been described that the membrane of rat pituitary tumor cells (GH_3) contains T_3 receptors and transport T_3 actively[10,11]. We report here a direct action of T_3 on GH_3 cells. The electrophysiological properties of GH cells are well documented[1-4,12,13], and are therefore good candidates for an investigation of the direct action of T_3 on the membrane potential, resistance and firing rate of action potentials.

Materials and methods. The GH_3/B_6 cells were grown in Ham's F 10 solution supplement with 15% horse serum and 2.5% fetal calf serum. The sera contained $2 \cdot 10^{-9}$ M T_3 and $1 \cdot 10^{-7}$ M T_4[14] giving a final concentration in the culture medium of $0.35 \cdot 10^{-9}$ M and $1.75 \cdot 10^{-8}$ M, respectively. Cells were cultured for 5–7 days in Petri dishes before being used for electrophysiological studies. Before each experiment the cells were washed twice with a balanced salt solution. This salt solution of Hank's (Gibco) was supplemented with 1 mg/ml glucose, 4 mM Ca^{++} (final concentration) and buffered with 10 mM HEPES and 4 mM $NaHCO_3$ at pH 7.2, but contained no hormones. Recording was started after at least 20 min of perifusion. Each washing procedure gave a dilution of approximately 30 times, giving a total dilution of 900 times. The perifusion had a flow velocity of 0.5 ml/min, while the content of the Petri dish contained approximately 2 ml; therefore an additional dilution of 100 times is achieved before the experiments started. So the final concentrations of T_3 and T_4 were less than $3.9 \cdot 10^{-15}$ M and $1.9 \cdot 10^{-13}$ M, respectively. Experiments were carried out under temperature-controlled conditions (30 °C).

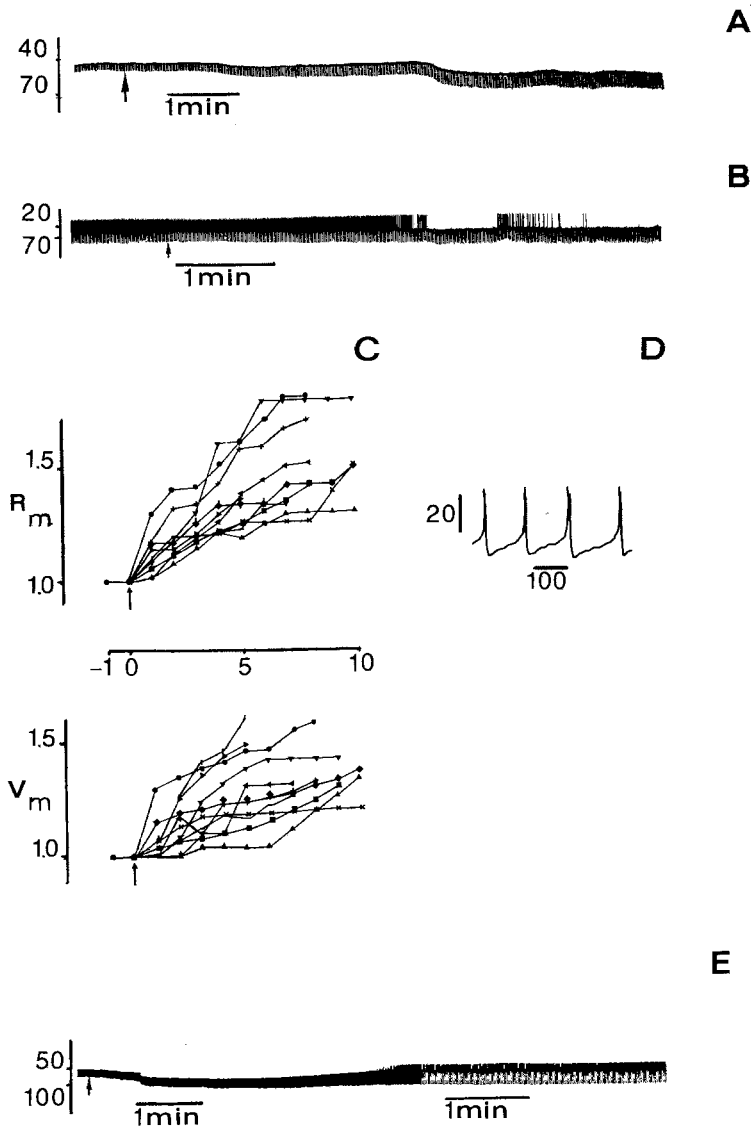
Stable intracellular recordings were made under direct visualisation at 200 × (inverted phase contrast microscope), using 3 M KAc-filled micropipettes with resistances of 100 MΩ–130 MΩ. A constant-current 'bridge' circuit was used that allowed simultaneous voltage recording and current passage (Mentor N950). The membrane potential was recorded with a rectilinear pen recorder (Gould 2400). Immediately after a cell was impaled a hyperpolarizing current (DC) was sent into it. This current was slowly diminished to zero, over a period of about 2 min, until the membrane potential V_m and resistance R_m were stable. R_m was measured by injecting into the cell current pulses of 0.1 nA, duration 100 ms, at a frequency of 1 s^{-1}; R_m was defined as the ratio of the resulting voltage pulse and the injected current, corrected for the same value before impalement.

