Non-genomic regulation of transmitter release by retinoic acid at developing motoneurons in *Xenopus* cell culture

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Accepted 12 February 2004
Journal of Cell Science 117, 2917-2924 Published by The Company of Biologists 2004
doi:10.1242/jcs.01153

Summary

Although the long-term effects of all-trans retinoic acid (RA) on neuronal growth and differentiation have been intensively studied, nothing is known about its effect on synaptic transmission. Here we show that RA rapidly and specifically enhances the spontaneous acetylcholine release at developing neuromuscular synapses in *Xenopus* cell culture using whole-cell patch-clamp recording. Acute addition of RA dose-dependently and reversibly enhances the frequency of spontaneous synaptic currents (SSCs). Application of the lipophilic RA analogue all-trans retinol or RA metabolites produced by light-induced decomposition failed to provoke similar changes in SSC frequency, indicating the specificity of RA-induced facilitation of spontaneous transmitter release. Protein synthesis inhibitors anisomycin or cycloheximide had no effect on RA-induced SSC frequency facilitation. Treating cells with pan RA receptor (RAR) selective agonist or RARβ-selective agonist, but not RARα-, RARγ- or retinoid X receptor (RXR)-selective agonists, mimicked the action of RA. These results suggest that RA acts through the activation of RARβ, to induce a rapid, non-genomic increase in the frequency of spontaneous transmitter release at developing neuromuscular synapses.

Key words: Retinoic acid, Non-genomic, Transmitter release, Neuromuscular junction, Development

Introduction

Retinoids are a family of molecules derived from vitamin A and include the biologically active metabolite, all-trans retinoic acid (RA). RA, which is widely distributed in the developing embryo, plays an essential role in embryogenesis by modulating the growth and differentiation of a variety of cell types, particularly in the developing nervous system. Many experimental approaches have suggested a regulatory role for RA in the development of the nervous system. RA is involved in the early axial patterning of the limbs and determination of the anterior-posterior patterning (Maden, 2002). It also increases the survival of developing motoneurons, stimulates neurite outgrowth and directs axons extending from embryonic spinal cord explants in vitro (Wuarin et al., 1990; Maden et al., 1998; Prince and Carlone, 2003). Vitamin A deprivation in the quail embryo results in a failure to extend neurites into the periphery of motoneurons, and disruption of the retinoid signaling pathway in the adult rat leads to a loss of motoneurons (Corcoran et al., 2002; McCaffery et al., 2003). High levels of RA as well as cytoplasmic proteins, which specifically bind RA and nuclear receptors for RA, have been detected in the embryo, particularly in the spinal cord (Wagner et al., 1990; Horton and Maden, 1995). Recently, it has also been suggested that RA, as an inductive signal, can direct embryonic stem cells to differentiate into motoneurons. Furthermore, the ES cell-derived motoneurons can populate the embryonic spinal cord, extend axons, and form synapses with target muscles (Sockanathan and Jessell, 1998; Wichterle et al., 2002)

The cellular effects of RA are elicited by changes in gene expression as an outcome of the interactions of RA with its cellular binding proteins and retinoid nuclear receptors. The retinoid receptors include the retinoic acid receptors (RAR) and the retinoid X receptors (RXR), which belong to the steroid-thyroid-retinoid hormone receptor superfamily and are the transducers of the RA signal at the level of the genome (Chambon, 1994; Kastner et al., 1997). Both receptor types comprise three subtypes (RARα, β, γ and RXRα, β, γ) encoded by different genes and displaying distinct patterns of expression (De luca, 1991). These receptors belong to the steroid/thyroid hormone nuclear receptor superfamily with RAR forming heterodimers with RXR whereas RXR, either as a homodimer or by forming heterodimers with orphan receptors, acts as a transcription factor. These transcription factors bind to retinoid response elements in the promoters of target genes and activate gene expression (the so-called 'genomic' effect) in the presence of ligands (Mangelsdorf and Evans, 1995). In addition to being considered a morphogenetic molecule with long-term effects, RA is now also thought to be a rapid neuromodulator. For example, it has been suggested that RA rapidly regulates potassium channel currents in human lymphocytes and uncouples gap junctions at electrical synapses of cultured retinal horizontal cells (Sidell and Schlichter, 1986; Zhang and McMahon, 2000).

The activity of neuromuscular transmission at developing synapses is crucial in synaptic maturation and competition, as well as in the differentiation of postsynaptic properties

(Kidokoro and Saito, 1988; Lo and Poo, 1991; Balice-Gordon and Lichtman, 1993). Although all the evidence to date supports the notion that RA is essential for neuronal growth and differentiation, the role of RA in synaptic activity is not understood. It is known that RA receptors are present in developing motoneurons and high levels of RA have been detected in the embryo, particularly in the spinal cord (Wagner et al., 1990; Horton and Maden, 1995). Here we examine the acute effect of RA on synaptic transmission in cultured *Xenopus* nerve muscle–a simple and easily accessible model–providing insight into related mechanisms. Cultures derived from *Xenopus* embryos offer several advantages in studying the early events of synaptogenesis. First, previous studies of neuromuscular synapses in *Xenopus* cell cultures provide detailed descriptions of the morphological and physiological events associated with the timing of development. Second, *Xenopus* myoblasts do not fuse to form polynucleated myotubes in culture but remain mono-nucleated as long as they survive, providing good conditions for whole-cell patch-clamp recording. Third, the cells remain viable for many hours in the open air at room temperature on the microscope stage, which is ideal for electrophysiological recordings (Tabti and Poo, 1991). Because the activity of neuromuscular transmission at developing synapses is crucial in synapse formation and RA is present in the *Xenopus* embryo, this study explores the roles of RA in synaptic activity during motoneuron development. Results from this study demonstrate that through activation of RARβ, RA induces a rapid, non-genomic enhancement in the frequency of spontaneous transmitter secretion at the developing neuromuscular synapses.

