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μ-Opioid Receptor Inhibition of Calcium Current: Development of Homologous Tolerance in Single SH-SY5Y Cells After Chronic Exposure to Morphine *In Vitro*

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SUMMARY

The human SH-SY5Y neuroblastoma cell line displays morphological, neurochemical, and electrophysiological characteristics of sympathetic neurons. μ-Opioid receptors mediate inhibition of the N-type calcium current present in these cells. Here we have studied the effects of chronic incubation with morphine (1 μM for 3–7 days) *in vitro* on the inhibition of this current induced by μ-opioid agonists and noradrenaline. In untreated control cells the μ-opioid agonists morphine (1 μM) and [D-Ala^2,*N*-MePhe^4,Gly-ol] enkephalin (DAMGO) (10 nM to 1 μM), and noradrenaline (10 nM to 10 μM) inhibited the calcium current to a similar extent. The maximal effects of DAMGO and noradrenaline were not additive. Chronic exposure to morphine had no effect on the maximum amplitude of the calcium current evoked or on its voltage sensitivity. However, the concentration-response curve to DAMGO was shifted to the right in a parallel manner, with a 7-fold increase in the IC_{50} value but no change in the maximum inhibition produced. In contrast, the maximum inhibition in response to morphine appeared to be substantially reduced. Noradrenaline inhibited the calcium current equally in untreated and morphine-tolerant cells. Thus, it is concluded that morphine-induced tolerance to inhibition of the N-type calcium current occurs at the single-cell level and is homologous to the μ-opioid receptor. Also, morphine appears to be an agonist of lower efficacy than DAMGO. The results are consistent with tolerance being due to a functional reduction in the μ-opioid receptor reserve, probably by disruption of the receptor/GTP-binding protein interaction.

Although it has been known for many years that repeated exposure to μ-opioid receptor agonists, such as morphine and heroin, *in vivo* or *in vitro* leads to tolerance (or desensitization) to their acute actions, the adaptive mechanisms underlying tolerance at the cellular and molecular levels are not yet clearly understood. It is still uncertain whether tolerance can develop at the level of the single cell in the absence of other neuronal input or whether, as recently suggested (1), on-going excitatory amino acid-mediated synaptic transmission is required for the induction of morphine tolerance *in vivo*. The effect of chronic exposure to morphine on single isolated cells *in vitro* can best be studied using a cultured homogeneous neuronal population that is responsive to opioids.

SH-SY5Y cells, the neuroblastoma subclone of the human SK-N-SH cell line, are a homogeneous population of cells that, when differentiated, have morphological, neurochemical, and electrophysiological properties characteristic of sympathetic neurons (2–5). These cells express μ-opioid receptors that are negatively linked to adenylate cyclase (4, 6, 7). Previous studies in our laboratory have shown that μ-opioid receptor activation also inhibits the I_{Ca} present in differentiated SH-SY5Y cells, through a PTX-sensitive G protein (5, 8, 9). Both the peak and the sustained components of the I_{Ca} in these cells have been identified as being mediated through the N-type of high voltage-activated calcium channel (10, 11), because they require large depolarizing pulses for activation and are irreversibly inhibited by ω-Conus toxin but are unaffected by the dihydropyridine L-type calcium channel agonists and antagonists (5, 12).

Here we have studied whether chronic exposure to morphine can induce tolerance to the inhibitory actions of μ agonists and NA on the N-type I_{Ca} in individual cultured SH-SY5Y cells in the absence of other external influences such as neurotransmission. Preliminary accounts of these findings have been presented (13, 14).

Materials and Methods

Cell culture and neuronal differentiation. SH-SY5Y cells were cultured as described previously (12). Briefly, cells were grown in nonconfluent monolayer cultures and were induced to differentiate by

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ABBREVIATIONS: I_{Ca}, calcium channel current; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol]enkephalin; EGTA, ethylene glycol bis(β-aminoethyl)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NA, noradrenaline; PTX, pertussis toxin; G protein, GTP-binding protein.

exposure to retinoic acid (10 or 30 μM) for 6–12 days. Retinoic acid inhibits cell division, induces neuronal characteristics, and up-regulates the μ-opioid receptor in SH-SY5Y cells (3–5). Small cells (<20 μm diameter) with short processes (<2 times the cell diameter) were chosen for study, to ensure adequate control of the membrane potential in voltage clamp.

