Journal of Neuroscience Research 71:670–678 (2003)

Thyroid Hormones Regulate the Frequency of Miniature End-Plate Currents in Pre- and Prometamorphic Stages of the Tadpole Tail

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Thyroid hormones (THs), primarily 3,3′,5-triiode-$_L$-thyronine (T_3), have been clearly established as natural inducers of apoptosis during metamorphosis of anuran embryos. We decided to use this phenomenon to test the hypothesis that, prior to genomic activation, T_3 has acute actions in the neuromuscular junction (NMJ) of the tail of amphibian embryos. We detected a dramatic increase in the production of miniature end-plate currents (MEPCs) 2–5 min after continuous application of T_3 (250 nM) using focal recordings under voltage clamp. Furthermore, this increase in the spontaneous release of neurotransmitter, evaluated by the MEPC frequency, was maintained for several hours. Reverse-T_3, the "inhibitory" form of THs, prevented this increase in MEPC frequency, suggesting that this is probably a highly specific action of T_3. In addition, the elevation in MEPC frequency induced by T_3 was unchanged in the presence or absence of extracellular calcium. The T_3-mediated increase in MEPC frequency was blocked by niflumic acid, a nonsteroidal antinflammatory fenamate used to prevent the apoptotic volume decrease observed in many systems. The present study demonstrated that T_3 induces a remarkable nongenomic action in the NMJ of the tadpole tail at pre- and promatamorphic stages.

Key words: thyroid hormones; niflumic acid; apoptotic volume decrease; spontaneous miniature end-plate current

In the absence of action potentials, spontaneous fusion of vesicles in the nerve terminal causes release of the contents of a single vesicle (Fatt and Katz, 1952; Del Castillo and Katz, 1954). This process is critical for organizing sites of neuronal communication in the developing neuromuscular junction (NMJ; Saitoe et al., 2001). The spontaneous release of neurotransmitter in the tadpole tail occurs stochastically (Quiñonez et al., 1996), but the kinetics of release are dependent on the specific metamorphic stage of the animal (Rojas and Lasalde 2000; Rojas et al., 2001). Metamorphosis in tadpoles comprises three periods (Dodd and Dodd, 1976): Premetamorphosis (pre-mp) occurs prior to the formation of a functional thyroid gland; prometamorphosis (pro-mp) occurs at the time when the gland develops; and proper metamorphosis (mp) is when metamorphosis proceeds (Leloup and Buscaglia, 1977). Metamorphosis in tadpoles is initiated by 3,3′,5-triiode-$_L$-thyronine (T_3), which induces apoptotic removal of the tadpole tail (Brown et al., 1996).

A prominent characteristic of apoptosis is cell shrinkage (Wyllie et al., 1980). The loss of cell volume that occurs during apoptosis contrasts with the events that are exhibited during necrosis, when cells generally swell, lose their membrane integrity, and eventually rupture, causing the subsequent inflammatory response. Numerous mechanisms have been suggested to explain the loss of cell volume that occurs during apoptosis (Grinstein et al., 1983; Arends and Wyllie, 1991; Barbiero et al., 1995; Benson et al., 1996; Bortner et al., 1997; Yu et al., 1997; Colom et al., 1998; Wang et al., 1999; Okada et al., 2001). DNA fragmentation is one possibility, but this phenomenon occurs relatively late during the course of apoptosis, after cell shrinkage has already begun (Arends and Wyllie, 1991). Changes in activity of the Na^+/H^+ exchanger and the Na^+-K^+-2Cl cotransporter have been also proposed (Grinstein et al., 1983). The nongenomic apoptotic volume decrease (AVD), which begins prior to cell fragmentation, is now known to be coupled to the release of cellular K^+ (Barbiero et al., 1995; Benson et al., 1996; Bortner et al., 1997), presumably via K^+ channels (Yu et al., 1997; Colom et al., 1998; Wang et al., 1999; Okada et al., 2001). To have a net cellular efflux of water, which leads to cell shrinkage, chloride is released in parallel with K^+, to maintain electroneutrality (Maeno et al., 2000; Okano and Maeno, 2001; Yu et al., 2001). Using a series of both Cl^- and K^+ channel blockers, Maeno et al. (2000) concluded that nongenomic AVD occurs prior to apopto-

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Received 3 September 2002; Revised 15 October 2002; Accepted 16 October 2002

sis and is mediated by the activation of both K^+ and Cl^- channels. These findings are consistent with the hypothesis that AVD precedes apoptosis.

The tadpole tail is removed by apoptosis, which is initiated by thyroid hormones (THs) released by the thyroid gland. It is reasonable to suppose that the initial actions of T_3 are nongenomic, similar to those exhibited in other process and tissues (for review see Davis and Davis, 1996). We decided, based on this premise, to determine whether T_3 produced nongenomic actions in the NMJ of tadpoles during pre-mp and pro-mp, two critical stages of development. Our results indicate that acute application of T_3 dramatically increased the frequency of miniature endplate currents (MEPCs) in pre-mp and pro-mp animals, although the effects were most pronounced in pre-mp animals. The effect of T_3 on the kinetics of spontaneous neurotransmitter release is specific, in that it is blocked by the "inactive" T_3 analog, reverse- T_3 (RT_3), and is independent of extracellular calcium levels. The enhanced frequency of MEPCs may be linked to chloride channel activation, insofar as the increase in MEPC frequency was blocked by a low concentration of niflumic acid (NFA).

