Whole-cell patch-clamp recordings were performed together with time-resolved measurements of membrane capacitance ($C_m$) in nerve terminals acutely dissociated from neurohypophysis of adult rats to investigate modulation of Ca$^{2+}$ currents and secretion by activation of opioid receptors. Bath superfusion of the $\kappa$-opioid agonists U69,593 (0.3–1 $\mu M$), dynorphin A (1 $\mu M$), or U50,488H (1–3 $\mu M$) reversibly suppressed the peak amplitude of Ca$^{2+}$ currents 32.7 $\pm$ 2.7% (in 41 of 56 terminals), 37.4 $\pm$ 5.3% (in 5 of 8 terminals), and 33.5 $\pm$ 8.1% (in 5 of 10 terminals), respectively. In contrast, tests in 11 terminals revealed no effect of the $\mu$-opioid agonist [D-Pen$^2$,5]-enkephalin (1–3 $\mu M; n = 7$) or of the $\delta$-agonist Tyr-$\delta$-Ala-Gly-N-Me-Phe-Gly-$\alpha$ (1 $\mu M; n = 4$) on Ca$^{2+}$ currents. Three components of high-threshold current were distinguished on the basis of their sensitivity to blockade by $\alpha$-conotoxin GVIA, nicardipine, and $\alpha$-conotoxin MVIIIC: N-, L-, and P/Q-type current, respectively.

Administration of U69,593 inhibited N-type current in these nerve terminals on average 32%, whereas L-type current was reduced 64%, and P/Q-type current was inhibited 28%. Monitoring of changes in $C_m$ in response to brief depolarizing steps revealed that the $\kappa$-opioid-induced reductions in N-, L-, or P/Q-type currents were accompanied by attenuations in two kinetically distinct components of Ca$^{2+}$-dependent exocytotic release. These data provide strong evidence of a functional linkage between blockade of Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels and inhibitory modulation of release by presynaptic opioid receptors in mammalian central nerve endings.

**Key words:** $\kappa$-opioid receptor; Ca$^{2+}$ currents; membrane capacitance; secretion; neuroendocrine nerve terminals; patch-clamp
The use of patch-clamp techniques to record Ca\(^{2+}\) currents in single neurosecretory endings of the rat neurohypophysis, simultaneously with time-resolved membrane capacitance (\(C_m\)) measurements as a monitor of exocytotic activity, enabled us to directly examine the involvement of Ca\(^{2+}\)-dependent processes in the mechanism of opioid-induced presynaptic inhibition. The present experiments were designed to identify the specific types of Ca\(^{2+}\) channels that serve as targets of inhibitory modulation by opioid receptors in neurohypophysial nerve endings and to decipher the role of this channel regulation in opioid depression of release.

**MATERIALS AND METHODS**

**Preparation of isolated nerve endings.** Male rats (150–250 gm) were rendered unconscious with CO\(_2\) and decapitated rapidly by guillotine. After isolation of the pituitary, the anterior and posterior lobes were separated by dissection, the isolated neural lobe was collected, and isolated neurohypophysial nerve endings were prepared as described previously (Cazalis et al., 1987; Stuenkel, 1994) by brief homogenization of the neural lobe in 100 \(\mu\)l of buffer containing (in mM): sucrose 270, EGTA 2, and HEPES 10, with pH adjusted to 7.0 with Tris. The resulting homogenate was directly aliquoted onto a glass coverslip forming the basis of a specially designed superfusion and recording chamber of elliptical shape and 100 \(\mu\)l solution volume. After allowing time for adherence to the chamber bottom, we superfused the nerve endings under laminar flow with a physiological saline (PS) solution containing (in mM): NaCl 140, KHCO\(_3\) 5, CaCl\(_2\) 2.2, MgCl\(_2\) 1, glucose 10, and NaOH-HEPES 10, with pH adjusted to 7.2. For recording of Ca\(^{2+}\) currents and \(C_m\) responses, the superfusion was changed from PS to one normally consisting of (in mM): TEA 137, CaCl\(_2\) 10, MgCl\(_2\) 2, glucose 19, and HEPES 10, with pH adjusted to 7.2 with Tris. All studies were performed on spherical nerve endings having diameters of 5–12 \(\mu\)m to facilitate patch-clamp recording.

**Recording of Ca\(^{2+}\) currents.** Whole-cell patch-clamp recording techniques (Hamill et al., 1981; Lindauer and Neher, 1988) were used to evoke and record Ca\(^{2+}\) currents and measure changes in \(C_m\) (see below) under voltage clamp from single neurohypophysial nerve endings. Whole-terminal recordings (as appropriately termned) were made at room temperature (23–25°C) using patch pipettes fashioned from 1.5 mm outer diameter capillary glass (WP Instruments), coated to within 100 \(\mu\)m of the tip with Sylgard elastomer, and fire-polished to resistances of 3–8 M\(\Omega\). The standard pipette recording solution contained (in mM): N-methyl-D-aspartate (NMDA) 140, MgATP 2, GTP 0.2, and Tris-EGTA 0.25, adjusted to pH 7.1 and 300 mOsm.

Current recordings and \(C_m\) measurements were obtained using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) that provides compensation circuitry to correct for pipette and whole-cell \(C_m\) and series resistance. Electrode capacitance was compensated before transition to whole-terminal recordings. Whole-terminal capacitance (0.8–3.5 pF) and 65–80% of the series resistance (3–35 M\(\Omega\)) were compensated to eliminate membrane-charging transients and voltage and temporal errors, respectively. The evoked Ca\(^{2+}\) currents were low-pass filtered (5–10 kHz) before digitization at 5 kHz and corrected for linear leak currents and capacitative transients by digital subtraction of an appropriately scaled current elicited by a series of small hyperpolarizing voltage commands, using a P/N routine. Voltage-clamp protocols, data acquisition, and analyses of current and \(C_m\) recordings were performed using Pulse Control V4.5 Apple computer-based software routines generously distributed by Dr. Richard Bookman (Herrington et al., 1995).

