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Effects of D-600 on Sodium Current in Squid Axons

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Summary. The effects of the calcium antagonist D-600 (methoxyverapamil) on the excitatory inward sodium current, I_{Na}, of internally perfused squid giant axons were studied under voltage-clamp conditions. We observed little or no effect of the drug when it was added to the external solution at concentrations of 10–200 μM. Furthermore, it did not produce a frequency, or use-dependent block of I_{Na} when repetitive voltage-clamp pulses were used at rates of 2–5 Hz. However, it did produce use-dependent blockade of I_{Na} when it was placed internally at a concentration of 200 μM. These results in conjunction with other studies suggest that D-600 is a selective blocker of calcium channels in squid axons when the drug is placed in the external solution. Its effects, when placed in the internal solution, are similar to those of permanently charged local anesthetic derivatives, which also produce use-dependent block of I_{Na}.

Key Words calcium antagonists · sodium channels · voltage clamp · squid axons

Introduction

Previously, Kohlhardt, Bauer, Krause and Fleckenstein (1972) demonstrated that D-600 (methoxyverapamil) blocked the inward calcium current in mammalian myocardial fibers without affecting the transient excitatory inward current, I_{Na}. Shortly thereafter, Baker, Meves and Ridgeway (1973) reported that the drug also blocked the entry of calcium ions into depolarized squid axons. However, they also demonstrated that D-600 blocked the I_{Na} component. This result appeared to be confirmed for cardiac tissue by subsequent reports (Rosen, Ilvento, Gelband & Merker, 1974; Bayer, Kalusche, Kaufman & Mannhold, 1975). In particular, Bayer et al. (1975) reported a frequency-dependent effect of D-600 on the maximal rate of rise of the upstroke of the cardiac action potential, which is consistent with an effect of the drug on I_{Na}.

We report here a lack of effect of externally applied D-600 on I_{Na}, using internally perfused squid axons. Application of the drug to the external solution at concentrations of 10–200 μM produced at most a 5–10% reduction of I_{Na}, which may have been attributable to axon deterioration. We also did not observe a frequency, or use-dependent, effect under these conditions. Frequency-dependent block of I_{Na} was observed when D-600 was added to the internal perfusate at a concentration of 200 μM.

Results similar to ours with external D-600 were briefly noted by Terakawa (1981) in conjunction with his study on bi-ionic action potentials in squid axons.

Materials and Methods

Experiments were performed on giant axons dissected from the mantles of squid (*Loligo pealei*). Standard axial wire voltage clamp and internal perfusion techniques were used (French & Wells, 1977; Clay & Shlesinger, 1983). Axons were internally perfused with a solution containing 50 mM KF, 200 mM K-glutamate, 25 mM K_2HPO_4, and 505 mM sucrose. They were superfused with artificial seawater (ASW) containing 430 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 10 mM Tris-HCl buffer. The pH of all solutions was adjusted to 7.2 at room temperature. The racemic form of D-600 was added directly to either the internal or external solution at a final concentration of 10 μM. Solutions with 100 or 200 μM D-600 were obtained by preparing a concentrated solution of the drug (100 mg/ml) in ethanol and carrying out the appropriate dilution. Ethanol was added to some of the control solutions at the same level as in the corresponding test solutions (0.05 or 0.1%). We did not observe effects of ethanol on membrane currents at these levels. The temperature used in these experiments ranged between 6 and 9 °C. In any single experiment the temperature was maintained constant to within 0.1 °C. The voltage-clamp protocol consisted of sequential depolarizing steps of several msec duration from a holding potential of −80 mV (inside negative) to −50, −40, . . . , +30 mV. After each depolarizing step, two hyperpolarizing steps of appropriate amplitude were applied to obtain leakage and capacitative currents. A rest interval of 3 sec was used between each step. Frequency-dependent effects were measured with a voltage-clamp