To study the effects of chronic exposure, morphine (1 μM) was included in the culture medium for 3–7 days. This treatment does not alter the growth characteristics of SH-SY5Y cells.[3] Using an antibody raised against morphine (Abuscreen; Roche) and a separation technique that removed the more polar morphine metabolites (15), the concentration of morphine in the medium bathing the cells was shown to fall by approximately 20% over the 3 days between feeding of the cells. No difference was found in the degree of tolerance seen at 3 days, compared with 7 days; therefore, the results obtained after 3–7 days of morphine treatment have been pooled.

Solutions and electrophysiological recording. The voltage-sensitive I_{Ca} was recorded with the whole-cell patch-clamp technique (16). Cells were superfused (5 ml/min) at room temperature (18–22°) with a solution containing (in mM) NaCl, 140; CsCl, 5.4; $BaCl_2$, 10.8; $MgCl_2$, 1; D-glucose, 10; and HEPES, 40; with 0.5 μM tetrodotoxin. In experiments where the current-voltage relationship for I_{Ca} was determined, NaCl was replaced with equimolar tetraethylammonium. Recording electrodes (resistance of 4–8 MΩ, coated with Sylgard and fire-polished) contained (in mM) CsCl, 100; $MgCl_2$, 5; EGTA, 5; ATP, 2; and HEPES, 40. In some initial experiments, 0.25 mM cyclic AMP was also present. The pH of both the intracellular and extracellular solutions was adjusted to 7.3 with CsOH.

I_{Ca} was recorded using an Axopatch 1D amplifier, which has the ability to subtract leak current and compensate for capacitive currents and series resistance. At the end of each experiment, the adjustment of the leak subtraction was verified by superfusion of the cell with a solution containing 100 μM $CdCl_2$, which abolishes I_{Ca} in SH-SY5Y cells (12). All currents were filtered at 5 kHz and recorded on video tape after A/D conversion by a pulse code modulator. For subsequent analysis, currents were played out from the tape onto a chart recorder or filtered at 1 kHz and then digitized at 3 kHz for computer analysis. Where averaged currents are shown, these were obtained by averaging three to six consecutively evoked currents.

Results are expressed as the mean ± standard error and were analyzed statistically using Student's unpaired t test. A probability value of $p < 0.05$ was considered to be statistically significant. Concentration-inhibition curves were fitted to the data by logistic (Hill equation), nonlinear regression analysis (GRAPHpad).

Drugs used. All drugs were added in known concentrations in the superfusate. The following drugs were used: DAMGO (Cambridge Research Biochemicals), idazoxan (Reckitt and Colman), morphine sulfate (National Institute on Drug Abuse), naloxone hydrochloride, NA bitartrate, and tetrodotoxin (Sigma).

[3] D. V. Hall, E. P. Seward, and G. Henderson, unpublished observations.

Results

Acute actions of μ-opioid agonists. The N-type I_{Ca} was evoked by stepping from a holding potential of −90 mV to +10 mV for 500 msec, because the inward I_{Ca} is maximal at this stepping potential (see Fig. 3). Under the whole-cell recording conditions used, T- or L-type I_{Ca} were not seen in SH-SY5Y cells (see also Ref. 12). I_{Ca} was not maintained but decayed from an initial peak during the voltage step (Fig. 1A). Morphine (1 μM), applied acutely, inhibited both the peak I_{Ca} and the current remaining just before the end of the voltage step (Fig. 1). The effect of morphine was maximal within 2 min of administration and was reversed either on washout or by ad-