MATERIALS AND METODS

Choosing the Animal Stage of Development

Experiments were carried out in *Rana castebeiana* tadpoles between stages 42 and 57 of development, according the table of Nieuwkoop and Faber (NF) used for *Xenopus laevis*, in combination with the more general anuran table of Gosner (Nieuwkoop and Faber, 1956; Gosner, 1963). Tadpoles were purchased from Carolina Biological Supply (Miami, FL) throughout the year. Anatomical development, including the relative body distances and the appearance of bud limbs, was carefully observed in determining the developmental stages of tadpoles. In all cases, the lengths of the body, tail, and head were evaluated and considered to differentiate the two major groups of animals utilized in these experiments: pre-mp and pro-mp. Pre-mp tadpoles belong to the stage of development prior to the formation of a functional thyroid gland up through stage 53 (NF42–53; Nieuwkoop and Faber, 1956), and pro-mp tadpoles belong to the stage of development with a functional thyroid gland, but without any metamorphosis, from stage 54 through stage 57 (NF54–57; Nieuwkoop and Faber, 1956). In the determination of MEPC frequency, we used the same batch of animals that was used for comparison in the pre-mp and pro-mp stages, because the average value gave a slightly different value from controls used in subsequent experiments. Thus, to obtain more reliable results, we always used animals derived from the same batch of tadpoles for experiments involving the same protocols, to minimize differences in these results.

Animal Maintenance and Surgical Procedures

Tadpoles were housed in appropriate tanks with filtered running water in a room maintained at 22°C. The tails of *Rana castebeina* tadpoles from the pre-mp and pro-mp stages of development, as described above, were dissected, and the NMJ was directly exposed to normal saline solution containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 10 HEPES, pH 7.20. Briefly, for 10–15 min, the tadpoles were placed in a cool (3–5°C) normal saline solution to allow removal of the tail skin. After the animals were decapitated, the tails without skin were separated and placed in the recording chamber. Normal saline solution was used as the extracellular medium (Quiñonez et al., 1996). Individual muscular fibers from isolated tails were exposed and were easily observed with a stereoscopic microscope attached to a CCD camera. Experiments were not performed in living animals, and all the procedures used were adequate to ensure minimal discomfort to animals. The animals were acquired and cared for in accordance with the guidelines published in the NIH *Guide for the Care and Use of Laboratory Animals* (revised 1985) and the principles presented in the *Guidelines for the Use of Animals in Neuroscience Research*. The animal protocol used was approved by the institutional committee for handling and care of laboratory animal (IACUCC).

Micropipette Preparation, Solutions, and Drugs

Soft glass (catalog No. B150-86-10; Sutter Instrument Co., Novato, CA) was used to make micropipettes in a puller (model PP-830; Narishige, Japan). Micropipettes were prepared in two stages, with characteristics similar to those used in patch-clamp experiments (Quiñonez et al., 1996). The micropipette tips were fire polished using a microforge (model MF-830; Narishige, Japan) to reach a diameter of 2–4 μm. Micropipettes were then filled with the normal saline solution described above. Micropipette resistance was between 5 and 8 MΩ when the micropipette was placed in the extracellular solution. When a solution with nominally zero calcium was used, only the $CaCl_2$ was omitted, and no calcium buffer was used.

Focal Recording of MEPCs

Focal macropatch recording was used to measure synaptic currents (Parnas et al., 1982; Dudel, 1983; Quiñonez et al., 1996; He et al., 1999; Ratner et al., 2000). The synaptic currents were obtained using the loose patch technique by lightly placing a 2–5-μm-diameter fire-polished, glass electrode directly over a single, spatially isolated varicosity nerve terminal. The lumen of the patch electrode was filled with the same solution as the bathing medium, and the seal resistance was about 1 GΩ. Because the seal can be lost easily if the muscle twitches under the electrode, in the majority of experiments we used TTX (100 nM) in the bath and pipette solution for blocking the possible action potential production in the nerve terminal and the muscle fiber. Recording of MEPCs were done at the resting membrane potential (–70 mV), and the room temperature was held between 22°C and 24°C. The Geneclamp-500 amplifier (Axon Instruments Inc., Foster City, CA) was used with a head stage that had 1 GΩ of feedback resistance. Microelectrodes were positioned on the NMJ to obtain the focal recording, as described previously (Quiñonez et al., 1996; Forti et al., 1997). A waiting period of 4–5 min was included to allow the preparation to stabilize and recover from the mechanical manipulation. All MEPC recordings used for the analysis had a rise time of less than 400 μsec, using throughput to disk acquisition in a detected-signals algorithm active in the EDR program, which was kindly provided by Dr. John Dempster (http://innovol.sibs.strath.ac.uk/physpharm/sw.shtml). A VHS recorder adapted to record PCM signals was used for long-time

continuous recording acquisition. The amplified signals were filtered at ≥5 kHz, and a playback of acquired recordings was used to analyze the data with a PC computer running at 500 MHz across a National Instruments I/O board (model PCI-6025). Final analysis was carried out after computer acquisition using the WCP software, also provided by Dr. John Dempster (see URL above).