After a stable period of at least 1 min, T_3 was ejected onto the cell. The T_3 solution in the ejection pipette had a concentration of $1 \cdot 10^{-10}$ M and was prepared in the following way: 6.73 mg T_3 (Sigma) was dissolved in a mixture of 0.1 g BSA (Sigma) and 1 ml 1 N NaOH, and filled up to 100 ml with H_2O, giving a $1 \cdot 10^{-4}$ M T_3 stock solution. Further dilutions were made with the bathing fluid. The only binding protein in this solution was BSA (MW = 69 kDa) which had a concentration of $1.44 \cdot 10^{-11}$ M in the solution with a concentration of $1 \cdot 10^{-10}$ M T_3. Jørgensen[15] gives for Human Serum Albumin two affinities, K, for T_3: $7.35 \cdot 10^3$ M^{-1} and $8.25 \cdot 10^5$ M^{-1}. Assuming that these K values are approximately the same for BSA, one finds, using simple binding kinetics, that the fraction of T_3 bound is less than 0.1%. So, we conclude that all T_3 in the ejection fluid was in the free form. A small volume (2–5 nl) of the T_3 solution (final concentration $1 \cdot 10^{-10}$ M) was delivered by pressure via a micropipette located near to the recorded cell. The ejected volume per second was estimated from the time necessary to empty a standard capillary (ID 0.94 mm, length 10 cm, tip diameter 3 µm) over a known length using a pressure of 0.3 bar. Control experiments were done with vehicle fluid with a BSA concentration of $1.44 \cdot 10^{-9}$ M and with a concentration of T_3 $1 \cdot 10^{-12}$ M. No alterations in V_m, R_m or firing frequency were seen. All data are presented as mean ± standard deviation.

Results. Stable intracellular recordings were obtained, sometimes for more than 2 h. Forty cells, 15 silent and 25 spontaneously active, were tested with triiodothyronine. The resting potential V_m and the input resistance R_m were respectively −51 mV ± 4 and 198 MΩ ± 66. All tested cells showed a hyperpolarization of V_m (the average value increased to −71 mV ± 3) and an increase of R_m (average 268 MΩ ± 81). In the silent cells (e.g. fig. A) V_m was −52 mV ± 5 and R_m 171 MΩ ± 57; after ejection of $1 \cdot 10^{-10}$ M T_3, V_m increased to −73 mV ± 3 and R_m to 234 MΩ ± 65 within 100 s. V_m and R_m in the spontaneously active cells (fig. B) increased from −51 mV ± 4 to −70 mV ± 3 and from 215 MΩ ± 68 to 289 MΩ ± 84 after ejection of $1 \cdot 10^{-10}$ M T_3. The changes in membrane potential and resistance were significant ($p < 0.001$ using the two-sample t-test[16]). The frequency of firing of spontaneously active cells (fig. B) diminished in association with these membrane changes and the cells became silent in a few minutes. Figure C illustrates for 11 randomly chosen cells the time course of V_m and R_m, normalized to a control value (= 1) of R_m or V_m in the period 1 min before T_3 application. This time-course is typical for all cells, except for two cells which exhibited a different behaviour. One silent cell (fig. E), with a V_m of −54 mV, hyperpolarized to −84 mV and then depolarized to about −70 mV before firing spontaneously 7 min after the delivery of T_3. One spontaneously active cell hyperpolarized when treated with T_3, but then showed an increase in firing rate.

