BRIEF COMMUNICATION

3,4-DIAMINOPYRIDINE

A POTENT NEW POTASSIUM CHANNEL BLOCKER

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ABSTRACT 3,4-diaminopyridine has been found to act very potently in selectively blocking the potassium channels of squid axon membranes. The apparent dissociation constants for this action are estimated to be 5.8 μM and 0.7 μM for external and internal applications, respectively, the potency being about 50 times higher than that of 4-aminopyridine. The block depends upon the membrane potential, time, and stimulus frequency. 3,4-diaminopyridine shows great promise as a useful tool for the study of membrane ionic channels.

Pharmacological dissection of the ionic channels of excitable membranes has progressed considerably as a result of the discovery of tetrodotoxin (TTX), a highly specific blocker of sodium channels in nerve and muscle (1). An equally potent and useful chemical for blocking potassium channels has not yet been found. Tetraethylammonium (TEA) blocks potassium channels (2) but only at high concentrations and less specifically. In addition, TEA is effective only when applied internally for certain nerves such as squid giant axons (2). 4-aminopyridine (4-AP) was found to block potassium channels in squid axons by either external or internal application at millimolar concentrations (3,4). Recent studies have also shown that contraction of smooth and skeletal muscles is enhanced by both 4-AP and 3,4-diaminopyridine (3,4-DAP) (5,6). We report here that in squid giant axons 3,4-DAP blocks potassium channels at micromolar concentrations in a highly specific manner.

Giant axons of the squid, *Loligo pealei*, both internally perfused and intact, were voltage clamped by the double axial wire technique (7). Series resistance was partially compensated, and leakage and capacity currents were subtracted electronically from the membrane current records. External solutions were maintained at a constant temperature between 7° and 9° C. 3,4-DAP (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was dissolved in either external or internal solutions. Artificial sea water had the following composition (mM): Na^+, 450; K^+, 10; Ca^+, 50; HEPES (*N*-2-hydroxyethylpiperazine-N^1-2-ethane sulfonic acid) buffer, 5; Cl^-, 560. The pH was adjusted to 8.0. Internal perfusate had the following composition (mM): Na^+, 50; K^+, 350; glutamate, 350; F^-, 50; sucrose, 33; phosphate buffer, 30. The pH was adjusted to 7.3. For voltage clamp experiments, the membrane potential was held at −70 mV for

intact axons and −80 mV for internally perfused axons, and various depolarizing or hyperpolarizing pulses were applied.

3,4-DAP was very effective in prolonging the falling phase of the action potential when applied externally to an intact axon (Fig. 1 A and B). In this experiment, the resting membrane became depolarized by 12 mV in 100 μM 3,4-DAP. The average depolarization was 13±3 mV in four experiments with 100 μM 3,4-DAP, and spontaneous repetitive action potentials were often observed. Despite the marked prolongation of the action potential, the membrane potential at its peak was unchanged by 3,4-DAP. The undershoot after the action potential, normally observed in squid axons, became a depolarizing after-potential in 3,4-DAP, as seen in Fig. 1 B. These results suggest a marked reduction of potassium current (I_K) with little change in sodium current (I_{Na}). Voltage clamp experiments have shown that this is indeed the case. Fig. 1 C illustrates a family of membrane currents in response to step depolarizations from a holding potential of −70 mV to levels ranging from −40 mV to +100 mV in 20 mV increments before application of 3,4-DAP. After application of 100 μM 3,4-DAP (Fig. 1 D), the currents measured at the end of each 8-ms pulse (primarily I_K) were almost entirely eliminated, while peak transient currents (I_{Na}) were unaffected.

