Lack of Effects of Carbachol on the Na-Ca Exchange Mechanism in Frog Atrial Muscle Treated with Goniopora Toxin

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Abstract—Carbachol (CCh) at a concentration of 10^{-7} M completely inhibited the twitch contraction of frog atrial muscle. However, when the preparation was treated with goniopora toxin (GPT), a selective inhibitor of Na channel inactivation, and the twitch contraction was augmented through the activation of Na-Ca exchange by this toxin, the reduction in contractile amplitude by CCh was only about 30% even at higher concentrations. The maximum rate of rise of the twitch contraction was not affected by CCh in GPT-treated preparations. The time course of configuration change of the twitch contraction after application of CCh in GPT-treated muscle indicated that the attenuation of amplitude preceded the shortening of duration. The residual contraction under the combined treatments with GPT and CCh was abolished by removal of external Ca^{2+} or addition of TTX. CCh only moderately shortened the action potential which was prolonged by GPT, and further addition of TTX abolished the action potential. From these results, we suggest that CCh does not influence the twitch contraction which is augmented via the activation of the Na-Ca exchange mechanism in frog atrial muscle.

In cardiac muscles, Ca ions required for generation of twitch contraction are influxed through the slow channel and Na-Ca exchange mechanism (1). Muscarinic agents produce a negative inotropic effect by reducing the slow inward Ca current directly at low concentrations and indirectly due to an increase in K current at higher concentration (2–7). However, it is not clear whether the Na-Ca exchange mechanism is affected by muscarinic agents.

Previously, we reported that goniopora toxin (GPT), a polypeptide isolated from coral, prolonged the action potential and produced a positive inotropic effect in mammalian and frog cardiac muscles (8–11). The underlying mechanisms are the inhibition of Na channel inactivation (10, 12), causing the increase in Na influx and the subsequent activation of the Na-Ca exchange mechanism.

In the present study, we investigated the effects of carbachol (CCh) on the twitch contraction which was augmented and prolonged by GPT. The results obtained suggest that CCh fails to affect the Na-Ca exchange mechanism which is activated under treatment with GPT.

Materials and Methods

For mechanical recordings, thick muscle bundles with a diameter ranging from 800 to 1000 μm were dissected from the left atrium of *Rana catesbeiana*. The preparations were vertically mounted in a muscle bath of 20 ml capacity containing modified Ringer’s solution. The atrial appendage was connected by a thread to the lever of a force-displacement transducer and the isometric tension was recorded. The resting tension was adjusted to 100 mg. The atrium was electrically driven with two platinum electrodes. Square wave pulses of 6 msec duration at a voltage 1.5
times the threshold at 0.1 Hz were used. The bathing medium was aerated with 95% O₂-5% CO₂. At least 60 min were allowed for equilibration before the start of experiments. The composition of the modified Ringer’s solution was as follows (mM): NaCl, 98.3; KCl, 2.5; CaCl₂, 1.0; NaHCO₃, 14.7; and glucose, 20.0. Propranolol (10⁻⁷ M) was added to the bath to eliminate the activation of β-adrenergic receptors. Calcium-free solution was obtained by omitting calcium and adding a chelating agent, EGTA [ethylene glycol bis(β-aminoethylether)-N,N′,N′,N′-tetraacetic acid], at a concentration of 10⁻³ M.

For electrophysiological experiments, thin muscle bundles (200–300 μm) were dissected, and the double sucrose-gap technique was used, as described previously (11). The chamber was composed of three compartments separated by two cuffs (2.0 mm long) of isotonic sucrose solution. The test node in the central compartment was adjusted to 200 to 300 μm. Two lateral compartments at each end of the preparation were filled with isotonic KCl solution (127 mM). A pair of low resistance Ag/AgCl electrodes in the lateral KCl pools was used to inject the current and to measure the potential. Another Ag/AgCl electrode was placed in the upstream end of the central compartment. The composition of the normal bathing solution was as follows (mM): NaCl, 110.0; KCl, 2.5; CaCl₂, 1.0; HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid), 3.0; and glucose, 20.0. The pH of the solution was 7.3. All experiments were done at a temperature of 16±0.5°C.

GPT was prepared as described by Hashimoto and Ashida (13). Carbachol (Sigma, St. Louis, U.S.A.), propranolol (Nakarai, Kyoto) and tetrodotoxin (Sankyo, Tokyo) were purchased.

**Results**

CCh at concentrations over 3×10⁻⁹ M produced a negative inotropic effect in a concentration-dependent manner, and at 10⁻⁷ M CCh, the twitch contraction disappeared (Fig. 1A). However, when the contraction was augmented by 10⁻⁸ M GPT, the inhibitory effect of CCh was much reduced; the inhibition was approximately 30% even at 10⁻⁶ M CCh (Fig. 1B), and further inhibition was not observed at higher concentration (Fig. 2, filled circles). Duration of 50% relaxation of the twitch contraction was shortened by CCh in the absence and the presence of GPT (Fig. 2, triangles). However, the extent of shortening in GPT-treated preparations was much smaller than that in toxin-untreated preparations.