Materials and Methods

Cell culture

Xenopus nerve-muscle cultures were prepared as previously reported (Lo and Poo, 1991). Briefly, the neural tube and the associated myotomal tissue of 1-day-old, stage 20-22 (Nieuwkoop and Faber, 1967) *Xenopus* embryos were dissected and dissociated in the Ca^{2+} and Mg^{2+}-free Ringer's solution supplemented with 0.15 mM EDTA. The dissociated cells were plated and used for experiments after incubation at room temperature (20-25°C) for 1 day. The culture medium consisted of 50% (v/v) Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES at pH 7.6), 49% L-15 Leibovitz medium (Sigma), and 1% fetal bovine serum (Life Technologies), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate). One day after cell plating, functional synapses were rapidly established between cultured spinal neurons and embryonic muscle cells. The present study utilized synapses where myocytes were innervated by single co-cultured spinal neurons.

Electrophysiology and data analysis

The whole-cell recording and excised membrane patch-clamp methods followed those described previously (Hamill et al., 1981). Patch pipettes (Hilgenberg) were dragged with a two-stage electrode puller (PP-830, Narishige), and the tips were polished immediately before the experiment using a microforge (MF-830, Narishige). Spontaneous synaptic currents (SSCs) were detected from innervated myocytes by whole-cell recording in the voltage-clamp mode. Recordings were made at room temperature in Ringer's solution, and the solution inside the recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.2). Evoked synaptic currents (ESCs) were elicited by stimulating presynaptic neurons at the soma with a heat-polished glass microelectrode (tip opening ~1-2 μm) filled with Ringer's solution. For supra-threshold stimulation of the neuron, a square current pulse of 0.3 milliseconds in duration and ~2-8 μA in amplitude was applied through the pipette. Such currents generally induce twitch contraction of the muscle cell when they are applied to the soma of the innervating neuron. Data were collected using a patch-clamp amplifier WPC-100 (E.S.F. Electronic) and pClamp 8.0 (Axon). Signals were filtered at 10 KHz (Digidata 1322, Axon). Spontaneous synaptic currents were detected and analyzed using the Mini Analysis Program version 5.0 (Synaptosoft). To measure the changes in neurotransmitter release quantitatively, a time course of SSC frequency was first constructed on a minute-to-minute basis. The SSC frequencies for a 6-minute period right before drug application was averaged as a control. The changes in SSC frequency were measured by averaging a 6-minute period recording starting from the highest number after drug application (Liou et al., 2003), and the results were expressed as mean±s.e.m. The statistical significance was evaluated by Student's paired *t*-test. For comparison of SSC amplitude distribution, the composite graph of cumulative frequency of all SSC events was constructed, and only synapses with a total number of events exceeding 180 were used for analysis. The statistical difference between these graphs was tested using the Kolmogorov-Smirnov test.

Chemicals

All-trans retinoic acid, AM-580, retinol, anisomycin and cycloheximide were obtained from Sigma. CIRD Galderma (Sophia-Antipolis, France) synthesized the following receptor agonists: CD367 (RAR panagonist), CD2314 (activates RARβ), CD666 (activates RARγ) and CD3640 (RXR panagonist). All retinoic acid and synthetic derivatives were dissolved as a 100 mM stock in dimethyl sulfoxide (DMSO) and then diluted into working concentrations with Ringer's solution just prior to use. The maximal final DMSO concentration was less than 0.03%. All drugs were applied directly to the culture media at the times indicated.

Results

Facilitation of spontaneous ACh quantal secretion by RA

In *Xenopus* nerve-muscle cultures, functional synaptic transmission can be detected within minutes of nerve-muscle contact (Xie and Poo, 1986; Evers et al., 1989), although morphological maturation of the synapse takes many days (Buchanan et al., 1989). SSCs are readily detectable from the innervated muscle cell with whole-cell voltage-clamp recordings. These currents must be caused by spontaneous ACh secretion from the neuron as they were eliminated by bath application of D-tubocurarine and unaffected by tetrodotoxin, which blocks action potentials in neurons (Xie and Poo, 1986). Bath application of RA at 10 μM dramatically enhanced spontaneous transmitter release, revealed by a marked increase in the frequency of spontaneous synaptic events. The increase in SSC frequency produced by RA was rapid and reached a plateau within ~8-15 minutes after application of RA, and the effect persisted for more than 30 minutes (Fig. 1A). On average, the frequency increased by 19.5-fold (±5.9, n=9) over the control SSC frequency. The RA-induced increase in the frequency of spontaneous ACh secretion showed a high dependence on the concentration of RA of between 1 and 30 μM (Fig. 1D).