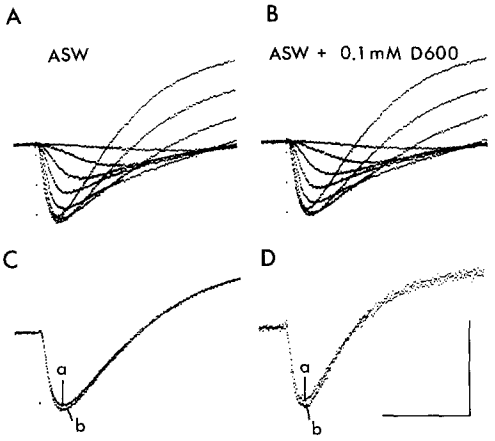

Fig. 1. Effects of external D-600 on membrane currents in squid axons. (*A*): Control results. (*B*): Test results obtained after 100 μM D-600 added to the external artificial seawater (ASW). Voltage-clamp pulses to −50, −40, . . . +30 mV from a holding potential of −80 mV. Corrections were made for leakage and capacitative currents. (*C*): Records for $V = +10$ mV for 100 μM D-600 (*a*) and control (*b*). (*D*): Records from a different axon for $V = +10$ mV for 200 μM D-600 (*a*) and control (*b*). (*A–C*): Axon C83.27. (*D*): Axon C83.33. The horizontal scale corresponds to 3 msec for *A–D*. The vertical scale corresponds to 5 mA · cm^{-2} for *A–C*; 3 mA · cm^{-2} for *D*

pulse of fixed duration and amplitude applied at a repetition rate of 2–5 Hz.

Results

Effects of External D-600 on I_{Na}

Representative results of our experiments with D-600 added to the external solution are shown in Fig. 1*A–D*. The records in Fig. 1*A* are superimposed measurements of membrane current in ASW in response to 9 msec duration voltage clamp steps to −50, −40, . . . +30 mV. These results demonstrate the transient inward sodium current (I_{Na}) and the outward potassium current (I_K), as originally described by Hodgkin and Huxley (1952). Results of the same protocol are shown in Fig. 1*B* following superfusion with ASW containing 100 μM D-600. These results show very little effect of the drug on either I_{Na} or I_K. A direct comparison of the control and the test record for the step to +10 mV is provided by Fig. 1*C*. The test record (labeled *a*) shows a diminution by about 5% of peak I_{Na} in comparison with the control record (labeled *b*), which may have been attributable to axon deterioration. Similar results are shown from another preparation in Fig. 1*D* with 200 μM D-600 added to the ASW, which is the same concentration of the drug used by Baker et al. (1973). Results similar to those in Fig. 1*C* and *D* were obtained from a total of five axons using 10 μM D-600, a total of three axons using 100 μM D-600, and a total of three axons using 200 μM D-600. We also did not observe a frequency-dependent block of I_{Na} in a total of six axons with either 100 or 200 μM D-600. These experiments consisted of single voltage-clamp pulses to a fixed voltage, usually 0 or +10 mV, applied sequentially at a rate of 2 Hz. We also looked for a use-dependent effect of D-600 on I_{Na} with pulse frequencies of 5 Hz. Under these conditions we noted a ~20% diminution of peak I_{Na} in control axons, which may represent either an artifact due to electrode polarization or a true frequency-dependent effect intrinsic to I_{Na} channels. This effect did not appear to be augmented by D-600.

Fig. 2. Effects of internal D-600 on membrane currents in squid axons. (*A*): Control records superimposed from 60 consecutive sweeps of voltage-clamp pulses to $V = +10$ mV with a holding potential of −80 mV. Repetition rate was 2 Hz. (*B*): Test records. Same protocol as in *A* with 200 μM D-600 added to the internal perfusate. Axon C83.36. Horizontal scale represents 4 msec; vertical scale represents 2 mA · cm^{-2}

Effects of Internal D-600 on I_{Na}

The addition of D-600 to our internal perfusate at a concentration of 10 μM did not produce an effect on membrane current in a total of five axons. However, 200 μM internal D-600 produced a clear effect, as illustrated by Fig. 2*A–B*. The result in Fig. 2*A* consists of superimposed oscilloscope sweeps of 60 voltage-clamp pulses of 8 msec duration to +10 mV applied sequentially to a control preparation at a rate of 2 Hz. The membrane current from all of the pulses in this sequence superimposed almost exactly. There was perhaps a slight diminution of I_K near the end of this experiment, which may have been attributable to the effects of potassium ion accumulation in the periaxonal space. The record in Fig. 2*B* illustrates results of the same procedure following the addition of 200 μM D-600 to the internal perfusate. The first record in the 60-pulse sequence was nearly the same as the record in Fig. 2*A*, which indicates little tonic block of I_{Na}. However, peak I_{Na} was reduced in a sequential manner to about 30% of its control value by the subsequent

30 pulses. This result demonstrates a phasic, or a use-dependent block of I_{Na} by D-600. Little further change in membrane current was observed during the latter 30 pulses of the 60-pulse sequence. The control membrane current response was recovered following a 10–20 sec rest period. Similar results were obtained from a total of three axons.