Perfusion of Solutions and Protocols for T_3, RT_3 , and NFA Application

Small steel pins were used to place NMJs in the bottom of a perfusion chamber containing Sylgar. NMJs were then perfused with normal solution for several minutes prior to experimental recording. Normal and experimental solutions were prepared using chemicals obtained from Sigma (St. Louis, MO), and solutions were applied continuously during the experiments. Briefly, after 20–25 min of applying normal saline solution (control recording), experimental solutions that included T_3 (250 nM), RT_3 (1 μM), or 2-[3-(trifluoromethyl)-anilo]nicotinic acid (niflumic acid, or NFA; 1 μM) were continuously superfused on the tissue (experimental recording). In each case, T_3, RT_3, and NFA were applied at concentrations sufficient to ensure the maximal effect.

Under each condition, an acquisition time of at least 20 min was used to evaluate sufficient stationary frequency in a steady-state situation. In experiments in which the effects of two components were evaluated, one component (i.e., T_3 or NFA) was initially applied and, after recording of its actions, a mixture of both components was applied. The sequence in which the components were applied is indicated in the figures. In all cases, T_3, NFA, and the mixture were continuously perfused throughout the experiment at a variable rate between 2 and 20 ml/min. Normal saline solution (during control recording) or the various experimental solutions (during experimental recordings) including T_3, RT_3, NFA, or the mixtures of T_3 and NFA were applied as quickly as possible, to produce the exchange. Subsequently (normally 2–3 min later), the speed of exchange was reduced, to minimize mechanical perturbation during recording.

Statistical Analysis

In the majority of experiments that included T_3, we observed that the frequencies of MEPCs were initially highest, but then declined to a stationary value where frequencies were estimated. In the figures, each individual point represents the average of unique experiments containing at least 250 MEPCs. The frequency of MEPCs was calculated in each individual experiment after the acquisition of more than 5 min of activity using an algorithm from the WCP software (provided by Dr. John Dempster). Frequency was evaluated as the inverse of the average interval between two consecutive MEPCs. At least 250 MEPCs were used to obtain the average MEPC frequency in each experiment. The distribution of MEPC frequency under each condition was obtained using the cumulated probability option (Van der Kloot, 1991). Results were statistically analyzed using one-way ANOVA for multiple populations with the Prism software (v.3; GraphPad Software Inc.). Statistical significance (P value) was calculated using the Wilcoxon signed-rank test. The values obtained are always indicated as the average ± standard error, with the number of values averaged (n) shown in parentheses.

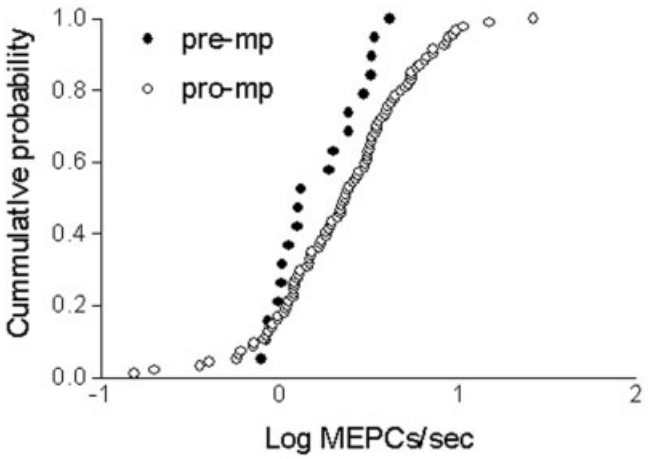

Fig. 1. Spontaneous release of neurotransmitter (spontaneous minis) in the NMJ varies in different developmental stages of the tadpole. Pro-mp animals exhibited a greater frequency of MEPCs than did pre-mp animals, which had not yet developed thyroid glands. The average frequency in pro-mp tadpoles was 3.355 ± 0.3664 (94), and the average frequency in pre-mp tadpoles was 1.900 ± 0.2419 (19). Data are expressed as mean ± SE (N), with $P < 0.0001$, as determined by the Wilcoxon signed-rank test.

RESULTS

Differences Between Pre- and Prometamorphic Tadpoles

The developmental stages of tadpoles were determined as described in Materials and Methods. The frequency of MEPCs differs in the NMJ of pre-mp and pro-mp tadpoles. Figure 1 shows the distribution MEPCs frequency in tadpoles at these two developmental stages. The average frequency of MEPCs was significantly higher in pro-mp tadpoles than in pre-mp tadpoles, which had not yet developed thyroid glands. The average frequency obtained was 1.90 ± 0.24 (19) for pre-mp animals and 3.35 ± 0.36 (94) for pro-mp animals ($P < 0.001$).

T_3 Acutely Increases the Spontaneous Release of ACh in the NMJ of Tadpoles

The acute nongenomic action of T_3 on the NMJ of the tadpole has been previously established using focal recordings (Rojas and Lasalde, 2000; Rojas et al., 2001). With use of the focal recording synaptic currents from a single synaptic area (spot of release; Quiñonez et al., 1996; Forti et al., 1997), the release data had no contamination from multiple synaptic contacts (Quiñonez et al., 1996). Our results indicate that T_3 increased the spontaneous release of neurotransmitter within 5 min after continuous application, as measured by an increase in the frequency of MEPCs (Fig. 2).