The increase in frequency of SSC events after RA treatment could be due either to an increase in presynaptic release of neurotransmitter or to greater postsynaptic sensitivity to the

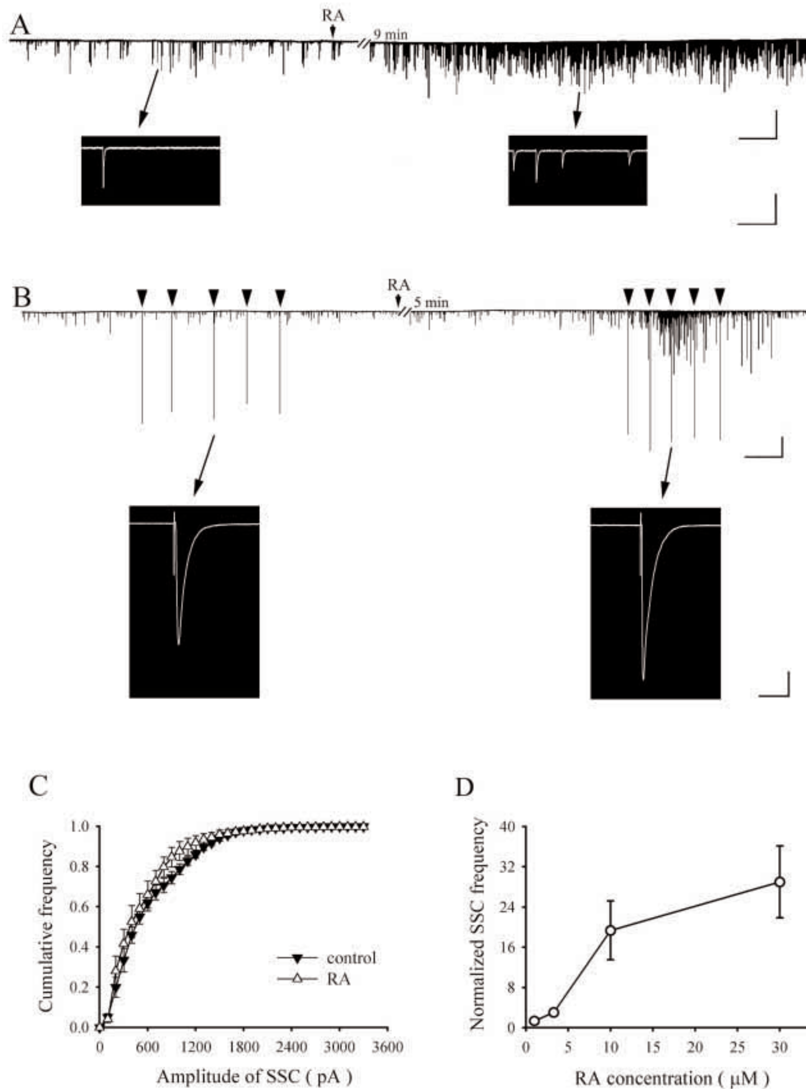

Fig. 1. Effect of retinoic acid (RA) on the spontaneous ACh quantal release at *Xenopus* neuromuscular synapses. (A) The continuous trace depicts the membrane current recorded from an innervated myocyte in day-1 *Xenopus* cell culture, using the whole-cell recording method (V_H= –70 mV, filtered at 10 kHz). Downward events are inward currents resulting from quantal ACh secretion. Samples of current events are shown below at higher time resolution. Scale bars are 1 nA, 20 seconds for the slow traces, and 1 nA, 50 milliseconds for the fast traces. (B) The presynaptic neuron was stimulated to fire action potentials and ESCs recorded in the myocytes are shown as downward deflections at the times marked by arrowheads. Shown below at higher time resolution are ESCs before and after RA application. Scale bars are 1 nA, 20 seconds, and 1 nA, 20 milliseconds for the slow and fast traces, respectively. (C) Amplitude distribution of all SSC events before and after RA treatment. Events during a period ~10-15 minutes after the application of RA were analyzed. The cumulative frequency refers to the proportion of total events with amplitudes smaller than the given amplitude. Each value represents mean±s.e.m. from 10 experiments. There was no significant difference between the two distributions (P>0.05, Kolmogorov-Smirnov test). (D) Concentration-response relationship for RA on the potentiation of SSC frequency. The mean SSC frequency ~10-15 minutes after the application of RA at different concentrations was normalized for each synapse by setting the mean SSC frequency before the RA addition to 1. Each value represents the mean and vertical line representing the s.e.m. for 5-17 experiments.

neurotransmitter. Increased postsynaptic ACh sensitivity could explain the increase in the SSC frequency, because previously undetectable small ACh quanta may emerge after exposure to the neurotransmitter. As shown in Fig. 1C, we found no detectable change in the distribution of the SSC amplitude (P>0.05, Kolmogorov-Smirnov test), suggesting that it is unlikely that ACh sensitivity was increased. The absence of any change in the rise time and the decay time of the SSC events after significant elevation of SSC frequency had occurred suggests that these factors did not affect the properties of postsynaptic ACh channels (Table 1). Thus, the primary action of RA at these synapses seems to be a presynaptic modulation of transmitter secretion mechanisms.

