Chemical Modification by Maleimide of Toxic and Nontoxic Ouabain Actions on Neuromuscular Transmission in the Frog

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Abstract A dual action of ouabain on the Mg^{2+} -blocked frog neuromuscular transmission was studied in two different experimental procedures by measuring the mean quantal content (m) of endplate potentials evoked during repetitive nerve stimulation. The nontoxic action of low-dose ($1\ \mu M$) ouabain was observed as a counterclockwise pivoting of the linear stimulation frequency versus the $\log m$ relation, termed frequency augmentation-potential (FAP), whereas the toxic effect of higher doses ($\geq 10\ \mu M$) of ouabain was recorded as a slow exponential increase in m . Since neural Na^+, K^+ -ATPase consists of two or three isoforms, the nontoxic action of low-dose ouabain was anticipated to be a consequence of the selective inhibition of ouabain-sensitive $\alpha(+)$ ($\alpha 2$ and $\alpha 3$ or neural type) Na^+, K^+ -ATPase activity. Such a possibility was assessed by applying ϵ -maleimidocaproic acid (MCA), a membrane-impermeant sulfhydryl reagent. MCA $15\ \mu M$ was found in the present study to suppress the nontoxic effect of ouabain without altering the toxic effect. The results strongly suggest that the toxic and nontoxic actions of ouabain are the result of actions on different entities. The ouabain-sensitive neural type Na^+, K^+ -ATPase, which is abundant in neural tissues, seems to play an important role in modulation of transmitter release observable as FAP.

Key words: maleimide, neuromuscular transmission, ouabain, potentiation, transmitter release.

Cardiac glycosides such as digoxin and ouabain have been reported to potentiate the amount of transmitter quanta released from motor nerve terminals [1–4] (for review see ref. [5]). In the case of ouabain, the effect seems to be divided into two categories; a saturable or nontoxic effect observed at lower concentrations ($\leq 1\ \mu M$) and an exponential or toxic effect at higher doses ($\geq 10\ \mu M$) [3]. In agreement

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with the generally accepted view of ouabain action [5, 6], the toxic action of ouabain on neuromuscular transmission, that is an exponential increase in the evoked release of transmitter quanta, is ascribed to an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [7]. Because higher doses of ouabain required for the toxic effect, this could be interpreted as a result of the blockade of α ($\alpha 1$ or renal type) Na^+, K^+ -ATPase having a low affinity for ouabain [7–14] (for review see ref. [15]).

The frequency augmentation-potential (FAP: formerly termed as frequency facilitation [16]) is a useful electrophysiological and neuropharmacological technique for the analysis of drug actions on neuromuscular transmission [16–19]. In contrast to the toxic effect, studies on the nontoxic actions of ouabain and similarly acting chemicals on the FAP relation [7, 19] suggested that the nontoxic ouabain action on FAP relation is a unique Ca^{2+} -independent potentiation of the transmitter release process [7, 17, 19] related to a selective inhibition of ouabain-sensitive $\alpha(+)$ ($\alpha 2$ and $\alpha 3$ or neural type) Na^+, K^+ -ATPase activity [8–12, 15].

N-Ethylmaleimide (NEM), a well-known sulfhydryl reagent, has been reported to differentially modify the activity and the apparent affinity for ouabain of Na^+, K^+ -ATPase subtypes; ouabain-sensitive $\alpha(+)$ Na^+, K^+ -ATPase is more susceptible to NEM than α Na^+, K^+ -ATPase [8, 10, 20] (for review see ref. [15]). Since the nontoxic action of ouabain on the FAP relation seems to be attributed to selective inhibition of $\alpha(+)$ Na^+, K^+ -ATPase [7, 19], it is of interest to investigate whether ϵ -maleimidocaproic acid (MCA), a membrane-impermeant maleimide [21, 22], exerts any specific inhibitory effect on the nontoxic ouabain action.

METHODS

The material used in the present paper was a sciatic nerve-sartorius muscle preparation of the Japanese frog, *Rana nigromaculata*. The neuromuscular transmission was suppressed by applying low Ca^{2+} , high Mg^{2+} Ringer's solution consisting of 115 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl_2 , and 4–8 mM MgCl_2 . The pH of the solutions was adjusted to 7.2–7.4 by 5 mM HEPES buffer. Similarly to the previous papers [7, 16–19, 22], 200 samples of the endplate potentials (EPPs) were recorded by a conventional extracellular microelectrode technique and were processed with an on-line computer system to calculate the mean quantal content of EPPs (m) by the variance method of Poisson analysis.