The acute effect of T_3 on the quantal release of neurotransmitter, as evaluated by the frequency of

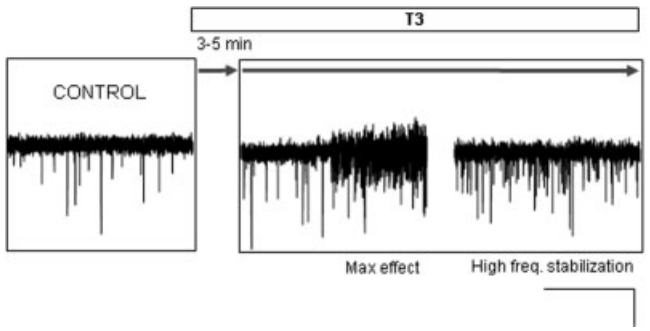

Fig. 2. T_3 increases MEPC frequency in the NMJ of the pre-mp tadpole tail. The MEPC frequency increased significantly within 2–5 min after the application of T_3 (250 nM; middle trace) compared with control (left). In this experiment, the MEPC frequency was 6.57 per sec under control conditions (left) and 17.578 per sec 7 min after application of T_3 (right). The period during which MEPCs frequency was maximally increased by T_3 is indicated in the middle trace (labeled as "max effect"). The arrow and the upper box represent continuous application of T_3 (250 nM). Representative traces from 2 sec of recording are shown and were acquired at 200 μsec/point and filtered using a low-pass filter of 2.5 kHz. Horizontal scale = 1 sec, vertical scale 25 pA.

MEPCs, lasted for more than 1 hr and was present in both pre-mp and pro-mp embryos. The effect of T_3 (250 nM) in the external solution was observed in all of the 134 patches analyzed. The developmental stage of the embryos in which this effect was observed was consistent with stages 42–57 (FN42–57), according the table of Nieuwkoop and Faber (1956).

T_3 Acutely Increases the Spontaneous Release of ACh in the NMJ of Pre-mp and Pro-mp Tadpoles: A Specific Action

T_3 increased the frequency of MEPCs in both pre-mp and pro-mp tadpoles, and no differences in the time course of the actions of T_3 were detected between these stages of development. The action of T_3 in the presynaptic region of the NMJ is highly specific in both pre-mp and pro-mp tadpoles. In pre-mp tadpoles (stages FN44–53), the application of the inactive RT_3 (RT_3), at a concentration of 1 μM, dramatically reduced the frequency of MEPCs to equal to or below control levels (Fig. 3A). The MEPCs increased from 4.62 ± 0.64 (111) in control to 11.56 ± 0.85 (30) in response to exposure to T_3 (250 nM). The MEPC frequency was further decreased to 1.17 ± 0.15 (23) after exposure to RT_3 (1 μM). Similarly, in pro-mp tadpoles (stages FN54–55), the application of T_3 increased the frequency of MEPCs from 3.06 ± 0.3 (31) to 9.41 ± 0.77 (29), whereas RT_3 at 1 μM dramatically reduced the frequency of MEPCs to 1.59 ± 0.16 (27), which was equal to or less than control levels (Fig. 3B). The decrease in frequency of MEPCs to below control levels occurred both when RT_3 was applied alone and when it was given prior to any other treatment. Furthermore, this effect was observed in both pre-mp and pro-mp animals, the reduction of frequency being more dramatic in pro-mp animals. The possible implications of this phenomenon are discussed in more detail below.

Presynaptic Action of T_3 Is Independent of Extracellular Calcium Levels

Calcium ions are one of the major regulators of neurotransmitter release in the NMJ, as well as in other fast and slow synapses (Kita and Van der Kloot, 1977; Rosenmund and Stevens, 1996; Mochida et al., 1998). In view of this, we investigated whether extracellular calcium plays a role in the presynaptic actions of T_3. In the absence of external calcium, T_3 increased the frequency of MEPCs in pre-mp tadpoles (Fig. 4, Table I). However, control levels of MEPC frequency were significantly decreased in the absence of extracellular calcium ($P < 0.001$; Table I). However, the ratio of the average frequency of MEPCs in T_3 and control samples (T_3/C) was about 2.5, and this did not differ in the presence (11.56/4.62 = 2.502) or absence (4.389/1.72 = 2.552) of calcium. The consistency of the T_3/C ratio in the presence or absence of external calcium seems to suggest further that extracellular calcium does not play a direct role in the effect of T_3 on the frequency of MEPCs.

NFA Reduces the Frequency of MEPCs

The spontaneous release of neurotransmitter is highly sensitive to hypertonicity (Fatt and Katz, 1952; Hubbard et al., 1968; Mochida et al., 1998; Kashani et al., 2001). Therefore, it was of interest to determine whether the actions of T_3 could be inhibited by NFA, a chloride channel blocker that has been previously used to prevent AVD in other experimental systems (Maeno et al., 2000; Okano and Maeno, 2001). To address this issue, we designed experiments to determine whether NFA had any effect on the frequency of MEPCs in the NMJ of pro-mp tadpoles. To our surprise, we found that NFA alone, and at a low concentration (1 μM), strongly reduced the frequency of MEPCs (Fig. 5). This decrease in MEPC frequency resembles the effects of RT_3 observed in pro-mp tadpoles. The reduction in the frequency of MEPCs in response to NFA did not produce any change in the amplitude of MEPCs, suggesting that postsynaptic actions were not involved and that the actions of NFA were most likely to be presynaptic.

