Hyperpolarization by Noradrenaline in Guinea pig Liver Cells: Effects of Ouabain and External Ca$^{2+}$

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Abstract The mechanism of hyperpolarization of the membrane caused by noradrenaline (NA) was investigated in guinea pig liver cells. The hyperpolarization produced by NA was accompanied by a reduction of membrane resistance. Both hyperpolarization and reduction of membrane resistance were suppressed by ouabain ($5 \times 10^{-6}$ M). However, when the external Na$^+$ was reduced to 38 mm, the NA response reappeared even in the presence of ouabain. The hyperpolarizing NA response may be divided into two phases, an early phase and a late phase. In a Ca$^{2+}$-free solution, the NA response gradually disappeared. However, the early phase of hyperpolarizing response was more resistant to a removal of external Ca$^{2+}$ than the late phase. Manganese ion also suppressed the late phase more strongly than the early phase. It was concluded that the hyperpolarization induced by noradrenaline is due to an increase in K$^+$ conductance and that this may be a result of an increase in intracellular Ca$^{2+}$ concentration. The early phase of hyperpolarizing NA response is probably due to release of bound Ca$^{2+}$ and the late phase to influx of Ca$^{2+}$ from the external solution. Suppression of the NA response by ouabain may be explained by assuming that the Ca$^{2+}$-activated K$^+$ conductance is blocked by an increase in intracellular Na$^+$ concentration.

It has been reported that the noradrenaline-induced hyperpolarization in guinea pig liver cells is due to an increase in K$^+$ conductance (Haylett and Jenkinson, 1972; Egashira, 1980). This conclusion is mainly based on the observation that there is a simultaneous reduction of the membrane resistance during hyperpolarization and that the degree of hyperpolarization roughly correlates with the initial membrane potential. However, changes in the membrane potential with varying external K$^+$ concentrations during noradrenaline (NA) application are smaller than those theoretically expected from the changes in K$^+$ equilibrium potential. Furthermore, the maximum decrease of the membrane resistance occurs when the membrane is being hyperpolarized and the membrane...
resistance tends to recover before the peak of hyperpolarization, i.e., there is no precise correlation between the time-course of the membrane resistance change and the hyperpolarization (EGASHIRA, 1980). Therefore, it is likely that, in addition to the increase in K⁺ conductance, some other process, such as activation of an electrogenic pump may also be involved in the NA-induced hyperpolarization. In fact, such a possibility has been considered by HAYLETT and JENKINSON (1972).

Underlying mechanisms for electrogenesis of the membrane potential of liver cells are still not well understood. It is generally agreed that the diffusion potential of a classical Goldman type is insufficient to explain the membrane potential and that an electrogenic active transport may partly be contributing to the membrane potential (CLARET et al., 1973; HELLER and VAN DER KLOOT, 1974; SHIBA et al., 1977; WILLIAMS et al., 1971). One of the aims of the present experiments was, therefore, to investigate the possible contribution of an electrogenic Na-pump to the hyperpolarizing component of the biphasic NA response. The other aim was to determine the role of external Ca²⁺ in the NA response by using an electrophysiological technique, since the importance of Ca²⁺ in the NA response has recently been emphasized not only in the increase in K⁺ conductance (HAYLETT, 1976; JENKINSON et al., 1978), but also in the biochemical reactions of the liver (ASSIMACOPOULOS-JEANNET et al., 1977; DE WULF and KEPPENS, 1976; EXTON et al., 1978; KEPPENS et al., 1977).