FAP measurements. The action of a nontoxic dose of ouabain ($1 \mu\text{M}$) on transmitter release was studied with the FAP technique [7, 17–19, 22]. To obtain the FAP relation, the stimulation frequency (f) was increased stepwise from 0.5 to 6.25 Hz, and m was calculated at each f after allowing at least 40 s for equilibration. As described already [7, 18, 19, 22], the FAP relation was measured twice in each test solution, and the obtained m s were normalized, taking the average of control $m_{0.5}$ (m recorded at 0.5 Hz stimulation in the control solution) as unity. The release

parameters of FAP were computed from the following equation fitted by the linear regression analysis; i.e.

$$\log m = \log m_0 + kf, \quad (1)$$

where m_0 is m extrapolated to $f=0$ and k is the slope constant [7, 16–19, 22].

Effect of MCA on FAP. As the first step of the experiment, two FAPs were recorded in the control solution. Subsequently, 5–15 μM MCA was applied to the neuromuscular preparation for 30 min. After 30 min of washout of the reagent, the FAP relation was studied twice to calculate the MCA/control ratio of k . Because of a tremendous increase in the occurrence of spontaneous miniature endplate potentials (MEPPs) [22], experiments with MCA above 15 μM were impractical.

Nontoxic effect of ouabain. The nontoxic effect of ouabain on the vesamicol- or MCA-treated preparation was studied by measuring FAP relations in the absence and presence of 1 μM ouabain. Because of the slow development of the nontoxic ouabain action [7], the FAP measurement was started 1 h after the application of 1 μM ouabain. Furthermore, to allow a recovery from an augmented post-tetanic potentiation in the presence of 1 μM ouabain, the preparation was rested at least 10 min before initiating the second FAP recording.

Toxic effect of ouabain. High doses of cardiac glycosides such as digoxin and ouabain ($\geq 10 \mu\text{M}$) increase the frequency of MEPPs and potentiate the amplitude or mean quantal content of EPPs evoked by a train stimulation in time- and dose-dependent manners [1–5]. In the previous paper [7], such an aggravating effect of ouabain on the transmitter release was termed “the toxic ouabain effect,” which is approximated by the following equation when $t \geq t_d$: i.e.,

$$\log m_t = (t - t_d)/\tau, \quad (2)$$

where m_t is the normalized m at t min after the application of ouabain, and t_d and τ are the delay of onset and the time constant of the exponential development of the toxic ouabain action, respectively.

The effect of treatment with 15 μM MCA on the toxic ouabain action was assayed by measuring the time-dependent increase in m . The procedure for 15 μM MCA pretreatment was the same as the case of FAP measurement. Because of the dose-dependent increase in the MEPP frequency [22], accurate measurement of m became difficult when the concentration of MCA exceeded 15 μM . The sciatic nerve was stimulated intermittently at an interval of 10 min with a 5 min train stimulation of 1 Hz, and m was calculated from 200 EPPs elicited by the 101–300th pulses [7]. All the data were normalized taking the average of three control m s as unity. Also, the normalized results from different preparations were processed with the statistical linear regression analysis to estimate t_d and τ .

Analysis of drug actions on FAP. In the previous paper, it was assumed as a working hypothesis that the slope constant of FAP relation (k) is determined by the amount of W , a structure or component in the nerve terminal membrane responsible for producing FAP, and that the value of k in the untreated control preparation

(k_c) is proportional to the amount of W in this state ($[W]_c$) [18, 22]. In the present investigation, it is presumed that ouabain binds with the ouabain binding site in W and potentiates the efficacy of W by a factor (a): i.e.,

$$[W]_c = [W]_o + [WO]_o \quad \text{and} \quad k_o/k_c = ([W]_o + a[WO]_o)/[W]_c,$$

where $[W]_o$, $[WO]_o$, and k_o are the amounts of free and ouabain-bound Ws and the value of k , respectively, in the presence of a nontoxic dose of ouabain. Since $[WO]_o = [W]_o[O]/o$, we find:

$$k_o/k_c - 1 = (a - 1)[O]/(o + [O]), \quad (3)$$

where $[O]$ and o are the concentration and dissociation constant of ouabain, respectively.