**Capacitance measurements.** High-resolution membrane capacitance measurements were obtained with whole-terminal patch-clamp recording to monitor \(C_m\), which directly reflects secretory activity. The fusion of synaptic vesicles or granules to the plasma membrane during exocytosis results in an increase in the total plasma membrane surface area (with corresponding increases in \(C_m\)) provided that the rate of exocytosis is much greater than the rate of endocytosis. Time-resolvable changes in \(C_m\) were monitored using an adaptation of the phase-tracking method of Fidler and Fernandez (1986). Briefly, changes in whole-terminal \(C_m\) were measured by adding a 30 nA mV., 1.2 kHz sine wave to the holding potential (usually −90 mV), and the resulting current output signal of the voltage clamp was analyzed at two orthogonal phase angles using a software-based, phase-sensitive detector with high time resolution. Current signals were sampled 16 times per sinusoidal period (19.1 kHz sampling rate), and a \(C_m\) value was computed every 13.3 msec. The \(C_m\) signal was shifted 90° from changes in access resistance (\(R_a\)) and membrane conductance (\(G_m\)), eliminating interference with the \(C_m\) measurement. Imposing defined changes in \(R_a\) by periodically inserting a 500 k\(\Omega\) resistor between the bath electrode and electrical ground, allowed for immediate calculation by computer of the correct phase angle for the phase-sensitive detector. Lack of projection of the resistor-induced \(R_a\) changes in the \(C_m\) trace ensured correct alignment of the phase-sensitive detector. Calibration of the \(C_m\) trace was obtained by computer-controlled unbalancing of the whole-cell capacitance circuitry of the amplifier to provide a series of 100 fF signals. Data acquisition and the software-based phase-sensitive detector were controlled by software developed for the Apple Macintosh computer (Pulse Control 4.5) and generously distributed by Dr. Richard Bookman (Herrington et al., 1995).

**Results**

**Opioid regulation of Ca\(^{2+}\) currents in neurohypophysial nerve terminals**

Whole-terminal recordings (\(n = 67\)) were obtained using bath and pipette solutions of composition that suppressed K\(^{+}\) and Na\(^{+}\) conductances, with Ca\(^{2+}\) (10 mM) as the charge carrier. Step depolarizations of 5–200 msec were made to various test potentials from a holding potential (\(V_h\)) of −90 mV to evoke Ca\(^{2+}\) currents and associated increases in \(C_m\) (see below). The Ca\(^{2+}\) currents that we recorded consisted of inactivating and sustained components of high-voltage activated current, with a threshold for activation of −40 mV and peak amplitudes obtained at +10 mV, but contained no rapidly inactivating low-threshold T-type component. The amplitude of the Ca\(^{2+}\) currents, measured at the peak of the current–voltage curve, averaged 128.9 ± 10.9 pA (\(n = 67\)), yielding a mean current density of 71.8 ± 7.2 nA/F for these nerve endings. Overall, the Ca\(^{2+}\) currents we measured corresponded closely to those described previously in acutely isolated rat neurohypophysial nerve endings (Lemos et al., 1994; Stuenkel, 1994) with regard to their amplitudes, voltage-dependent activation, and kinetic properties.

Binding studies and immunohistochemical localization of mRNA coding for the various opioid receptor subtypes indicate that opioid receptors on the nerve endings of rat neurohypophysial magnocellular secretory neurons are predominantly, if not
exclusively, of the \( \kappa \)-type (Herkenham et al., 1986; Sumner et al., 1990; Mansour et al., 1995). Guided by these considerations, we first looked for regulation of the evoked \( \text{Ca}^{2+} \) current by agonists acting at \( \kappa \)-opioid receptors. Bath superfusion of the \( \kappa \)-opioid agonists dynorphin A (1 \( \mu \)M), U50,488H (1–3 \( \mu \)M), or U69,593 (0.3–1 \( \mu \)M) reversibly suppressed the peak amplitude of \( \text{Ca}^{2+} \) currents in 45 of 67 terminals examined. The inhibitory response to a particular agonist varied considerably among the group of nerve endings that showed \( \kappa \)-opioid sensitivity. For example, the maximal response to U69,593 ranged from 9 to 66% inhibition of the control current, while exceeding 50% suppression in \( \text{Ca}^{2+} \) current in approximately one-third (12) of 41 responsive nerve endings. However, comparisons of the effects of U69,593, dynorphin A (\( n = 5 \)), and U50,488H (\( n = 2 \)) in the same nerve ending revealed equivalent inhibitory responses to these agonists (Fig. 1A). Moreover, when the data were pooled for a particular agonist, no significant differences were observed in the maximal inhibition of \( \text{Ca}^{2+} \) current produced by U69,593 (32.7 ± 2.7%; 41 of 56 terminals), dynorphin A (37.4 ± 5.3%; 5 of 8 terminals), and U50,488H (33.5 ± 8.1%; 5 of 10 terminals). Although the predominant effect of the \( \kappa \)-opioid agonists was to decrease a rapidly inactivating current (\( \tau = 142 ± 6 \) msec; \( n = 10 \)), more-sustained components were also reduced in many of the terminals. We simplified the interpretation of experimental outcomes by using U69,593 for assessing \( \kappa \)-opioid regulation of \( \text{Ca}^{2+} \) currents and \( C_m \) responses (see below), avoiding potential problems associated with the susceptibility of dynorphin peptides to breakdown and cross-reactivity with multiple opioid receptor subtypes.