NFA Blocks and Prevents the T_3-Induced Increase in MEPC Frequency

Under normal conditions, the tail NMJ undergoes T_3-induced apoptosis during tadpole metamorphosis (Brown et al., 1996). During this time, the cells exhibit typical signs that are associated with cell shrinkage, which is the fundamental and universal characteristic of programmed cell death. Our results indicate that NFA blocks the nongenomic action of T_3 on the frequency of MEPCs (Fig. 6A). NFA (1 μM) reverses the effects of T_3 by reducing the frequency of MEPCs to below control levels.

We also asked whether NFA could prevent the effects of T_3 on MEPC frequency if it were applied prior

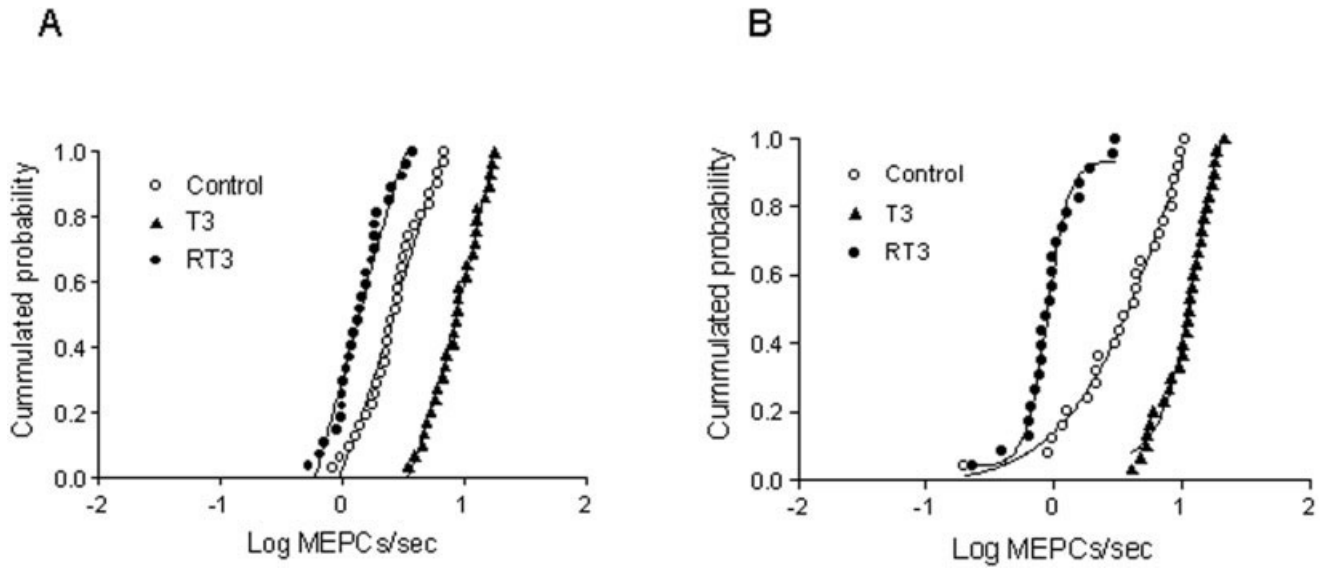

Fig. 3. Effects of T_3 and RT_3 on the frequency of MEPCs in the NMJ of pre-mp and pro-mp tadpole tails. **A:** Pre-mp (FN42–53) tadpole tails were incubated with normal medium (control; open circles), T_3 (250 nM; triangles), or RT_3 (1 μM; solid circles) as described in Materials and Methods. **B:** Pro-mp (FN54–57) tadpole tails were similarly incubated with normal medium (control; open circles), T_3 (250 nM; triangles), or RT_3 (1 mM; solid circles) as described. Each point in the figure represents independent experiments with between 250 and 700 MEPCs. Average values obtained under each condition are shown in Table I.

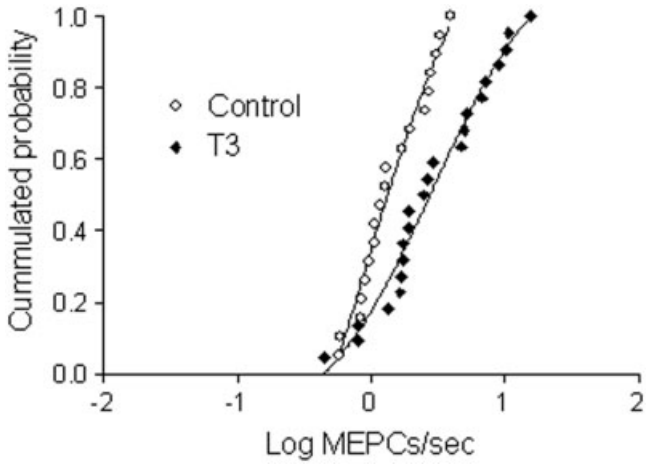

Fig. 4. Effects of T_3 on the frequency of MEPCs are independent of extracellular calcium levels. The NMJs of pro-mp tadpole tails were incubated in calcium-free medium in the absence (control) or presence (250 nM) of T_3. Each point in the figure represents independent experiments with between 250 and 700 MEPCs. Average values obtained under each condition are shown in Table I.

to T_3. In fact, either superfusion of NFA prior to T_3 exposure or treatment with a mixture of NFA and T_3 inhibited the effects of T_3 on MEPC frequency (Fig. 6B). Our data suggest that, under these conditions, NFA interferes with the T_3-induced increase in the frequency of MEPCs by blocking a key step in the mechanism of action of T_3.