METHODS

Details of the preparation and incubation of the liver slices are the same as previously described (EGASHIRA, 1980). The control solution contained (mM): NaCl 103, KCl 4.7, CaCl₂ 2.6, MgCl₂ 1.1; NaHCO₃ 25, NaH₂PO₄ 1.2, d-glucose 2.8; Na pyruvate 4.9, Na fumarate 2.7 and Na glutamate 4.9. This solution had a pH of 7.4 at 38°C when bubbled with 5% CO₂ in O₂. In some experiments, Na⁺ was replaced with Mg²⁺ and sucrose, keeping the Cl⁻ concentration normal. In 0 mM [Ca²⁺], solution, MgCl₂ was increased to 22 mM, and 0.5 mM EGTA was added. When Mn²⁺ was added to the reservoir at a concentration of 2 mM, NaHCO₃ was reduced to 13 mM and NaH₂PO₄ was replaced with equimolar NaCl, and the solution was bubbled with 97% O₂ and 3% CO₂. Ouabain was added to the reservoir at a concentration of 5 × 10⁻⁶ M.

Noradrenaline was prepared just before each experiment by diluting with the test solution and a fixed amount (0.1 ml) was added directly to the perfusing solution to give a peak concentration of 10⁻⁵ M. In some experiments, the concentration of NA (10⁻⁵ M) was kept constant by adding to the reservoir.

The method of external polarization was used to produce electrotonic potentials for measuring the changes in membrane resistance, as described in a previous paper (EGASHIRA, 1980). Constant current pulses of 100 msec were applied at 0.4–0.8 Hz. Membrane potentials and electrotonic potentials were measured by using a conventional microelectrode technique at a distance of 100–
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200 µm from the nearest polarizing electrode.

RESULTS

Effects of ouabain

In the control solution, the membrane potentials obtained from 18 preparations ranged from -45 to -62 mV, with a mean of -54.7±1.3 mV (S.E. of the mean). An application of NA produced a biphasic response, i.e., a small initial depolarization followed by a slow hyperpolarization in many preparations, but in some preparations it produced only hyperpolarization. There was a simultaneous decrease in the amplitude of electrotonic potentials (Fig. 1a). The magnitude of NA-induced hyperpolarization ranged from 1 to 15 mV, with a mean of 7.2±0.7 mV. The degree of hyperpolarization depended on the membrane potential before NA application (Fig. 2, closed circles). The hyperpolarization was larger when the initial membrane potential was smaller, confirming a previous report (EGASHIRA, 1980).

To investigate the possible contribution of an electrogenic active transport to the NA-induced hyperpolarization, the effects of ouabain were examined.

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**Fig. 1.** Blockade of the NA response by ouabain (5×10⁻⁶ M) and its recovery in Na⁺-deficient (38 mM) solution. Arrows indicate transient NA applications (10⁻⁵ M). Top traces show current pulses (100 msec, 0.8 Hz) for polarization of the membrane. a: NA response in control solution (141 mM Na⁺). b: 5 min after and c: 15 min after ouabain. d: 15 min after and e: 35 min after in 38 mM Na⁺ solution containing ouabain. f: recovery 30 min after in normal solution. All records were obtained from the same cell.

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When ouabain (5×10^{-6} M) was applied, the membrane potential gradually decreased. The mean value of the membrane potential measured about 15 min after ouabain application was -45.8±1.2 mV, which was about 10 mV lower than that in a control solution without ouabain (P<0.001).

The NA-induced hyperpolarization was greatly suppressed by ouabain, and the hyperpolarization was only a mean of 1.4±0.4 mV after treatment with ouabain for 15 min. Although the reduction was statistically significant (P<0.001), the degree of suppression varied from preparation to preparation. In Fig. 2, the relationship between the NA-induced hyperpolarization and the membrane potential was plotted before and after ouabain treatment from 18 preparations. In the presence of ouabain, potential dependency of the NA response was less and the reversal potential extrapolated from the regression line was reduced from the control value of -74.4 to -52.6 mV. The decrease in the reversal potential may be due to the loss of intracellular K⁺ concentration caused by the inhibition of Na⁺-K⁺ pump.

In some preparations, the NA response was completely abolished without large changes in the membrane potential and resistance, as shown in Fig. 1c.