In a separate set of experiments on 11 nerve terminals, the effects of administration of agonists selective for either \( \mu \)- or \( \delta \)-opioid receptors on \( \text{Ca}^{2+} \) currents were compared with those produced by a subsequent application of U69,593 (0.3–1 \( \mu \)M) to determine whether opioid-induced suppression of these currents resulted specifically from the activation of \( \kappa \)-opioid receptors. These tests revealed no effect of the \( \mu \)-opioid-selective agonist DAMGO (1–3 \( \mu \)M; \( n = 7 \); Fig. 1B) or of the \( \delta \)-agonist DPDPE (1 \( \mu \)M; \( n = 4 \); Fig. 1C) on \( \text{Ca}^{2+} \) currents, although reductions in current by the \( \kappa \)-opioid were registered in more than half (\( n = 8 \)) of the same preparations. The graph depicted in Figure 1E summarizes the results of all experiments in which we tested for regulation of nerve terminal \( \text{Ca}^{2+} \) currents by agonists having selectivity for different subtypes of opioid receptors. The inhibitory effects of the five \( \kappa \), \( \mu \)-, or \( \delta \)-opioid-selective agonists tested were compared by plotting the averaged amount of \( \text{Ca}^{2+} \) current evoked in the presence of a particular agonist (represented by open bars), expressed as a percentage of the predrug control current. Current amplitudes were reduced significantly (\( p < 0.05 \)) by U69,593, dynorphin A, and U50,488H but not by DAMGO and DPDPE. Furthermore, recomputation of the effects of each \( \kappa \)-opioid agonist using only those data obtained from opioid-responsive terminals (filled bars) demonstrated equivalent inhibitory efficacy of the three \( \kappa \)-opioids examined. In additional experiments, inhibitory responses to U69,593 were tested for sensitivity to blockade by naloxone or nor-BNI, a \( \kappa \)-opioid receptor-selective antagonist (Portoghese et al., 1987), to confirm their mediation via activation of the corresponding subtype of opioid receptor. In all eight nerve endings examined, the ability of U69,593 to inhibit the evoked \( \text{Ca}^{2+} \) current was blocked by coadministration of naloxone (1 \( \mu \)M; \( n = 4 \)) or nor-BNI (1 \( \mu \)M; \( n = 4 \); Fig. 1D). These data, when taken together, indicate that only the \( \kappa \)-subtype of opioid receptor is functionally coupled to \( \text{Ca}^{2+} \) channels in rat neurohypophysial nerve terminals.

Identification of high-threshold currents sensitive to \( \kappa \)-opioids

To identify the types of high-threshold \( \text{Ca}^{2+} \) channels that are modulated by presynaptic \( \kappa \)-opioid receptors, we examined the ability of U69,593 to reduce \( \text{Ca}^{2+} \) current before and after selective blockade of N-, L-, and P/Q-type current components (\( n = 11 \)). The traces in Figure 2A illustrate the inhibitory effect of U69,593 (1 \( \mu \)M) on \( \text{Ca}^{2+} \) currents evoked by 50 msec steps to +10 mV from a \( V_m \) of −90 mV. Application of U69,593 (for 1 min) reduced the peak amplitude of the \( \text{Ca}^{2+} \) current from 260 to 110 pA (150 pA), and this effect was reversed after washout of the
These data suggest that only N- and L-type Ca\(^{2+}\) majority of terminals that were studied (5 of 11, 45.5%) possessed sensitivity to U69,593 of pharmacological tracing effect of U69,593 that remained after nicardipine. The pairs of antagonists yielded pharmacologically distinguished N-, L-, and P/Q-type current components in control conditions and in the presence of U69,593. The changes in the rate of endocytosis), and these depend not only on Ca\(^{2+}\) entry. It was shown for these peptidergic nerve endings that this transient increase that was independent of Ca\(^{2+}\) current was greatly reduced after application of each of these blockers. A, lower. Subtraction of the corresponding currents evoked before and after application of antagonists revealed systematic examination of their modulation by \(\kappa\)-opioids (see below). Despite variation in the depolarization-evoked \(C_m\) increases that were measured among individual nerve endings, each \(C_m\) response was resolvable into several kinetically and functionally distinct components, as has been described in detail in an earlier report (Giovannucci and Stuenkel, 1997). Briefly, depolarizations of single nerve endings achieved with 5 msec steps produced a transient \(C_m\) increase that was independent of Ca\(^{2+}\) entry. It was shown for these peptidergic nerve endings that this transient \(C_m\) signal does not represent Ca\(^{2+}\)-dependent exocytotic activity and is most likely related to a charge redistribution.

**Figure 2.** \(\kappa\)-Opioids inhibited GVIA-sensitive, nicardipine-sensitive, and MVIIC-sensitive Ca\(^{2+}\) current components. A, upper. Recordings from a single nerve terminal show the effects of U69,593 (traces marked with asterisks) on Ca\(^{2+}\) currents evoked by 50 msec steps to +10 mV from a holding potential of ~90 mV before the application and in the presence of Ca\(^{2+}\) channel-type selective blockers. Sequential administration of GVIA (0.5 \(\mu M\)), nicardipine (10 \(\mu M\)), and MVIIC (1 \(\mu M\)) completely abolished whole-terminal current. The ability of U69,593 to suppress Ca\(^{2+}\) current was greatly reduced after application of each of these blockers. A, lower. Subtraction of the corresponding currents evoked before and after application of antagonists yielded pharmacologically distinguished N-, L-, and P/Q-type current components in control conditions and in the presence of U69,593. B, Relative contribution of pharmacologically distinguished N-, L-, and P/Q-type current components to the total whole-terminal current and their sensitivity to the inhibitory effect of U69,593. Bars in the graph represent the mean \(\pm\) SEM of the normalized current amplitudes obtained from the number of terminals shown, examined using the experimental protocol depicted in A.

Opioid regulation of Ca\(^{2+}\)-dependent exocytosis in nerve terminals

The changes in \(C_m\) that are measured in response to brief depolarizations reflect increases in surface membrane area caused by the fusion of vesicles (provided that the rate of exocytosis exceeds the rate of endocytosis), and these depend not only on Ca\(^{2+}\) entry (Fig. 3) and buffering but also on the pattern and duration of stimulation (Lindau and Neher, 1988; Lim et al., 1990; Horrigan and Bookman, 1994; Giovannucci and Stuenkel, 1997). Single-step depolarizations to potentials that yielded maximal inward Ca\(^{2+}\) currents were associated with \(C_m\) responses in virtually all nerve endings examined, and the evoked increases in \(C_m\) measured in 43 of these were sufficiently stable to allow systematic examination of their modulation by \(\kappa\)-opioids (see below). Despite variation in the depolarization-evoked \(C_m\) increases that were measured among individual nerve endings, each \(C_m\) response was resolvable into several kinetically and functionally distinct components, as has been described in detail in an earlier report (Giovannucci and Stuenkel, 1997). Briefly, depolarizations of single nerve endings achieved with 5 msec steps produced a transient \(C_m\) increase that was independent of Ca\(^{2+}\) entry. It was shown for these peptidergic nerve endings that this transient \(C_m\) signal does not represent Ca\(^{2+}\)-dependent exocytotic activity and is most likely related to a charge redistribution.