DISCUSSION

The acute action of THs that has been demonstrated in several tissues (see Davis and Davis, 1996) is poorly understood. Likewise, there is a disparity between the understandings of the basic mechanism of TH actions in the nervous system and the beneficial effects that THs are known to provide. In this work, we used THs, primarily T_3, that have been recognized as natural inducers of apoptosis during metamorphosis of anuran embryos to describe the initial steps of its mechanisms of action.

Early experiments were designed to detect any potential differences in the frequency of MEPCs at different stages of development. It would have been difficult to follow the possible variations in MEPC frequency during sequential stages of development. To simplify this task, we first separated animals into two general groups, pre-mp and pro-mp, that could be clearly distinguished by their morphology. In the initial experiments, we were able to observe differences in frequency of MEPCs from tadpoles at these stages of development; pro-mp animals exhibited a significantly higher frequency of MEPCs compared with pre-mp animals. The differences in MEPCs frequency observed between the pre-mp and the pro-mp developmental stages may depend on the level of TH receptor expressed in these stages and the endogenous level of TH activity in the nerve terminal of the NMJ. Although the mechanisms underlying these differences are not understood, evaluation of TH or THR levels will be essential to define the basis for this developmental difference in MEPC frequency. The variability observed in MEPC frequency may be due to subtle differences in develop-

TABLE I. Summary of TH Actions on Pre-mp and Pro-mp Tadpoles in the Presence and Absence of Extracellular Calcium★

		Frequency (MEPC/sec)								
		Control			T_3 (250 nM)			RT_3 (1 μM)		
		Mean	SE	N	Mean	SE	N	Mean	SE	N
2Ca	Pro-mp[a]	4.62	0.64	111	11.56	0.85	30	1.17	0.15	23
2Ca	Pre-mp[b]	3.06	0.30	31	9.41	0.77	29	1.59	0.16	27
0Ca	Pro-mp[c]	1.72	0.24	19	4.39	0.85	22	NA	NA	

★The results obtained in pre-mp and pro-mp tadpoles are expressed as the average MEPCs ± the standard error (SE) and the total number of experiments (N). The effects of T_3 in the presence or absence of external calcium is shown only for pre-mp tadpoles. Statistically significant differences are indicated by superscript letters.

[a]MEPCs frequencies in pre-mp: control vs. T3 and RT3 was significantly different ($P<0.0001$).

[b]MEPCs frequencies in pro-mp: control vs. T3 and RT3 was significantly different ($P<0.0001$).

[c]MEPCs frequencies in pro-mp control vs. T3 was significantly different ($P<0.0001$) in each set of experiments.

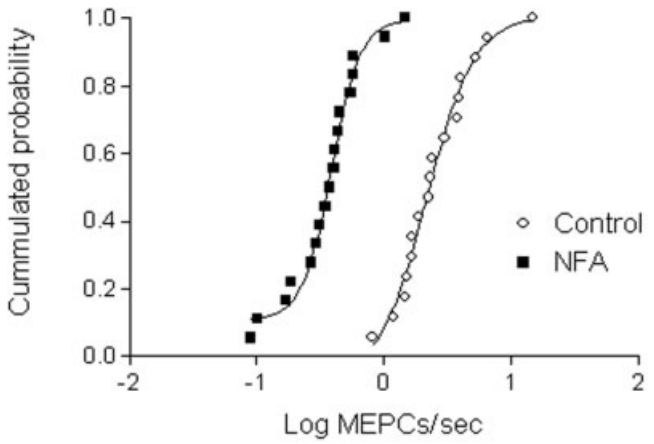

Fig. 5. Presynaptic actions of niflumic acid (NFA) in the pre-mp NMJ. The NMJs of tadpole tails were incubated in the absence (control) or presence of NFA (1 μM) as described in Materials and Methods. NFA significantly reduced the spontaneous release of neurotransmitter by decreasing the MEPC frequency to equal to or below control levels. Each point in the figure represents independent experiments with between 250 and 400 MEPCs. The average values of the calculated frequencies [MEPCs/sec ± SE (N)] were 3.466 ± 0.80 (17) for control and 0.4471 ± 0.08 (18) for NFA-treated samples. This difference was significant, as determined by the Wilcoxon signed-rank test, at $P < 0.0002$.

mental stages of the tadpoles, which were evaluated mainly based on the external morphology as represented in the developmental tables of Nieuwkoop and Faber (1956) and by Gosner (1963).

The acute increase in MEPC frequency observed in the NMJ of pre-mp and pro-mp tadpoles in response to T_3 suggests a nongenomic action, because the effect consistently occurs between 2 and 5 min after the application of T_3. This relatively rapid onset is unlikely to result from activation of gene expression and/or protein synthesis. This effect was observed in all of the 379 experiments in which T_3 was used. The nongenomic actions of T_3 are usually considered to occur in both excitable and nonexcitable adult cells, but not in embryonic tissues (Davis and Davis, 1996). However, our findings show that the nongenomic actions of T_3 could play a role during the earlier stages of development, specifically in synapses formed in the NMJ of pre-mp and pro-mp tadpole tails.