Fig. 2. Relationship between NA (10^{-5} M)-induced hyperpolarization (ordinate) and membrane potential before NA application (abscissa). Amplitude of the hyperpolarization is plotted against membrane potential in each experiment. Closed circle: without; and open circle: with ouabain (5×10^{-6} M), obtained from 18 preparations. A regression line is expressed by Y=0.36X+26.8 for closed circles and Y=0.19X+10.0 for open circles.

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When the external Na\(^+\) concentration was reduced to 38 mM by substituting with MgCl\(_2\)+sucrose, keeping the Cl\(^-\) concentration constant, the hyperpolarizing NA response gradually reappeared even in the presence of ouabain (Fig. 1d, e). Under these conditions the depolarizing NA response was absent or very small. In the absence of ouabain, a reduction of Na\(^+\) to 38 mM depolarized the membrane by a few mV, and suppressed the depolarizing component, and increased the hyperpolarizing component produced by NA (Egashira, 1980). Thus, the NA response observed in the Na\(^+\)-deficient solution was essentially the same in the presence and in the absence of ouabain.

Figure 3 shows the time-course of the blockade of NA response by ouabain and its reappearance in Na\(^+\)-deficient solution with regard to both the changes in membrane resistance (closed circles) and the hyperpolarizing response induced by NA (open circles). In this experiment, NA was transiently applied every 10 min. It was clear that there was a good correlation between the degree of hyperpolarization and the maximum decrease in membrane resistance. From these results, it may be suggested that the suppression of the NA response by ouabain is not simply due to a direct action on an electrogenic Na\(^+\)-pump.

![Fig. 3. Effects of ouabain on NA (10\(^{-5}\) M) response and its recovery in Na-deficient solution. Ordinate: right (open circles), amplitude of NA-induced hyperpolarization (mV); left (closed circles), resistance change caused by NA (Rm%). Abscissa: time in minutes. NA was transiently applied every 10 min. There is a good correlation between resistance change and hyperpolarization.](image-url)
Effects of removing external Ca\textsuperscript{2+}

Effects of the removal of external Ca\textsuperscript{2+} were studied. When Ca\textsuperscript{2+} was omitted from the solution, 0.5 mM EGTA was added, and Mg\textsuperscript{2+} was increased to 22 mM in order to reduce depolarization of the membrane and a fall in the membrane resistance. In this solution, the membrane was slowly depolarized by only a few mV.

NA (10\textsuperscript{-5} M) was transiently applied repeatedly every 8 min while the external Ca\textsuperscript{2+} was removed for 40 min, after observing constant responses to NA. Such experiments were performed on 5 preparations. One of the results is shown in Fig. 4A and B.

In the control solution (2.5 mM Ca\textsuperscript{2+}), NA caused a typical response, accompanied by a reduction of the membrane resistance (Fig. 4A-a). On removal of the external Ca\textsuperscript{2+}, both the changes in membrane potential and membrane resistance became smaller (A-b), and was finally abolished after about 30 min (A-c). On readmission of 2.5 mM Ca\textsuperscript{2+}, the NA response quickly reappeared and its recovery was nearly perfect (A-d). The change of the NA-induced reduction of membrane resistance was plotted with time after Ca\textsuperscript{2+} removal and Ca\textsuperscript{2+} readmission, in Fig. 4B. After removing the external Ca\textsuperscript{2+}, the maximum reduction of membrane resistance in response to NA applied every 8 min gradually became smaller and after about 30 min the response was finally abolished. The recovery of response following readmission of 2.5 mM Ca\textsuperscript{2+} was very fast compared with the rate of disappearance of the response in Ca\textsuperscript{2+}-free solution. From these results, it was suggested that Ca\textsuperscript{2+} ion was essential for the NA response.

Fig. 4. Effects of removing external Ca\textsuperscript{2+} on NA (10\textsuperscript{-5} M) response. A: a: NA response in control solution (2.5 mM Ca). b: 9 min after and c: 33 min after in 0 mM Ca\textsuperscript{2+}. d: recovery in normal solution. Arrows indicate transient NA application. B: Time-course of effects of changing the external Ca\textsuperscript{2+} on NA-induced resistance reduction. NA was transiently applied every 8 min.