We examined the effects of RA on impulse-evoked synaptic currents (ESCs) by stimulating presynaptic neurons at the cell body with a microelectrode to fire action potentials. Postsynaptic recordings of ESCs were made at different times before and after addition of RA. An example of one recording is shown in Fig. 1B. We found that application of RA significantly increased the amplitude of ESCs. The average ESC amplitudes before and ~10-16 minutes after RA application were 4.9±0.3 nA and 6.2±0.2 nA (n=4, P<0.05, Student's t-test), respectively. Because no change was observed for the amplitude of SSCs (Fig. 1C), the increase in ESC amplitude is likely to result from an increased depolarization-evoked ACh release from the presynaptic

Table 1. Effect of RA, bleached RA, DMSO vehicle and retinol treatment on the properties of spontaneous synaptic currents (SSCs)

	Control	RA	Bleached RA	Vehicle	Retinol
Amplitude (pA)	568.3±73.2	606.5±168.0	574.7±70.3	472.8±57.8	529.1±112.6
Rise time (milliseconds)	1.8±0.3	2.2±0.3	1.5±0.2	2.1±0.2	1.6±0.1
Decay time (milliseconds)	3.9±0.5	4.4±0.6	4.0±0.6	4.2±0.3	3.5±0.4

*Data were collected from 6-28 experiments and presented as mean±s.e.m.

nerve terminal, rather than increased postsynaptic responsiveness.

Specificity of RA action

As RA treatment resulted in an increase in SSC frequency, we then investigated the specificity of RA action. RA is a fat-soluble substance that is structurally related to vitamin A (all-trans retinol) and susceptible to light-induced decomposition. We first investigated if RA itself, and not the products of its chemical metabolites (for example 5,6-epoxyretinoic acid and retinoyl-β-glucuronide), was responsible for the facilitation of spontaneous transmitter release. To approach this problem, we destroyed RA by exposing a 10-μM solution to strong light (100-W fiber-optic light source) overnight. The complete decomposition of RA was witnessed when the yellowish RA solution turned into a clear so-called 'bleached RA' solution (Zhang and McMahon, 2000). Treating cells with bleached RA solution failed to elicit significant change on the frequency of SSC events, and the SSC frequency after bleached RA treatment was 0.9±0.1 (*n*=7) compared to a normalized control value of 1 (Fig. 2A,D). Moreover, the characteristics of SSC events were unaffected by the presence of bleached RA solution (Table 1). RA is a lipophilic, low molecular weight (300 Da) substance that may nonspecifically disrupt the lipid bilayer of the plasma membrane. We further examined the specificity of RA on the potentiation of SSC frequency. Adding 10 μM all-trans retinol (retinol), a structurally similar compound with greater membrane disrupting effects than RA (Ortiz et al., 1992; Achkar et al., 1996), had no effect on the frequency of spontaneous transmitter release (Fig. 2C,D) with only a 1.1-fold (±0.3) increase over the control value (*n*=7). This suggests that RA-induced facilitation of spontaneous transmitter release is not a consequence of nonspecific membrane disruption. The subsequent addition of RA after retinol treatment exerted a similar enhancement of SSC frequency as RA alone (Fig. 2C,D), 26.3±14.6 times greater than the control value (*n*=5). These results suggest that RA is responsible for the facilitation of spontaneous neurotransmitter release.