opioid. After the current recovered from the inhibition by U69,593, GVIA (0.5 \(\mu M\)) was administered for 40 sec. GVIA irreversibly suppressed the peak amplitude of the control current from 260 to 75 pA (185 pA, representing 71.2% of the control current), and after GVIA the fraction of current that was inhibited by U69,593 was reduced from 150 to 48 pA (representing a 68% blockade). The application of nicardipine (10 \(\mu M\)) subsequent to the establishment of the irreversible blockade by GVIA reduced peak current amplitude further from 75 to 29 pA (equivalent to 17.7% of the control current), and this was associated with a further reduction in the inhibitory response to U69,593 (from 48 to 17 pA). Administration of MVIIC (1 \(\mu M\)) in the presence of nicardipine abolished whole-cell current and any effect of U69,593 that remained after nicardipine. The pairs of traces depicted in Figure 2A (lower) were obtained by subtraction of corresponding currents evoked before and after application of antagonists and illustrate sensitivity to U69,593 of pharmacologically distinguished N-, L-, and P/Q-type current components. Overall, in 3 of 11 terminals (28.9%) tested, \(Ca^{2+}\) currents showed sensitivity to blockade by selective \(Ca^{2+}\) channel blockers similar to that illustrated for the nerve terminal in Figure 2. The majority of terminals that were studied (5 of 11, 45.5%) possessed only N- and L-type \(Ca^{2+}\) channels, whereas the remaining 3 terminals expressed L- and P/Q-type but not N-type channels. It should be noted, however, that all three pharmacologically distinct current components demonstrated sensitivity to \(\kappa\)-opioids.

These data suggest that \(\kappa\)-opioid receptors are negatively coupled to all types of \(Ca^{2+}\) channels in rat neurohypophysial nerve terminals, including a GVIA-sensitive (N-type) channel, a nicardipine-sensitive (L-type) channel, and a MVIIC-sensitive (P/Q-type) channel.

To quantify the relative contribution of individual current components to the total whole-terminal current and its \(\kappa\)-opioid-sensitive portion, we averaged measurements of current amplitude blocked by each antagonist that were obtained from the terminals examined using the same protocol as described for the experiment in Figure 2A. This analysis revealed that 59 \(\pm\) 8% (\(n = 8\)) of high-threshold \(Ca^{2+}\) current was contributed through GVIA-sensitive N-type channels, 25 \(\pm\) 4% (\(n = 6\)) through dihydropyridine-sensitive L-type channels, and 13 \(\pm\) 5% (\(n = 3\)) through pharmacologically defined P/Q-type channels. Administration of U69,593 inhibited N-type current on average ~32%, whereas nicardipine-sensitive current (L-type) was reduced ~64%, and MVIIC-sensitive (P/Q-type) current was inhibited ~28% (Fig. 2B).
within the membrane (Giovannucci and Stuenkel, 1997). Depolarizations of longer duration (50–200 msec) to between 0 and +30 mV produced more-prolonged Ca$^{2+}$-dependent increases in $C_m$ that could be resolved into two kinetically distinct phases (Fig. 3A): an immediate step-like jump (seen in all 43 terminals) reflecting fusion of an immediately releasable pool of “predocked vesicles” followed by a larger, slow $C_m$ increase of several seconds duration indicative of fusion and release from a distinct, larger readily releasable pool of vesicles (Lindau et al., 1992; Horrigan and Bookman, 1994; Hsu and Jackson, 1996; Giovannucci and Stuenkel, 1997). This latter component of Ca$^{2+}$-dependent exocytosis was observed in 19 of the 43 nerve terminals studied. The presence of the transient $C_m$ change together with slower Ca$^{2+}$-dependent $C_m$ components obscures the correct estimation of the immediate step-like exocytotic $C_m$ jump. Therefore, the transient $\Delta C_m$ evoked by a 5 msec step was digitally subtracted from the $C_m$ changes evoked with longer depolarizations to yield a measure of Ca$^{2+}$-dependent exocytosis. All data described subsequently were obtained from transient $\Delta C_m$-subtracted records.

The properties of the immediate and slow $C_m$ responses measured here, as illustrated in Figure 3A, for example, were similar to those described for these peptidergic nerve endings in previous studies (Lim et al., 1990; Seward et al., 1995; Hsu and Jackson, 1996; Giovannucci and Stuenkel, 1997). In contrast to the transient $\Delta C_m$, both the immediate jump and slowly increasing $C_m$ responses were markedly altered by manipulations that resulted in attenuation of depolarization-evoked Ca$^{2+}$ entry. Administration of Cd$^{2+}$ (100 $\mu$M) to the external medium was found to abolish the inward Ca$^{2+}$ currents and any corresponding immediate or slow $C_m$ responses, without appreciable change in the transient $C_m$ components [$n = 2$; see also Giovannucci and Stuenkel (1997), their Fig. 3A]. Conversely, depolarization-evoked inward currents were greatly enhanced, whereas both immediate and slow $C_m$ responses were reduced when Ba$^{2+}$ (10 mM) was substituted for external Ca$^{2+}$ ($n = 2$). These results support the notion that the immediate step and slowly increasing $C_m$ changes reflect exocytotic activity that is dependent on Ca$^{2+}$ influx. In additional experiments ($n = 8$), the dependence of these $C_m$ responses on Ca$^{2+}$ entry was examined further by comparing the relationships of the magnitude of the evoked Ca$^{2+}$ current and associated changes in $C_m$ (measured in the same nerve ending) as a function of the step depolarization (Fig. 3). Depolarizing pulses of 200 msec duration were applied in a random order to membrane potentials between −40 and +60 mV from a $V_h$ of −90 mV, and all changes in $C_m$ were allowed to return to baseline (typically requiring 60–90 sec) before we initiated the next depolarization. To facilitate comparisons between current–voltage and $C_m$–voltage relationships, we normalized the magnitude of the time-integrated Ca$^{2+}$ currents and the amplitudes of immediate and slow $C_m$ responses measured in a given nerve ending to the corresponding maximal responses obtained, and the results from all experiments were pooled, yielding the plots shown in Figure 3C. The inward Ca$^{2+}$ current began to activate at −30 mV and reached a maximum at +10 mV, at which point the time-integrated Ca$^{2+}$ current averaged 12.0 ± 2.3 pC ($n = 8$). The corresponding immediate step increases in $C_m$ ($n = 8$) closely followed the current–voltage profile and also reached a maximal value (18.4 ± 5.0 fF) at +10 mV (Fig. 3C). When steps were made to potentials ≥ 0 mV (Figs. 3A, C), a slowly increasing $\Delta C_m$ was observed after the immediate $C_m$ response in six of the terminals. With a higher threshold, the plot of the slow $C_m$ increase as a function of test potential showed a rightward shift compared with the voltage relationships of the evoked Ca$^{2+}$ and immediate $C_m$ responses but also peaked at +10 mV (57.6 ± 17.8 fF). On the other hand, voltage steps to test potentials positive to +50 mV evoked no inward currents and failed to induce immediate or slow $C_m$ responses in any of the nerve endings (Fig. 3C). These results support the conclusion that both the immediate and slow $C_m$ responses measured here are tightly coupled to elevations in [Ca$^{2+}$], produced by influx through voltage-sensitive Ca$^{2+}$ channels. Nevertheless, the normalized amplitude of the immediate $C_m$ increase exceeded that of the slow $C_m$ response at all test potentials, suggesting that the initial component of exocytotic release may have a different requirement for triggering by elevations in [Ca$^{2+}$], than the slow phase of release (Lim et al., 1990; Lindau et al., 1992; Seward et al., 1995; Hsu and Jackson, 1996; Giovannucci and Stuenkel, 1997).