In the previous papers [18, 22], we assumed that vesamicol inhibits W by reacting with the vesamicol binding site in W. The ratio of k s before and after the application of vesamicol (k_c/k_v) is given by the following equations: i.e.,

$$[W]_c = [W]_v + [WV] \quad \text{and} \quad k_c/k_v = [W]_c/[W]_v,$$

where $[W]_v$ and $[WV]$ are the amounts of free and vesamicol-bound Ws, respectively, in the presence of vesamicol. Since $[WV] = [W]_v[V]/v$, we obtain the famous Dixon equation:

$$k_c/k_v - 1 = [V]/v, \quad (4)$$

where $[V]$ and v are the concentration and dissociation constant of vesamicol, respectively. If ouabain is ineffective when it binds with WV, the ratio of vesamicol-treated k s in the presence and absence of ouabain (k_{vo}/k_v) can be expressed by the following equations:

$$[W]_v = [W]_{vo} + [WO]_{vo} \quad \text{and} \quad k_{vo}/k_v = ([W]_{vo} + a[WO]_{vo})/[W]_v,$$

where $[W]_{vo}$ and $[WO]_{vo}$ are the amounts of free and ouabain-bound Ws in the presence of vesamicol and ouabain. Since $[WO]_{vo} = [W]_{vo}[O]/o$, we obtain:

$$k_{vo}/k_v - 1 = (a - 1)[O]/(o + [O]).$$

Because the ouabain binding site is independent of the vesamicol binding site, vesamicol is expected to exert no influence on either a or o . Consequently, we find:

$$k_{vo}/k_v = k_o/k_c. \quad (5)$$

During a 30-min application of MCA, a membrane-impermeant protein-modifying reagent [21, 22], a part of W is inactivated so that k is suppressed from k_c to k_i , namely,

$$[W]_c = [W]_i + [W^*] \quad \text{and} \quad k_i/k_c = [W]_i/[W]_c,$$

where $[W]_i$ and $[W^*]$ are the amounts of intact and chemically modified forms of W, respectively. Because chemical modification by MCA is an irreversible reaction,

the k_i/k_c ratio can be expressed as the following log-linear function when $[I] < i$ [22]:

$$\log k_i/k_c = -[I]/i, \quad (6)$$

where $[I]$ and i are the concentration and inactivation constant of MCA, respectively. At the same time, MCA modifies a part of the ouabain binding site in $[W]_i$ and turns into $[W^-]$, a fraction of $[W]_i$ incapable of reacting with ouabain. Since chemical modification of the ouabain binding site is also an irreversible reaction, $[W^-]$ can be approximated by the equation:

$$[W^-] = [W]_i[I]/j,$$

where j is the inactivation constant of MCA for the ouabain binding site. The ratio of k s in the presence and absence of ouabain in MCA-treated preparation (k_{io}/k_i) can be formulated as:

$$k_{io}/k_i = ([W]_{io} + a[W^-]_{io} + [W^-])/[W]_i,$$

where $[W]_{io}$ and $[WO]_{io}$ are, respectively, the amounts of intact and ouabain-bound W s in MCA-treated preparation. From the above equation we yield for $[I] < j$:

$$k_{io}/k_i - 1 = (1 - [I]/j)(a - 1)[O]/(o + [O])$$

or

$$k_{io}/k_i - 1 = (1 - [I]/j)(k_o/k_c - 1). \quad (7)$$

Eq. (7) suggests that if MCA affects the ouabain receptor, the fractional nontoxic effect of ouabain on k should be depressed by a factor $(1 - [I]/j)$ in the MCA-treated muscle, as was the case for vesamicol-binding protein [22].

Chemicals. Organic chemicals tested in the present study were ϵ -maleimidocaproic acid (MCA: Sigma, St. Louis, MO, USA), ouabain octahydrate (Sigma, USA), and AH5183 (vesamicol: courtesy of Glaxo, Ware, Hertfordshire, UK). MCA and ouabain were dissolved in distilled water, and vesamicol in ethanol. These solutions were stocked in a refrigerator. The final concentration of ethanol in the test solutions was 0.1%.

