FLUCTUATION OF ACETYLCHOLINE SENSITIVITY IN SKELETAL MUSCLES WITH DEVELOPMENT, DENERVATION AND GLYCEROL TREATMENT

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Abstract—The variation of acetylcholine (ACh) sensitivity induced by development, denervation and glycerol treatment was studied using skeletal muscles of frogs and rats. In the course of metamorphosis from tadpole to adult, sensitivities (affinity) of rectus abdominis muscles of frogs to ACh and n-butyltrimethylammonium (BTMA) increased, as did the maximum responses (intrinsic activity) to BTMA, whereas the sensitivities to ACh-competitive antagonist decreased. The increasing phenomena were similar to the supersensitivity seen with denervation. The slope of dose-inhibitory response curve for curare-like agents changed from 1.0 to 1.5 with development of muscles. These results suggest changes in ACh receptors. With 4 or 10% glycerol treatment of chronically denervated diaphragm muscles, the responses to ACh decreased, whereas those to ATP increased. The maximum responses and sensitivity of ACh dose-response curves on glycerinated muscles decreased dose dependently with glycerol. ACh log dose-response curves of muscles treated with 2% glycerol shifted in parallel with d-tubocurarine application and pA2 values were observed to be similar to those of non-treated muscles. The responses of glycerinated muscles to ACh were potentiated by 1.5 μM neostigmine less than those seen in non-treated muscles. The increase in ACh sensitivity with development may be in close analogy to the supersensitivity seen with denervation, and the pattern of the increase in ACh sensitivity seen with development or denervation was the reverse of that seen with glycerol treatment. The sensitivity of ACh receptor is probably closely related with the glycerol treatment.

The responses to acetylcholine (ACh) on skeletal muscles in levels of development, denervation and glycerol treatment are considered to reflect the sensitivity of ACh receptors to ACh. The process of denervation supersensitivity is similar to that of development in ACh reactions. As denervation supersensitivity developed, the action potential becomes resistant to tetrodotoxin (TTX) and sodium-dependent TTX-resistant action potentials have also been found in the developing rat myoblast (1). The properties of receptors presented in neonatal diaphragm muscles were found to be similar to those of receptors in denervated muscles, as shown with d-tubocurarine (d-TC) inhibition and isoelectric focusing (2). The ACh responses of skeletal muscles treated with various concentrations of glycerol from 2% (expressed as 'partially glycerinated') to 50% are of interest as the reverse phenomenon is found in cases of denervation. From these points of view, we studied the dose-response curves induced by ACh on isolated tissues in the situation of development, denervation and glycerol treatment, and also whether glycerol was effective as a solvent for the extraction of ACh binding protein.
MATERIALS AND METHODS

Experiments were performed during metamorphosis of the Rana catesbiana weighing from 6–26 g to 106–378 g in each growing stage: GS-I (the hind legs and fore ones had not appeared), GS-II (the hind legs began to appear), GS-III-IV (the hind legs were more developed), GS-V (a tail resorption) and the adult GS-F. Isolated rectus abdominis muscles were suspended in 5 ml Ringer's solution. ACh (Daiichi Pharm.), n-butyltrimethylammonium bromide (BTMA), d-tubocurarine chloride (d-TC; Nakarai), cyclohexyl thionothiol phosphorylcholine bromide (NP 245) reported to be a potent curare-like compound (3), and neostigmine methylsulfate (NeS; Merck) were used. The dose-response curves induced by BTMA were plotted as the percent to an irreversible mechanical contraction induced by 0.1 M CuCl₂ (4) in order to study the change in the maximal response.

In glycerination experiments, isolated rectus abdominis muscles of Rana nigromaculata weighing 20–40 g and chronically denervated diaphragm muscles of male rats (Wistar strain) weighing 150–250 g were used. Glycerination procedures were as follows: after being contracted by 0.55 mM ACh and then washed out, the muscles were fixed to prevent irreversible contractions, and were then immersed in 10 ml glycerol-Ringer's solution at 0 °C for 24 hr in the case of rectus abdominis muscles, and in 10 ml glycerol-Tyrode solution at 11 °C for 3–20 hr in the case of denervated diaphragm muscles, respectively. Glycerol-Ringer's solution and glycerol-Tyrode solution consisted of a prescribed concentration of glycerol and the residual volume of Ringer's (NaCl 111, KCl 2, CaCl₂ 0.9 and NaH₂PO₄ 1.7 mM) or modified Tyrode (NaCl 137, KCl 5, CaCl₂ 2, MgCl₂ 1, NaH₂PO₄ 1, NaHCO₃ 12 and glucose 11 mM) solutions. These muscles were washed out for 1–2 hr with Ringer's or modified Tyrode solution and then were allowed to respond to ACh. The other muscles were washed out with 150 mM KCl solution containing 10 mM MgCl₂ or modified KCl solution (KCl 150, MgCl₂ 10, NaH₂PO₄ 1, NaHCO₃ 12 and glucose 11 mM) and then allowed to respond to adenosine 5'-triphosphate disodium salt (ATP; Daiichi Pharm.).