To determine whether the activation of $\kappa$-opioid receptors had an effect on Ca$^{2+}$-dependent exocytosis, we tested the ability of U69,593 (1 $\mu$M) and dynorphin A (1 $\mu$M), administered at con-
Figure 4. Activation of \( \kappa \)-opioid receptors attenuates immediate and slow \( C_m \) responses evoked by steps inducing smaller \( \Ca^{2+} \) influx. \( C_m \) increases (\( A_1, A_2, B_1, B_2 \)) and corresponding \( \Ca^{2+} \) currents (\( A_1, B_1 \)) recorded in response to 200 msec depolarizations to +10 mV (A) and +30 mV (B) from a holding potential of –90 mV. Administration of U69,593 appreciably reduced \( \Ca^{2+} \) influx (32.1%, from 18.53 to 12.58 pC) evoked by depolarization to +10 mV but had little effect on the corresponding immediate \( C_m \) response (9% reduction, from 49 to 44 fF, indicated by dotted lines). Depolarization to +30 mV induced much smaller \( \Ca^{2+} \) influx (9.1 pC) that was attenuated further by activation of \( \kappa \)-opioid receptors (to 6.23 pC, representing 31.5% blockade). Under this condition of reduced driving force for \( \Ca^{2+} \) influx, inhibition of the immediate \( C_m \) response by the \( \kappa \)-opioid agonist was markedly increased (47%, from 36 to 19 fF). Opioid administration also reduced the slowly increasing \( C_m \) component recorded in this nerve ending, and this effect of U69,593 was greater on the response measured at +30 mV (33.9%, from 118 to 78 fF) compared with that recorded at +10 mV (17.7%, from 192 to 158 fF). C, Bar graph summarizes the effects of U69,593 on the immediate step and slowly increasing components of \( C_m \) responses. Bars represent the mean ± SEM of the normalized \( C_m \) responses pooled from \( \kappa \)-opioid-responsive terminals (number shown in numerator, out of the total number of terminals examined, shown in denominator) for immediate and slow \( C_m \) responses evoked at command potentials of +10 or +30 mV, expressed as a percent of the corresponding predrug control values. Reduction of depolarization-evoked \( \Ca^{2+} \) influx to submaximal levels resulted in an increased probability for modulation of the immediate \( C_m \) response by \( \kappa \)-opioid receptor activation from 32% (13 of 41) at +10 mV to 100% (6 of 6) at +30 mV, without significantly (\( p = 0.19 \); NS indicates not significant) altering the magnitude of the inhibitory effect of U69,593 on these initial exocytotic events. In contrast, the inhibitory effect of U69,593 on the slow \( C_m \) responses was significantly increased under conditions in which the driving force for \( \Ca^{2+} \) influx and the net amount of depolarization-evoked \( \Ca^{2+} \) influx was reduced (asterisk indicates \( p < 0.001 \)).

centrations near-maximal for \( \Ca^{2+} \) current inhibition, to modulate depolarization-evoked immediate \((n = 43) \) and slow \((n = 19) \) increases in \( C_m \). The effects of U69,593 were examined in 41 individual nerve endings, of which 19 exhibited a slowly increasing phase of \( C_m \) in addition to the immediate step \( C_m \) response. The traces in Figure 4 show \( \Ca^{2+} \) currents (\( A_1 \)) and corresponding \( C_m \) responses (\( A_1, A_2 \)) elicited by a 200 msec step to +10 mV from a \( V_h \) of –90 mV. Bath application of U69,593 (1 \( \mu \)M) for 30 sec decreased the peak amplitude of the \( \Ca^{2+} \) current from 193 to 121 pA (Fig. 4A1), resulting in a 32% reduction in the depolarization-evoked \( \Ca^{2+} \) influx (from 18.5 to 12.6 pC). The immediate step-like increase in \( C_m \) was only slightly attenuated (9%, from 49 to 44 fF) by \( \kappa \)-opioid administration in this terminal (Fig. 4A2); however, the magnitude of the subsequent slowly increasing phase of \( C_m \) was appreciably reduced (19%, from 193 to 157 fF; Fig. 4A3). In three additional terminals in which we used the same protocol to examine for mediation of agonist effects via \( \kappa \)-opioid receptors, both the inhibition in evoked \( \Ca^{2+} \) current and corresponding attenuation in immediate or slow \( C_m \) responses produced by U69,593 (1 \( \mu \)M) were blocked reversibly by administration of nor-BNI (1 \( \mu \)M; Fig. 5). Overall, the administration of U69,593 reduced immediate step increases in \( C_m \) in 13 of 41 nerve endings examined (25.2 ± 4.2%; range, 8–40%), when measurements were obtained using step depolarizations to test potentials yielding maximal \( \Ca^{2+} \) currents (Fig. 4C). Under these conditions, an inhibition in the evoked \( \Ca^{2+} \) current was observed in 31 of the nerve terminals (30.1 ± 3.0%; range, 9–67%), including 11 of those in which reductions in the immediate \( C_m \) response were registered (26.2 ± 5.0%; range, 8–62%). In contrast, administration of the \( \kappa \)-opioid agonist reduced the slowly increasing \( C_m \) responses evoked by depolarizing steps to the peak of the current–voltage curve in 16 of the 19 terminals that showed this additional exocytotic component. In 14 of these 16 terminals, U69,593 reduced the evoked \( \Ca^{2+} \) current by an average of 33.7 ± 5.1% (range, 9–66%), a value close to the mean reduction observed in the peak amplitude of the slow \( C_m \) response (33.0 ± 3.5%; range, 6–47%). Interestingly, in the other two terminals, opioid-induced reductions in the slow \( C_m \) response (and in the immediate step increases that preceded them) were observed without appreciable changes in the \( \Ca^{2+} \) current. Application of U69,593 had no effect on either the evoked \( \Ca^{2+} \) currents or the associated slow (and immediate) \( C_m \) responses in the remaining three terminals. Tests with dynorphin A (1 \( \mu \)M) in seven terminals, including five that were screened initially with U69,593, revealed a similar profile of inhibitory effects on depolarization-evoked \( \Ca^{2+} \) currents and the corresponding immediate and slow \( C_m \) responses. Administration of the \( \kappa \)-opioid peptide routinely suppressed inward currents (five of seven terminals) and the associated slowly increasing phase of \( C_m \) (three of three terminals), whereas significant inhibitory effects on immediate \( C_m \) responses were observed in only a single terminal. Direct comparisons of the effects of U69,593 and dynorphin A in the same nerve ending \((n = 5) \) demonstrated nearly equivalent inhibitory responses to these agonists (Fig. 6). Moreover, when the data were pooled for all terminals that responded to a particular agonist, no significant differences were observed in the maximal inhibition of \( \Ca^{2+} \) current produced by U69,593 (30.1 ± 3.0%; \( n = 31 \)) and dynorphin (37.4 ± 5.3%; \( n = 5 \)) and in agonist-induced attenuation
of the slow $C_m$ response produced by U69,593 (33.7 ± 3.1%; $n = 16$) and dynorphin (37.9 ± 4.1%; $n = 3$).

