ANION PERMEABILITY OF THE INHIBITORY SUBSYNAPTIC MEMBRANE OF THE SPINAL MOTONEURON OF THE TOAD

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Summary 1. Single microelectrodes filled with solutions containing various anions were inserted into toad spinal motoneurons and used for both the electrophoretic injection of anions and for intracellular recording.

2. Judging from the effect of anion injections on the IPSP's, the activated inhibitory subsynaptic membrane was found to be permeable to Br^- , I^- , Cl^- , NO_2^- , NO_3^- , N_3^- , ClO_4^- , SCN^- , OCN^- , ClO_3^- , and HCO_2^- ions.

3. BrO_3^- , F^- , HSO_3^- , HCO_3^- , CH_3CO_2^- , SO_4^{2-} , H_2PO_4^- , HPO_4^{2-} and citrate ions were found to be impermeable through the activated inhibitory subsynaptic membrane. The ineffectiveness of injection of HS^- ions on the IPSP was confirmed.

4. The time constants of the IPSP recovery after injections were evaluated for nine penetrating anions. Then a sequence was determined for the order of their relative ability to penetrate the motoneuron membrane: It is $\text{Br}^- > \text{OCN}^- > \text{NO}_3^- > \text{NO}_2^- > \text{SCN}^- > \text{Cl}^- > \text{HCO}_2^- > \text{I}^- > \text{ClO}_3^-$.

5. Results obtained in the present investigation were compared with those from studies on cat spinal motoneurons and goldfish Mauthner cells.

COOMBS, *et al.* (1955) observed that the inhibitory postsynaptic potential (IPSP) of the spinal motoneuron of the cat changed from a hyperpolarizing type to a depolarizing one when Cl^- , Br^- , NO_3^- , and SCN^- ions were injected intracellularly. These results were explained by assuming that the activated inhibitory subsynaptic membrane is permeable to these ions. On the other hand, it was found that the injection of HCO_3^- , CH_3CO_2^- , SO_4^{2-} , $\text{H}_2\text{PO}_4^{2-}$ ions had no effect on the IPSP. Since the permeable ions are smaller in the hydrated form

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than the impermeable ions, it was postulated that the activated inhibitory subsynaptic membrane is converted into a sieve-like structure having pores of a standard size. These results have been confirmed and extended to many other anions by ARAKI *et al.* (1961) and ITO *et al.* (1962). In their extensive investigation, it has been shown that anions are definitely separated into two categories, *i.e.*, the one that can penetrate and the ones that cannot penetrate through the activated inhibitory subsynaptic membrane of spinal motoneurons of the cat. Research along this line has been further extended to the IPSP's of other central synapses, including those in the Mauthner cells of the goldfish (ASADA, 1963), in the neurons of the snail (KERKUT and THOMAS, 1964), in the neuromuscular junction of the crayfish (TAKEUCHI and TAKEUCHI, 1967) and in the cortical neuron of the cat (KELLY *et al.*, 1969).

In the present investigation, anion permeability of the activated inhibitory subsynaptic membrane of the spinal motoneuron of the toad has been tested from the comparative physiological point of view. Furthermore, the time constant for diffusion of injected anions from the motoneuron has been evaluated according to the time course of the recovery of the IPSP after anion injection. It will be shown that between spinal motoneurons of the cat and toad, there is no essential difference in anion permeability of the inhibitory subsynaptic membrane but there are discrepancies in the relative anion permeability of the non-synaptic membrane.

METHODS

Toads anaesthetized with Nembutal (25 mg/kg) were used. They were firmly suspended in a rigid metal frame. The spinal cord was exposed and the 9th and 10th ventral and dorsal roots were cut and mounted on Ag-AgCl electrodes for stimulation. In some cases, muscle nerves such as the tibial and peroneal nerves were prepared for orthodromic stimulation, the dorsal roots being kept intact. In some later experiments excised spinal cords were used. They were kept in a Ringer's solution through which a gas mixture consisting of 95% O₂ and 5% CO₂ was continuously bubbled.

Glass microelectrodes were filled with the solutions listed in Table 1, which contained various anions as either potassium or sodium salts. Potassium salts were preferred, unless their solubility was very slight. The electrical resistance of the microelectrode used was usually 10–20 MΩ.

