

# Methadone Block of K<sup>+</sup> Current in Squid Giant Fiber Lobe Neurons

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**ABSTRACT** Voltage-dependent ionic currents were recorded from squid giant fiber lobe neurons using the whole-cell patch-clamp technique. When applied to the bathing solution, methadone was found to block  $I_K$ ,  $I_{Na}$ , and  $I_{Ca}$ . Both  $I_{Na}$  and  $I_{Ca}$  were reduced without apparent change in kinetics and exhibited  $IC_{50}$ 's of ~50–100 and 250–500  $\mu$ M, respectively, at +10 mV. In contrast,  $I_K$  was reduced in a time-dependent manner that is well fit by a simple model of open channel block ( $K_D = 32 \pm 2 \mu$ M, +60 mV, 10°C). The mechanism of  $I_K$  block was examined in detail and involves a direct action of methadone, a tertiary amine, on K channels rather than an opioid receptor-mediated pathway. The kinetics of  $I_K$  block resemble those reported for internally applied long chain quaternary ammonium (QA) compounds; and recovery from  $I_K$  block is QA-like in its slow time course and strong dependence on holding potential. A quaternary derivative of methadone (*N*-methyl-methadone) only reproduced the effects of methadone on  $I_K$  when included in the pipette solution; this compound was without effect when applied externally.  $I_K$  block thus appears to involve diffusion of methadone into the cytoplasm and occlusion of the open K channel at the internal QA blocking site by the protonated form of the drug. This proposed mode of action is supported by the pH and voltage dependence of block as well as by the observation that high external K<sup>+</sup> speeds the rate of drug dissociation. In addition, the effect of methadone on  $I_K$  evoked during prolonged (300 ms) depolarizations suggests that methadone block may interfere with endogenous K<sup>+</sup> channel inactivation. The effects of temperature, methadone stereoisomers, and the methadone-like drugs propoxyphene and nor-propoxyphene on  $I_K$  block were examined. Methadone was also found to block  $I_K$  in GH3 cells and in chick myoblasts.

## INTRODUCTION

Methadone represents a class of synthetic  $\mu$ -opioid-receptor agonists including propoxyphene, commonly known as Darvon (Eli Lilly, Indianapolis, IN), that are similar to morphine in their receptor-binding properties and analgesic actions. Although the pathways underlying opioid receptor-mediated analgesia are amply documented, opioid agonists can induce a multitude of effects other than analgesia (Martin, 1984). Some of these effects cannot be reversed by opioid receptor antagonists, and therefore may be unrelated to opioid receptor occupancy by the drug (Oron et al., 1992; Calligaro et al., 1988; Walker et al., 1985). Such nonopioid effects have been reported for both methadone (Choi and Viseskul, 1988; Leander and McCleary, 1982; Stickney, 1977) and propoxyphene (Barraclough and Lowe,

1982; Strom et al., 1985). Various nonopioid cardiovascular effects associated with propoxyphene poisoning have been particularly well documented and include bradycardia, asystole, bundle branch block, widening of QRS complex, reduced myocardial contractility and hypotension (Amsterdam et al., 1981; Heaney, 1983; Sorensen et al., 1985).

In several cases the mechanism underlying the nonopioid action of opioid agonists has been shown to involve a direct interaction between the drug and voltage-dependent Na channels. For example, propoxyphene was initially found to exert a local anesthetic-like effect on cardiac action potentials (Nickander et al., 1979; Holland and Steinberg, 1979). Later investigation revealed that propoxyphene and its major metabolite nor-propoxyphene block Na current, in a manner similar to local anesthetics; exhibiting both use dependence and slow recovery from block (Whitcomb et al., 1989). Whitcomb et al. (1989) further demonstrated that lidocaine, a local anesthetic, both speeds recovery of  $I_{Na}$  from propoxyphene block and is clinically effective at reversing some nonreceptor mediated effects associated with propoxyphene poisoning (QRS widening

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and bradycardia). Thus, it appears that propoxyphene, like lidocaine, can directly block the Na channel pore and that propoxyphene toxicity may be attributed, in part, to direct Na channel block. The  $\kappa$ -opioid receptor agonists U-50488H and U-54494A also block Na current in a nonreceptor-mediated fashion (Alzheimer and ten Bruggencate, 1990; Zhu and Im, 1992). Interestingly, the anticonvulsant action of U-54494A appears to depend upon its ability to directly block Na channels as well as its ability to bind to  $\kappa$ -opioid receptors (Fischer et al., 1993). Finally, a variety of opioid agonists including methadone have been shown to inhibit the binding of batrachotoxin to Na channels, indicating that many opioids can bind to Na channels with  $K_d$ s in the  $\mu$ M range (Creveling et al., 1985).

These examples demonstrate not only that some opioid agonists can directly block Na channels but also that such a mechanism can contribute significantly to the clinical side effects or even the primary actions of these drugs. However, the ability of opioid agonists to interact directly with ion channels other than Na channels has not been well characterized. The question of whether opioid agonists can directly block  $K^+$  or  $Ca^{2+}$  channels is of particular interest, because activation of opioid receptor-coupled pathways typically lead to an enhancement of  $I_K$  or inhibition of  $I_{Ca}$  (reviewed by North, 1986). Thus, nonopioid actions on  $I_K$  and  $I_{Ca}$  might be expected to modulate a drug's opioid-receptor mediated response. Such an effect, even if it were not clinically important, could prove relevant to the interpretation of studies involving opioid-receptor mediated pathways.

The present study demonstrates that methadone blocks voltage dependent  $K^+$ ,  $Ca^{2+}$  and  $Na^+$  currents in squid neurons. Propoxyphene and norpropoxyphene are similar to methadone in their ability to block  $I_K$  in this preparation. Several lines of evidence suggest that these effects are not mediated by opioid receptors but rather involve direct interactions between drug and ion channel. The mechanism of  $I_K$  block by methadone was examined in detail and is adequately described by a model developed for block by internal quaternary ammonium (QA) compounds (Armstrong, 1971). The results of this study reveal some interesting aspects of methadone action that may reflect interaction of the aromatic portion of this compound with the inner mouth of the K channel pore. These effects include temperature-dependent and stereospecific interaction of methadone with its binding site as well as interference with endogenous K channel inactivation. In addition, the characteristics of  $I_K$  block and the ability of methadone to block  $I_K$  in other tissues (GH3 cells and chick myoblasts) raise the possibility that  $I_K$  block, might contribute to the nonopioid toxicity of methadone-like opioid receptor agonists such as propoxyphene.

## METHODS

### Cells

Giant fiber lobe neurons from the squid *Loligo opalescens* were isolated and maintained as previously described (Gilly and Brismar, 1989).  $K^+$  and  $Ca^{2+}$  currents were recorded from cells which had been in culture <3 d, whereas  $Na^+$  currents were recorded after 3–5 d (Brismar and Gilly, 1987). GH3 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD) and maintained under standard conditions (Matteson and Armstrong, 1986). Chick myoblasts were isolated from day 4 limb buds, plated on coverslips coated with collagen (3 mg/ml in 0.012 N HCl; Vitrogen 100, Collagen Corp., Palo Alto, CA) and maintained for 2 d before use as small myotubes (Miller and Stockdale, 1986).

### Voltage Clamp

Currents were recorded with the whole-cell patch-clamp technique (Hamill et al., 1981) using an EPC-7 amplifier (Medical Systems, Greenvale, NY). Electrodes were pulled from 0010 glass (Garner Glass, Claremont, CA) and coated with Sylgard (Dow Corning, Midland, MI). Currents were filtered using an 8-pole Bessel filter (Frequency Devices Inc., Haverville, MA) with a corner frequency of 10 kHz and were digitized at 20 kHz with a 14 bit A/D converter. The bath solution was grounded through an agar bridge filled with 10K-ASW (see solutions below), and the liquid junction potential between internal and external solutions ( $-10$  mV) has been ignored. Currents were leak-subtracted with a P/4 procedure (Armstrong and Bezanilla, 1974). Pipette access (series) resistance was electronically compensated and the residual access resistance (0.4 to 1 M $\Omega$ ) was estimated from compensation settings and used to correct the pipette voltage ( $V_p$ ) at which current was recorded. The corrected pipette voltage,  $V_m$ , was used in plotting current-voltage relationships and in determining the voltage and dose dependence of steady state  $I_K$  block. Residual series resistance error was <10 mV for all data presented in this paper. Recordings involving squid neurons were carried out at 10°C unless otherwise indicated. In experiments involving temperature changes,  $T$  was measured directly with a thermistor probe in the bath solution. Recordings from GH3 and chick cells were carried out at 20°C.

### Solutions and Current Isolation

Drug application and solution exchange were accomplished by perfusing the chamber with 10–20 vol of drug solution (requiring 1–3 min). No sign of delay in the onset of block or washout of block was observed after solution changes.

Solutions used for recording from squid neurons were designed to isolate  $I_K$ ,  $I_{Na}$ , or  $I_{Ca}$ . These solutions were adjusted to 980 mosM (internal) or 1000 mosM (external), and, unless otherwise indicated, pH was adjusted to 7.4 at 10°C.

$K^+$  current was recorded using a variety of internal and external  $K^+$  concentrations. The convention "external//internal" is used to indicate these concentrations in each experiment. The standard external solution (10K ASW) contained (in millimolar) 10 KCl, 450 NaCl, 10  $CaCl_2$ , 50  $MgCl_2$ , and 15 HEPES. 1 mM  $CdCl_2$  and 200 nM TTX were included to block  $Ca^{2+}$  and  $Na^+$  currents, respectively. High  $K^+$  solutions (50–400 mM K) were made by substituting KCl for NaCl on an equimolar basis. The in-

ternal (pipette) solution contained (in millimolar) 25 KF, 20 KCl, 10 K<sub>2</sub>-EGTA, and 15 HEPES; K-glutamate was added to yield total K<sup>+</sup> levels of 100–400 mM and sucrose was added to adjust the osmolarity. In some experiments a 50 K internal solution was used that contained (in millimolar) 420 *N*-methyl-D-glucamine, 400 glutamic acid, 5 K-glutamate, 25 KF, 20 KCl, 10 EGTA, and 10 HEPES. For experiments involving changes in external pH both internal and external solutions contained 50 mM HEPES.

Na<sup>+</sup> current was recorded with an external solution containing (in millimolar) 470 NaCl, 50 MgCl<sub>2</sub>, 10 CaCl<sub>2</sub>, 10 HEPES, 5 CsCl, and 1 CdCl<sub>2</sub>. The internal solution contained 230 TMA-glutamate, 120 Na-glutamate, 50 NaF, 25 NaCl, 25 TEA-Cl, 10 Na<sub>2</sub>-EGTA, and 10 HEPES. Ca<sup>2+</sup> current was recorded with an external solution containing (in millimolar) 30 CaCl<sub>2</sub>, 450 TRIZMA 7.2 (Sigma Chemical Co., St. Louis, MO), 50 MgCl<sub>2</sub>, 10 HEPES, 5 CsCl, and 200 nM TTX. The internal solution contained 350 TMA-glutamate, 70 TMA-Cl, 50 TMA-F, 10 EGTA, and 10 HEPES.

Recording solutions for GH3 and chick myoblasts were as follows: external = 130 NaCl, 5 KCl, 5 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 HEPES, 200 nM TTX; and internal = 85 K-glutamate, 10 KCl, 25 KFI, 10 EGTA, 10 HEPES. All solutions were adjusted to 280 mosM and pH 7.4.