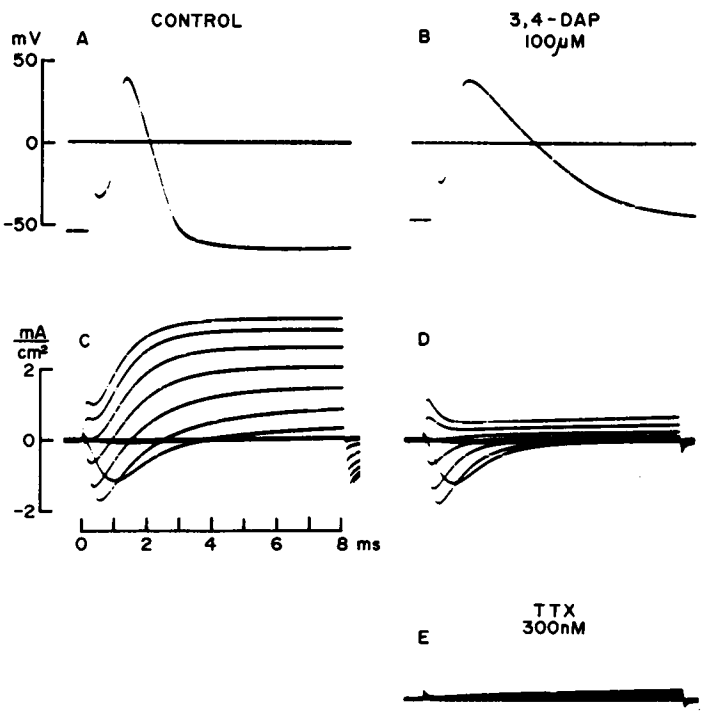

FIGURE 1 Effects of 3,4-DAP on action potentials (A and B) and membrane currents associated with step depolarizations (C–E) recorded from an intact axon. A and C are control records before addition of 3,4-DAP. B and D were recorded from the same axon after the addition of 100 μM 3,4-DAP to the external bathing solution. E, membrane currents after external application of both 3,4-DAP and TTX (300 nM). Holding potential −70 mV. Temperature, 8°C.

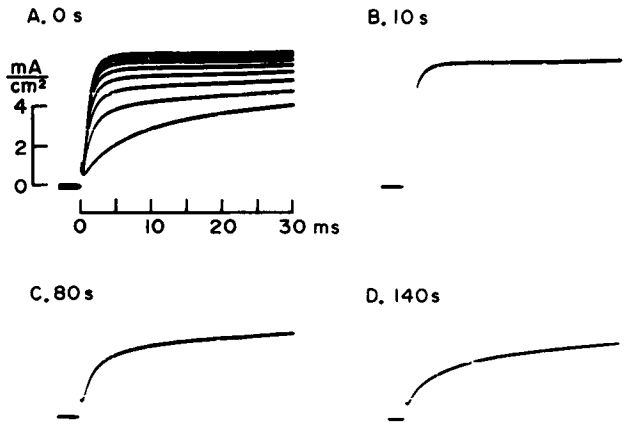

FIGURE 2. Removal of block by repetitive depolarizing pulses (A) and the reestablishment of block under resting conditions (B–D). Internally applied 3,4-DAP (10 μM), externally applied TTX (300 nM). A, superimposed records of membrane currents during a conditioning train of eight depolarizing pulses at a frequency of 2 Hz. B–D, membrane currents in response to test pulses at different time intervals after the conditioning train. Both conditioning and test pulses depolarized the membrane to +100 mV for 30 ms from a holding potential of −80 mV. Temperature, 8°C.

Fig. 1 E shows the residual I_K after application of 300 nM TTX to the same axon. With I_{Na} completely blocked by TTX, it can be seen that 3,4-DAP almost entirely eliminated I_K.

In squid giant axons, 4-aminopyridine (4-AP) block of potassium channels is dependent upon voltage, time, and stimulus frequency (8,9). This was shown by experiments in which the block was relieved by long-lasting depolarization or repetitive depolarizing pulses. Similar behavior of the potassium current was observed with 3,4-DAP. Fig. 2 A shows the results of an experiment with internal application of 10 μM 3,4-DAP in the presence of TTX outside. Repetitive depolarizing pulses to +100 mV and 30 ms in duration were applied at a frequency of 2 Hz. The first pulse resulted in the smallest potassium current amplitude and each succeeding pulse caused a progressively larger current. The steady-state current level reached 60% of the control after 20 pulses. The time-course of block removal (measured as current amplitude at the end of the 30-ms pulse) proceeded as a single exponential function with a time constant of 2.3 s. At higher 3,4-DAP concentrations of 1–2 mM (internal or external application), the block could not be removed by repetitive pulses.