The microelectrode was connected to a current generator through a 100 MΩ resistor, and anions were injected into the motoneuron by the passage of currents of $1-5 \times 10^{-8}$ A for 30–120 sec. The intracellular potentials were recorded through the same electrode that served for the injection. Following the application of the current, a transient change in the tip potential of the microelectrode was frequently observed and this artifact disturbed the measurement of the membrane potential

Table 1. Solutions used for filling microelectrodes.

Substance	Concentration (M)	Substance	Concentration (M)
KBr	1	NaBrO ₃	1.8
KI	1	NaF	0.8
KCl	1	NaHCO ₂	2
KNO ₂	1	NaHSO ₃	3
KNO ₃	2	KHCO ₃	1
NaN ₃	2	KCH ₃ CO ₂	1
NaClO ₄	1	K ₂ SO ₄	0.6
KSCN	2	KH ₂ PO ₄	1
NaHS	1	K ₂ HPO ₄	1
KOCN	1	K-citrate	2
NaClO ₃	1		

change immediately after the cessation of the current. In order to compensate for this artifact potential, potential changes were recorded by applying the same current just outside the motoneuron. Usually, electrodes with large artifact potentials were not used.

For intracellular potential recording, a cathode follower with a negative capacitance compensation was used and the output was connected to a oscilloscope through differential AC and DC amplifiers. The current passed through the microelectrode for injections was recorded by another differential DC amplifier. The resting potential was registered by an inkwriting recorder.

All experiments were carried out at room temperature, 15–19°C.

RESULTS

Penetrating anions

The injections of Br⁻, I⁻, Cl⁻, NO₂⁻, NO₃⁻, N₃⁻, ClO₄⁻, SCN⁻, OCN⁻, ClO₃⁻ and HCO₂⁻ ions by a hyperpolarizing current of $1-5 \times 10^{-8}$ A for 30–120 sec readily resulted in a reversal of the IPSP. Figure 1 shows an example of the reversal of the IPSP by the injection of NO₂⁻ ions. Record A shows the control IPSP recorded from a motoneuron evoked by stimulation of the 10th dorsal root. Records B–G were obtained at successive intervals after application of 2×10^{-8} A for 60 sec. Immediately after the cessation of the current, the reversal of the IPSP was so great that it attained the level of the threshold depolarization of the motoneuron and a spike potential was initiated as shown in Record B. Thereafter, the reversed IPSP decreased in size (C and D) and eventually returned to the original hyperpolarizing type (E–G). A diphasic stage was observed when the reversed IPSP turned back to a hyperpolarization, as pointed out by COOMBS *et al.* (1955) and ARAKI *et al.* (1961). Records I–K in Fig. 1 were obtained with high and low amplification after the second injection of NO₂⁻ ions by a current of

1×10^{-8} A for 60 sec in the same motoneuron, the antidromic spike being evoked at the end of the sweep.

Fig. 1. Effects of injection of NO_2^- ions on the IPSP. A shows the control IPSP from a motoneuron evoked by stimulation of the 10th dorsal root. B, C, D, E, F and G were obtained at 8, 16, 36, 48, 70, and 260 sec, respectively, after the application of an inward current of 2×10^{-8} A for 60 sec through the intracellular microelectrode filled with a 1 M KNO_2 solution. H-K show the effect of the second injection of NO_2^- ions on the same cell. H shows the IPSP together with antidromically evoked spike potential before, and I-K show it after, the passage of a hyperpolarizing current of 1×10^{-8} A for 60 sec. Note the double beam recording, the upper and lower beams being in high and low amplification, respectively.

The change in the size of the IPSP by the injection of NO_2^- ions together with that of the spike potentials and of the resting potential is plotted in Fig. 2. The size of the IPSP was measured by the amplitude at a fixed time corresponding to the peak of the largest depolarizing IPSP. As is seen in Fig. 2, the IPSP was recovered in an approximately exponential way. The recovery of the IPSP after the injection of NO_2^- ions was almost complete in the second trial. On the other hand, the final value of the IPSP after the first trial was slightly different from the original one, as was often observed after injections of penetrating anions in the present investigation.

Fig. 2. Plot of the sizes of IPSP's, antidromically evoked spike potentials (S.P.) and resting potentials (R.P.) before and after injections of NO_2^- ions. The plots were obtained from the series partly illustrated in Fig. 1.

