The Time-dependent and Dose-dependent Effects of the Sulfhydryl Blocker N-Ethylmaleimide on the Tonic Tension in Bullfrog Atrium

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Abstract The kinetics of the inhibitory action by N-ethylmaleimide (NEM) on I_{Ca} -independent tonic tension in bullfrog atrial muscle was studied under voltage-clamped conditions, using the double sucrose gap method. The higher the concentration of NEM became, the larger was the rate of decrease of the tonic tension. In addition, the tension decreased with time of exposure to NEM. When the rate of the decrease of the tonic tension was plotted as a function of NEM concentration, the curve showed saturation kinetics of the Michaelis-Menten type with respect to NEM concentration, following the characteristics of a carrier-mediated process. Moreover, in the presence of excess Ca or half quantity of Na in Ringer solution, the curve showed sigmoidal kinetics. This probably indicates that the condition of either an excess of Ca or a reduction of Na in Ringer solution is apparently a cooperative effector of the inhibitory action of NEM, suggesting that the carrier protein responsible for the generation of tonic tension may be an allosteric protein. Finally, the relationship between I_{Ca} -independent tonic tension and the Na-Ca exchange mechanism which is supposed to be a powerful candidate for generating tension was discussed.

Usually, the cell membrane contains special binding proteins for drugs and hormones. These binding proteins located on the outer surface of the membrane are composed of binding sites (receptors) or loci. As the first steps for the action of N-ethylmaleimide (NEM) used in a series of experiments (AOMINE and ABE, 1978a, b), it is supposed that the drug irreversibly binds with high affinity to specific receptors composed of sulfhydryl groups. In bullfrog atrium, we have shown the general characteristics of action of NEM on the membrane currents, voltages and tension components, using the double sucrose gap method under voltage-clamped or unclamped conditions in previous papers. Especially for the tension components, NEM caused an initial increase and a late decrease in I_{Ca} -dependent phasic tension, but it caused a consistent reduction in I_{Ca} -independent

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tonic tension.

Therefore, if the effects of NEM on the atrial muscle result from the chemical modification of the sulfhydryl groups of binding proteins on the atrial cellular membrane, we can expect that the NEM-receptor interaction in the atrium should obey certain rules.

In the present work, special attention was paid to an inhibitory action of NEM on I_{Ca} -independent tonic tension in order further to characterize the mode of action of NEM. It was confirmed that the decrease in I_{Ca} -independent tonic tension by NEM was explained by the Langmuir adsorption isotherm, suggesting a carrier-mediated process. A preliminary report of part of this work has been presented elsewhere (AOMINE and ABE, 1977).

MATERIALS AND METHODS

Preparation and the experimental conditions were the same as reported previously (AOMINE and ABE, 1978a).

RESULTS

A NEM concentration-dependent decrease of tonic tension

As reported in previous papers (AOMINE and ABE, 1978a, b), NEM at 10^{-3} to 10^{-4} M led to a transient enhancement of twitch and I_{Ca} -dependent phasic tension of the bullfrog atrial muscle, followed by a late inhibition. For the case of I_{Ca} -independent tonic tension, 10^{-3} M NEM caused a gradual depression with time of exposure, whatever the ionic circumstances (Na-poor or Ca-rich solution) tested.

In the present work, the effects of NEM at various concentrations on the tonic tension were examined (Fig. 1). Figure 1 shows the inhibitory effects of representative concentrations (10^{-4} , 2×10^{-4} , 4×10^{-4} and 10^{-3} M) of NEM on the tonic tension. The inhibitory action of NEM on the tonic tension was significant at around 10^{-4} M NEM. However, NEM at 10^{-5} M hardly affected the tonic tension. The tonic tension linearly decreased with time of exposure to NEM, though at 10^{-3} M the time course of decrease of the tension showed a slight sigmoidal curve. It seems that this sigmoidal curve is concerned with the diffusion of extracellular fluid containing NEM; it takes a little time for the fluid containing NEM to reach the cellular surface. The higher the concentration of NEM became, the larger was the rate of the decrease of tonic tension. For example, at 30 min the ratios of the decrease by 10^{-4} , 2×10^{-4} , 4×10^{-4} and 10^{-3} M NEM were about 23, 38, 61 and 96%, respectively. From these results, the rate of the decrease of tonic tension within the exposure time of 30 min was plotted as a function of NEM concentration (Fig. 2A). The rate of decrease of tonic tension after addition of NEM showed saturation kinetics of the Michaelis-Menten type with respect to NEM concentration, exhibiting the characteristics of a carrier-mediated process. The Lineweaver-

Fig. 1. Time courses of the decrease of I_{Oa} -independent tonic tension in various concentrations of NEM. Panel A, B, C and D represent the time courses of the tonic tension after addition of 10^{-4} , 2×10^{-4} , 4×10^{-4} and 10^{-3} M NEM, respectively. NEM was added to the atrial muscles at zero time and data are presented relative to the control 100%. Each point represents the mean value of 5-10 determinations.