Despite a routine suppression of the slow phase of exocytosis, activation of $\kappa$-opioid receptors had variable and inconsistent effects on the immediate step-like increases in $C_m$ measured in response to depolarizations that yielded maximal $\Ca^{2+}$ currents. Given the close correlation observed between the opioid-induced inhibition in evoked $\Ca^{2+}$ currents and in slow $C_m$ responses, what might account for the lack of correspondence between changes in $\Ca^{2+}$ currents and the immediate $C_m$ increases that constitute the initial secretory event? One possibility is that such an outcome might derive from fundamental differences in the dependency of the initiation and maintenance of vesicular exocytosis on increases in $\Ca^{2+}$ concentration within functionally distinct intraterminal domains (Lindau et al., 1992; Horrigan and Bookman, 1994; Hsu and Jackson, 1996; but see Seward et al., 1995). We hypothesized that if the immediate $C_m$ response reflects exocytosis of predocked vesicles (Lindau et al., 1992; Horrigan and Bookman, 1994), then activation of $\Ca^{2+}$ channels by depolarizations to the peak of the current–voltage curve may increase submembrane $\Ca^{2+}$ concentration within spatially restricted domains close to the release sites beyond the level of saturation for vesicle fusion. Under these conditions, a modest reduction in the evoked $\Ca^{2+}$ current by $\kappa$-opioids might not decrease submembrane concentrations of $\Ca^{2+}$ near the release sites below the level for saturation and, therefore, would have little if any effect on the immediate $C_m$ response. To test this hypothesis, we examined the effects of U69,593 on $\Ca^{2+}$ currents and $C_m$ responses that were evoked by stepping to more positive command potentials (+30 mV) closer to $E_{Ca}$, thereby reducing the driving force for $\Ca^{2+}$ influx (Fig. 4B). We relied on this approach to manipulate the magnitude of $\Ca^{2+}$ entry induced by depolarization rather than to modify influx by altering $[\Ca^{2+}]_i$, because the latter manipulations induce changes in basal levels of $[\Ca^{2+}]_i$ in these nerve endings with dramatic effects on the functional readiness of different vesicular pools (Stuenkel, 1994), thereby precluding valid comparisons between values obtained under control and experimental conditions. With delivery of single depolarizing steps to +30 mV, the amplitudes of the evoked currents and associated immediate and slow $C_m$ responses were reduced on average 45.8 ± 10.5% ($n = 6$), 20.7 ± 5.7% ($n = 6$), and 45.8 ± 6.4% ($n = 3$), respectively, compared with the corresponding values measured in the same terminals after depolarizations to +10 mV. Under this test condition, all response parameters recorded in each nerve terminal were inhibited by the $\kappa$-opioid receptor agonist, including the immediate step $C_m$ increases in three terminals that did not show modulation by the $\kappa$-opioid when evoked by steps to +10 mV. For this entire subgroup of nerve terminals ($n = 6$), no significant difference was observed between the inhibitory effect of U69,593 on the peak $\Ca^{2+}$ currents evoked at +10 and +30 mV (34.2 ± 4.0% and 29.5 ± 5.2%, respectively), nor was there any difference in the extent of inhibition of the nonactivating current component measured at the end of the 200 msec pulse (32.2 ± 4.7% and 34.3 ± 4.4% at +10 and +30 mV, respectively). However, the U69,593-induced inhibition of the immediate $C_m$ increase tended to be greater for responses evoked at +30 mV (34.3 ± 3.9%) compared with responses evoked at +10 mV (14.1 ± 9.9%) and was comparable in magnitude with the $\kappa$-opioid inhibition of slow $C_m$ increases (33.7 ± 3.1%; $n = 16$) measured in response to depolarizations (+10 mV) that yielded maximal $\Ca^{2+}$ currents. Similarly, under the condition of reduced $\Ca^{2+}$ driving force, the inhibitory effect of U69,593 on the slow $C_m$ response was markedly increased in two of three nerve terminals that exhibited this additional component of $\Ca^{2+}$-dependent exocytosis. Figure 4C provides a graphical comparison of the inhibitory effects of U69,593 on the immediate step and slowly increasing $C_m$ responses evoked by depolarizations to +10 or +30 mV, expressed as a percent of the corresponding predrug control responses. The height of each bar represents the opioid effect computed by pooling results obtained only from nerve terminals that showed modulation of the particular $C_m$ response. The principal effect of reducing depolarization-evoked $\Ca^{2+}$ influx to submaximal levels was to increase the probability for modulation of the immediate $C_m$ response by activation of $\kappa$-opioid receptors (from 32 to 100%, Fig. 4C), without significantly altering the magnitude of
the inhibitory effect of U69,593 on the initial exocytotic events evoked by depolarizing steps yielding peak inward currents compared with submaximal Ca\(^{2+}\) influx (25.2 ± 4.2% at +10 mV compared with 34.3 ± 3.9% at +30 mV; p = 0.19). An additional effect of this manipulation was to significantly increase the extent to which the slowly increasing C\(_m\) component can be inhibited by \(\kappa\)-opioid receptor activation (79.2 ± 20.8% at +30 mV compared with 33.7 ± 3.1% at +10 mV; p < 0.001). Taken together, the results of these experiments suggest that activation of \(\kappa\)-opioid receptors on the endings of rat neurohypophysial magnocellular neurons reduces the secretion of the neurohormones AVP and OT by modulating several kinetically distinct components of exocytotic vesicular release.