The effect of T_3 is very specific, because the frequency of MEPCs was reduced in response to RT_3, either alone or when given prior to any other treatments. This finding suggests that some endogenous mechanism maintains the frequency of MEPCs under normal conditions and that this mechanism is inhibited by RT_3. This endogenous mechanism most likely controls the basal level of MEPC frequency in pro-mp tadpoles. The most plausible origin of increases in MEPC frequency is elevations in the level of intraterminal T_3 in these animals (Dodd and Dodd, 1976; Leloup and Buscaglia, 1977; Brown et al., 1996). Our data are consistent with the idea that RT_3 reduces the T_3-mediated spontaneous release of neurotransmitter to a basal level. Controls reflect the normal MEPC frequency that is produced by the endogenous level of T_3. The differences observed in the frequency of MEPCs in pre-mp and pro-mp tadpoles during control recordings may be explained by developmental differences in T_3 levels, with this leading to the basal differences in MEPC frequency.

The application of T_3 in the absence of external calcium also increased the frequency of MEPCs in pro-mp tadpoles. This finding suggests that the effect of T_3 on the MEPC frequency is not dependent on extracellular calcium levels and indicates that T_3 is not acting on the presynaptic calcium channel, at least not through modulation of calcium entry into the nerve terminal. The average ratio of MEPC frequency in T_3 and control samples (T_3/C) was approximately 2.5, and this ratio did not differ in the presence or absence of extracellular calcium. The fact that this ratio was unaffected by external calcium levels further suggests that external calcium does not directly participate in the effect of T_3 on MEPC frequency. In addition, the increase in frequency induced by T_3 was dependent only on the original MEPC frequency value under control conditions. If T_3 is the endogenous regulator of MEPC frequency, this could indicate that the T_3 concentration used in these experiments (250 nM) is rel-

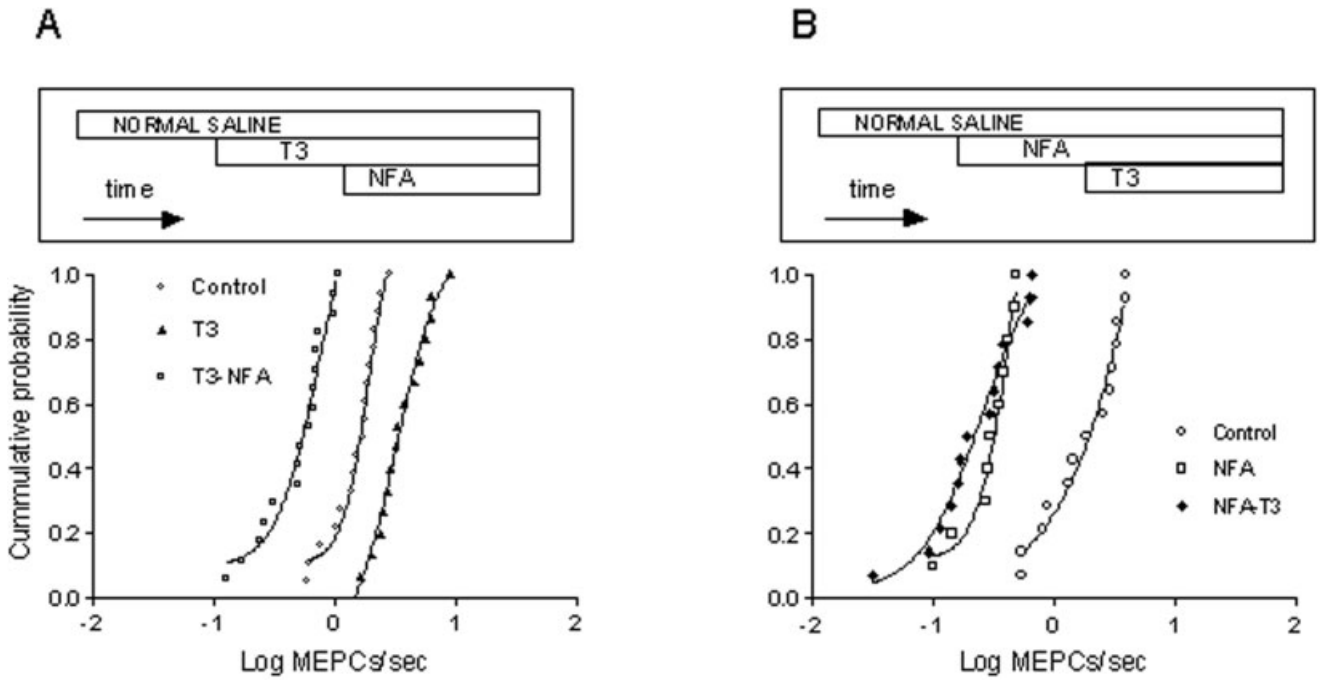

Fig. 6. NFA both blocks and prevents the presynaptic effect of T_3 on MEPC frequency in the pre-mp NMJ. **A:** Tadpole tails were first exposed to normal saline (control; circles), then to T_3 (250 nM; triangles), which increased the frequency of MEPCs. NFA (1 μM; squares) was then added in the continued presence of T_3. **B:** Tadpole tails were first exposed to normal saline (control; circles), then to NFA (1 μM; squares). T_3 (250 nM; diamonds) was then added in the continued presence of NFA. The box in the upper portion of each panel summarizes the experimental protocol used in each case. Each point represents independent experiments with between 250 and 600 MEPCs. In A, the total average values of the calculated frequencies [MEPCs/sec ± SE (N)] were 1.621 ± 0.15 (18) for control, 4.003 ± 0.51 (15) for T_3 alone, and 0.5628 ± 0.07 (17) for the mixture of NFA-T_3 (1 μM/250 nM). In B, the total average values of the calculated frequencies [MEPCs/sec ± SE (N)] were 4.003 ± 0.51 (15) for control, 0.421 ± 0.15 (18) for NFA, and 0.5628 ± 0.07 (17) for the mixture of NFA (1 μM) and T_3 (250 nM). Except for the last two values (NFA alone and NFA plus T_3), these values were significantly different from each other, as determined by the Wilcoxon signed rank test, at $P < 0.0002$.

atively greater than the endogenous levels of T_3 in pro-mp tadpoles, which have already developed thyroid glands.