### Drugs

Methadone stereoisomers, propoxyphene-HCl, and norpropoxyphene maleate were provided by the National Institute of Drug Abuse (NIDA). (±)*N*-methyl-methadone was synthesized by reacting equimolar amounts of (±)methadone (Sigma Chemical Co.) and methyl iodide in ethyl acetate overnight at room temperature. The resulting crystals were washed in cold ethyl acetate, and recrystallized twice from MeOH/H<sub>2</sub>O. *N*-methyl-methadone was applied through addition to the pipette solution. Control currents were obtained immediately after attaining the whole-cell configuration (allowing ionic equilibration), drug affected cur-

rents were recorded when block kinetics had stabilized, generally within 15 min after attaining the whole-cell configuration.

## RESULTS

### Methadone Block of Ionic Currents Is Not Mediated by μ-Opioid Receptors

When applied externally to squid neurons at concentrations of 50–500 μM, methadone blocks voltage sensitive K<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>2+</sup> currents (Fig. 1). *I*<sub>Na</sub> (Fig. 1 *B*) and *I*<sub>Ca</sub> (Fig. 1 *C*) are reduced without appreciable change in kinetics, whereas *I*<sub>K</sub> (Fig. 1 *A*) is reduced in a distinctly time-dependent manner during a single depolarization. Morphine, like methadone, is an opioid receptor agonist that preferentially binds to μ-type receptors. Application of 350 μM morphine to squid neurons, however, has no detectable effect on *I*<sub>K</sub> (Fig. 1 *D*), *I*<sub>Na</sub>, or *I*<sub>Ca</sub> (data not shown), nor does naloxone, a potent opioid antagonist, inhibit the ability of methadone to block *I*<sub>K</sub> in squid neurons (data not shown). Thus, the blocking action of methadone described here appears to be unrelated to the drug's activity as a μ-opioid receptor agonist. Indeed, in the case of *I*<sub>K</sub> block, several lines of evidence presented below suggest that methadone directly interacts with the K channel at an internal site; a mechanism that does not involve binding of methadone to any opioid receptor.

The molecular structure of methadone (Fig. 1 *E*) suggests that its ability to block *I*<sub>K</sub>, *I*<sub>Na</sub>, and *I*<sub>Ca</sub> may involve direct interaction with ion channels. Methadone is a membrane permeant tertiary amine that is structurally similar to local anesthetics (LA) such as tetracaine (Fig.

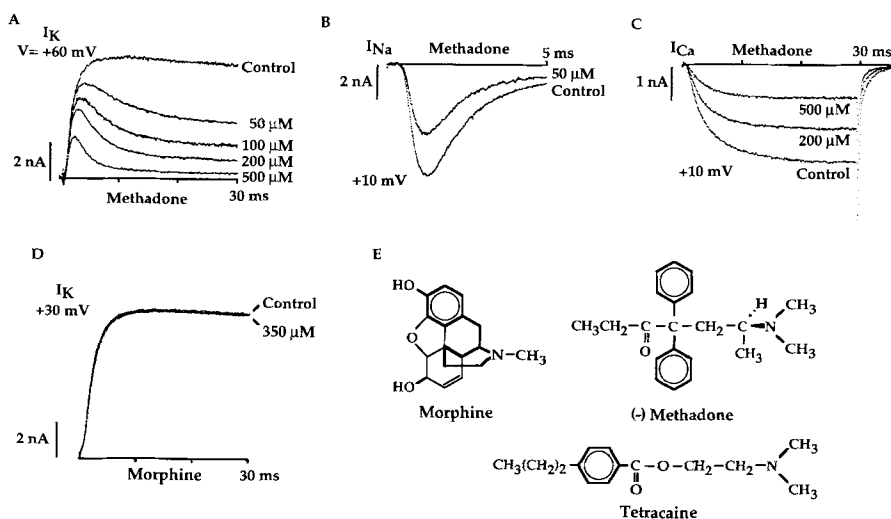


FIGURE 1. Externally applied methadone blocks *I*<sub>K</sub>, *I*<sub>Na</sub>, *I*<sub>Ca</sub> in squid GFL neurons. Currents were isolated as described in the methods section. (A) *I*<sub>K</sub> evoked by 30-ms depolarizations to +60 mV from a holding potential of -60 mV in the presence of indicated concentrations of (-)methadone (10K/100K). Control records, in the absence of drug, were obtained immediately before application or after washout of each drug solution. Some rundown of control *I*<sub>K</sub> was observed over the 80-min time course of the experiment thus drug records were scaled based upon the decline in amplitude of their corresponding controls. The scale bar corresponds to the illustrated control which was obtained in conjunction with

the 100 μM drug trace. The order in which the drug traces were recorded, along with their scaling factors, are: 200 μM (0.83), 100 μM (1.0), 500 μM (1.0), 50 μM (1.38). (B) *I*<sub>Na</sub>, recorded in response to 5-ms depolarizations to +10 mV (*H*<sub>p</sub> = -80 mV) before and during application of 50 μM (±)methadone. (C) *I*<sub>Ca</sub> evoked by 30-ms depolarization to +10 mV (*H*<sub>p</sub> = -80 mV) in 30 mM external Ca<sup>2+</sup> in the presence of the indicated concentrations of (±)methadone. (D) *I*<sub>K</sub> evoked by 30-ms depolarizations to +30 mV is unaffected by application of 350 μM morphine, the three superimposed traces include control, drug and washout (E) The molecular structure of (-)methadone (chiral center indicated) is compared to that of morphine and the local anesthetic tetracaine.

1 E) or, in its charged form, to quaternary ammonium ions (QA). Like methadone, some LA's such as tetracaine and dibucaine have been reported to block  $K^+$ ,  $Na^+$ , and  $Ca^{2+}$  currents (Carmeliet et al., 1986; Narahashi et al., 1969; Sugiyama and Muteki, 1994). Similarly, QA ions such as C9 (Armstrong, 1971) can also block both  $I_K$  and  $I_{Na}$  (Rojas and Rudy, 1976). Mechanisms underlying  $Na^+$  channel block by LA and  $K^+$  channel block by QA compounds have been studied extensively (reviewed by Armstrong, 1975; Stanfield, 1983; Hille, 1992), and the blocking action of methadone on  $I_{Na}$  and  $I_K$ , are suggestive of similar mechanisms that do not involve occupancy of opioid receptors. Although methadone is thought to be capable of achieving a morphine-like tertiary structure that allows it to bind to opioid receptors (Gero, 1954), morphine is not a tertiary amine and clearly differs from local anesthetics (Fig. 1 E). Thus, it is not surprising that morphine failed to reproduce the LA-like action of methadone on ionic currents in squid neurons.

The bulk of this paper is devoted to a detailed analysis of the effects of methadone on  $I_K$ . First, a general picture of the effects on  $I_{Na}$  and  $I_{Ca}$  is presented.

#### $I_{Na}$ and $I_{Ca}$ Block

Effects of methadone on  $I_{Na}$ , recorded in response to 5-ms depolarizations in the presence of 244 mM internal  $Na^+$  are illustrated in Fig. 2. In 100  $\mu M$  external

( $\pm$ )methadone both inward and outward  $I_{Na}$ , are substantially reduced relative to the control (Fig. 2 A). Maintained outward currents under these conditions are carried by  $Na^+$  (Gilly and Brismar, 1989). Although currents were recorded in 1 mM  $Cd^{2+}$  to reduce  $I_{Ca}$ , residual  $I_{Ca}$  is probably responsible for the inward current flowing at the end of the largest depolarization in methadone.

$I_{Na}$  was studied in isolation by examining prepulse-sensitive current (Fig. 2 B) (Gilly and Brismar, 1989). Fig. 2 B indicates that prepulse-sensitive  $I_{Na}$  evoked at +10 and +60 mV is blocked by methadone with no apparent change in kinetics and that the degree of block is not appreciably voltage dependent (see also Fig. 2 C). Similarly the steady state inactivation-voltage relationship (not shown) is not affected by methadone. These results are consistent with a local anesthetic-like "tonic" (resting) block of Na channels similar to the effect of tetracaine (Hille, 1977). An approximate value for the  $IC_{50}$  for  $I_{Na}$  block can be estimated from Figs. 1 and 2 to lie between 50 and 100  $\mu M$  at +10 mV.

Unlike many local anesthetics, methadone produces little use-dependent block of  $I_{Na}$ . When evoked by repetitive depolarization to +70 mV (5-ms pulses at 4 hz,  $H_p = -80$  mV) in 50  $\mu M$  ( $\pm$ )methadone,  $I_{Na}$  showed only a 20% use-dependent reduction in peak amplitude (data not shown). Repetitive depolarization at this rate to +10 mV had no detectable effect on peak  $I_{Na}$ .

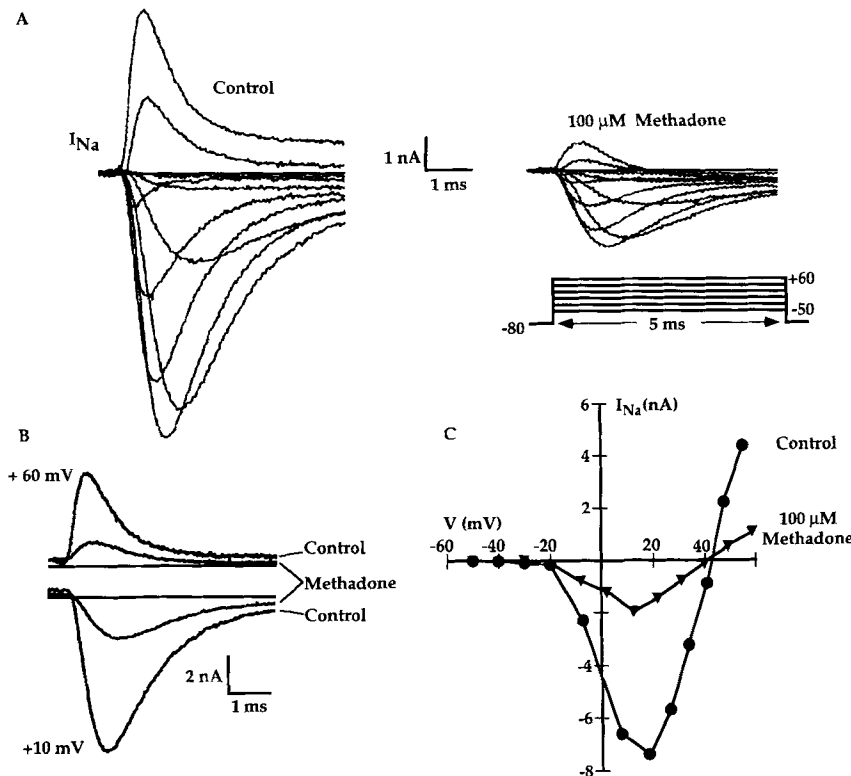


FIGURE 2.  $I_{Na}$  block by methadone. (A) Families of  $I_{Na}$ , evoked by 5-ms depolarizations (-50 to +60 mV in 10-mV steps) before (control) and during application of 100  $\mu M$  ( $\pm$ )methadone. Inward and outward  $Na^+$  current are seen because 244 mM  $Na^+$  is present in the internal solution. (B) Prepulse-sensitive  $I_{Na}$ , recorded at +10 and +60 mV in the presence and absence of 100  $\mu M$  ( $\pm$ )methadone. A 50-ms prepulse to -15 mV preceded each of the voltage steps. This completely inactivates the transient component of  $I_{Na}$ . Prepulse-sensitive  $I_{Na}$ , was obtained by direct subtraction of currents obtained with and without a prepulse. (C) Peak  $I_{Na}$ -voltage relationships for prepulse-sensitive current.