**DISCUSSION**

This work provides the first direct examination of the relationship between opioid-induced suppression of Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels and inhibitory modulation of neurotransmitter or neuropeptide release in mammalian nerve terminals. This became possible because of the development of an anatomically unique preparation of neurosecretory terminals from the rat neurohypophysis. These nerve endings maintain cytoplasmic constituents, release neuropeptides in a Ca\(^{2+}\)-dependent manner, and respond to receptor-mediated modulatory influences (Cazalis et al., 1987; Nordmann et al., 1987).

Opioids have been shown to act at \(\kappa\)- and, possibly, \(\mu\)-opioid receptors (Zhao et al., 1988a) to inhibit depolarization-evoked release of AVP and OT from isolated rat neural lobes and single neurohypophysial nerve terminals (Zhao et al., 1988b; Dayanithi et al., 1992; Kato et al., 1992). In some reports, \(\kappa\)-opioid agonists and nonselective opioids (etorphine) also reduced the associated rise in Ca\(^{2+}\), measured with Fura-2, but the relationship between the changes in intraterminal [Ca\(^{2+}\)] and inhibition of K\(^+\)-stimulated release of neuropeptides from isolated endings or intact neural lobes showed only a weak correlation (Dayanithi et al., 1992; Kato et al., 1992). To clarify the role of Ca\(^{2+}\)-dependent processes in opioid-induced inhibition of release, we examined the effects of \(\mu\)-, \(\delta\)-, and \(\kappa\)-opioid-selective agonists on Ca\(^{2+}\) currents in single neurohypophysial nerve terminals using the whole-terminal recording configuration, while simultaneously monitoring changes in whole-terminal C\(_m\) as an assay of Ca\(^{2+}\)-triggered exocytotic release (Lindau and Neher, 1988; Lim et al., 1990).

Inhibition of Ca\(^{2+}\) current by the \(\kappa\)-opioid agonists dynorphin A, U69,593, or U50,488H was observed in 67% of neurohypophysial nerve terminals, and these responses were blocked by naloxone and the \(\kappa\)-selective antagonist nor-BNI. Ca\(^{2+}\) currents were unaffected by either \(\mu\)- or \(\delta\)-opioid agonists, although reductions in current by \(\kappa\)-opioids were registered in most (8 of 11) of the same preparations. Thus, only \(\kappa\)-opioid receptors are negatively coupled to Ca\(^{2+}\) channels in these neurosecretory endings.

The Ca\(^{2+}\) currents we recorded were high-threshold and consisted of both inactivating and sustained components, but these currents contained no rapidly inactivating low-threshold T-type current. Our findings that GVIA irreversibly blocked ~59%, nicardipine suppressed 25%, and MVIIC (applied subsequent to GVIA and in the presence of nicardipine) blocked 13% of the total current are consistent with earlier reports that such terminals only express N-, L-, and Q-type channels (Wang et al., 1993; Lemos et al., 1994). However, only one-fourth of the terminals examined demonstrated this profile of sensitivity to Ca\(^{2+}\) channel blockers, whereas approximately one-half seemed to express only N- and L-type Ca\(^{2+}\) channels, and the remainder possessed only L- and P/Q-type channels.

Somatic recordings obtained from rat peripheral sensory (Schoeber et al., 1991; Moises et al., 1994; Rusin and Moises, 1995) and nucleus tractus solitarius neurons (Rhim and Miller, 1994) have identified several high-threshold Ca\(^{2+}\) channels that are modulated by opioid receptors. In these neurons, \(\mu\)-, \(\delta\)-, and \(\kappa\)-selective agonists to a lesser extent, inhibit Ca\(^{2+}\) current contributed by GVIA-sensitive N-type and pharmacologically distinct P- and Q-type channels but spare L- and T-type currents, thereby regulating the principal Ca\(^{2+}\) channel types involved in exocytosis at central synapses and peripheral sites of release (Luebke et al., 1993; Takahashi and Momiyama, 1993; Wheeler et al., 1994; Dunlap et al., 1995). Studies in cortical synaptosomes suggest a similar pattern of coupling of presynaptic opioid receptors to Ca\(^{2+}\) channels, in that here both N- and L-type channels contribute to depolarization-evoked Ca\(^{2+}\) entry, whereas the
inhibitory effects of \( \kappa \)-opioid agonists on \([Ca^{2+}]_i\) and exocytosis are blocked by GV1A but not by dihydropyridines (Adamson et al., 1989; Xiang et al., 1990). However, L-type channels have been shown in chromaffin cells to be more efficiently coupled to exocytosis than N- and P-type channels (Artalejo et al., 1994) and similarly play an important role in neurohemorrhage release in rat neurosecretory endings (Stuenkel and Nordmann, 1993). It is not surprising, therefore, that L channels are the target for \( G \)-protein modulation in melanotrophs from rat pituitary gland (Ciranna et al., 1996) and that they are most sensitive to modulation by \( \kappa \)-opioids in isolated neurohypophysial nerve terminals as reported here. Administration of U69,593 in a saturating concentration inhibited L-type current on average by \( \sim 64\% \), whereas N- and P/Q-type currents were reduced by \( \sim 32\% \) and \( \sim 28\% \), respectively. The present results also serve to emphasize the necessity for direct examination of isolated terminal \( Ca^{2+} \) currents to establish the involvement of particular \( Ca^{2+} \) channel subtypes in opioid regulation of exocytosis. Thus, recordings from magnocellular supraoptic neurons reveal that activation of \( \mu \)-opioid receptors inhibits N- and P-type but not L-type \( Ca^{2+} \) channels, yet these recordings fail to demonstrate functional coupling between \( \kappa \)-opioid receptors and \( Ca^{2+} \) channels (Soldo and Moises, 1996).