NFA reduced the frequency of MEPCs, suggesting that some endogenous mechanism that is inhibited by NFA controls the basal level of MEPC frequency. Our data suggest that the endogenous level of T_3 could play a key role in controlling the frequency of MEPCs in these tadpoles. Along these lines, it is also possible that the elevated levels of T_3 in these animals play a role in the spontaneous release of neurotransmitter.

At low concentrations, NFA is thought to block mainly chloride channels (Okada et al., 2001). However, we cannot rule out the possibility that NFA may also have some blocking effects on K channels. The reduction in MEPC frequency in the presence of NFA suggests that a chloride channel may be either directly or indirectly responsible for the T_3-induced increase in MEPC frequency. Our results suggest the hypothesis that T_3 could also induce AVD. Such changes in cell volume could play a role in the control of spontaneous neurotransmitter release. This idea is consistent with previous observations that hypertonic solutions are important activators of the spontaneous release of neurotransmitter (Fatt and Katz, 1952; Hubbard et al., 1968; Mochida et al., 1998; Kashani et al., 2001) and that this process is calcium independent (Chen and Grinnel, 1995, 1997). Interestingly, it has been demonstrated that a reduction in cellular volume induced by water movement causes chloride and potassium ions to exit the cell, to compensate for the intracellular changes in osmotic concentration (Okada and Maeno, 2001). We hypothesize that this chloride ion movement could be linked to the control of spontaneous neurotransmitter release in our system, because the chloride channel blocker nonsteroidal antinflammatory fenamate NFA reduced the frequency of MEPCs to below control levels, as summarized schematically in Figure 7. In many other systems (see Maeno et al., 2000; Okano and Maeno, 2001), this chloride channel blocker inhibits the cell shrinkage associated with apoptosis (Maeno et al., 2000).

These findings raise the question of whether spontaneous neurotransmitter release is important in neuronal communication or whether it merely results from leakage from other synaptic machinery. This question was recently addressed by Saitoe and colleagues (2001). These investigators found that spontaneous neurotransmitter release is crucial for organizing sites of neuronal communication in

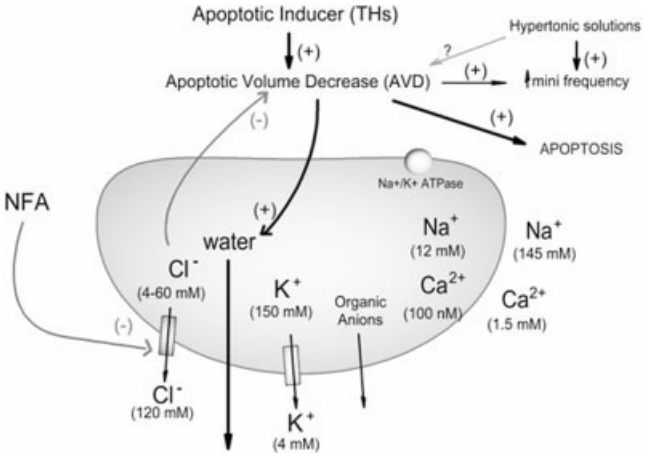

Fig. 7. Schematic model representing a hypothetic possible interaction among T_3, ionic movements, and apoptosis in the tadpole NMJ. Approximate values of the intracellular and extracellular levels of various ions in the frog NMJ are shown. Similarly to hypertonic solutions, T_3 increases the frequency of MEPCs. NFA blocks the production of MEPCs, producing the hypothetic blockage of the mechanism that induces apoptotic volume decrease (AVD).

the NMJ of *Drosophila* during development. This finding suggests that the spontaneous release of neurotransmitter may play an important role in various physiological processes in the NMJ and in other synapses, including those from the CNS.

The present findings suggest that T_3, the natural inducer of apoptosis in the tadpole tail NMJ, may play a key role in regulating the spontaneous release of neurotransmitter by producing an "elevated" tone of MEPCs at resting levels in synapses from tadpoles that are approaching metamorphosis. However, it is not clear whether the effect of T_3 on the frequency of MEPCs is required for apoptosis in the tadpole tail.

The major conclusion from these findings is that T_3 induces a nongenomic effect in the NMJ of tadpoles. A very tempting speculation is that the nongenomic action of T_3 in the tadpole tail could be part of the mechanism involved in the loss of cell viability reesulting from AVD, similarly to what has been proposed for other nonexcitable biological systems (Okada et al., 2001). Given that this biological system exhibits apoptosis that is tightly controlled by T_3, this study suggests the hypothesis that the nongenomic actions of T_3 observed in this study are part of the mechanism by which cell death is initiated in this system.

ACKNOWLEDGMENTS

We especially thank Dr. John Dempster for providing the WCP and EDR programs. We thank Ms. Annette Rivera for secretarial assistance. This work was supported by National Institutes of Health grants RR03035-16 (L.V.R.) and 2GMRO1563710-06 (J.A.L.).

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