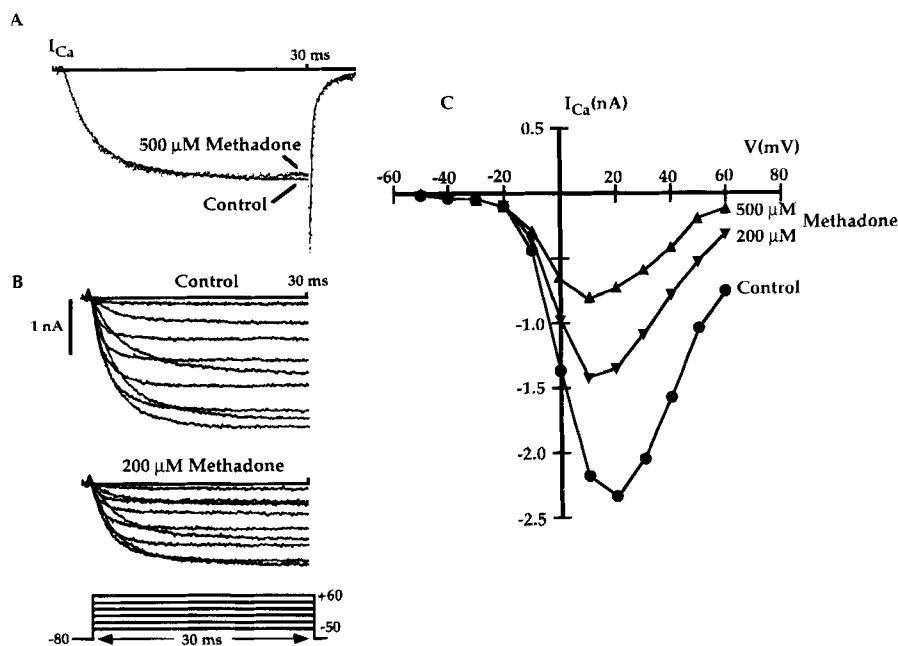


FIGURE 3.  $I_{Ca}$  block by methadone. (A) Normalized  $Ca^{2+}$  currents recorded at +10 mV in the presence and absence of 500  $\mu M$  ( $\pm$ )methadone (from Fig. 1 C) exhibit identical kinetics. (B) Families of  $I_{Ca}$  evoked by 30-ms depolarizations to a range of potentials (-50 to +60 mV in 10-mV steps) in the presence and absence of 200  $\mu M$  ( $\pm$ )methadone. (C)  $I_{Ca}$ -voltage relationships obtained at the indicated methadone concentrations. The control and 200  $\mu M$  methadone plots correspond to the records in B. Control current was recorded after washout of 200  $\mu M$  methadone from the external solution. 500  $\mu M$  methadone was applied following the control.

$Ca^{2+}$  currents were recorded in response to 30-ms depolarizations in the presence of 30 mM external  $Ca^{2+}$  and absence of both internal and external  $K^+$  and  $Na^+$ . As suggested by the records illustrated in Fig. 1 C, methadone does not alter  $I_{Ca}$  kinetics, and this is better demonstrated by comparison of scaled traces recorded at +10 mV, before (control) and during application of 500  $\mu M$  methadone illustrated in Fig. 3 A.

$I_{Ca}$  records evoked over a range of depolarizations in the presence and absence of 200  $\mu M$  methadone are shown in Fig. 3 B. The  $I_{Ca}$ -voltage relationships corresponding to these data and to those obtained in 500  $\mu M$  methadone are plotted in Fig. 3 C. In this case (unlike that for  $I_{Na}$ )  $I_{Ca}$  block appears to be voltage dependent. Little or no block is observed at -10 and -20 mV but block around the peak of the current-voltage relationship ( $\sim +20$  mV) is significant and exhibits an approximate  $IC_{50}$  between 200 and 500  $\mu M$ . The voltage dependence of  $I_{Ca}$  block was not studied in further detail.

#### Methadone Is an Open Channel Blocker of $I_K$

Control  $I_K$  (Fig. 4 A) was evoked in response to a 25-ms depolarization to +80 mV (HP = -70 mV) and represents maximal activation of  $K^+$  conductance ( $g_K$ ). After application of 100  $\mu M$  (-)methadone,  $I_K$  begins to activate normally in response to depolarization but is then blocked in a time-dependent manner, declining exponentially to a steady state level that represents 27% of control (Fig. 4 A). This behavior is well described by a simple model of open-channel block, that assumes no block at rest (Fig. 4 B). Control current is modeled according to the scheme presented by Gilly and Armstrong (1982). Steady state  $I_K$  in methadone represents

an equilibrium between open and blocked channels with an equilibrium constant  $K_D = (L/\kappa)$  where  $\kappa$  and  $L$  represent the rates of drug block and dissociation from the open channel and  $\kappa$  is proportional to the methadone concentration.

The effect of varying concentrations of drug seen in Fig. 1 A is well fit by the assumption that the blocking rate ( $\kappa$ ) is directly proportional to the drug concentration. This point is illustrated in Fig. 4 C by a Hill plot derived from the data in Fig. 1 A. A linear fit to this data indicates a Hill coefficient of 1.2, implying that methadone blocks the K channel by binding with 1:1 stoichiometry. Given the above model, the apparent  $K_D$  for block can be determined from the relationship  $K_D = [f_o/(1-f_o)] \times [\text{methadone}]$  where  $f_o$  is the steady state fractional block ( $I_{drug}/I_{control}$ ). Thus, the apparent  $K_D$  for methadone block is  $38 \pm 4 \mu M$  (mean  $\pm$  SEM) from the records in Fig. 1 A or  $32 \pm 2 \mu M$  ( $n = 17$ ) based upon data from 12 cells.

#### Methadone Blocks K Channels at an Internal Site

The model of open-channel block used to account for the effects of methadone is the same as that used to describe the action of internal QA on  $I_K$  (Armstrong, 1971). This similarity, along with the ability of methadone to induce LA-like block of  $I_{Na}$ , suggests that methadone, like QA and LA, may act at an internal site. To test this hypothesis, the effects of a permanently charged and presumably membrane-impermeant quaternary derivative of methadone (( $\pm$ )*N*-methyl-methadone) were examined.

When applied externally, 1 mM *N*-methyl-methadone has no effect on  $I_K$ ,  $I_{Na}$ , or  $I_{Ca}$  (not illustrated). How-

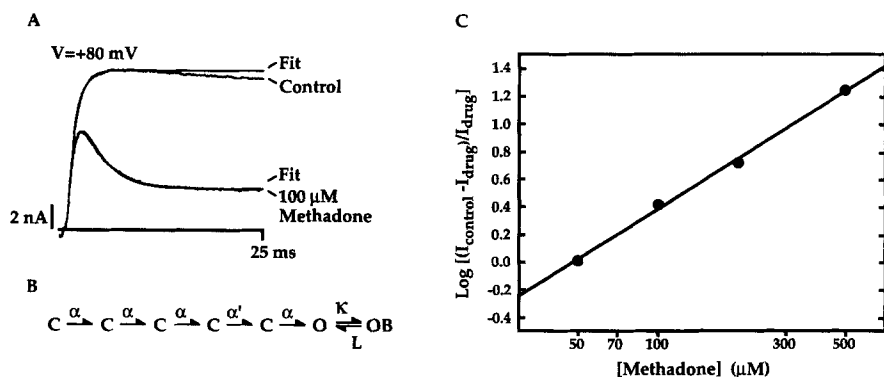


FIGURE 4.  $I_K$  is blocked by methadone in a manner consistent with open-channel block. (A)  $K^+$  current evoked at +80 mV in response to a 25 ms depolarization in the presence and absence of 100  $\mu\text{M}$  (—) methadone (100K//200K) is compared to predictions (solid curves) of the model illustrated in the accompanying kinetic diagram (B). An instantaneous “jump” in current is observed upon depolarization and the baseline was set to the inflection of the current trace (initially sampled at 10- $\mu\text{s}$  intervals). The model for control  $I_K$  is conventional

(based on Gilly and Armstrong, 1982), and the rate constants are (in  $\text{ms}^{-1}$ )  $\alpha = 9$ ,  $\alpha' = 0.9$ ,  $L = 0.096$ ,  $\kappa = 2.6 \text{ ms}^{-1} \text{ mM}^{-1}$ . C represents a closed state, O the open state and OB is the methadone-blocked state. (C) Methadone blocks the potassium channel by binding with 1:1 stoichiometry. A Hill plot of steady state block data obtained in conjunction with the experiment depicted in Fig. 1 A demonstrates that points fall on a best fit straight line with a slope (Hill coefficient) of 1.21 ( $R = .99$ ). Determination of steady state block involved comparison of  $I_K$  at the end of a 30-ms depolarization in methadone ( $I_{\text{drug}}$ ) to the corresponding control data ( $I_{\text{control}}$ ). Control and drug records for each drug concentration were obtained over a range of  $V$  (+60 to +80 mV). The resulting  $I$ - $V$  relationships were compared at +60 mV after correcting  $V$  for series resistance error (see Methods).

ever,  $I_K$  is strongly reduced in the presence of internal 500  $\mu\text{M}$  ( $\pm$ ) *N*-methyl-methadone (added to the pipette solution) (Fig. 5). Control current in Fig. 5 A was evoked immediately after attaining the whole-cell recording configuration before drug could diffuse throughout the cell. After 15 min perfusion time a pattern of time-dependent block similar to that seen with externally applied methadone was evident. The kinetics of  $I_K$  in 500  $\mu\text{M}$  *N*-methyl-methadone are similar to those in 500  $\mu\text{M}$  (—) methadone (Fig. 1 A), suggesting that both substances are equally potent at blocking  $I_K$ . Similarly, the values for steady state fractional block ( $f_o$ ), obtained from these records, are 0.067 and 0.060 for 500  $\mu\text{M}$  *N*-methyl-methadone and (—) methadone, respectively.

These results indicate that permanently charged methadone is ineffective when applied externally and suggest that methadone as a tertiary amine permeates the membrane in an uncharged form, becomes protonated in the internal medium and acts internally as the charged form. The high  $\text{pK}_a$  exhibited by methadone (9.64) (Kaufman et al., 1975) also implies that the charged form is the active species, because nearly all

drug molecules (>99%) should be charged at physiological pH. The pH and voltage dependence of methadone block (see below) further support this conclusion.

#### Methadone-blocked $K^+$ Channels Can Inactivate

Further characterization of the action of methadone is easier to understand if interpreted in the framework of a plausible model. The simple model of open channel block presented in Fig. 4 accurately describes the kinetics of  $I_K$  in the presence of methadone only if control  $I_K$  exhibits little or no inactivation. However,  $K^+$  currents recorded from many GFL neurons do inactivate to some extent, and for these cells methadone's action could not be accounted for by the simple model. This section shows that such records can be fit when the model is extended to allow inactivation of both open and methadone-blocked channels.

In the absence of drug,  $K^+$  current evoked by prolonged (300 ms) depolarization of GFL neurons slowly inactivates with a time constant of  $\sim 50$  ms at 10–12°C (Llano and Bookman, 1986). The degree of inactiva-

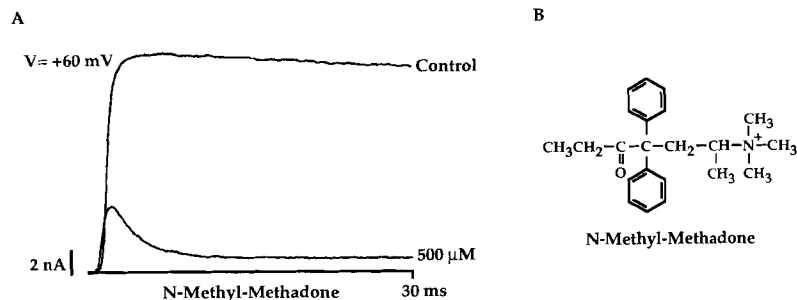


FIGURE 5. Internally applied *N*-methyl-methadone reproduces the effect of methadone on  $I_K$ . (A)  $I_K$  evoked at +60 mV immediately after attaining the whole-cell configuration (control) and after 15 min with a pipette solution containing 500  $\mu\text{M}$  ( $\pm$ ) *N*-methyl methadone (10K//50K). (B) The chemical structure of *N*-methyl-methadone.

tion varies from cell to cell, and typically the current remaining at the end of a 300-ms depolarization represents from 20 to 80% of peak  $I_K$  (unpublished data). In cells that exhibit minimal inactivation, the decay of peak  $I_K$  during a 20-ms depolarization is negligible and the effects of inactivation can be ignored when analyzing the much faster block by methadone. This point is illustrated by the example in Fig. 4 A where  $I_K$ , recorded in the presence of methadone, reaches a steady state level as predicted by the model. However, in a cell that displays significant inactivation of control  $I_K$  (Fig. 6 A) this is not the case.  $I_K$  recorded during a 20-ms depolarization in methadone continues to decline after block has apparently reached the equilibrium predicted by the simple model above.