Denervation; the left lateral phrenic nerves of etheranaesthetized rats were extracted 1–3 cm and dissected at the plexus cervicalis. Segments (0.3 × 1.5 cm) of the muscles 5–17 days after denervation were isolated and the right lateral innervated muscles were used as a control.

The responses to ACh, d-TC, NeS and ATP were obtained isotonically at a room temperature (22–26°C), load 650 mg, magnification ×8 (rectus abdominis muscles of frogs) and at 30°C, 500 mg, ×10 (diaphragm muscles of rats). Ringer's solution was used for the rectus abdominis muscles, modified Tyrode solution for diaphragm muscles, and corresponding KCl solution above mentioned, respectively.

RESULTS

Responses induced by acetylcholine and n-butyltrimethylammonium with or without various drugs on rectus abdominis muscles of frogs (Rana catesbiana) in various stages of metamorphosis

In the early developmental stages, ACh induced dose dependently phasic contractions which were not followed by tonic contractions. The contractions were blocked dose
dependently by d-TC (1 and 10 \(\mu g/ml\)) and potentiated by NeS (5 \(\mu g/ml\)) as shown in Fig. 1. These observations suggested the existence of ACh receptors and also acetylcholinesterase (AChE) even in the early developmental stage. With growth, tonic contractions were generated by ACh, and the sensitivity to ACh increased, whereas the AChE activity did not increase.

In experiments on tadpoles, 0.1 M CuCl\(_2\) produced much larger contractions even at the earlier growth stage than did 0.1 M K\(_2\)SO\(_4\), which produced only smaller ones than the maximal dose of ACh in some cases. The CuCl\(_2\) was therefore used as a convenient tool for a standard of contractions, although mechanisms of irreversible contractions are still unknown (4).

BTMA has been reported by Kimura (5) to have the same site of actions as ACh, but not to be hydrolyzed by AChE. According to growth from GS-III to GS-IV, GS-V and GS-F, as shown in Fig. 2, BTMA dose-response curves shifted to the left, and their maximum responses gradually increased.

The responses induced by ACh in the two or three doses to the muscles of GS-II-III and GS-F were studied with or without d-TC (1.44 \(\mu M\)) or NP245 (0.543 \(\mu M\)). Each

![Graphs showing ACh responses with and without d-tubocurarine or neostigmine on rectus abdominis muscles of frogs in various stages of metamorphosis.](image)

Fig. 1. Typical records of ACh responses with and without d-tubocurarine or neostigmine on rectus abdominis muscles of frogs in various stages of metamorphosis. Stage I; tadpole before hind legs emerge, Stage II-III; when hind legs emerge, Stage V; small frog of which tail was completely resorbed. Note tonic contraction following after the phasic one with growth.
response represents a percentage of the maximum responses to ACh in each stage and is transformed logistically (6) in Fig. 3 a and b. These results indicated that despite increase in ACh sensitivity, the inhibitory responses to d-TC or NP245 appeared to decrease with growth because ACh dose-response curves in GS-II—III shifted to the left with application of the antagonist to a greater extent than did those in GS-F. This suggests the decrease of sensitivity of the muscles to these antagonists with growth.

In rectus abdominis muscles of adult Rana nigromaculata, the slope of NP 245 in dose-inhibitory response curve was reported not to deviate significantly from the theoretical value 1.5 (3). The slope in the case of tadpole as shown in Fig. 4 was estimated to be 1.1,
which did not deviate from a theoretical value 1.0 according to the calculation previously reported (6); linear regression: \( F_0 = 0.973 < \frac{F}{F_{0.05}} = 2.86 \) \((p = 0.05)\), deviation from the slope (1.0): \( t_0 = 0.816 < t_{3.182} = 3.182 \) \((p = 0.05)\). It is, however, not still explicable that the slope of NP 245 dose inhibitory response curves changed from 1.0 to 1.5 with growth.

**Effects of glycerol treatment on chronically denervated diaphragm muscles of rats compared with rectus abdominis muscles of adult frogs (Rana nigromaculata)**

Chronically denervated or innervated diaphragm muscles were treated with 50% glycerol for 20 hr at 5°C. The muscles responded previously to 0.1 M K\(_2\)SO\(_4\) and 5.5 \(\mu\)M ACh. Glycerol treated muscles did not contract with the same concentration of K\(^+\) and ACh, but did contract with application of 6.6 mM ATP as shown in Fig. 5. Since the responses induced by ACh reached almost the same shortening as that induced by K\(^+\) ion, they were regarded as the maximum level of the denervation supersensivity (7). Both innervated and denervated muscles treated with 50% glycerol were contracted by ATP to the same extent.

The contractions induced by 5.5 \(\mu\)M ACh or 6.6 mM ATP on rectus abdominis muscles of frogs were expressed as a percentage of the contraction caused by 0.1 M K\(_2\)SO\(_4\) in Fig. 6. With glycerol treatment, the responses to ACh disappeared and those to ATP appeared gradually at about 10% glycerol. With ethanol treatment, almost the same tendency was observed, except that the response to ATP disappeared at a lower concentration of ethanol than glycerol. These results suggest that treatment with over 20% ethanol denaturates muscle proteins.