While an injection of penetrating anions produced a great change in the IPSP, no significant change in the spike potential or in the EPSP was observed, unless the resting potential of the motoneuron was depressed by the damaging effect of the injection. The ineffectiveness of the penetrating anions on the spike potential and EPSP was also observed when nonpenetrating anions were injected.

The toxic effect of N_3^- ions reported by ARAKI *et al.* (1961) was found to be rather slight in the present investigation and the reversal of the IPSP after injection was demonstrated in three cells: The recovery of the IPSP followed an exponential curve approximately. Though the injection of ClO_4^- ions was technically difficult (ARAKI *et al.*, 1961), it was possible to observe the reversal of the IPSP after their injection into four cells.

The effect of the above-mentioned anions upon the IPSP indicates that they can penetrate the activated inhibitory subsynaptic membrane. These penetrating ions are, therefore, labelled by plus signs in Table 2.

Table 2. Effects of anion injections on the IPSP.

Ion	Relative hydrated size	IPSP effect	No. of injections	No. of cells
Br ⁻	0.94	+	12	4
I ⁻	0.94	+	4	4
Cl ⁻	0.96	+	9	7
NO ₂ ⁻	1.02	+	17	6
NO ₃ ⁻	1.03	+	14	5
N ₃ ⁻	1.07	+	3	3
ClO ₄ ⁻	1.09	+	5	4
SCN ⁻	1.11	+	9	3
HS ⁻	1.13	—	5	2
OCN ⁻	1.14	+	5	3
ClO ₃ ⁻	1.14	+	5	3
BrO ₃ ⁻	1.32	—	3	2
F ⁻	1.33	—	2	2
HCO ₂ ⁻	1.35	+	4	4
HSO ₃ ⁻	1.47	—	2	2
HCO ₃ ⁻	1.65	—	5	3
CH ₃ CO ₂ ⁻	1.80	—	3	2
SO ₄ ²⁻	1.84	—	4	3
H ₂ PO ₄ ⁻	2.04	—	2	2
HPO ₄ ²⁻	2.58	—	2	2
C ₆ H ₅ O ₇ ³⁻	3.08	—	5	3

Nonpenetrating anions

After the injection of BrO₃⁻, F⁻, HSO₃⁻, CH₃CO₂⁻, SO₄⁻, H₂PO₄⁻, HPO₄²⁻, citrate and HS⁻ ions, the IPSP remained nearly the same or increased slightly in size. In Fig. 3, the effect of the injection of CH₃CO₂⁻ ions is shown. Record A gives the control IPSP produced by the stimulation of the 9th dorsal root. An antidromic spike is shown at the right of the sweep. An inward current of 1.8×10^{-8} A was applied through the microelectrode. B and C are specimen records obtained at 20 and 120 sec, respectively, after the cessation of the current. The sizes of the IPSP, spike potential and resting potential are plotted in Fig. 4. As is seen in this figure, the IPSP increased in size immediately after the cessation of the current and then went back to its original size within a few minutes. However, there was no significant change in the spike potential and the resting potential. The increase in the hyperpolarization of the IPSP after the injection may be attributable to the depletion of Cl⁻ ions from the cell by the hyperpolarizing current as suggested by ARAKI *et al.* (1961).

Since COOMBS *et al.* (1955) could not exclude the possibility that HCO₃⁻ ions were partly responsible for changing the IPSP in the depolarizing direction, this ion species was carefully tested by ARAKI *et al.* (1961). The latter authors found that in the first series of successive injections of HCO₃⁻ ions in one cell

Fig. 3. Effect of injection of CH_3CO_2^- ions on the IPSP. A shows the control IPSP evoked by the stimulation of the 9th dorsal root, followed by an antidromically evoked spike potential. They are recorded in high and low amplification in the upper and lower beams, respectively. B and C were obtained at 20 and 120 sec, respectively, after the application of an inward current of 1.8×10^{-8} A through the microelectrode filled with a 1M KCH_3CO_2 solution.

a small reduction of the IPSP was observed, the reduction being progressive with a maximum of about 30 sec after the injection. In the later series the IPSP turned into a depolarizing potential following a time course similar to that in the initial series. In the present investigation, however, these unique changes in the size of the IPSP were not produced in five trials of injection of HCO_3^- ions into three cells, and no appreciable change in the IPSP was observed.