Models of open channel block that include inactivation from both the open and blocked states were examined in an attempt to account for this discrepancy. In Fig. 6 A control  $I_K$  is well fit [Fit(A)] by a model (Fig. 6 B, kinetic diagram A) which assumes that inactivation proceeds from the open state (O) to an inactivated state (I) at a rate  $\gamma = 0.016 \text{ ms}^{-1}$ . On a short time scale (i.e., 20 ms) backward transitions from I to O can be neglected for the purposes of this model. The methadone trace in Fig. 6 A can be well fit [Fit(C)] by a scheme (kinetic diagram C) that assumes identical (control) rates of inactivation from both open and open-blocked states. An alternative model [kinetic diagram B, Fit(B)], that allows inactivation only from the open state clearly fails to reproduce the slow decline of  $I_K$ . Both models of block fit the initial rise and fall of  $I_K$  equally well [using identical rates of block ( $\kappa$ ) and unblock ( $L$ )]. The abil-

ity of the models to accurately account for the rapid block kinetics is consistent with the assumption of a single population of K channels underlying the control currents. In turn this implies that if more than one K channel subtype is significantly represented in GFL neurons (Nealey et al., 1993) then they are equally affected by methadone.

The results presented in Fig. 6 suggest that both blocked and unblocked K channels enter inactivated states at a similar rate during a 20-ms depolarization. Consequently, the model represented by kinetic diagram C is used throughout the remainder of this paper in order to fit the kinetics of  $I_K$  evoked by brief depolarizations in the presence of methadone. The model accurately reproduces the slow decline of  $I_K$  observed in methadone for a variety of cells that exhibit different degrees of inactivation. These fits are used in interpreting effects of membrane potential, pH, temperature and methadone stereo-isomers on the rates of block ( $\kappa$ ) and unblock ( $L$ ). However, it is important to point out that assumptions about inactivation do not affect the ability of the model to fit the initial kinetics of block and therefore do not affect determination of  $L$  and  $\kappa$ . Moreover, inactivation in the following experiments is far less prominent than in the case of Fig. 6 which represents an extreme example.

#### Voltage Dependence of $I_K$ Block

The degree of steady state block of  $I_K$  by methadone increases with depolarization over a range of voltages where  $K^+$  conductance ( $g_K$ ) is maximally activated, sug-

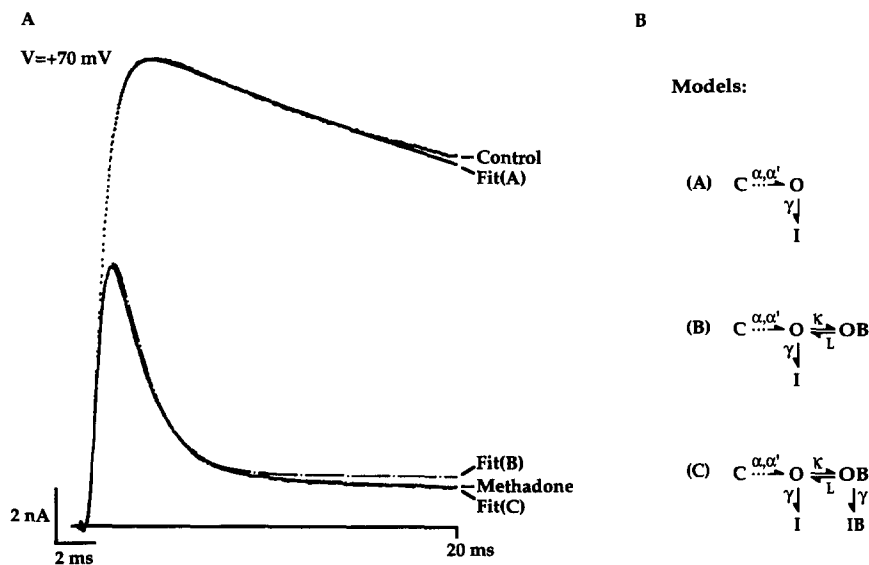


FIGURE 6. Blocked K channels can inactivate (A)  $I_K$  evoked at +70 mV before (control) and during application of 200  $\mu\text{M}$  methadone (200K//200K). Control current is well fit (fit A) by a model where channels proceed from an open state (O) to an inactivated state (I) at a rate  $\gamma$  (B, diagram A). The drug trace can be fit (fit C) by a model (diagram C) which assumes that both open and blocked channels inactivate at the control rate ( $\gamma$ ). Fit B is based upon a model that does not allow inactivation from the blocked state (diagram B), and can not reproduce the decline in steady state current observed. Control and methadone traces represent the averaged response to 20 and 36 depolarizations, respectively (5.5-s intervals,  $H_p = -60 \text{ mV}$ ). Individual current records were indistinguishable. (B) Models

incorporating both inactivation and block are indicated by kinetic state diagrams. IB is the blocked-inactivated state. As in Fig. 4 B the model incorporates five closed states. These states and the transitions among them are abbreviated by a single dashed arrow between C and O. The rate constants are (in  $\text{ms}^{-1}$ )  $\alpha = 9$ ,  $\alpha' = 1.5$ ,  $\gamma = 0.016$ ,  $L = 0.078$ ,  $\kappa = 2.4 \text{ ms}^{-1} \text{ mM}^{-1}$ .

gesting an intrinsic voltage dependence to the methadone-channel interaction (Fig. 7 A). A semilogarithmic plot of the apparent  $K_D$  as a function of membrane potential (Fig. 7 B) indicates an exponential dependence on voltage with an  $e$ -fold decrease in  $K_D$  per 102 mV depolarization. The direction and magnitude of this voltage dependence is similar to that determined for a variety of QA compounds in squid axon (reviewed by Yellen, 1987) and in Shaker  $K^+$  channels (Choi et al., 1993).

Because steady state block is determined by the rates of block ( $\kappa$ ) and unblock ( $L$ ), the voltage dependence of each rate constant was also examined.  $\kappa$  and  $L$  were determined by fitting the kinetics of  $I_K$  block at different  $V$  using model C described in conjunction with Fig. 6. Plots of  $L$  and  $\kappa$  in Fig. 7 B indicate an approximately symmetrical voltage dependence, with  $L$  decreasing  $e$ -fold per 210 mV depolarization and  $\kappa$  increasing  $e$ -fold per 200 mV. These results are consistent with the expected effects of depolarization on movement of a positively charged drug between the cytoplasm and a binding site that lies a small distance within the membrane electric field.

Voltage dependence of the blocking rate ( $\kappa$ ) is consistent with findings of Swenson (1981) concerning the action of various QA ions. However, the measurable voltage dependence of the rate of unblock ( $L$ ) in methadone contrasts with these previous findings. The voltage dependence of  $L$  may in part be explained by an interaction of methadone with external  $K^+$ . While Swenson observed no effect of external  $K^+$  on the kinetics of block by  $C_8TP$  during a depolarization, such an effect is seen for methadone. Fits obtained to  $I_K$  in the presence of methadone in 10 and 200 mM external  $K^+$  indicate

an increase in  $L$  with increasing  $[K^+]$ :  $L = 0.067 \pm .004 \text{ ms}^{-1}$  (mean  $\pm$  SEM,  $n = 4$ , +60 mV) in 10 mM  $K^+$  while  $L = 0.10 \pm .005 \text{ ms}^{-1}$  ( $n = 4$ , +60 mV) in 200 mM  $K^+$ . This  $K^+$  dependence is consistent with the idea that methadone occupies a site in the channel pore and can therefore interact with external  $K^+$ . The voltage dependence of  $L$  for methadone was determined in 50 mM  $K^+$  and may, therefore, involve an effect of membrane potential on movement of external  $K^+$  into the channel, rather than a direct effect of voltage on drug dissociation.

#### pH Dependence of $I_K$ Block

Changes in external pH ( $pH_o$ ) do not affect the ability of internally applied *N*-methyl-methadone to block  $I_K$  (data not shown). However methadone block of  $I_K$  is highly dependent upon  $pH_o$  in a way that is consistent with an internal site of drug action. Fig. 8 shows  $I_K$  evoked at +60 mV in the presence and absence (*control*) of 200  $\mu\text{M}$  external methadone at  $pH_o$  7.4 and 6.5. Control currents were not significantly affected by pH, but currents evoked in the presence of methadone at pH 6.5 exhibit both reduced levels of block and altered kinetics in comparison to those recorded at pH 7.4. Fits to these data, illustrated in Fig. 8, indicate that the effect of  $pH_o$  on methadone block can be accounted for by a 10.8-fold reduction in the rate of block ( $\kappa$ ) at pH 6.5 vs 7.4 whilst holding  $L$  constant.

Because the rate  $\kappa$  is directly proportional to drug concentration (Fig. 4), the 10.8-fold reduction of  $\kappa$  with reduced  $pH_o$  is equivalent to an identical 10.8-fold decrease in the concentration of methadone at the site of block. Since methadone has a high  $pK_a$  (Kaufman et

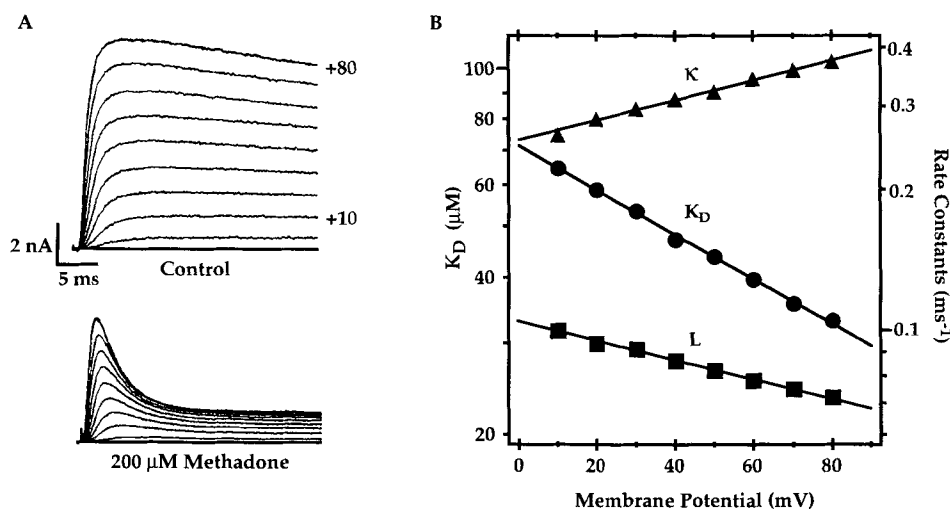


FIGURE 7.  $I_K$  block is voltage dependent. (A)  $I_K$  recorded over a range of activating voltages (0 to +80 mV) with, and without, 200  $\mu\text{M}$  methadone present (50K//100K).  $K^+$  conductance is maximally activated even for the smaller pulses. (B) Intrinsic voltage dependence of steady state methadone block corresponding to data in panel A is indicated by a semilogarithmic plot of  $K_D$  (left axis) as a function of activating voltage, where the apparent  $K_D = [f_o/(1 - f_o)] \times [\text{methadone}]$  and the fractional block ( $f_o = I_{\text{methadone}}/I_{\text{control}}$ ) were determined at the end of the 30-ms depolarization. The

best fit straight line indicates an  $e$ -fold change in  $K_D$  per 102 mV. Rates of block ( $\kappa$ ) and unblock ( $L$ ), plotted on the same graph (right axis), were obtained by fitting the data in A using model C shown in Fig. 6. Linear regressions to this data indicate an  $e$ -fold change in rate constant per 200 mV ( $\kappa$ ) or 210 mV ( $L$ ).