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In the experiment shown in Fig. 5, the effect of transient Ca\(^{2+}\) readmission to Ca\(^{2+}\)-free solution was studied in the absence and in the presence of NA. NA was added to the reservoir at a concentration of 10\(^{-5}\) M, and was applied for 3 min or continuously. After observing the control response to NA in normal solution (Fig. 5a), Ca\(^{2+}\) was removed. At the first application of NA, an appreciable response could still be observed 20 min after exposure to Ca\(^{2+}\)-free solution (b), but it was abolished in the second application of NA (c). Then, continuous administration of NA was started in Ca\(^{2+}\)-free solution. In the presence of NA, readmission of 2.5 mM Ca\(^{2+}\) for 3 min produced hyperpolarization accompanied by a reduction of the electrotonic potential (Fig. 5d). This response was qualitatively similar to the NA response in normal solution (Fig. 5a). After washing out NA, adding Ca\(^{2+}\) to Ca\(^{2+}\)-free solution produced almost no effect (Fig. 5e). These results may suggest that NA introduces the external Ca\(^{2+}\) into the hepatocyte, and that this in turn increases the K\(^{+}\) conductance, resulting in hyperpolarization.

To obtain further information about the contribution of Ca\(^{2+}\) to the NA response, NA was applied for 3 min at various intervals from the beginning of the removal of Ca\(^{2+}\). After each application of NA, the preparation was exposed to normal control solution for 30 min before the next experiment. An example of such an experiment is shown in Fig. 6. In the control solution, NA caused...
membrane hyperpolarization following a small depolarization, and reduced the membrane resistance. The membrane resistance recovered to some extent even in the presence of NA, while hyperpolarization remained (Fig. 6A-a) or slightly increased during NA application. The time-course of reduction of the membrane resistance was plotted for every 20 sec after each NA application (Fig. 6B). The resistance was rapidly decreased to the maximum level after the start of NA infusion; but it recovered to a certain extent between 60 and 120 sec, and then kept nearly constant.

After removal of Ca\textsuperscript{2+}, NA was applied 10 (A-b), 20 (A-c) and 30 min later (A-d). The preparation was incubated in normal solution containing 2.5 mM [Ca\textsuperscript{2+}]\textsubscript{o} for 30 min before removing Ca\textsuperscript{2+} each time. In Ca\textsuperscript{2+}-free solution, the early phase of hyperpolarizing response to NA was nearly the same independent of the exposure time to Ca\textsuperscript{2+}-free solution. On the other hand, the late phase of hyperpolarizing response was suppressed in spite of the continuous presence of NA (Fig. 6A and B). Thus, the early phase which is relatively independent of external Ca\textsuperscript{2+} could be differentiated from the late phase, which is preferentially abolished by Ca\textsuperscript{2+} removal.

Fig. 6. Effects of exposure time to Ca\textsuperscript{2+}-free solution on the NA response. Actual records are shown in A and time-course of the resistance change was plotted in B. NA concentration (10\textsuperscript{-5} M) was kept constant for 3 min. a: NA response in control solution. b: NA response 10 min, c: 20 min, and d: 30 min after incubation in Ca\textsuperscript{2+}-free solution. Before each exposure to NA, the preparation was incubated in normal solution containing 2.5 mM Ca\textsuperscript{2+} for 30 min.
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Fig. 7. Effects of Mn²⁺ (2 mM) on NA response. A: actual records and B: plot of resistance changes. NA was applied for 3 min. a: NA response in control solution. b: the first application of NA 5 min, and c: the second application 15 min after incubation in Mn²⁺ solution. Membrane resistance was gradually increased by Mn²⁺.