Discussion. The results show three rapid effects of T_3 on the cell membrane: hyperpolarization of V_m, increase of R_m, and suppression of action potentials. None of these effects were reversible during the experiment, possibly because the lipophilic hormone accumulated in the hydrophobic membrane. Ozawa and Kimura[3] reported that TRH causes a hyperpolarization accompanied by a decrease in R_m. They explained these phenomena on the basis of an increased K^+ permeability. Our results (the hyperpolarization and increased R_m by T_3) cannot be explained by an absolute increase in K^+ permeability alone because of the increase of R_m. However the ratio of membrane permeabilities of Na^+ and K^+ (P_{Na}/P_K) = p, is not negligible in secretory cells[17,18]. Using the Goldman-Hodgkin-Katz equation, the concentrations of Na^+ and K^+ in the Hank's solution and an estimate of $[Na^+]_i$ = 10 mM, $[K^+]_i$ = 135 mM, we calculated from the control value V_m = −51 mV and from V_m = −71 mV after T_3, p values of respectively 0.18 and 0.03. These p values are in the physiological range[18] and imply a strongly diminished Na^+ in relation to the K^+ permeability.

Various authors[1,3,13] have suggested that there is a relation between action potential firing rate and hormone release. If this is so then the suppression of action potentials reported here as response to T_3 could be a way of controlling secretion. It has been reported that the action potentials of GH_3 cells are mainly Ca^{++} dependent[1,3,13], and that Ca^{++} ions promote prolactin release from GH_3 cells[19]. Therefore, the relationship between our observations and hormone release might be an indirect one. In addition, GH_3 cell lines can also secrete growth hormone. If these GH-secreting cells differ electrophysiologically from prolactin-secreting cells, this might be the explanation for the two recordings that differ from all others. These complications, however, preclude as yet further speculations about the relationship between our observations and the secretory properties of the cells studied.



Effect of T_3 on the electrical properties of GH_3 cells. Arrow: time of ejection of T_3. *A* Silent cell: ordinate in mV, downwards more negative. The downward pulses were caused by injection of a current pulse (0.1 nA, 100 ms). The input resistance was calculated using Ohm's law. Time indicated by bar. *B* Spontaneously active cell: ordinate in mV. Downward pulses as in (A). Upward going pulses are action potentials. Time indicated by bar. *C* Time course of membrane resistance and potential of eleven typical cells; abcissa, time in minutes. Upper graph: ordinate values of input resistance R_m normalized to control value (= 1). Bottom graph: ordinate values of membrane potential V_m normalized to control value (= 1). Control value is the value of the input resistance or the membrane potential in the period of 1 min before T_3 application. *D* Action potentials: abcissa bar is 100 ms, ordinate is 20 mV. *E* The silent cell that starts to generate action potentials after application of T_3. Note two different time bases.

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1 Kidokoro, Y., Nature *258* (1975) 741.
2 Taraskevich, P. S., and Douglas, W. W., Neuroscience *5* (1980) 421.
3 Ozawa, S., and Kimura, N., Proc. natn. Acad. Sci. USA *76* (1979) 6017.
4 Dufy, B., Vincent, J.-D., Fleury, H., Du Pasquier, P., Gourdji, D., and Tixier-Vidal, A., Science *204* (1979) 509.
5 Maruyama, T., Shiino, M., and Rennels, E. G., Neuroendocrinology *32* (1981) 28.
6 Sterling, K., New Engl. J. Med. *300* (1975) 117.
7 Sterling, K., New Engl. J. Med. *300* (1975) 173.
8 Garcia, M. D., Escobar del Rey, F., and Morreale de Escobar, G., Endocrinology *98* (1976) 203.
9 Hervas, F., Morreale de Escobar, G., and Escobar del Rey, F., Endocrinology *97* (1975) 91.
10 Horiuchi, R., Cheng, S.-Y., Willingham, M., and Pastan, I., J. biol. Chem. *257* (1982) 3139.
11 Horiuchi, R., Johnson, M. L., Willingham, M., Pastan, I., and Cheng, S.-Y., Proc. natn. Acad. Sci. USA *79* (1982) 5527.
12 Dufy, B., Vincent, J.-D., Fleury, H., Du Pasquier, P., Gourdji, D., and Tixier-Vidal, A., Nature *282* (1979) 855.
13 Ozawa, S., Biomed. Res. *1*, suppl. (1980) 140.
14 Samuels, H. H., Tsai, J. R., and Cintron, R., Science *181* (1973) 1253.
15 Jørgensen, E. C., Thyroid hormones, in: Hormonal Proteins and Peptides, vol. 6. Ed. C. H. Li. Academic Press, New York 1978.
16 Campbell, R. C., Statistics for Biologist. Cambridge University Press, 1974.
17 Trifaro, J. M., A. Rev. Pharmac. Toxic. *17* (1977) 27.
18 Williams, J. A., J. theor. Biol. *28* (1970) 287.
19 Tixier-Vidal, A., and Gourdij, D., Physiol. Rev. *61* (1981) 974.

0014-4754/87/060596-03$1.50 + 0.20/0