The ineffectiveness of HS^- ions in converting the IPSP to a depolarizing response in spite of the small hydrated size of this ion species was reported by ITO *et al.* (1962) and later by ASADA (1963). This has been confirmed also in the toad spinal motoneuron in the present investigation.

Since the injections of the above-mentioned anions are ineffective in changing the IPSP in the depolarizing direction, it can be concluded that the activated inhibitory subsynaptic membrane is impermeable to these anions. They are labelled by minus signs in Table 2.

Fig. 4. Plots of the sizes of IPSP's, antidromic spike potentials (S.P.) and resting potentials (R.P.) before and after the injection of CH_3CO_2^- ions. The plots were obtained from records partly illustrated in Fig. 3.

Recovery of the IPSP after anion injection

With penetrating anions the time course of the IPSP after anion injection was assumed to be of an exponential nature. In order to test the presumed exponential time course, the sizes of the IPSP were measured as the algebraic difference from the values obtained at the end of the recovery and these were plotted logarithmically against the time after cessation of the current. Figure 5 illustrates the time course of the recovery of the IPSP as plotted in this way after two trials of NO_2^- injection as shown in Figs. 1 and 2. As is seen in Fig. 5, the points fall approximately along straight lines with time constants of 52 and 47 sec for the first and the second trials of NO_2^- injection, respectively. It was not possible to obtain reliable values for the time constant of the IPSP recovery after injections of N_3^- and ClO_4^- ions. For other anion species which penetrate through the activated inhibitory subsynaptic membrane, the time constants thus measured showed a considerable range as indicated in Table 3.

COOMBS *et al.* (1955) reported that the time course of the recovery of the IPSP after the penetrating anion injection is attributable to a fall of the injected anion concentration as the ions diffuse outward across the motoneuron membrane. Furthermore, it has been shown by ARAKI *et al.* (1961) that this diffusion of injected ions takes place for the most part across the non-synaptic membrane, the activated

Fig. 5. Logarithmic plot of the IPSP size in the course of recovery after the injection of NO_2^- ions. Plots are obtained from two trials in the cell that is partly illustrated in Figs. 1 and 2.

Table 3. Time constant of IPSP recovery.

Anion species	No. of cells	No. of trials	Mean time constant (sec)	Range (sec)	Relative ability to penetrate
Br^-	4	12	34.8 ± 4.5 (S.D.)	29–42	1.58
OCN^-	3	5	37.6 ± 2.8	35–42	1.46
NO_3^-	5	14	42.6 ± 5.8	30–56	1.29
NO_2^-	6	17	45.6 ± 4.3	36–57	1.20
SCN^-	3	9	49.0 ± 5.9	43–61	1.12
Cl^-	7	9	54.6 ± 7.1	41–63	1.00
HCO_2^-	4	4	63.0 ± 4.8	58–71	0.87
I^-	4	4	65.5 ± 7.6	55–76	0.83
ClO_3^-	3	5	85.2 ± 8.3	69–91	0.64

inhibitory subsynaptic membrane forming an insignificant part in this respect.

According to the constant-field theory (GOLDMAN, 1943; HODGKIN and KATZ, 1949), the time constant of the anion escape from the cell is related to the permeability coefficient of the membrane, to the ratio of the cell volume to surface area, and to the membrane potential. Motoneurons tested in the present investigation had large resting potentials (larger than 53 mV) and tolerated the injection for a considerable time, so that they could be assumed to be large cells. Therefore, the permeability coefficient of the membrane may be the only important factor in

determining the above-mentioned time constant. Thus, the time constant of the IPSP recovery can be assumed as reciprocally proportional to the permeability coefficient through the whole surface area of the motoneuron (ARAKI *et al.* 1961). The relative values of the permeability coefficients thus calculated are given in Table 3.