Effects of Mn²⁺

Effects of Mn²⁺ on the NA response were investigated, since Mn²⁺ is known to antagonize Ca²⁺ in many biological membranes. In this experiment, NaHCO₃ was reduced to half the control solution (13 mM), by replacing it with NaCl. The response to NA was qualitatively the same in this low HCO₃⁻ solution as in the control solution (Fig. 7: a in A and B). When 2 mM Mn²⁺ was applied, the NA response was gradually suppressed. After 5 min in Mn²⁺ solution, the response to the first application of NA became transient as observed in Ca²⁺-free solution; i.e., the late phase of the response was preferentially inhibited by Mn²⁺ (b in A and B). When NA was applied again 10 min later, only a very small response was produced; i.e., the early phase was also greatly suppressed and the late phase was abolished (c in A and B).

DISCUSSION

The factors determining the membrane potential of the liver cells are still not clear, as mentioned in the introduction. There may be some components due to active ionic transport, however, effects of ouabain greatly differ in different publications. In the rat, ouabain is reported to have no significant effect (BEIGELMAN and THOMAS, 1972; SHIBA et al., 1977), or to cause depolarization of 6.8 mV (CLARET et al., 1973). In the mouse, ouabain produces rapid depolarization by 1–2 mV followed by a slow decline (GRAF and PETERSEN, 1974). In the guinea pig, a large depolarization by ouabain (about 20 mV in 1 hr) has been reported (HELLER and VAN DER KLOOT, 1974). It seems that contribution of ouabain-
sensitive Na\(^+\)-pump may vary in different species or in different experimental conditions (Russo et al., 1977; Shibata et al., 1977). In the present experiments on the guinea pig liver slice, depolarization of about 10 mV is observed after treatment with ouabain (5 × 10\(^{-6}\) M) for 15 min. However, it is not clear whether this depolarization is due to a change in the ionic concentration gradient across the membrane, to a change in the ionic permeability, to a blockade of an electrogenic Na\(^+\)-pump, or to a combination of these factors.

In the present experiments, it is shown that ouabain strongly suppresses the NA response, although there are some variations in the degree of suppression in different preparations. This result may suggest that an ouabain-sensitive Na\(^+\)-pump contributes to the NA-induced hyperpolarization. However, the observation that the NA response reappears by reducing the external Na\(^+\) concentration seems contradictory to this idea. Since NA can produce hyperpolarization accompanied by a reduction of membrane resistance in the Na\(^+\)-deficient solution containing ouabain, it is more likely that the suppression of the response by ouabain is not due to its direct action on the electrogenic Na\(^+\)-pump. Haylett and Jenkinson (1972) have observed no effect of ouabain on the NA-induced hyperpolarization in the guinea pig liver. The reason for this discrepancy is not clear, but one of the reasons is probably too short an exposure to ouabain in their experiments. They applied NA only 3–4 min after ouabain (10\(^{-5}\) M) administration. In the present experiments, the clear response to NA can still be observed even after 15 min ouabain (5 × 10\(^{-6}\) M) application, although it is blocked after more than 30 min.

It is known in some cells that the K\(^+\) conductance is increased when the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) is increased (Krnjević and Lisiewicz, 1972; Meech, 1974, 1978; Eckert and Tillotson, 1978; Dos Reis et al., 1979; Okada et al., 1979). Thus, a decrease in membrane resistance induced by NA may also be related to a similar mechanism, since it is shown that the presence of Ca\(^{2+}\) is essential for this action, and that Ca\(^{2+}\) application in Ca\(^{2+}\)-free solution containing NA causes hyperpolarization with a reduction of membrane resistance. It is known that K\(^+\) ion content in the liver slice is affected by intracellular Ca\(^{2+}\) (Van Rossum, 1970). Subsequently, Haylett (1976) observed a concomitant increase in the effluxes of Ca\(^{2+}\) and K\(^+\) from liver slices of guinea pig by \(\alpha\)-adreno-receptor agonists, and suggested that a rise in the free [Ca\(^{2+}\)], may be responsible for the increase in K\(^+\) conductance. In the rat parotid, involvement of Ca\(^{2+}\) in K\(^+\) release has also been suggested (Batzri et al., 1973; Selinger et al., 1973), and it has been shown that a Ca\(^{2+}\)-ionophore, A23187, simulates the action of adrenaline on \(\alpha\)-receptors, causing a Ca\(^{2+}\)-dependent loss of K\(^+\) ion (Selinger et al., 1974). Furthermore, in the lacrimal cells, injections of Ca\(^{2+}\) produces hyperpolarization and resistance reduction which are very similar to the responses to adrenaline (Iwatsuki and Petersen, 1978).