The time dependence of the removal of block is shown in Fig. 2 A also. During the first pulse of the train, potassium current increased from an early level (measured at 8 ms) of 6.5% of control to 26.6% after 30 ms of depolarization. The time-course of the potassium current during this pulse was exponential, with a time constant of 10 ms. This is much slower than the normal gating kinetics of the potassium channel, and, by analogy with 4-AP (8,9), probably reflects the removal of 3,4-DAP molecules from the binding site, allowing potassium current to flow. The time-course of the currents elicited by subsequent repetitive pulses appears to have two phases: an early fast

phase that may be determined by the kinetics of unblocked K channels and a much slower phase that may reflect block removal.

Reestablishment of block under resting conditions is illustrated by Figs. 2 B–D. Single 30-ms depolarizing pulses to a membrane potential of +100 mV were applied at various times after the eight-pulse train shown in Fig. 2 A. The block was completely reestablished only after a rest interval of 140 s. Block reestablishment is much slower than block removal during repetitive stimulation, proceeding exponentially with a time constant of 55 s.

Fig. 3 shows the dose-response relationships for both internal (filled circles) and external (open circles) applications of 3,4-DAP. The potassium current in response to an 8-ms depolarizing pulse to +60 mV was measured after a steady state had been reached at each concentration (at least 10 min for internal application and at least 15 min for external application). Thus, these data represent the steady-state block of potassium channels in the resting membrane. Since the drug effect could be removed only partially, even after a very long washing period (about 15 min for internal application and more than 1 h for external application), doses were increased cumulatively. Curves were fitted to the data according to the Michaelis-Menten equation. The values for maximum response and apparent dissociation constants were estimated to be 100% and 0.68 μM for internal application, and 97% and 5.75 μM for external application, respectively.

The present study has demonstrated that the effects of 3,4-DAP and 4-AP on nerve are qualitatively very similar. Both drugs are specific for the potassium channel, effective by either internal or external application, and are less effective under conditions that would normally open the potassium channels. For either drug, the reestablishment of block under resting conditions is much slower than removal of block by depolarization. Therefore, low-frequency repetitive pulses can also remove the block.

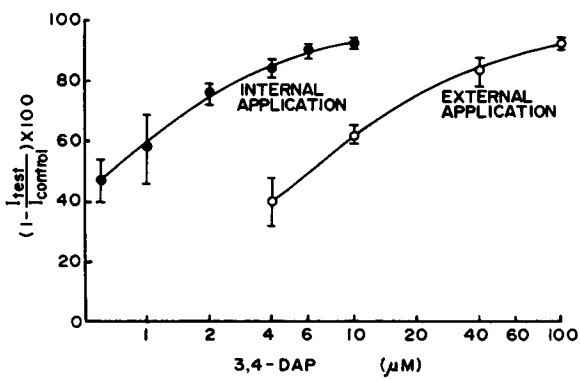

FIGURE 3 Dose-response relationships for external and internal applications of 3,4-DAP. Ordinate is a linear scale of the amount of reduction of potassium current in drug-treated axons compared to current in the normal axon treated only with TTX. Test pulses depolarized the membrane to +60 mV for 8 ms. The abscissa is a log scale of 3,4-DAP concentration. Filled circles represent mean data from four internally perfused axons (except 0.6 μM, three axons). Open circles are data from three intact axons. Bars represent standard deviations.

Thus, as in the case of 4-AP, 3,4-DAP probably binds to the closed potassium channel, is removed while the channel is opened by depolarization, and slowly rebinds to the channel after it is closed by repolarization.

The major difference between the two drugs is that 3,4-DAP has a greater affinity for the potassium channel than 4-AP. 4-AP applied externally at a concentration of 1 mM caused a 75% block of potassium current (8), whereas a concentration of only 20 μM would be required for 3,4-DAP to give the same degree of block (Fig. 3). Although the membrane potential of the test pulse was different in these experiments, being +100 mV for 4-AP and +60 mV for 3,4-DAP, the difference in potency is not due to this factor, because in other experiments we found only a 4.3% and a 3.2% decrease in block by increasing the test pulse from +60 to +100 mV in 10 μM and 4 μM 3,4-DAP, respectively.