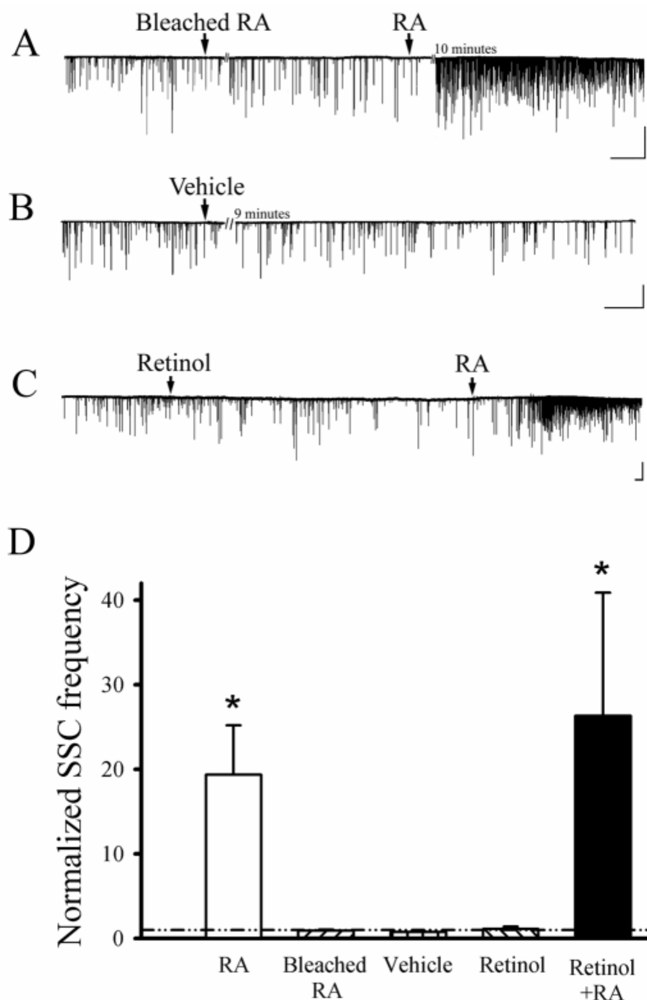

Fig. 2. RA itself is responsible for the potentiation of SSC frequency. RA was decomposed into 5,6-epoxyretinoic acid and glucuronide by exposing 10 μM RA solution to a strong light overnight and the complete decomposition was confirmed by disappearance of yellow color of the RA solution ('bleached RA'). The basal level of spontaneous ACh release was not affected by the addition of bleached RA (A), DMSO vehicle (B), or (C) retinol. Note the subsequent addition of RA ~20 minutes after retinol application caused a significant increase in SSC frequency. Scale bars, 1 nA, 30 seconds. (D) Summary of the change in SSC frequency. Plotted are the mean and s.e.m. from 7-9 separate experiments. *$P < 0.05$ compared with the control group (Student's *t*-test). The reference dotted line is shown for comparison.

Protein synthesis is not required for RA-induced facilitation of spontaneous transmitter release

RA effects mediated by modulation of gene expression are known to occur with a time lag of hours or even days and the action can last for a long time. However, our results show that the increase in SSC frequency produced by RA was rapid and dependent on the continued presence of the factor developing synapses in *Xenopus*. The SSC frequency was reduced almost to the control level within 5 minutes when the RA-containing medium was replaced with fresh medium after a 15-minute exposure to RA (Fig. 3). To further investigate this uncommonly rapid action, we tested the effect of protein synthesis inhibitors on RA-induced facilitation of spontaneous transmitter release. Protein synthesis inhibitors anisomycin (30 μM) or cycloheximide (30 μM) were added to the culture 45 minutes before the experiments. The SSC frequency facilitation effect of 30 μM RA was not hampered in the presence of anisomycin or cycloheximide (see Fig. 4; 29.4±10.5 and 24.7±12.7 normalized SSC frequencies for anisomycin and cycloheximide pretreatment respectively, *n*=7). This suggests that synthesis of new proteins is not necessary for RA-induced facilitation of spontaneous transmitter release.

Receptor-selective analogues and SSC frequency facilitation

How does RA enhance presynaptic efficacy? Classically, the effect of RA is mediated by the activation of two classes of nuclear receptor, retinoic acid receptors (RAR-α, –β, –γ) and retinoid X receptors (RXR-α, –β, –γ). We further propose that the SSC frequency facilitation induced by RA can also be

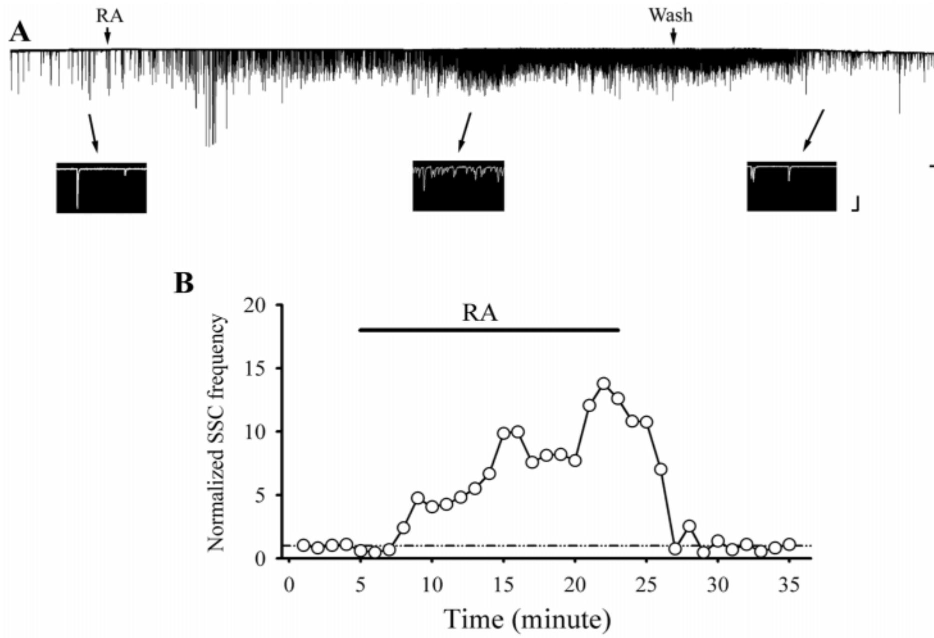

Fig. 3. SSC frequency facilitation induced by RA requires continued presence of the factor. (A) The continuous trace depicts the inward membrane current recorded from an innervated muscle cell in *Xenopus* culture (filtered at 10 kHz). The arrow marks the time of addition of RA to the culture medium and RA was removed by the substitution of the culture medium with Ringer's solution at the time indicated by 'wash'. Scale bars, 2 nA, 20 seconds and 1 nA, 50 milliseconds. (B) Changes in the SSC frequency (normalized to control frequency) with time during the addition and removal of 30 μM RA. The increased SSC frequency declined to control level within 5 minutes of washout of RA.