RESULTS

Vesamicol and nontoxic ouabain action

As reported previously, vesamicol and similarly acting chemicals on neuromuscular transmission in frog suppressed k , the stimulation frequency-related release parameter in Eq. (1), in a dose-dependent manner [18]. In the presence of $20 \mu\text{M}$ vesamicol, the k_v/k_c ratio was decreased to 0.66, from which v was estimated to be $38 \mu\text{M}$ [18]. Similarly, a nontoxic dose of ouabain ($1 \mu\text{M}$) was reported to have selective action on k ; the k_o/k_c ratio was calculated to be 1.57 [7].

In the present paper, the effect of $1 \mu\text{M}$ ouabain on the FAP relation was

Fig. 1. Nontoxic effect of ouabain in the presence of vesamicol. A: An example recorded from a single end-plate spot. B: Averaged data of 8 measurements. Open and filled symbols refer the data before and an hour after the application of $1\text{ }\mu\text{M}$ ouabain, respectively. $20\text{ }\mu\text{M}$ vesamicol was present throughout the experiment. All the data were normalized, taking the average of control $m_{0.5}$ as unity. Vertical bars represent SE.

studied in the presence of vesamicol to see whether vesamicol interferes with the nontoxic action of ouabain [7]. A typical example is shown in Fig. 1A. The averaged results from 8 preparations are presented in Fig. 1B. Because of seasonal variation in k [23], the value of k_v , $0.17 \pm 0.01\text{ s}$ (mean $\pm$ SE), obtained in the present study on winter frog was considerably higher than the $0.12 \pm 0.01\text{ s}$ already reported for the summer frog [7]. Ouabain $1\text{ }\mu\text{M}$ increased k to 0.27 ± 0.02 , from which the k_{vo}/k_v ratio was calculated to be 1.59. This agrees satisfactorily well with the k_o/k_c ratio (1.57) reported elsewhere [7]. Thus, the present results verify Eq. (5) and corroborate the previous report that the effect of $1\text{ }\mu\text{M}$ ouabain on k was independent of the value of k before the application of ouabain [7].

Differential effect of MCA on ouabain actions

NEM, a protein-modifying sulfhydryl reagent, shows a differential inactivating action for the subtypes of Na^+, K^+ -ATPase; α type is more resistant to NEM than $\alpha(+)$ type Na^+, K^+ -ATPase [8, 10, 20] (for review see ref. [15]). Since α and $\alpha(+)$ types of Na^+, K^+ -ATPase are, respectively, putative targets of the present toxic and nontoxic ouabain actions [7, 19], MCA, a membrane-impermeant maleimide [21, 22], is expected to exert a differential effect on these ouabain actions.

As reported previously [22], 30-min exposure to MCA suppressed k of the FAP relation in a dose-dependent manner. The averaged results obtained from 8 measurements are given in Fig. 2. In accordance with the expectation from Eq. (6), a log-linear relation was observed between $[I]$ and k_i/k_o . The value of i calculated from the reciprocal of its slope constant was $61\text{ }\mu\text{M}$. It was obvious from the results presented in Figs. 3 and 4 that MCA pretreatment dose-dependently impaired the

Fig. 2. Effect of MCA on FAP relation. The semi-log plot of the ratio of k_s before and after a 30-min application of MCA (k_i/k_c) against the concentration of MCA. Averages of 8 cases. The inactivation constant of MCA (i) was calculated from Eq. (6) to be $61 \mu\text{M}$.

fractional nontoxic action of ouabain as anticipated from Eq. (7). The value of j calculated from the extrapolation of the linear regression line in Fig. 4 was $30 \mu\text{M}$.

In the present paper, the effect of MCA pretreatment on the toxic ouabain action shown in Fig. 5 was studied on the summer frog. Because of a low susceptibility of summer preparation to ouabain [7], the concentration of ouabain for toxicity study was increased to $40 \mu\text{M}$, instead of $10 \mu\text{M}$ in the case of winter and early spring frogs [7]. The obtained results were compared with those of the corresponding separate control experiments (circles in Fig. 5). In contrast to the case of nontoxic ouabain action, $15 \mu\text{M}$ MCA pretreatment was found to exert no influence on the toxic ouabain action. In both untreated control and $15 \mu\text{M}$ MCA-treated summer preparations, t_d and τ were calculated from Eq. (2) to be 7.3 and 36 min, respectively (10 cases). A similar toxic ouabain effect could be observed on winter frog at a lower dose. $10 \mu\text{M}$ ouabain gave rise to an exponential increase in m in MCA-pretreated winter muscle which was indistinguishable from that in the corresponding untreated preparation [7]. t_d and τ were estimated to be 13 and 34 min, respectively (data not shown).