In addition to 3,4-DAP, several other aminopyridine derivatives were found to block potassium channels in a frequency-dependent manner. They ranked in order of decreasing effectiveness: 3,4-DAP, 4-AP, 2,3-diaminopyridine, 4-hydroxypyridine, 2,6-diaminopyridine, 3-hydroxypyridine, and 2-hydroxypyridine. In general, compounds which had neither hydroxyl nor amino substituents had no effect. These results suggest that the high potency of 3,4-DAP may be due to its increased hydrogen bonding ability.

The reason for the difference in potency between external and internal applications of 3,4-DAP is unclear. The pH difference between external (pH 8) and internal (pH 7.3) media is unlikely to be the explanation. If we assume, by analogy with local anesthetic block of the sodium channel (10), that only the uncharged species of the drug molecule cross the membrane and that only the charged species block the K channel from inside the membrane, then after external application of 3,4-DAP to an intact axon, the charged form would accumulate inside to reach a level about five times as high as in the external solution. This is because the pK_a of 3,4-DAP is 9.08 (11) and the internal pH is more favorable for the charged species than the external pH. Such an accumulation would make external application of drug appear more potent than internal application, which contradicts the observed decrease in potency with external application. Other differences between internal and external conditions, such as the high external concentration of calcium ions (50 mM) relative to the internal concentration (nearly zero), may contribute to the difference in potencies. The existence of two receptors with different affinities for the drug cannot be ruled out. 3,4-DAP shows great promise as a potassium channel blocker, especially in view of its high potency, high specificity for potassium channels, and effectiveness when applied from either side of the membrane.

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REFERENCES

1. NARAHASHI, T. 1974. Chemicals as tools in the study of excitable membranes. *Physiol. Rev.* **54**:813.
2. ARMSTRONG, C. M., and L. BINSTOCK. 1965. Anomalous rectification in the squid giant axon injected with tetraethylammonium chloride. *J. Gen. Physiol.* **48**:859.
3. MEVES, H., and Y. PICHON. 1975. Effects of 4-aminopyridine on the potassium current in internally perfused giant axons of the squid. *J. Physiol. (Lond.).* **251**:60P. (Abstr.).
4. YEH, J. Z., G. S. OXFORD, C. H. WU, and T. NARAHASHI. 1976. Interactions of aminopyridines with potassium channels of squid axon membranes. *Biophys. J.* **16**:77.
5. JOHNS, J., D. S. GOLKO, P. A. LAUZON, and D. M. PATON. 1976. The potentiating effects of 4-aminopyridine on adrenergic transmission in the rabbit vas deferens. *Eur. J. Pharmacol.* **38**:71.
6. HARVEY, A. L., and I. G. MARSHALL. 1977. The facilitatory actions of aminopyridines and tetraethylammonium on neuromuscular transmission and muscle contractility in avian muscle. *Naunyn-Schmeideberg's Arch. Pharmacol.* **299**: 53.
7. WU, C. H., and T. NARAHASHI. 1973. Mechanism of action of propranolol on squid axon membranes. *J. Pharmacol. Exp. Ther.* **184**:155.
8. YEH, J. Z., G. S. OXFORD, C. H. WU, and T. NARAHASHI. 1976. Dynamics of aminopyridine block of potassium channels in squid axon membrane. *J. Gen. Physiol.* **68**:519.
9. MEVES, H., and Y. PICHON. 1977. The effect of internal and external 4-aminopyridine on the potassium currents in intracellularly perfused giant axons. *J. Physiol. (Lond.).* **268**:511.
10. NARAHASHI, T., D. T. FRAZIER, and M. YAMADA. 1970. The site of action and active form of local anesthetics. I. Theory and pH experiments with tertiary compounds. *J. Pharmacol. Exp. Ther.* **171**:32.
11. ALBERT, A. 1963. Physical Methods in Heterocyclic Chemistry. Vol. 1. A. R. Katritzky, editor. Academic Press, Inc., New York. 73.