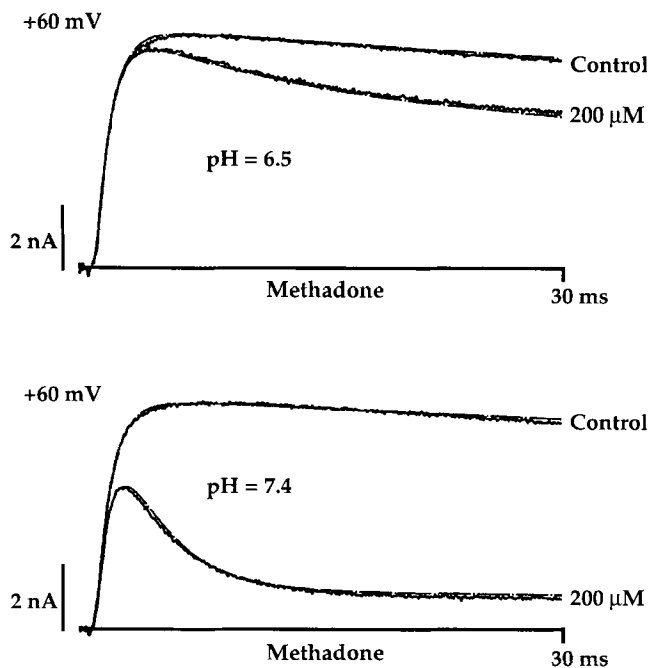


FIGURE 8. The strong pH-dependence of  $I_K$  block by methadone is shown by records obtained at +60 mV before (*control*) and during application of 200  $\mu\text{M}$  methadone at external pH of 6.5 or 7.4 (10K/50K). Control records show little dependence on pH. Fits to the data (corresponding to model C, Fig. 6 B) are superimposed on the current traces. The rate constants are (in  $\text{ms}^{-1}$ ):  $\alpha = 9.5$ ,  $\alpha' = 1.0$ ,  $\gamma = 0.003$ ,  $L = 0.055$ ,  $\kappa = 1.4 \text{ ms}^{-1} \text{ mM}^{-1}$  (pH 7.4);  $\alpha = 8.5$ ,  $\alpha' = 1.05$ ,  $\gamma = 0.005$ ,  $L = 0.055$ ,  $\kappa = 0.13 \text{ ms}^{-1} \text{ mM}^{-1}$  (pH 6.5).

al., 1975) and exists primarily in the charged form at physiological pH, a decrease in  $\text{pH}_o$  by 1.0 will have little effect (0.5% increase) on the concentration of protonated methadone ( $\text{Me}^+$ ) in the external solution. However, the same change in  $\text{pH}_o$  reduces the external concentration of uncharged (membrane permeant) methadone ( $\text{Me}$ ) by a factor of 10, resulting in a 10-fold decrease in the internal concentration of  $\text{Me}$ . The internal concentration of  $\text{Me}^+$  should also decrease by a factor of 10 because the ratio  $[\text{Me}^+]_i: [\text{Me}]_i$  is constant for constant internal pH. Thus, decreased block observed in reduced  $\text{pH}_o$  is consistent with the predicted effect of pH on the concentration  $\text{Me}^+$  at an internal blocking site.

The above arguments imply that the concentration of methadone at its blocking site depends upon both the applied drug concentration and the difference in pH between internal and external solutions. Therefore, we normally attempted to maintain equal  $\text{pH}_i$  and  $\text{pH}_o$  such that the blocking drug concentration and applied drug concentration were equivalent. However, a difference between internal and external pH of only 0.2 U should theoretically alter the ratio of internal to external methadone by factor of 1.58. Such a strong pH effect may account for variability in the degree of block

and rate of block ( $\kappa$ ) that were observed between experiments with the same applied drug concentration. Such variation, within an experiment, was minimized by including 15 mM HEPES buffer in both internal and external solutions. This precaution assured that pH did not vary with the amount of drug that was added to the stock solution and that changes in temperature affected internal and external pH equally.

#### Recovery of $I_K$ from Methadone Block

Recovery of  $I_K$  from block in the presence of 200  $\mu\text{M}$  methadone was examined using a double pulse procedure as illustrated in Fig. 9. During the initial 20 ms conditioning pulse,  $I_K$  activates to a peak and then declines to a steady state, representing equilibrium block of K channels by methadone (Fig. 9 A). After a 5,600-ms recovery interval at  $-90$  mV, a second (test) pulse evokes  $I_K$  with a reduced peak amplitude in comparison to the first, indicating that some fraction of  $\text{K}^+$  channels were still blocked (and therefore, unable to activate) at the start of the second depolarization. Full recovery of peak current requires  $\sim 30$  s at  $-90$  mV, but recovery is much faster at more positive holding potentials (Fig. 9 B).

Even at  $-70$  mV, recovery from block is at least an order of magnitude slower than would be expected if this process simply reflected the rate of drug dissociation from the open blocked state ( $L = \sim 0.1 \text{ ms}^{-1}$  at +60 mV). Furthermore the slight voltage dependence of  $L$  (increasing at more hyperpolarized potentials) is in the wrong direction to account for a slowing of recovery from block with hyperpolarization. Similar recovery behavior has been reported in connection with both QA block (Armstrong, 1971) of K channels and LA block of Na channels (Yeh and Tanguy, 1985). These phenomena have been attributed to a mechanism by which drugs are 'trapped' at their binding sites when channels enter a closed-blocked state. The above data are consistent with the idea that drug trapping plays an important role in recovery from methadone block. This issue will be explored in more detail in a later paper (Horrigan, F. T., and W. F. Gilly, manuscript in preparation).

#### Methadone Block Interferes with K Channel Inactivation

Results in Fig. 6 demonstrated that in cells which exhibit significant  $\text{K}^+$  channel inactivation,  $I_K$ , evoked by a 20-ms depolarization continues to decline after the establishment of block equilibrium. This observation is consistent with the idea that methadone binding does not affect the ability of K channels to enter an inactivated state. However, if methadone has no effect on K channel inactivation then  $I_K$  recorded in methadone should continue to decline during long depolarizations. The following section presents data showing this

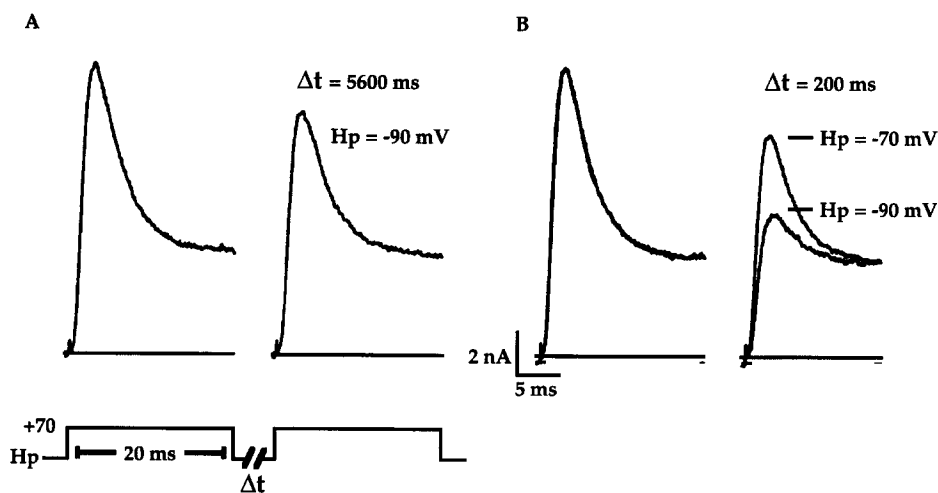


FIGURE 9. Recovery of  $I_K$  from methadone block is slow and dependent upon holding potential. (A)  $I_K$  evoked at +70 mV in the presence of 200  $\mu$ M (—) methadone using the illustrated double pulse procedure for a recovery interval ( $\Delta t$ ) of 5,600 ms at a holding potential of -90 mV (100K//100K). (B) From the same experiment, superimposed currents evoked by test pulses after 200 ms recovery intervals at -70 or -90 mV (as indicated) demonstrate that recovery is slower at more hyperpolarized holding potentials.

not to be the case and indicating that the model presented in Fig. 6 must be modified in order to account for the effects of methadone on  $I_K$  during prolonged depolarizations.

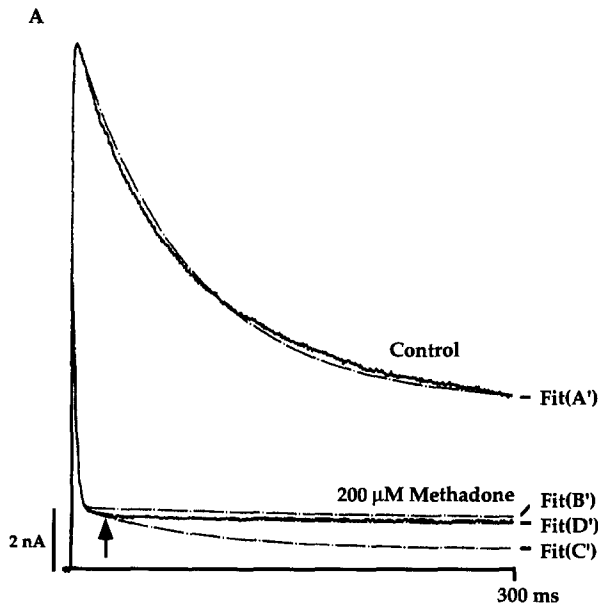
Fig. 10 A shows  $I_K$  evoked by 300 ms pulses in the presence and absence of 200  $\mu$ M methadone. The control record exhibits strong inactivation and the decay of  $I_K$  is approximated by a time constant of 46 ms (fit with model A' diagrammed in Fig. 10 B). This model is a simple extension of the one developed in conjunction with Fig. 6 A and accounts for incomplete inactivation. In the presence of methadone,  $I_K$  declines much more rapidly and quickly (2 ms time constant) reaches the blocked-equilibrium level predicted by model B' (fit B').  $I_K$  then continues to decay more slowly to a steady level as drug-blocked channels inactivate (OB  $\rightarrow$  IB), a feature previously discussed in conjunction with Fig. 6 B (model C). The time course of this secondary decline in  $I_K$  matches neither that of control  $I_K$  (i.e., normal inactivation) nor that of fit C' calculated with model C' (Fig. 10 B) for methadone block which accurately describes the decline of  $I_K$  for only the first 25 ms (Fig. 10 A, arrow). Both control  $I_K$  and fit C' decline by over 60% as K channels inactivate during the period between 25 and 300 ms. Such a large, slow decline in  $I_K$  clearly does not occur in the presence of methadone. This means that although drug-blocked channels can inactivate and enter IB after a strong depolarization, there is little increase in the number of inactivated-blocked channels after the first 25–50 ms.

This behavior can be accounted for by a simple modification of model C' (Fig. 10 B) that selectively increases the rate constant of the IB  $\rightarrow$  OB transition (model D' in Fig. 10 B) resulting in decreased stability of the inactivated-blocked (IB) state with little change in the initial rate of entry into this state. The  $I_K$  record in methadone of Fig. 10 A is fit very well (fit D') by assuming the IB  $\rightarrow$  OB rate ( $\epsilon$ ) is 12 times larger than the

I  $\rightarrow$  O rate ( $\delta$ ). This modification has little effect on the fit to the initial 25 ms of the drugged  $I_K$  trace. This can be seen by comparing fits C' and D' in Fig. 10 A and demonstrates that transitions leaving the inactivated states can be ignored during brief depolarizations. Possible transitions between inactivated states (I and IB) do not alter these general conclusions and were not included in the model.