DISCUSSION

It has been shown in the present investigation that the anion penetrability of the activated inhibitory subsynaptic membrane of the spinal motoneuron of the toad is essentially the same as that found in the spinal motoneuron of the cat reported by ARAKI *et al.* (1961) and ITO *et al.* (1962) and also in the Mauthner cell of the goldfish described by ASADA (1963). The exceptional permeability of HCO_2^- ions has been reinforced by the finding of the present study. In their investigation of the motoneuron of the cat, ARAKI *et al.* (1961) found that HCO_2^- ions were permeable through the activated inhibitory subsynaptic membrane in spite of their large hydrated size. To explain this exceptional behaviour, they suggested that the relative hydrated size of HCO_2^- ions might be smaller at 38°C, the body temperature of the cat, than at 25°C where the conductance measurement was made. Their alternative explanation was that the HCO_2^- ions might have an ellipsoidal shape with an axis smaller than the pore diameter. Since all present experiments in the toad spinal cord were performed at room temperature, 15–19°C, the exceptional behaviour of HCO_2^- ions cannot be explained by the former hypothesis. Therefore, the latter hypothesis seems to be acceptable. The same conclusion has been reported by ASADA (1963). The exceptional behaviour of HS^- ions, being ineffective on the IPSP despite the small hydrated size, reported by ITO *et al.* (1962) and described by ASADA (1963), has been confirmed in the present investigation. The most probable explanation for this phenomenon may be that HS^- ions cannot exist free in the cell as suggested by ITO *et al.* (1962).

The results of the present investigation as well as those reported by ARAKI *et al.* (1961), ITO *et al.* (1962) and ASADA (1963) indicate that anions are clearly separated into penetrating and non-penetrating ones through the activated inhibitory subsynaptic membrane. There are no transitional ions forming an intermediate zone. However, KERKUT and THOMAS (1964) have shown that BrO_3^- ions caused a very slight decrease in the reversal potential of the IPSP in the neurons of the snail, so this ion species may lie in an intermediate zone between the penetrating and non-penetrating anions. TAKEUCHI and TAKEUCHI (1967) have also demonstrated that BrO_3^- ions are slightly permeable in the crayfish inhibitory neuromuscular junction. The structure of the activated subsynaptic membrane of the snail neuron and of the crayfish neuromuscular junction may be somewhat different from that of the cat and toad motoneuron and of the goldfish Mauthner cell. Recently, significantly different results have been reported in the study on

anion permeability of cortical neurons of the cat (KELLY *et al.* 1969). All inorganic univalent anions tested, except IO_3^- , and organic univalent anions (mainly of aliphatic and aromatic acids) have been found to be capable of producing a reversal of cortical IPSP's by diffusion from microelectrodes.

Concerning the anion permeability of the non-synaptic motoneuron membrane, it is interesting to compare the time constant of the IPSP recovery listed in Table 2 with that reported by ARAKI *et al.* (1961). It has been found in the present investigation that the order of the relative permeability for the non-synaptic motoneuron membrane in the toad is $\text{Br}^- > \text{OCN}^- > \text{NO}_3^- > \text{NO}_2^- > \text{SCN}^- > \text{Cl}^- > \text{HCO}_2^- > \text{I}^- > \text{ClO}_3^-$, while that of the cat motoneuron membrane has been found by ARAKI *et al.* (1961) to be $\text{Br}^- = \text{SCN}^- > \text{Cl}^- > \text{NO}_2^- > \text{BF}_4^- > \text{I}^- > \text{NO}_3^- > \text{ClO}_3^- > \text{HCO}_2^-$. It should be noticed that Br^- ions are the most permeable, while the permeability to HCO_2^- and ClO_3^- ions is very low in both the cat and toad motoneuron membrane. However, discrepancies are found in the relative permeability to other penetrating anions between cat and toad motoneuron membranes, particularly for Cl^- , NO_3^- and SCN^- ions. For the halide anions, the sequence of $\text{Br}^- > \text{Cl}^- > \text{I}^-$ holds true in both cases. Since the sequence of relative anion permeability of the toad motoneuron membrane is not in keeping with their hydrated size, the simple pore hypothesis (BOYLE and CONWAY, 1941) does not seem to be applicable. This is also the case with the cat motoneuron membrane.

Various hypothesis have appeared to explain the relative ionic permeability of biological membranes (*e.g.*, HARRIS, 1956; SHANES, 1958; MULLINS, 1959, 1960; EISENMAN, 1965). The applicability of various hypothesis to the relative anion permeability of the cat motoneuron membrane has been discussed by ARAKI *et al.* (1961). They have come to the conclusion that no single hypothesis based upon the hydrated size or the naked ion size or hydration energy can explain the sequence of the relative anion permeability of the cat motoneuron membrane. As SHANES (1958) has suggested, the sequence of anion permeability may be accounted for in terms of hydration energies and naked ion diameters. It may be not impossible to explain the sequence of the relative anion permeability found in the present investigation if one takes Shane's hypothesis into account.

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