DISCUSSION

The initial step in the cascade of reactions caused by cardiac glycosides has been explained whereby the agents bind with the receptive site in the Na^+ pump and inhibit the exchange of intracellular Na^+ with extracellular K^+ ions. An elevation of the intracellular concentration of Na^+ as a consequence of Na^+ pump inhibition in turn increases the $[\text{Ca}^{2+}]_i$ via activation of Na^+ - Ca^{2+} exchange and finally potentiates the Ca^{2+} -dependent cellular processes [5, 6]. Obviously, the toxic

Fig. 3. Nontoxic effect of ouabain on MCA-pretreated preparation. Open and filled symbols represent the FAP relations obtained before and an hour after the application of $1\text{ }\mu\text{M}$ ouabain. The concentrations of MCA were, from A to D, zero, 5, 10, and $15\text{ }\mu\text{M}$, respectively. Normalized averages on 12 muscles in A (data from ref. [7]) and 7 cases in B-D. Vertical bars show SE.

action of high-dose ouabain ($\geq 10\text{ }\mu\text{M}$) on transmitter release (Fig. 5) could be described by the above scheme, for it is suppressed by an application of amiloride, an $\text{Na}^+\text{-Ca}^{2+}$ exchange blocker, and an intracellular loading of BAPTA, an intracellular Ca^{2+} chelator [7]. However, a potentiating action of ouabain on transmitter release still occurs in the Ca^{2+} -free environment [24–27]. Furthermore, the nontoxic action of $1\text{ }\mu\text{M}$ ouabain (Figs. 1 and 3) remains unchanged even

Fig. 4. Dose-dependent modification by MCA of the nontoxic ouabain effect on FAP relation. The data presented in Fig. 3 were analyzed according to Eq. (7). The inactivation constant of MCA for ouabain binding site (j) was estimated as $30 \mu\text{M}$.

Fig. 5. Ineffectiveness of MCA for the toxic ouabain effect. An exponential increase in m caused by an application of a toxic dose of ouabain (toxic ouabain effect) was measured on the preparation pretreated with $15 \mu\text{M}$ MCA (triangles), and was compared with that obtained from separate untreated control muscle (circles). Because of a seasonal difference in susceptibility to ouabain [7], the concentration of ouabain for toxicity study was increased to $40 \mu\text{M}$ in summer frog. Exchange with ouabain Ringer's solution took about 10 min as indicated by arrows. Recording of the ouabain effect started immediately after completion of the exchange of solution (time zero). Normalized averages obtained on 10 muscles. Vertical bars represent SE. For details see text.

after an application of amiloride or an intracellular loading of BAPTA [7]. These results suggest a dual mode of ouabain action: Ca^{2+} -dependent and -independent actions of ouabain.

It has been well documented that Na^+, K^+ -ATPase in neural tissues is composed of two or three subtypes: i.e., ouabain sensitive $\alpha(+)$ and less sensitive α Na^+, K^+ -ATPase [8–14] (for review see ref. [15]). The target of the nontoxic action of $1 \mu\text{M}$ ouabain seems to be $\alpha(+)$ Na^+, K^+ -ATPase, since the IC_{50} values reported for the inhibition of $\alpha(+)$ Na^+, K^+ -ATPase activity are below $1 \mu\text{M}$ [9–12]. The nontoxic action of ouabain has other characteristics in common with $\alpha(+)$ Na^+, K^+ -ATPase; namely, selective effects of erythrosin B [19, 28] and maleimide [8, 10, 15, 20].

On the other hand, the target of the toxic action of high-dose ouabain ($\geq 10 \mu\text{M}$) might be anticipated to be α Na^+, K^+ -ATPase having a low affinity for ouabain, because the IC_{50} of ouabain reported for α Na^+, K^+ -ATPase ($32\text{--}100 \mu\text{M}$) [9–12] roughly corresponds to the ouabain concentration required for producing the present toxic effect ($10\text{--}40 \mu\text{M}$). A low sensitivity for the chemical modification by maleimide is also a common feature shared with α Na^+, K^+ -ATPase [8, 10, 15, 20] and the present toxic ouabain effect (Fig. 5).

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