Model D' as described above predicts that in the presence of 200  $\mu$ M methadone most nonconducting channels are in the open-blocked (OB) state rather than the inactivated (I) or blocked-inactivated (IB) state after a prolonged depolarization. This prediction was tested by exploring differences in the kinetics of recovery from block and recovery from inactivation.

Although recovery from inactivation is slowed at more depolarized holding potentials (unpublished data), recovery from methadone block is accelerated at more depolarized potentials (Fig. 9) and in high external  $K^+$ . Thus, in 200 mM external  $K^+$  at a holding potential of -70 mV, recovery from drug block by 200  $\mu$ M methadone following a 20-ms depolarization is quite rapid, and significant recovery occurs during an 8.5-ms interpulse interval. (Fig. 11 A; compare  $I_{ss}[20]$  with  $I_{p2}[20]$ ). Under the same conditions, but in drug-free solution recovery from inactivation after a 300-ms depolarization is much slower, and little or no recovery occurs with an 8.5 ms recovery interval (Fig. 11 B; compare  $I_{ss}[300]$  with  $I_{p2}[300]$ ). Furthermore, Fig. 11 A shows that recovery of  $I_K$  after a 300-ms depolarization in methadone is as fast as recovery from the 20 ms depolarization. This result is examined in more detail in Fig. 11 C which plots the time course of recovery for a similar experiment with a holding potential of -50 mV. Recovery after a 300-ms depolarization in methadone (filled circles) follows the time course of recovery from methadone block produced by a short (26 ms) pulse (open circles) and is clearly distinct from the time course



of recovery from inactivation (*triangles*). Thus,  $I_K$  recovery after a prolonged depolarization in methadone is determined by recovery from the open-blocked (OB) state rather than by recovery from an inactivated state. This result is consistent with the notion that methadone block destabilizes the inactivated state of the K channel.

#### Stereospecificity of $I_K$ Block

The stereo-isomers of methadone exhibit an interesting difference in their ability to block  $I_K$  that is evident upon comparing records obtained during successive application each drug at 100  $\mu\text{M}$  concentrations (Fig. 12 A). Under these conditions the time constant of block (describing the exponential decline of  $I_K$ ) is indistinguishable for the two isomers, however steady state current recorded in (+)methadone is greater than that in (-)methadone. Comparison of the steady state block produced by (+)- and (-)-methadone at 100 and 200  $\mu\text{M}$  concentrations indicated that (+) methadone exhibits a  $2.1 \pm 0.2$ -fold greater apparent  $K_D$  than (-)methadone ( $N = 2$  cells). The kinetics of block suggest that the on rates ( $\kappa$ ) for these two compounds are similar since, at this drug concentration, the time constant of block  $\{\tau = 1/(\kappa[\text{drug}] + L)\}$  is primarily determined by  $\kappa$ . Fits to the data in Fig. 12 A confirm that (+)- and (-)-methadone differ primarily in their rates of unblock ( $L$ ).

B

Models:

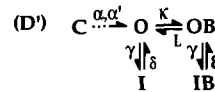
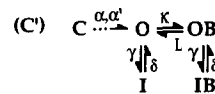
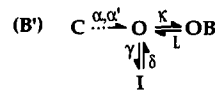
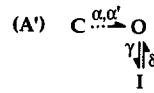


FIGURE 10. Methadone block interferes with  $K^+$  channel inactivation. (A)  $I_K$  evoked by a 300-ms depolarization to +70 mV in the presence and absence (control) of 200  $\mu\text{M}$  methadone (200K//200K). Control current is approximated (fit A') by a model (B, diagram A') which extends that used in Fig. 6 B to include a rate ( $\delta$ ) from the inactivated state (I) to the open state (O), an adjustment necessary to fit  $I_K$  (300 ms). Diagrams B' and C' (corresponding to fits B', C') represent alternative models of block incorporating control rates of inactivation and  $\delta$  both fail to reproduce the kinetics of  $I_K$  in the presence of drug. However, the data was well fit (fit D') by a model (diagram D') which assumes that the rate describing the transition from IB to OB ( $\epsilon$ ) is 12-fold larger than the control rate from I to O ( $\delta$ ). The rate constants are (in  $\text{ms}^{-1}$ ):  $\alpha = 9.0$ ,  $\alpha' = 1.4$ ,  $\gamma = 0.009$ ,  $\delta = 0.004$ ,  $\epsilon = 0.048$ ,  $L = 0.078$ ,  $\kappa = 3.0 \text{ ms}^{-1} \text{ mM}^{-1}$ .

#### Temperature Dependence of $I_K$ Block

Methadone block of  $I_K$  exhibits a significant temperature dependence (Fig. 12 B). Control currents, measured at 10 and 20°C (in the same cell) show a speeding of activation and inactivation kinetics with increasing temperature, however, peak  $I_K$  is essentially unchanged. Matching traces recorded in the presence of 100  $\mu\text{M}$  (-)methadone show a decrease in the time constant of block and an enhancement of steady state block with increased temperature. Further analysis of block kinetics indicated by the fits to  $I_K$  in Fig. 12 B reveals that the rates of block ( $\kappa$ ) and unblock ( $L$ ) increase by factors of 2.1 and 1.4, respectively, as temperature is elevated from 10 to 20°C. Based on this experiment, the  $K_D$  for block decreased 1.5-fold over this temperature range (from 40 to 26  $\mu\text{M}$ ). The time constant of block in this and other experiments was observed to decrease exponentially with temperature with a  $Q_{10}$  of  $2.0 \pm 0.1$  (mean + SEM,  $N = 3$  cells). These results are consistent with the experiment in Fig. 12 B and support the idea that the rate  $\kappa$  is temperature sensitive.

#### Methadone Blocks $I_K$ in GH3 Cells and Chick Myoblasts

It is conceivable that the direct interactions of methadone with K channels described thus far are unique to K channels in squid neurons. This possibility was tested by examining the effect of methadone on  $I_K$  in a rat pi-

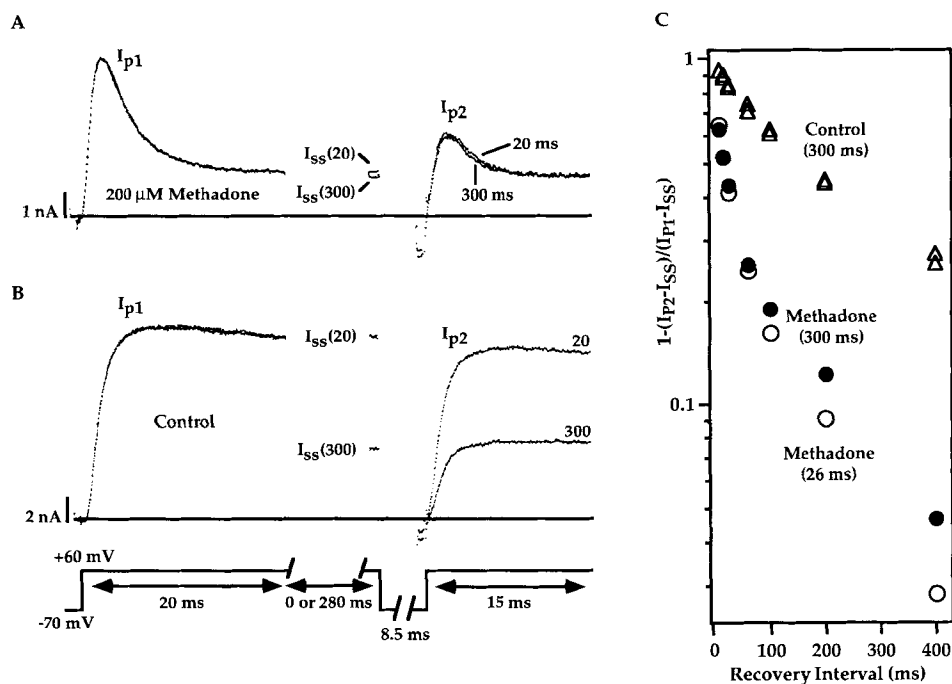


FIGURE 11. Recovery from block/inactivation. Recovery of peak current is shown using a double pulse protocol under conditions ((400K//400K), HP = -70 mV) which favor rapid recovery from block and slow recovery from inactivation. (A) During a depolarization in 200  $\mu\text{M}$  methadone,  $I_K$  reaches a steady state by 20 ms. The right half of the figure shows  $I_K$  at the end of a 20-ms pulse ( $I_{SS}[20]$ ) and at the end of a 300-ms pulse ( $I_{SS}[300]$ ) followed by  $I_K$  elicited in each case by a test pulse after a brief recovery interval (8.5 ms). A similar amount of peak  $I_K$  ( $I_{p2}$ ) recovers in both cases. (B) Shows a set of analogous records for control  $I_K$  (no methadone) which inactivate by >50% over 300 ms and show no recovery after an 8.5-ms interval. (C) Recovery

data obtained as indicated in A and B are compared by plotting the quantity  $1-(I_{p2}-I_{SS})/(I_{p1}-I_{SS})$  vs recovery interval. Currents were recorded in (200K//200K) with  $H_p = -50$  mV. Recovery in methadone following a 300-ms depolarization ( $\bullet$ ) shows the same time course as recovery from block measured after a 26-ms pulse ( $\circ$ ) and is clearly distinct from the time course of recovery from inactivation measured before application and after washout of methadone ( $\Delta$ ).

tuitary cell line (GH3) and in primary cultures of chick skeletal myotubes. In GH3 cells, as in squid neurons,  $I_K$  evoked at +60 mV was blocked in a time-dependent manner during a depolarization in the presence of 50  $\mu\text{M}$  (-)methadone (Fig. 13 A). If the model of block developed for squid neurons is assumed to be valid, an apparent  $K_D$  of  $22 \pm 7$   $\mu\text{M}$  (SE,  $n = 8$  cells,  $t = 20^\circ\text{C}$ ) is obtained based upon the action of 10 to 50  $\mu\text{M}$  methadone. Similarly, in chick myoblasts, the effect of 50  $\mu\text{M}$  ( $\pm$ )methadone on steady state  $I_K$  indicates an apparent  $K_D$  of  $20 \pm 4$   $\mu\text{M}$  (SE,  $n = 2$ , 1 cell, +40 mV,  $20^\circ\text{C}$ ). Although the mammalian or avian  $K_D$  values cannot be accurately assessed from this limited analysis, these rough indications of  $K_D$  are similar to more reliable values obtained for squid at the same temperature ( $K_D = 26$   $\mu\text{M}$  for (-)methadone at  $20^\circ\text{C}$  in Fig. 12 B).

#### $I_K$ Is Blocked by Propoxyphene and Norpropoxyphene

Effects of the methadone-like drug, D-propoxyphene, and its major metabolite norpropoxyphene were also examined on  $I_K$  in squid neurons and in GH3 cells. Both drugs induced time dependent block similar to that produced by methadone and the effect of norpropoxyphene on squid  $I_K$  is shown in Fig. 13 B. The apparent  $K_D$ s for steady state block in squid neurons were estimated from several experiments using 50–200  $\mu\text{M}$  drug concentrations to be  $54 \pm 10$   $\mu\text{M}$  (SE,  $n = 5$ , 4

cells) and  $43 \pm 11$   $\mu\text{M}$  (SE,  $n = 3$ , 2 cells) for propoxyphene and norpropoxyphene, respectively (+60 mV,  $10^\circ\text{C}$ ). In GH3 cells the apparent  $K_D$  for propoxyphene was estimated to be  $31 \pm 1$   $\mu\text{M}$  (SE,  $n = 4$ , 2 cells, +60 mV, at  $20^\circ\text{C}$ ) based upon the action of 20 or 40  $\mu\text{M}$  drug. Again, the same model developed for methadone was assumed to be applicable, and these values can only be taken as approximations.