attributed to classical steroid receptor activation and therefore, we investigated which subtype of receptor is involved in RA action. The roles of different subtypes of retinoid receptor on the SSC frequency facilitation were analyzed using receptor-selective synthetic retinoid agonists for the individual retinoid acid receptor subtypes at 10 μm concentration (see methods). Application of CD367 (RAR panagonist) yields similar results to RA (Figs 5 and 6) in facilitating spontaneous transmitter release 15.8±5.4 (n=7) times greater than the control level. In the presence of RARα- and γ-receptor agonists there was no change in the frequency of SSC events (1.0±0.2, n=7; and 1.5±0.4, n=7, compared to control for AM580 and CD3640 treatment, respectively). In contrast, treating cells with RARβ agonist significantly increased the SSC frequency with a profile similar to RA, a rapid onset (~8-12 minutes) and with 11.5±2.7 (n=7) times control SSC frequency potentiation at the plateau. The SSC frequency remained unchanged (1.0±0.1, n=7) after the addition of RXR nonspecific agonist CD3640 (Figs 5 and 6).

Discussion

To our knowledge, we are the first to provide evidence that RA, under acute conditions, increases the frequency of spontaneous neurotransmitter release and the amplitude of impulse-evoked synaptic currents in the peripheral nervous system. Recently, it has been suggested that vitamin A and RA function as essential competence factors for long-term synaptic plasticity within the adult brain. Mice deprived postnatally of vitamin A showed a profound impairment of hippocampal CA1 long-term potentiation and a virtual elimination of long-term depression (Misner et al., 2001). In addition, genetic modification of RARβ$^{-/-}$ and RXRγ$^{-/-}$ knockout mice displayed impaired hippocampal synaptic plasticity as well as compromised

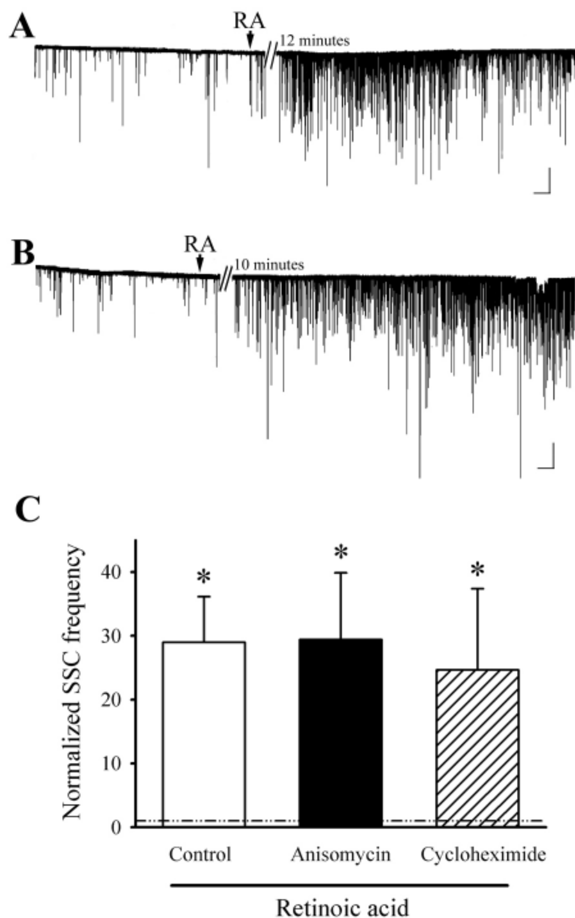

Fig. 4. Protein synthesis is not required for SSC frequency facilitation induced by RA. The culture was pretreated with 30 μM anisomycin (A) or 30 μM cycloheximide (B) for more than 45 minutes and the effect of RA on SSC frequency was then evaluated. Scale bars, 500 pA, 20 seconds. (C) Summary of data for the SSC frequency (±s.e.m.) at ~10-15 minutes after RA application under various drug pretreatment, normalized by mean control values from the same synapse before the addition of RA.