Fig. 2. (A) A relationship between the concentration of NEM and the rate of the decrease of the tonic tension by the drug at 30 min after addition of the drug in normal Ringer solution. Data are presented relative to the control 100% and each point represents the mean value of 5-10 determinations. (B) Lineweaver-Burk plot of data derived from (A). A half-saturation value of the maximal value is about 1.8 mM.

Burk plots of the data also supported the above view (Fig. 2B). The half-saturation value of the maximal decrease for exposure to NEM was about 1.8 mM.

Cooperative effect of either reduction of Na or excess of Ca on NEM-binding

In the previous work (AOMINE and ABE, 1978b), we have already observed that the effects of NEM on tension components were facilitated under the conditions of either a Na-poor or Ca-rich solution. In this paragraph, in order further to characterize the mode of the inhibitory action of the tonic tension by NEM, the effects of both Na-poor and Ca-rich solutions on NEM-kinetics were studied. Calcium-rich Ringer solution was made by newly adding 4 mM Ca to normal Ringer (Ca, 1 mM). A half-Na Ringer solution was used as a reduced Na solution, in which NaCl in the normal Ringer solution was reduced to 1/2 and replaced by isosmotic sucrose.

After the atrial muscle strips had been perfused with Ca-rich or Na-poor solution for 10 min, 10^{-3} M NEM was added to the respective solutions. Figure 3A shows the effect of excess of Ca on NEM kinetics. At 10, 20 and 30 min after the addition of NEM, the curves of the rate of decrease of tonic tension vs. the concentration of NEM became sigmoidal curves, differing from the hyperbolic curve not obeying the kinetics of the Michaelis-Menten equation in Fig. 2. Similarly, in 1/2-Na Ringer solution, the curves at 10, 20 and 30 min also were sigmoidal (Fig. 4A).

Fig. 3. (A) A relationship between the concentration of NEM and the rate of decrease of tonic tension by the drug at 10 (x), 20 (○) and 30 (●) min after addition of the drug in 5 mM Ca Ringer solution. Data are presented relative to the control 100% and each point represents the mean value of 3–4 determinations. (B) A Hill plot of data derived from (A). Symbols are the same as (A).

Kinetics for the inhibitory action of NEM

To account for the effect of NEM on the I_{Ca} -independent tonic tension, it was assumed that NEM binds to some receptor in the muscle membrane. It was fur-

Fig. 4. (A) A relationship between the concentration of NEM and the rate of decrease of tonic tension by the drug at 10 (x), 20 (o) and 30 (●) min after addition of the drug in 1/2-Na Ringer solution. Data are presented relative to the control 100% and each point represents the mean value of 3-5 determinations. (B) A Hill plot of data derived from (A). Symbols are the same as (A).

ther assumed that one or more molecules of NEM simultaneously react with this receptor *i. e.*,

where k_1 and k_2 are the association and dissociation rate constants respectively, $[\text{NEM}]$ the concentration of free NEM, and $[\text{NEM}_n \cdot \text{R}]$ the concentration of the NEM-receptor complex. n is the number of molecules. The unknown term $[\text{R}]$ could be considered as resulting from

$$[\text{R}] = [\text{R}_t] - [\text{NEM}_n \cdot \text{R}] \quad (2)$$

where $[\text{R}_t]$ is the total concentration of the receptors. At equilibrium,

$$\frac{[\text{NEM}_n \cdot \text{R}]}{[\text{R}_t]} = \frac{[\text{NEM}]^n}{[\text{NEM}]^n + K} \quad (3)$$

where $K = k_2/k_1$ is the equilibrium dissociation constant and $[\text{NEM}_n \cdot \text{R}]/[\text{R}_t]$ represents the fraction of receptors occupied by NEM. Here, the initial rate of the reaction, v , is in proportion to $[\text{NEM}_n \cdot \text{R}]$, and the maximal rate V_m is proportional to $[\text{R}_t]$. The maximal rate V_m was obtained from an estimation with eye in the respective figure. Then,

$$v = \frac{V_m \cdot [\text{NEM}]^n}{[\text{NEM}]^n + K} \quad (4)$$

where n is the so-called Hill number and also the number of molecules as mentioned

above, which are thought to represent the number of molecules that simultaneously become associated and dissociated from the receptor. Equation (4) may be expressed in log plotting

$$\log \left(\frac{v}{V_m - v} \right) / \log [\text{NEM}] \quad (5)$$

where Eq. (5) is the so-called Hill plot.

Figure 3B indicates that in 5 mM Ca Ringer solution the Hill number is about 3. In 1/2-Na Ringer solution, the number is about 2 (Fig. 4B). Naturally, for hyperbolic curves shown in Fig. 2A, the Hill plot gives a straight line with a unit slope. These increases in the Hill number suggest that either an excess of Ca or a reduction of Na in Ringer solution is apparently a cooperative effector for the NEM effect.