Discussion

External Application of D-600

Our experiments demonstrate a lack of a pronounced effect of D-600 on I_{Na} and I_K in squid axons when the drug was applied externally. It may have produced a slight diminution of inward current, as illustrated by the results in Fig. 1, although it is difficult to completely rule out the possibility that this effect was due to axon deterioration. The drug undoubtedly does affect I_{Na} if a sufficiently large concentration is used. For example, Terakawa (1981) reported a 20% block of peak I_{Na} with 1 mM external D-600. The point of our experiments is that the drug has little or no effect on I_{Na} at a concentration (200 μM) that is effective at blocking inward calcium current in squid axons. This same general conclusion appears to be valid for cardiac muscle membrane, as well, although the concentration range for which effects on I_{Na} occur in these preparations is considerably lower than it is in nerve. For example, Rosen et al. (1974) reported a reduction of the upstroke velocity of the cardiac action potential with 10 μM verapamil, and Bayer et al. (1975) reported a frequency-dependent reduction of the upstroke velocity using 6–16 μM D-600, which is suggestive of an effect of these drugs on I_{Na}. However, the drugs are effective blockers of inward calcium current in cardiac muscle at considerably lower concentrations than those used in these reports. For example, Kolhardt et al. (1972) demonstrated blockade of inward calcium current with 1 μM D-600 in their original experiments; Josephson and Sperelakis (1982) have reported similar findings in chick embryonic heart cells with 1 μM verapamil; and McDonald, Pelzer and Trautwein (1980) have reported effects on calcium current of mammalian heart ventricles using 2 μM D-600. Furthermore, Ebihara, Shigeto, Lieberman and Johnson (1980) have demonstrated a lack of an effect on I_{Na} in chick embryonic heart cells using 2.5 μM D-600. Consequently, the calcium antagonists appear to be selective blockers of the calcium current with respect to I_{Na} in the 1–2 μM concentration range. However, they do not appear to be selective in cardiac membrane with regard to potassium currents. For example, Kass and Tsien (1975) reported effects of 1 μM D-600 on potassium currents in cardiac Purkinje fibers. Similar results have been observed in chick embryonic heart cells (A. Shrier, *unpublished*). The experiments in this report demonstrate a lack of an effect of D-600 on I_K in squid axons.

Internal Application of D-600

The results in Fig. 2 demonstrate frequency-dependent block of I_{Na} of internally perfused axons by D-600 when the drug was added to the internal perfusate. We did not observe frequency-dependent block of I_{Na} when the drug was added to the external solution. Certain quaternary derivatives of the local anesthetic, lidocaine, produce similar effects in squid axons (Frazier, Narahashi and Yamada, 1970; Cahalan, 1978). These compounds apparently have difficulty in crossing biological membranes because they are permanently charged (Frazier et al., 1970). By contrast, neutral and amine anesthetics are active when they are applied either externally or internally (Frazier et al., 1970). They appear to reach their blocking site by diffusing across the membrane, when they are applied externally (Hille, 1977). D-600 is similar to some of the permanently charged forms of these compounds in both its chemical structure and its effects on I_{Na}, which suggests that its blocking site for I_{Na} is located on the inner surface of excitable cell membranes.

Comparison with Previous Work

As noted above, Baker et al. (1973) reported a significant tonic block of I_{Na} in squid axons with 200 μM D-600, whereas we report little or no effect of the drug under similar external conditions. Baker et al. (1973) used intact axons; we have used internally perfused axons. The difference between the two sets of results may be attributable to the fact that an externally applied drug can accumulate inside an intact axon, if it is able to cross the membrane, whereas the drug is swept away from the inside of the axon during internal perfusion. However, we observed little tonic block of I_{Na} when 200 μM D-600 was added to our internal perfusate. We observed significant blockade only when repetitive voltage-clamp pulses were applied. Consequently, we would predict little tonic block of I_{Na} with 200 μM external D-600 even if the drug were able to cross the membrane. We conclude that there may be a significant difference between perfused and intact axons concerning the effects of D-600, which warrants further study.

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