We tested for a functional linkage between opioid suppression of voltage-dependent \( Ca^{2+} \) channels and inhibition of release by comparing the effects of opioid-induced alterations in \( Ca^{2+} \) influx on kinetically distinct components of the neurosecretory response. The slow increases in \( C_m \) that we measured are thought to reflect the trafficking and membrane fusion of secretory vesicles from a readily releasable vesicular pool (Lindau et al., 1992; Horrigan and Bookman, 1994). Administration of U69,593 consistently attenuated the amplitude of these slow \( C_m \) responses evoked by depolarization to \( +10 \text{ mV} \) as well as potentials (\( +30 \text{ mV} \)) closer to \( E_{C_m} \). On the other hand, the immediate step-like \( C_m \) increases in response to depolarizations that induced maximal \( Ca^{2+} \) influx were not affected by the \( \kappa \)-opioids in more than two-thirds of the terminals examined, this despite the fact that \( Ca^{2+} \) currents were substantially reduced in three-quarters of these preparations. Interestingly, the effects produced by \( \kappa \)-opioid receptor activation mirror the relationship between changes in \( Ca^{2+} \) influx and release described in chromaffin cells, wherein manipulations that reduce \( Ca^{2+} \) currents (reducing \( [Ca^{2+}]_i \), or stepping closer to \( E_{C_m} \) ) decreased the delayed rise in \( C_m \) but had little effect on the immediate \( C_m \) jump after 100 msec depolarizations (Horrigan and Bookman, 1994). It was proposed that in those cells high levels of submembrane \( Ca^{2+} \) might saturate the \( Ca^{2+} \) receptors, resulting in a constant probability of release of the vesicles from the immediately releasable pool (IRP). Similarly, saturation of \( Ca^{2+} \) receptors might account for the variable effects of opiates on immediate responses observed here. In fact, when we evoked smaller \( Ca^{2+} \) currents by stepping closer to \( E_{C_m} \), the amplitudes of the immediate \( C_m \) responses were correspondingly reduced, indicating that \( Ca^{2+} \) levels at the release sites were decreased to a level below saturation. Under these conditions, the immediate \( C_m \) responses were reliably decreased by U69,593. The ability of \( \kappa \)-opioids to modulate any associated slowly increasing \( C_m \) component was also related to the size of the depolarizing step used to evoke these responses and the corresponding \( Ca^{2+} \) currents. Thus, this slowly increasing phase of \( C_m \) was inhibited to a much greater extent (being abolished in two-thirds of the terminals) when U69,593 was applied under conditions in which the \( Ca^{2+} \) driving force was reduced (\( +30 \text{ mV} \)) compared with the effects measured on responses evoked by stepping to test potentials that yielded maximal \( Ca^{2+} \) currents. This experimental outcome is in keeping with recent findings that suggest that maintenance of exocytotic activity in these nerve endings (presumably reflected in the slowly increasing \( C_m \) response) may involve a replenishment of vesicles into the IRP that is directly dependent on the level of intraterminal \( [Ca^{2+}]_i \) (Giovannucci and Stuenkel, 1997).

Overall, our results provide strong evidence that activation of \( \kappa \)-opioid receptors modulates several components of exocytotic release in rat neurohypophyseal nerve endings, including both vesicle fusion and the recruitment of vesicles into an IRP. However, the molecular mechanism(s) whereby \( \kappa \)-opioid receptors produce these presynaptic inhibitory actions remains to be elucidated. Our findings support the hypothesis that modulation of \( Ca^{2+} \) dependent processes plays a role in \( \kappa \)-opioid-induced inhibition of exocytosis in neurohypophysial nerve endings. However, they do not preclude the possibility that the relationship between opioid-induced reduction of voltage-dependent \( Ca^{2+} \) influx and presynaptic modulation of release could be more complex and that the inhibition of \( Ca^{2+} \) channels may be selectively involved in regulatory mechanisms that govern distinct aspects of the exocytotic process. In this regard, recent findings obtained in rat hippocampal slice preparations that \( \mu \)-opioid agonists reduced the occurrence of spontaneous, \( Ca^{2+} \)-independent synaptic events raise the possibility of a direct opioid effect on the intracellular machinery that regulates vesicle exocytosis (Cohen et al., 1992; Capogna et al., 1993; Rekling, 1993). The possibility of a direct depressant action of opioids on the secretory apparatus is also supported by recent findings obtained in the GLC8 small-cell lung carcinoma cell line, wherein it was found that activation of endogenous \( \delta \)-opioid receptors not only inhibited the depolarization-induced release of \([H] \)serotonin but also the \( Ca^{2+} \)-dependent secretion of the labeled transmitter induced by thapsigargin and ionomycin (Sher et al., 1997). Our finding in a few terminals that administration of \( \kappa \)-opioids attenuated depolarization-evoked \( C_m \) responses despite little or no decrease in the associated \( Ca^{2+} \) currents raises the additional possibility that opioids might regulate vesicle fusion and/or trafficking independently of \( Ca^{2+} \) current modulation. In any event, the present results suggest a potential mechanism whereby endogenous release of prodynorphin-derived opioid peptides, known to be colocalized in AVP- and OT-containing terminals (Zhao et al., 1988b; Russell et al., 1995), can exert inhibitory modulation of neuropeptide release from the neurohypophysis.

REFERENCES


Solbo BL, Moises HC (1996) Ca\(^{2+}\) channels in rat supraoptic neurons are modulated by \(\mu\) but not \(\delta\) or \(\kappa\)-opioid receptors. Soc Neurosci Abstr 22:1752.