Based on the isotope experiments, Weiss and Putney (1978) proposed the
concept that Ca\(^{2+}\) released from the intracellular store mediates the increase in K\(^{+}\) permeability in the liver, because the first in a series of the Rb\(^{+}\) release responses is independent of extracellular Ca\(^{2+}\), while Ca\(^{2+}\) is required to obtain a subsequent response. Similar results have been obtained in the parotid and lacrimal glands (Putney, 1977; Parod and Putney, 1978a,b). In the present experiments, it is shown that the early phase of NA-induced hyperpolarizing response is more resistant to removal of the external Ca\(^{2+}\), and to application of Mn\(^{2+}\), than the late phase. Thus, it is possible that Ca\(^{2+}\) involved in the early phase may be tightly bound or located intracellularly, whereas a sustained influx of external Ca\(^{2+}\) may mediate the late phase of hyperpolarization.

There is a discrepancy between the degree of hyperpolarization and the increase in membrane conductance in the response to NA. The membrane conductance tends to recover while the hyperpolarization is still increasing or maintained. Thus, some contribution of an electrogenic pump to the hyperpolarization cannot be neglected, particularly in the late phase of response. However, if such a pump is involved, this pump should be sensitive to the external Ca\(^{2+}\). Another possibility to explain the discrepancy is that the changes of Na\(^{+}\) and Cl\(^{-}\) conductance caused by NA are biphasic; i.e., an increase is followed by a decrease. These problems are not investigated in the present experiments.

Suppression of the NA response by ouabain and reappearance of the NA response in low Na\(^{+}\) solution are difficult to explain from the present experiments. Since the reversal potential for the NA-induced hyperpolarization is reduced by ouabain, it is expected that the intracellular K\(^{+}\) concentration is reduced and the intracellular Na\(^{+}\) concentration (\([\text{Na}^{+}]_i\)) is increased. An increase in the \([\text{Na}^{+}]_i\), due to blockade of Na\(^{+}\)-pump may gradually increase the \([\text{Ca}^{2+}]_i\), as a result of a Na\(^{+}\)-Ca\(^{2+}\) exchange process (Baker, 1972). A slow accumulation of intracellular Ca\(^{2+}\) may inactivate the K\(^{+}\) permeability as suggested in the rat parotid gland (Putney, 1978). However, the recovery of NA response in Na\(^{+}\)-deficient solution cannot be explained with this hypothesis because \([\text{Ca}^{2+}]_i\), would be further increased by removal of external Na\(^{+}\). Furthermore, the presence of a Na\(^{+}\)-Ca\(^{2+}\) exchange has been questioned in the rat liver (Cittadini and Van Rossum, 1978). As a tentative hypothesis, it is speculated that intracellular Na\(^{+}\) may compete with Ca\(^{2+}\) at the site where Ca\(^{2+}\)-induced opening of K\(^{+}\) channel takes place. Then, the decrease in \([\text{Na}^{+}]_i\), in Na\(^{+}\)-deficient solution would lead to recovery. This hypothesis is in accord with the observation that the Ca\(^{2+}\)-activated K\(^{+}\) efflux is inhibited by an increase in \([\text{Na}^{+}]_i\), in the red cell ghosts (Porzig, 1977). Further experiments are necessary to clarify this possibility in liver cells.

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