The effect of glycerol treatment on diaphragm muscles of rats showed the same tendency as on rectus abdominis muscles of frogs as shown in Fig. 7. ACh dose-response curves of these muscles treated with several concentrations of glycerol are shown in Fig. 8. With
increase in the glycerol concentrations, the maximum height of contractions decreased and the graded curves shifted to the right. These phenomena are opposite those seen with denervation supersensitivity of diaphragm muscles. The denervation supersensitivity, that is the gradual increase of affinity and intrinsic activity of the same curves with days after phrenicotomy, was attributed to the increase of ACh receptor (8, 9). Thus, the results seen with the glycerol treatment, probably reflect the decrease in sensitivity of ACh receptors.

FIG. 6. Effects of increasing concentrations of glycerol (circles) or ethanol (triangles) on contractions induced by 550 μM ACh (blackening symbols) or 6.6 mM ATP (open symbols) on rectus abdominis muscles of frogs. Each point showed the average of 4 experimental values with ± S.E.. Note ATP-induced contraction following after the disappearance of ACh-induced contraction of both glycerol- and ethanol-treated muscles.

FIG. 7. Effects of increasing concentrations of glycerol on the contractions induced by 30.8 μM ACh (●) or 6.6 mM ATP (○) in chronically denervated rat diaphragm muscles. Each point showed the average of 4 experimental values with ± S.E.. Note ATP-induced contraction following after the disappearance of ACh-induced contraction of glycerol-treated muscles.

FIG. 8. ACh log dose-response curves of chronically denervated rat diaphragm muscles treated with each different concentration of glycerol. Each point showed the average of 4 experimental values with ± S.E..
Effects of ACh-related drugs on the glycerol muscles

To determine whether or not residual ACh receptors in glycerinated muscles with 2% glycerol are intact, the responsiveness of these muscles to d-TC and NeS was studied. From ACh dose-response curves of glycerinated muscles in the absence and presence of d-TC (0.36, 0.72 and 1.44 μM), log (A/Ao−1) was plotted against log dose of d-TC according to the method of Arunlakshana and Schild (10). The lines of both glycerol treated muscles and untreated muscles did not differ significantly, indicating that these muscles had almost the same or slightly smaller pA2 values, so to speak, the same affinity for d-TC. These results suggest that residual ACh receptors in glycerol treated muscles were intact.

The ACh dose-response curves of glycerol-treated muscles treated with 1.5 μM NeS for 30 min were compared with those of untreated muscles as shown in Fig. 10. NeS caused a much lesser shift in the curve to the left seen in the glycerol treated muscles. These results...
suggested that 2% glycerol-treatment decreased AChE activity in the tissues.

It is known that monohydric alcohols potentiate the responses to ACh (11). The contractions induced by ACh (5.5 μM) on chronically denervated rat diaphragm muscles were potentiated by acute administration of 1% glycerol, accompanied with a slight contraction. The same effects were also observed after treatment with 1.5 μM NeS for 1 hr, suggesting that the potentiation was not the result of the anti-cholinesterase action of glycerol. The acute effects of 1-2% glycerol on muscles treated with 2-3% glycerol were no potentiation or rather slight decrease of the response to ACh, but the response to ACh was potentiated only after washout of the muscles as shown in Fig. 11. Thus, partial disappearance or lowering of membrane function is apparent in glycerol treated muscles.

DISCUSSION

In the developmental process from tadpole to the fully grown stage, the increase of ACh sensitivity and the generation of the tonic contraction may be considered to be due to the increase of the efficacy for ACh and the multiplication of muscle cells, respectively. With growth, it was also observed that the sensitivity to d-TC or NP 245 decreased. The slope
of NP 245 dose-inhibitory response curve changed from 1.0 to 1.5, which meant the change of apparent binding order between ACh receptor and NP 245. The gradual increases of the intrinsic activity and affinity of ACh dose-response curves on rat diaphragm muscle after denervation were attributed by Elmqvist et al. (7) to the increase in the number of receptors in the muscle membrane. Our results, therefore, that development increased gradually the intrinsic activity and the affinity of BTMA dose-response curve may be likewise explicable in terms of the increase in the amount of ACh receptors.

The ACh responses on the glycerol-treated muscles were observed to decrease gradually with increase in the concentrations of glycerol. The residual ACh receptor under 2% glycerol treatment was not considered to differ from that of untreated muscles, since two $d$-TC lines of Schild plot overlapped. On the other hand, denervation supersensitivity was regarded to occur due to the increase in the dimension of ACh receptor area (suggested by Miledi), not due to the increase of sensitivity of ACh receptor itself (8, 9). Contrary to this, the decrease of ACh responses on glycerol treated muscles may reflect the homogeneous decrease in the number of ACh receptors in the muscle membrane, and not the decrease of sensitivity of ACh receptors. This assumption is supported by findings with the acute effects of glycerol; the sensitization of ACh responses in untreated muscles and the loss of the sensitization in glycerol treated muscles. These experimental results suggested that ACh receptors may to some extent be solubilized by glycerol.

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