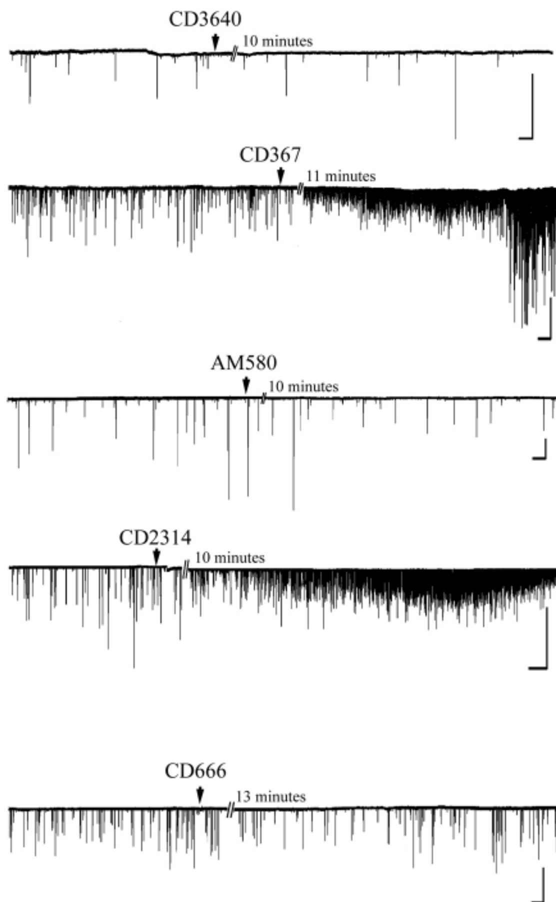

Fig. 5. Effect of various subtype-specific synthetic compounds on the SSCs in *Xenopus* cell cultures. The continuous traces depict the membrane currents recorded from the innervated myocytes before and after bath application of various agonists (10 μM). Downward deflections are SSCs (V_H= –70 mV). Scale bars, 1 nA, 20 seconds.

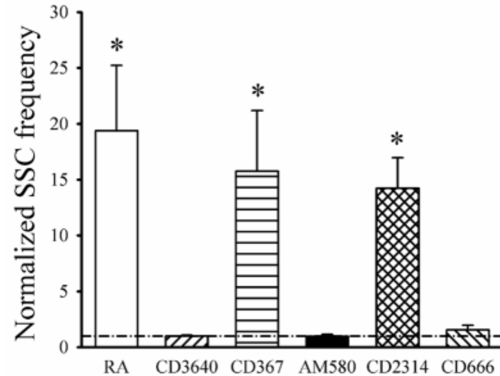

Fig. 6. Summary of the change in SSC frequency resulting from addition of various retinoid derivatives. Bar graph represents mean increase in SSC frequency induced by a series of retinoid derivatives; the name for each is indicated below the bars. Each bar shows the mean (±s.e.m.) compared to control from ~7-9 cell cultures. For comparison, the horizontal dashed line defining basal activity as 1 is shown. *$P<0.05$ compared to the RA treatment group (Student's *t*-test).

learning during behavioral tests (Chiang et al., 1998). However, functional and histological analysis of these animals revealed that the deficit in learning and memory might be due to either a reduction in hippocampal nuclei size or to impaired cholinergic transmission resulting from vitamin A deficiency-induced decrease in choline acetyltransferase synthesis (Cocco et al., 2002). These results suggest that modulation of glutamate release from the hippocampus by RA is an indirect effect. Several observations in this study indicate that RA acts by specific and receptor-mediated mechanisms. First, it has been suggested that membrane disruptions such as a change in fluidity or the microenvironment of membrane proteins might enable steroid hormones to regulate certain physiological functions (Clarke et al., 1990; Shivaji and Jagannadham, 1992). Application of all-trans retinol, a structurally similar compound with more membrane disrupting effects than RA, but lacking the capacity for binding and activation of the retinoic acid receptors, had no effect on the frequency of spontaneous transmitter release. This suggests that RA-induced SSC frequency facilitation is not a consequence of nonspecific disruptions in the lipid bilayer of the plasma membrane (Ortiz et al., 1992; Achkar et al., 1996). Second, retinoic acid contains many double bonds and is susceptible to light-induced decomposition. However, our results show that the chemical metabolites of RA failed to mimic the effect of RA, indicating that RA itself is responsible for the SSC frequency facilitation. Finally, this specificity was further confirmed by the fact that only receptor-selective agonists such as CD2314, a RARβ-selective compound, and CD367, an RAR panagonist, induce SSC frequency in the developing neuromuscular synapse.

Our results also provide new evidence for a non-classical, non-genomic mechanism for RA action in regulation of synaptic transmission at developing motoneurons. The retinoid receptors RAR and RXR belong to the steroid-thyroid-retinoid hormone receptor superfamily. Ligands of this superfamily may exert their action in living cells by the well-known genomic (classical) pathway, involving hormone binding to cytosolic receptors and subsequent modulation of gene expression followed by protein synthesis. Alternatively, pathways could be operating that do not act on the genome, therefore indicating non-genomic action (Losel et al., 2003). Although there is increasing evidence that steroids, thyroid hormones, and the metabolites of vitamin D_3, 1α, 25-dihydroxyvitamin D_3, can act with a rapid onset rather than their classical mode of genomic steroid action, very little is known of the RA effects on targets other than those classically described. Several convincing results provided by our study suggest that the SSC frequency facilitation induced by RA is incompatible with the classical genomic model of RA action. First, only a short timeframe was needed for the onset and termination of RA action. The time course of RA action on SSC frequency facilitation is much more rapid than the previously reported transcriptional effects of RA on cell differentiation. In addition, the frequency of SSC events return to control levels rapidly (~5-10 minutes) after the withdrawal of RA from culture medium. The rapid onset of facilitation following RA addition and the immediate decay of facilitation after RA removal suggests that the intracellular effector pathway does not involve a permanent alteration in the secretory machinery, which would have been associated

with a traditional differentiation effect of RA. A second important clue to the non-genomic regulation of synaptic transmission induced by RA came from the finding that inhibitors of protein synthesis did not dampen the RA-induced SSC frequency facilitation. Previous studies based on *Xenopus* or crayfish neuromuscular junctions and *Aplysia* sensory to motor synapses have shown that local protein synthesis at synaptic sites may provide a potential mechanism for modulation of synaptic activity (Martin et al., 1997; Beaumont et al., 2001; Zhang and Poo, 2002). However, we found that inhibition of protein synthesis with 30-μM anisomycin or cycloheximide had no effect on the rapid potentiation of spontaneous ACh secretion induced by RA. Thus the most likely action of RA is a direct modulation or a post-translational modification of effector proteins that are involved in the secretory machinery.