#### DISCUSSION

The present study demonstrates that methadone blocks  $I_K$ ,  $I_{Na}$ , and  $I_{Ca}$  in squid neurons.  $K^+$  current is blocked with an apparent  $K_D$  of  $32 \pm 2$   $\mu\text{M}$  (at +60 mV,  $10^\circ\text{C}$ ) and  $Na^+$  current is similarly sensitive ( $IC_{50} = 50$ – $100$   $\mu\text{M}$ , at +10 mV) while  $Ca^{2+}$  current exhibits a  $IC_{50}$  between 200 and 500  $\mu\text{M}$  (+10 mV).

The mechanism of  $I_K$  block was examined in detail and involves a direct interaction of methadone with K channels rather than an opioid receptor coupled pathway. Methadone is a tertiary amine, structurally similar to both local anesthetics (LA) and (in its charged form) to quaternary ammonium ions (QA). In line with this structural similarity,  $I_K$  is reduced during a single depolarization in a time dependent manner that is well described by the model of open channel block developed by Armstrong (1971) to describe the effects of

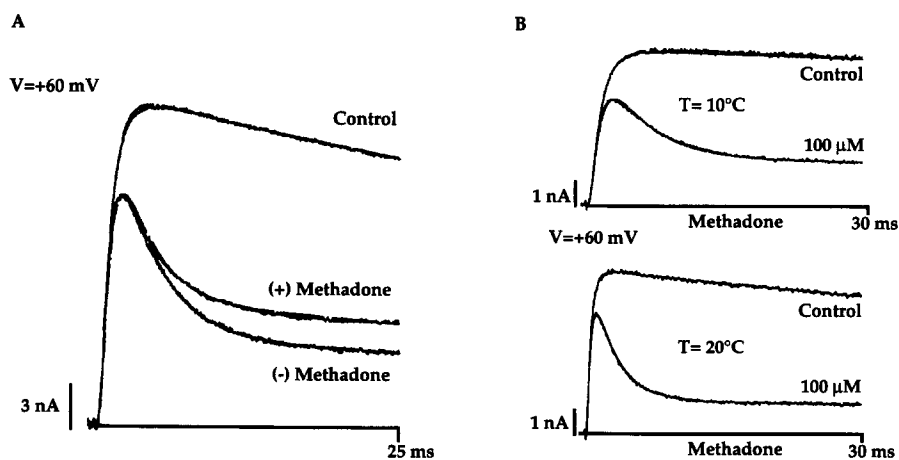


FIGURE 12. Methadone block of  $I_K$  is stereospecific and temperature dependent. (A) Control current, at +60 mV, is compared to  $I_K$  evoked during sequential application of 100  $\mu\text{M}$  (-) and (+) methadone (10K/100K). Fits to the data (corresponding to model C, Fig. 6 B) are superimposed on the current traces. Rate constants are (in  $\text{ms}^{-1}$ ):  $\alpha = 9.0$ ,  $\alpha' = 1.3$ ,  $\gamma = 0.009$ ; (-)methadone:  $\kappa = 2.2 \text{ ms}^{-1} \text{ mM}^{-1}$ ,  $L = 0.062 \text{ ms}^{-1}$ ; (+)methadone:  $\kappa = 2.3 \text{ ms}^{-1} \text{ mM}^{-1}$ ,  $L = 0.142 \text{ ms}^{-1}$ . (B) The temperature dependence of  $I_K$  block by methadone is shown by comparing effects of 100  $\mu\text{M}$  (-) methadone at 10 and 20°C. Currents were evoked at +60 mV (10K/50K). The control record, at 10°C, was obtained immediately before application of methadone. Drug traces were obtained during a single drug application, before and after raising the temperature to 20°C. The control at 20°C was obtained after washout of drug from the external solution. Fits to the data (corresponding to model C, Fig. 6 B) are superimposed on the current traces. Rate constants are (in  $\text{ms}^{-1}$ ):  $\alpha = 9$ ,  $\alpha' = 0.9$ ,  $\gamma = 0.002$ ,  $L = 0.060$ ,  $\kappa = 1.5 \text{ ms}^{-1} \text{ mM}^{-1}$  (10°C);  $\alpha = 21$ ,  $\alpha' = 2.2$ ,  $\gamma = 0.006$ ,  $L = 0.084$ ,  $\kappa = 3.2 \text{ ms}^{-1} \text{ mM}^{-1}$  (20°C).

done at 10 and 20°C. Currents were evoked at +60 mV (10K/50K). The control record, at 10°C, was obtained immediately before application of methadone. Drug traces were obtained during a single drug application, before and after raising the temperature to 20°C. The control at 20°C was obtained after washout of drug from the external solution. Fits to the data (corresponding to model C, Fig. 6 B) are superimposed on the current traces. Rate constants are (in  $\text{ms}^{-1}$ ):  $\alpha = 9$ ,  $\alpha' = 0.9$ ,  $\gamma = 0.002$ ,  $L = 0.060$ ,  $\kappa = 1.5 \text{ ms}^{-1} \text{ mM}^{-1}$  (10°C);  $\alpha = 21$ ,  $\alpha' = 2.2$ ,  $\gamma = 0.006$ ,  $L = 0.084$ ,  $\kappa = 3.2 \text{ ms}^{-1} \text{ mM}^{-1}$  (20°C).

internally applied QA. The pH and voltage dependence of  $I_K$  block and the effectiveness of internally applied *N*-methyl-methadone suggest that methadone permeates the cell membrane and blocks K channels at the internal QA-site. Consistent with this scheme, the slow kinetics and strong voltage dependence of recovery from block are similar to those described for QA, and the rate of unblock ( $L$ ) and recovery from block are accelerated by increases in external  $\text{K}^+$ . Taken together these results indicate that methadone block of  $I_K$  in squid neurons does not involve interaction with an opioid receptor but rather a direct occlusion of the K channel pore.

The ability of methadone to block  $I_{\text{Na}}$  and  $I_{\text{Ca}}$  also appear consistent with a mechanism involving direct LA- or QA-like interaction of the drug with Na and Ca channels.  $I_{\text{Na}}$  is blocked without apparent kinetic alteration or voltage dependence, similar to "tonic" block by LA molecules. Repetitive depolarization induces an additional 20% decrease in  $I_{\text{Na}}$ , consistent with use-dependent (phasic) LA block.  $I_{\text{Ca}}$  is also blocked without apparent change in kinetics but with a marked volt-

age dependence.  $I_{\text{Ca}}$  evoked at the smallest activating depolarizations in Fig. 3 are not appreciably affected by methadone, suggesting that Ca channels are not blocked at rest. However,  $I_{\text{Ca}}$  is blocked more strongly during larger depolarizations. The voltage dependence of block appears roughly coincident with  $I_{\text{Ca}}$  activation suggesting that Ca channels, like K channels, may be blocked from an open state. However further experiments would be required to resolve this issue.

Although the mechanism of  $I_{\text{Na}}$  and  $I_{\text{Ca}}$  block were not examined in detail, several lines of evidence support the idea that the ability of methadone to block  $I_{\text{Na}}$  and  $I_{\text{Ca}}$  as well as  $I_K$  do not involve opioid receptor coupled pathways. Morphine, a  $\mu$ -opioid agonist, had no effect on  $I_K$ ,  $I_{\text{Na}}$ , or  $I_{\text{Ca}}$  in squid neurons, and naloxone, an opioid antagonist, did not prevent  $I_K$  block by methadone. These results indicate that the observed actions of methadone do not involve  $\mu$ -opioid receptors but do not immediately rule out the possibility that other opioid receptors ( $\kappa$ - and  $\delta$ -) might be involved. Previous studies indicate that methadone binds to  $\mu$ -,  $\kappa$ - and  $\delta$ -receptors with dissociation constants of 4.2 nM, 15.1

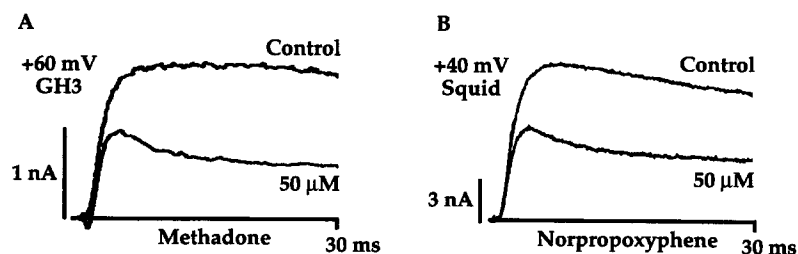


FIGURE 13. (A)  $I_K$  evoked by a 30-ms depolarization to +60 mV in a GH3 cell is blocked in a time-dependent manner after application of 50  $\mu\text{M}$  (-) methadone. (B)  $I_K$  evoked by a 30-ms depolarization to +40 mV in a squid neuron is blocked by 50  $\mu\text{M}$  norpropoxyphene.

nM, and 1.63  $\mu\text{M}$  respectively in guinea pig brain (Maganan et al., 1982). However, the involvement of  $\kappa$ - and  $\delta$ -receptors seems unlikely since morphine and naloxone also bind to these receptors with similar or higher affinity than methadone (in guinea pig). In addition, the half-blocking concentration for methadone block of  $I_K$ ,  $I_{Na}$ , and  $I_{Ca}$  in our experiments ( $\sim 30$ – $500 \mu\text{M}$  at  $10^\circ\text{C}$ ) far exceed the dissociation constants for methadone at opioid receptors ( $0.004$ – $1.6 \mu\text{M}$ ). This disparity in sensitivity suggests that the effects of methadone on squid neurons do not involve opioid receptors. Although it is conceivable that opioid receptors in squid (if they exist) might have a much lower affinity for methadone than do mammalian receptors, this possibility seems unlikely to account for the relatively low sensitivity of  $\text{K}^+$  channels to methadone because methadone is similarly effective at blocking  $I_K$  in squid, mammalian (GH3) and avian (chick myocyte) preparations.

#### *Mechanism of $I_K$ Block: Structure–Function Relationships*

Previous studies of  $I_K$  block by internally applied, long chain QA ions have concluded that block by these compounds involves two distinct binding interactions. The charged QA headgroup, is thought to enter and occlude the pore, while the hydrophobic tailgroup of these molecules interacts with the inner mouth of the channel. Changes in either group can alter binding (Armstrong, 1971; Swenson, 1981; Choi et al., 1993).

In comparison with these previously studied compounds, methadone and *N*-methyl-methadone are unusual in that they are potent blockers despite their small dimethyl- or trimethyl-amine headgroups. For example, internal tetramethyl ammonium blocks  $I_K$  in squid axons much less effectively ( $K_D = 180 \text{ mM}$ ) than does tetraethyl ammonium (TEA) ( $K_D = 1 \text{ mM}$ ) (French and Shoukimas, 1985). Similarly, long chain trimethyl amines studied by Armstrong (1971) and Swenson (1981) were relatively ineffective in comparison to analogous triethyl and tripropyl amines. The most effective trimethyl amines were those with aromatic tail groups. These results suggest that the relatively high affinity of methadone is likely to be due to interactions of the  $\text{K}^+$  channel with the extensive aromatic tail of the drug rather than with the dimethylamine head group. The temperature dependence and stereospecificity of methadone block may therefore reflect characteristics of this interaction.