DISCUSSION

The results presented here together with those previously reported (AOMINE and ABE, 1978b) suggested that an essential feature of the I_{Ca} -independent tonic tension was a carrier-mediated process. In normal Ringer solution, the curve for the rate of decrease of tonic tension vs. the concentration of NEM was hyperbolic, exhibiting a simple Michaelis-Menten-type kinetics. Furthermore, in either Ca-rich or Na-poor conditions, the carrier showed sigmoidal kinetics. That is, both conditions apparently cooperate as effectors of the inhibitory action of NEM. On the other hand, it is undeniable that the I_{Ca} -dependent phasic tension is also a carrier-mediated process, since NEM caused an initial increase and a late decrease in the tension.

In sheep and calf ventricular muscles, KAVALER (1959) found that the sustained mechanical tension was obviously composed of two different compounds: an initial hump corresponded to the tension that would have been developed as a consequence of a normal action potential, and a subsequent maintained tension clearly related to the prolongation of depolarization. This finding is the first description of "tonic tension." His results were confirmed by various experiments in which action potentials were also artificially lengthened or shortened in mammalian and frog hearts (MORAD and TRAUTWEIN, 1968; KAWATA *et al.*, 1969; WOOD *et al.*, 1969; BRAVERY and SUMBERA, 1970). Under voltage-clamped conditions, VASSORT and ROUGIER (1972) demonstrated that the mechanical activity of frog auricular trabeculae can be separated into two components depending on two sources of activator-calcium: one, the "phasic" component, depends on the slow inward current and is sensitive to external Ca ions; the other, the "slow" component, depends on the membrane potential and duration, and it accounts for the contraction observed in the absence of a slow inward calcium current and depends on intracellular Ca ions. In addition, VASSORT (1973) proposed that a displacement of Ca by Na from some intracellular binding sites by a variation of membrane potential or internal Na causes the "slow" component.

KAVALER (1974) studied the origin of the tonic tension elicited by application of 4-sec and 87-mV depolarizing pulses from the resting potential. Although this magnitude of depolarization seems to be insufficient for complete elimination of phasic tension, he observed that the contraction of the frog ventricle was immediately and continuously sensitive to the extracellular Ca level for a fixed time course of membrane depolarization. Eventually, he concluded that the contraction of the frog ventricular muscle was largely determined by a continuous, voltage-dependent influx of Ca into the cell, with little or no contribution from mechanisms involving release of Ca from intracellular stores. We, in the present work, recorded that I_{Ca} -independent tonic tension elicited by 140-mV and 2-sec depolarizing pulses from the resting potential. It therefore seems that the tonic tension by this depolarization hardly contains I_{Ca} -dependent phasic tension.

The difference between frog and mammalian hearts in their mechanical response (especially, tonic tension) to changes of depolarization is probably due to the existence in mammalian myocardium of sarcoplasmic reticulum filled by Ca influx, such an organelle being absent or poorly developed in the frog heart. In the frog atrium, however, tonic tension was affected by caffeine which was capable of releasing Ca ions from sarcoplasmic reticulum (KIMOTO *et al.*, 1974; Aomine, unpublished observation) and by cyanide that released the stored Ca from mitochondria (Aomine, unpublished observation). Hence, the existence of sarcoplasmic reticulum in the frog heart could not be ignored entirely. In the present paper, however, we want to discuss the possibility of the participation of the cell surface membrane for the generation of tonic tension.

The main observations in the present work are the following: (1) I_{Ca} -independent tonic tension is in large part a clearly carrier-mediated process; (2) the carrier may possibly be an allosteric protein which changes the ability of ligand binding. Indeed, in the present work, the Hill number in Ca-rich and Na-poor solutions represents 3 and 2, respectively (Figs. 3B and 4B). That is, these facts mean that the number of ligands (here, NEM) capable of binding to a receptor becomes 3 or 2 in Ca-rich or Na-poor conditions, respectively, from 1 in normal conditions. Recently, HORACKOVA and VASSORT (1976) have reported that more than 2 Na ions (probably 4 or more) are exchanged for each Ca ion, by experiments in which both Na and Ca ions in the solution are reduced at a constant Ca/Na². Their conclusion may be indirectly supported by the results in the present work. We observed that the binding ratio of NEM to a receptor was changed by the alteration in ionic circumstances. The binding ratio of Na or Ca to a receptor however was not examined. Taking into consideration the results in the present work, however, we can understand that the binding ratio of Na ions for the carrier proteins which may be related to the occurrence of tonic tension is able to change in response to the ionic circumstances, as HORACKOVA and VASSORT (1976) suggested.

Assuming that the I_{Ca} -independent tonic tension is mainly generated by the Na-Ca exchange mechanism at the level of the cell surface membrane, it seems that a

carrier protein responsible for the generation of tonic tension may be an allosteric protein. It is suggested that the protein which participated in the generation of tonic tension might have these properties, even with changes within the physiological conditions.

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