Figure 3 shows the effects of CCh on the maximum rate of rise of twitch contraction. In normal preparations, the rate was reduced by CCh in a concentration-dependent manner (Fig. 1A). However, in GPT-treated preparations, the maximum rate of force development was not affected by CCh, suggesting that CCh had no effect on the rising phase of twitch contraction in GPT-treated preparations. Thus, CCh attenuated the peak amplitude of twitch contraction and shortened the duration in GPT-treated preparations. Such configuration change of twitch contractions was clearly observed in the time course after addition of a high concentration of CCh. Figure 4 shows a representative result in which the GPT-treated preparation was stimulated every 10 sec and twitch contractions before and at the 3rd, 5th and 10th stimulations after application of 3×10⁻⁷ M CCh were superimposed. In this case, however, the inhibition of peak amplitude was more rapidly developed than...
the shortening of the duration. These results indicate that there are at least two different mechanisms underlying the configuration change of twitch contraction in GPT-treated preparations.

Figure 5 shows the effects of CCh on electrical activity. In normal preparations, CCh shortened the action potential markedly and reduced the amplitude, resulting in a transient spike. In the GPT-treated preparation, action potential was markedly prolonged. CCh also shortened the action potential duration and reduced the amplitude, but the action potential that was longer than the control one persisted even after CCh. The residual components after application of CCh in GPT-treated and untreated preparations were completely abolished by 10^{-6} M tetrodotoxin, suggesting that the residual components were induced through Na channels.
Fig. 4. Time course of the effects of CCh on twitch contraction in a preparation treated with $10^{-8}$ M GPT. The preparation was continuously stimulated at intervals of 10 sec. The contractions elicited by the 3rd, 5th and 10th stimulation after application of $3 \times 10^{-7}$ M CCh are superimposed. Note that the inhibition of peak amplitude preceded the shortening of duration by CCh.

Fig. 5. Effects of CCh on action potential of frog atrial muscle. A: GPT-untreated preparation. Left panel shows the action potential before applying CCh, and the middle panel shows the spike-like action potential elicited 4 min after application of $10^{-7}$ M CCh. Right panel shows the abolishment of action potential after further application of $10^{-6}$ M tetrodotoxin (TTX). B: Left panel shows the prolonged action potential in the $10^{-8}$ M GPT treated preparation. Middle panel shows the action potential 4 min after application of $10^{-6}$ M CCh. The right panel shows the effects of further application of $10^{-6}$ M TTX.

Discussion

It is well-known that the twitch contraction of frog atrial muscle is elicited by slow inward calcium current ($I_{Ca}$) and that the $I_{Ca}$ is markedly reduced by muscarinic agents (3, 4). In the present study with GPT-untreated preparations, also, CCh attenuated the twitch contraction and inhibited the action potential. In contrast, such effects of CCh were much reduced in GPT-treated preparations.

Previously, we reported that GPT prolonged the action potential of frog atrial muscle by inhibiting the inactivation process of the Na channel and induced the positive inotropic effect due to the secondary activation of the Na-Ca exchange mechanism (9, 10). In the present study, also, GPT prolonged the action potential and twitch contraction, and it increased the contractile amplitude. CCh at high concentrations showed the inhibitory effects on such parameters of GPT-affected twitch contraction and action potential, but the contraction and action potential were highly resistant to CCh as compared with those of toxin-untreated preparations. These results indicate that CCh cannot produce a potent negative inotropic effect in the toxin-treated muscle.

It is interesting to note that the twitch contraction which remained after treatments with GPT and CCh was abolished by TTX or Ca removal. The action potential was also abolished by TTX in the preparations which were treated with GPT and CCh. These results are much the same as those observed in the preparations treated with GPT alone (10). Thus, it is likely that the twitch contraction observed after treatments with GPT and CCh is also produced mainly through a Na-Ca exchange mechanism, as suggested in GPT-treated preparations (9, 10).

Although the effects were not so evident, CCh produced muscarinic effects on GPT-treated muscles. Close inspection of the configuration of twitch contraction has revealed that the inhibitory action of CCh on the contractile amplitude developed more rapidly than the shortening action on the contractile duration. This unparallel change would reflect the different time courses of two muscarinic effects of CCh; that is, a reduction of $I_{Ca}$ and an increase in outward potassium current (2-7). As mentioned above, the twitch contraction of GPT-treated preparations is caused by Ca influx which is mediated through Na-Ca exchange and also in part through the slow channel (9, 10). Therefore, we considered that CCh
initially and/or at low concentrations attenuated the peak amplitude of contraction due to the reduction of $I_{Ca}$, and/or at higher concentrations it attenuated both the peak amplitude and duration of twitch contraction due to the increase in outward potassium current, because the prolonged action potentials in GPT-treated muscles were shortened by CCh. These effects of CCh were actually observed in Figs. 1, 2 and 4.

From these results, we deduced that the Na-Ca exchange mechanism may not be affected directly by muscarinic agents. Ikemoto and Goto (4) have come to the same conclusion from the results that the tonic contraction induced by large depolarization under voltage clamp conditions was not affected by acetylcholine.

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