The temperature dependence of block suggests that the binding of methadone to the  $\text{K}$  channel is not a diffusion limited process. The rate of block ( $\kappa$ ) was found to increase significantly with temperature ( $Q_{10} = \sim 2$ ) whereas diffusion of symmetrical QA ions exhibits a  $Q_{10}$  ranging from 1.12 to 1.19 (Robinson and Stokes, 1970). This temperature dependence is consistent with the idea that methadone binding might be limited by a

hydrophobic interaction between the tailgroup and the channel. Similarly, Swenson (1981) found that  $I_K$  block by some long chain QA derivatives is highly temperature dependent.

Stereoisomers of methadone differ in their ability to block steady state  $I_K$  with the (+) form exhibiting a higher apparent  $K_D$  than the (–) form. This difference mainly reflects a higher rate of unblock (L) for (+) methadone. Opioid receptor mediated effects of methadone also exhibit a greater potency for the (–)-form of the drug. However, the difference in apparent  $K_D$  at opioid receptors is 8–50-fold (Leimbach and Eddy, 1954; Sullivan et al., 1975; Jaffe and Martin, 1990) as opposed to only about a twofold for the effect described here. This modest stereospecificity is not inconsistent with the proposal that methadone directly blocks  $\text{K}^+$  channels since some local anesthetics exhibit a similar degree of stereospecificity in their interaction with  $\text{Na}$  channels (Clarkson, 1989; Wang, 1990). The (+) and (–) forms of methadone differ in their arrangement about a chiral carbon that is located between the putative head and tail groups of the molecule (Fig. 1*E*). Thus, the effects of these stereoisomers on  $I_K$  may reflect a difference in the relative orientation of the head and tail groups that, in turn, affects the ability of either or both of these groups to bind to the  $\text{K}^+$  channel. Choi et al. (1993) have determined that the head and tail group binding of long chain TEA derivatives is not strictly independent, consistent with the idea that the conformation of the region that links the head and tail may affect the ability of these binding determinants to simultaneously interact with their binding sites.

#### *Inactivation and Block*

Methadone block and  $\text{K}$  channel inactivation were found to interact in an interesting manner. A series of increasingly complex models are presented in Figs. 4, 6, and 10 in order to describe the time course of  $I_K$  evoked during short (20 ms) and prolonged (300 ms) depolarizations in methadone. In cells that exhibit minimal inactivation (Fig. 4), currents recorded in methadone can be fit by a simple model of open channel block. In  $200 \mu\text{M}$  methadone,  $I_K$  is rapidly blocked during a 20-ms depolarization and attains a steady state, representing block equilibrium. However, in cells that display significant inactivation,  $I_K$  continues to decline after the establishment of predicted block equilibrium and attains a steady state level only after prolonged depolarization ( $>50 \text{ ms}$ ). The magnitude and kinetics of the initial decline below block equilibrium are consistent with the assumption that both open and drug-blocked channels inactivate at the same rate during the first 20 ms of a depolarization (Fig. 6). On the other hand, establishment of a steady state current level within 50 ms (Fig. 10) suggests that inactivation is

inhibited, because control  $I_K$  inactivates continuously during a 300-ms pulse. To resolve this apparent contradiction, we propose that whereas methadone-blocked channels (OB) inactivate to a blocked-inactivated state (IB) at a normal rate, the reverse transition from the inactivated to the noninactivated state is accelerated when the channel is drug blocked. By enhancing the reverse transition, methadone shifts the equilibrium between OB and IB toward the OB state and effectively inhibits inactivation. The time constant for this equilibrium is inversely proportional to the sum of the forward and reverse rate constants and is, therefore, reduced by methadone. Consequently, equilibrium is achieved rapidly and an overt sign of inactivation is only visible during the first 50 ms or so of a depolarization in methadone. During a 20-ms depolarization, when few channels have entered the IB state, the IB to OB rate is unimportant and inactivation is determined primarily by the rate from OB to IB. A model based on this proposed mode of action fits the entire time course of  $I_K$  evoked during a 300-ms pulse (Fig. 10). Furthermore, the rapid recovery of  $I_K$  after a 300-ms depolarization in methadone (Fig. 11) provides independent support for the conclusion that methadone block destabilizes the inactivated state.

One factor that may limit our ability to provide a quantitative assessment of the effect of methadone on inactivation is the possibility that  $I_K$  represents a heterogeneous population of channels. Multiple K channel types have been described in GFL neurons and in squid axons based on single-channel conductance (Nealey et al., 1993; Llano et al., 1988). Although detailed activation and inactivation properties of these channels and their relative expression levels are unknown, a consensus exists that a single type of 20 pS  $K^+$  channel underlies the bulk of macroscopic "delayed rectifier"  $I_K$  in both axons (Perozo et al., 1991) and cell bodies (Llano and Bookman, 1986; Nealey et al., 1993). Analyses presented in this paper are based on the assumption that  $K^+$  channels in GFL neurons are a single population with uniform activation kinetics, drug affinity and inactivation rates. The ability of the model to fit the kinetics of activation and block of  $I_K$  lends support to the first two assumptions. However, variability in the degree of inactivation seen from cell to cell suggests that channels with different inactivation properties might exist and the basis for incomplete inactivation of  $I_K$  in squid is not known (Perozo et al., 1991). Although uncertainty as to the degree of non-inactivating versus incompletely inactivating  $K^+$  channels does exist, all channels appear to be equally sensitive to methadone, as evidenced by a linear Hill plot over a large concentration range (Fig. 4 C). Moreover, the fraction of  $I_K$  blocked by methadone was not dependent on the amount of inactivation in any given cell. If all channels are equally

blocked by methadone, then general lines of reasoning followed here are still valid, but quantitative details derived from analysis of  $I_K$  kinetics during 300-ms depolarizations may require modification. For example, if control  $I_K$  is assumed to represent a mixture of non-inactivating and completely inactivating channels then the data in Figs. 6 and 10 are still best fit by a model which assumes that methadone speeds the IB to OB transition (for the inactivating channels) without affecting the OB to IB rate. However, both control and methadone-blocked inactivation rates would differ from those used in Fig. 10.

The effect of methadone on  $K^+$  channel inactivation is interesting for several reasons. Open channel block of K channels at the internal QA site has been closely compared to the inactivation process and inspired the "ball and chain" model of inactivation (Armstrong and Bezanilla, 1977). This model has been verified in the case of *Shaker*  $K^+$  channels where an  $NH_2$ -terminal domain of the channel protein forms an inactivation particle that can block the inner mouth of the open pore much like QA ions (Hoshi et al., 1990). Indeed internally applied TEA slows this N-type inactivation as though QA ions and the inactivation particle compete for a common binding site (Choi et al., 1991). C-type inactivation in K channels, on the other hand, is thought to involve the outer mouth of the pore and is unaffected by internal TEA. Thus, the ability of methadone to inhibit inactivation of  $I_K$ , together with methadone's internal site of action, suggests that inactivation of squid  $K^+$  channels during 300-ms depolarizations may involve the inner mouth of the pore.

In contrast to the action of internal TEA on *Shaker* K channels, the effect of methadone on inactivation can not be accounted for by a simple competition model. In other words, methadone binding does not prevent inactivation but rather destabilizes the inactivated state. The selective effect of methadone on the inactivated-blocked (IB) to open-blocked (OB) transition suggests that inactivation and the reverse transition may represent processes that involve distinct binding interactions. This idea is consistent with studies of the *Shaker* B potassium channel which indicate that alteration of residues in a hydrophobic domain of the amino terminal inactivation particle can alter the rate of inactivation particle dissociation without effecting its on-rate (Zagotta et al., 1990). Isakov et al. (1991) found similar results when hydrophobic residues in the intracellular S4-S5 linker were mutated and proposed that these residues formed part of a receptor for the inactivation particle. It is tempting to speculate that the hydrophobic tailgroup of methadone may interfere with the binding of an inactivation particle to such a hydrophobic binding site in the inner mouth of the channel and in this way destabilizes the inactivated state.

### Implications Toward Drug Toxicity

An important question is whether nonopioid block of  $K^+$  channels by methadone or propoxyphene contributes to the clinical side effects of these drugs. Although the present study does not directly address this issue, our results taken together with previous findings suggest that such a contribution may be possible for propoxyphene but is less likely for methadone. Methadone, as detailed above, exhibits a  $K_D$  for opioid receptor binding that is in the nanomolar to low micromolar range, well below the apparent  $K_D$  for  $K^+$  channel block observed in squid neurons, GH3 cells or chick muscle (20–26  $\mu\text{M}$  at 20°C, +60 mV). Consequently, opioid receptor mediated effects of methadone, including side-effects unrelated to analgesia, would occur at drug concentrations that are insufficient to induce direct  $K^+$  channel block. In fact, the majority of methadone's side effects are reported to be similar to those of morphine and are reversed by opioid antagonists such as naloxone (Jaffe and Martin, 1990). In contrast, propoxyphene can induce nonopioid cardiotoxic effects in addition to opioid receptor-mediated side effects (Baraclough and Lowe, 1982; Amsterdam et al., 1981; Heaney, 1983; Sorensen et al., 1985; Strom et al., 1985). Although our results indicate that propoxyphene and methadone are similar in their ability to block  $I_K$  in squid neurons and in GH3 cells, propoxyphene is a relatively weak opioid receptor agonist with an analgesic potency 20 to 40 times less than that of (–)methadone (Robbins, 1955). Thus, in contrast to methadone, propoxyphene may be capable of binding to opioid receptors and  $K^+$  channels at similar drug concentrations; thereby raising the possibility that  $K^+$  channel block might contribute to the the clinical side effects of propoxyphene.

Several lines of evidence suggest that drug concentrations achieved in mammals during propoxyphene poisoning may approach levels that are sufficient to block  $K^+$  channels in squid neurons or GH3 cells. We estimate that  $K_D$ 's for  $K^+$  channel block by propoxyphene

and norpropoxyphene are  $\sim 50$  and  $\sim 40$   $\mu\text{M}$  respectively in squid neurons at 10°C and  $\sim 30$   $\mu\text{M}$  for propoxyphene in GH3 cells at 20°C. In addition, the temperature dependence of  $I_K$  block exhibited by methadone and by a variety of QA compounds (Swenson, 1981) suggests that our measurements may underestimate the efficacy of propoxyphene at 37°C. In comparison, plasma concentrations of propoxyphene and its major metabolite norpropoxyphene associated with lethality have been reported to fall generally between 2.5 to 25  $\mu\text{M}$  in humans (Finkle et al., 1976; Caplan et al., 1977). Similarly, in vitro studies of propoxyphene's effect on canine purkinje fiber action potentials achieve half maximal effects at concentrations of  $\sim 20$  to 60  $\mu\text{M}$  (Holland and Steinberg, 1979). It is likely that the ability of propoxyphene to directly block Na channels is at least in part responsible for the non-opioid effect of propoxyphene on cardiac excitability (Whitcomb et al., 1989). However, it is difficult to predict, a priori, how a combination of  $I_{Na}$ ,  $I_K$  block by propoxyphene might effect the highly complex cardiac action potential and it is, therefore, uncertain whether or not previously reported effects of propoxyphene on cardiac action potentials (Nickander et al. 1979; Holland and Steinberg, 1979) are also consistent with an effect of the drug on  $I_K$ .

Although additional studies would be required to determine whether  $K^+$  channels in cardiac tissue are sufficiently sensitive to propoxyphene to play a role in toxicity, the mechanism of block suggests that such a possibility merits consideration. We find that methadone blocks  $I_K$  in squid neurons, GH3 cells, and chick myoblasts with similar effectiveness, indicating that this action is not specific to K channels in squid. This apparent lack of tissue specificity may reflect methadone's proposed action at the internal QA site since sensitivity to QA ions or local anesthetics is a common feature of many K channels including those in cardiac tissue (Carmeleit et al., 1986; Courtney and Kendig, 1988; Josephson, 1988; Ono et al., 1989; Orito et al., 1994).

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