Much of our knowledge of the non-genomic effect of nuclear receptor superfamily comes from the studies of steroid hormones. Although it has been suggested that the rapid effects are likely to be transmitted via putative membrane receptors, such non-classical receptors are not yet well characterized and only limited information about their identity is available (Losel and Wehling, 2003). It has been suggested that RA reduces gap-junctional conductance in cultured retinal horizontal cells by binding to the external RARβ/γ binding site (Zhang and McMahon, 2000). In addition, a study conducted with human lymphocytes has shown that RA blocks K^+ channels by reversibly binding to a blocking site outside the channel (Sidell and Schlichter, 1986). Although results from our study provide evidence that activation of the 'RARβ' receptor is responsible for RA-induced SSC frequency facilitation, its identity requires further investigation. How does RA enhance presynaptic efficacy? It is well known that the intracellular calcium ($[Ca^{2+}]_i$) level in the nerve terminal exerts a dominant effect on the rate of spontaneous transmitter release (Augustine et al., 1987). This increase in $[Ca^{2+}]_i$ may be due to influx of Ca^{2+} from the extracellular fluid or to release of Ca^{2+} from intracellular stores. Many experimental approaches have suggested a role for steroid hormones in intracellular Ca^{2+} regulation. Aldosterone induces a rapid increase in intracellular protein kinase C (PKC) activity and a rise in Ca^{2+} in human distal colon cells (Doolan et al., 1998). In cultured skeletal muscle cells, 1,25-dihydroxyvitamin D3 produced a rise in $[Ca^{2+}]_i$ by promoting a non-genomic release of Ca^{2+} from internal stores via activation of phospholipase C and D and 1,4,5-trisphosphate (IP_3) and by calcium influx through L-type and store-operated Ca^{2+} channels (Capiati et al., 2000). It has been suggested that RA modulates PKC activity by competing with acidic phospholipids to bind to the C2-domain of PKC. Furthermore, results from amino acid alignments and crystal structure analysis suggest the existence of an RA binding site on the PKC molecule (Radominska-Pandya et al., 2000; Ochoa et al., 2003). However, it remains to be seen whether RA-induced SSC frequency facilitation results from direct modulation of voltage-dependent Ca^{2+} channels or from liberated Ca^{2+} from intracellular stores via activation of a second-messenger signaling cascade.

Apart from its known effect in patterning both the anteroposterior and dorsoventral axes, RA also has considerable significance as a neural differentiation factor. During development, RA receptors are present in developing motoneurons, and high levels of RA have been detected in the spinal cord (Wagner et al., 1990; Horton and Maden, 1995). In the present study, we provide the first physiological evidence that RA enhances the spontaneous transmitter release at the developing neuromuscular synapse. What is the functional significance of potentiating ACh secretion by RA during the early phase of synaptogenesis? Neuronal activity at developing synapses is crucial in synapse maturation and competition as well as in the differentiation of postsynaptic properties (Balice-Gordon and Lichtman, 1993; Lo and Poo, 1991). The potentiation of spontaneous ACh release at developing neuromuscular synapses may have a profound developmental significance. Several studies indicate that the gene expression and secretion of neurotrophic factors NT-3 and NT-4 in the neuromuscular junction are regulated by synaptic activity (Liou and Fu, 1997; Xie et al., 1997). Activity-dependent secretion of neurotrophic factors is also important in synaptic activity regulation and may be involved in Hebbian-type homosynaptic potentiation (Poo, 2001). Furthermore, SSCs at developing neuromuscular junctions in *Xenopus* cultures are capable of eliciting action potentials and spontaneous contractions in muscle cells (Chow and Poo, 1985). This frequent supra-threshold excitation produces a global influence on the development of contractile properties of the postsynaptic muscle cell (Kidokoro and Saito, 1988). In addition, spontaneous synaptic potentials are accompanied by a localized influx of ions at the subsynaptic site of the muscle, including Ca^{2+} (Decker and Dani, 1990). Local Ca^{2+} accumulation and the consequent Ca^{2+}-dependent enzymatic reactions are likely to play an important role in regulating the development of postsynaptic structure.

In conclusion, our results suggest that RA rapidly enhances the activity and efficacy of developing neuromuscular synapses. RA may therefore have a significant role in initiating the consecutive and complex cross-interaction between presynaptic motoneurons and postsynaptic muscle cells that then lead to the maturation of the neuromuscular synapse.

The authors are most grateful for the continuous help provided by Professor W.-M. Fu and Y.-J. Chan. Retinoid agonists were generous gifts from Dr Michel Serge of Galderma R & D (Sophia-Antipolis, France). This work was supported by the University Integration Program from the Ministry of Education and by a grant from the National Science Council of Taiwan (NSC 92-2320-B-110-010).

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