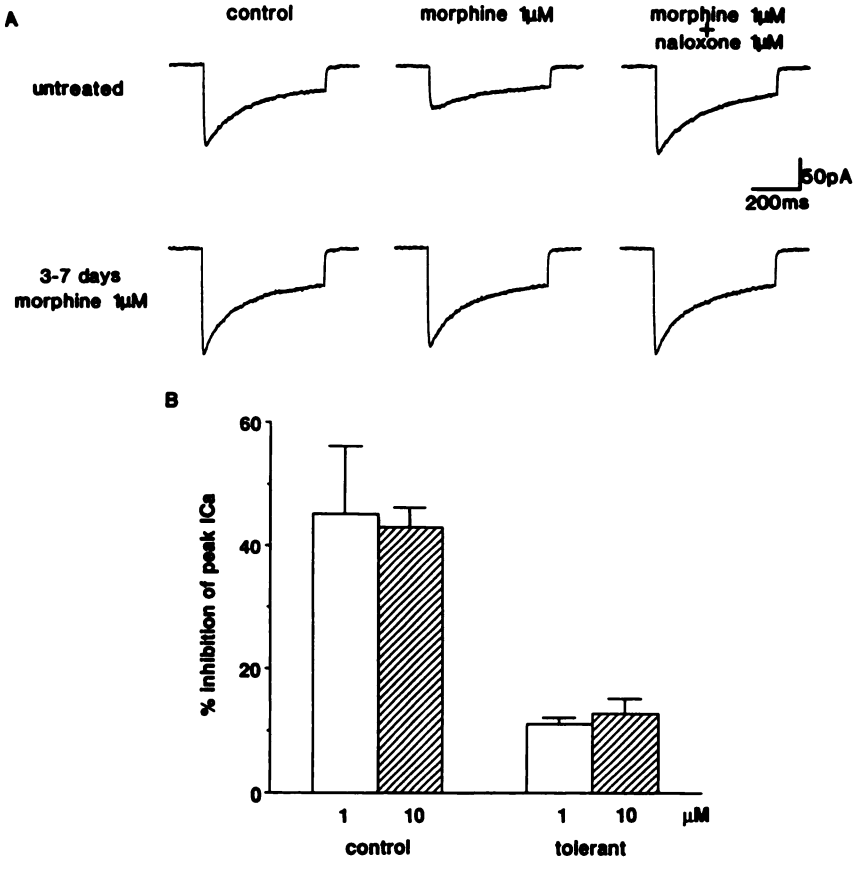

Fig. 1. Inhibition of I_{Ca} by morphine. A, Averaged whole-cell I_{Ca} in a single, morphine-naive cell (*upper traces*) and in another cell after incubation with morphine (1 μM) for 3–7 days (*lower traces*). I_{Ca} was evoked by stepping from a holding potential of −90 mV to +10 mV for 500 msec every 20 sec. Morphine (1 μM), when applied acutely, substantially inhibited I_{Ca} in the control cell but had much less effect in the chronically treated cell. In both cases the inhibitory action of morphine was reversed by naloxone (1 μM). B, Mean inhibition of I_{Ca} evoked by morphine (1 μM) (n = 6) (*open columns*); that evoked by morphine (10 μM) (n = 4) (*stripped columns*) in control, morphine-naive cells (*left*) and in chronically treated cells (*right*). *Vertical bars*, standard errors.

dition of the opioid receptor antagonist naloxone (1 μM). Increasing the concentration of morphine acutely applied to 10 μM produced no further inhibition (Fig. 1B).

The μ-opioid receptor-selective agonist DAMGO (1 nM to 1 μM) inhibited peak I_{Ca} in a concentration-dependent (Fig. 2) and naloxone-sensitive manner. The calculated IC_{50} was 11.8 nM, and the Hill slope was not significantly different from unity. The maximum inhibition elicited by DAMGO (1 μM) (47 ± 7%; $n = 6$) was not significantly different from that produced by morphine (1 μM) (45±11%; $n = 6$).

As well as depressing peak I_{Ca} amplitude, morphine and DAMGO also slowed the rate of rise of the current, as measured by the time taken to reach a peak. Thus, the time to peak was virtually doubled by maximally effective concentrations of morphine and DAMGO (Table 1).

Effects of chronic exposure to morphine. In these studies, in order to remove any residual acute effect of the morphine included in the tissue culture incubation media, recordings were obtained from chronically treated cells bathed in morphine-free superfusate. Under these conditions, the activity of morphine is reversed within 2–3 min. DAMGO and morphine then were acutely applied within 5 min of washout of the media.

The electrophysiological properties of I_{Ca} appeared to be unchanged by chronic (3–7 day) exposure to morphine. The mean amplitudes of peak I_{Ca} in control (87 ± 7 pA; range, 45–155 pA; $n = 26$) and chronically treated cells (100 ± 9 pA; range, 52–213 pA; $n = 23$) were not significantly different. The times to peak I_{Ca} (Table 1) and the decay of the current (Fig. 1A) during the voltage pulse were similar. When a full current-voltage curve was determined for I_{Ca}, it was identical in untreated and morphine-treated cells (Fig. 3).

Chronic exposure to morphine induced tolerance to DAMGO (Fig. 2). The DAMGO concentration-response curve was shifted to the right in a parallel manner, with a 7-fold decrease in sensitivity at the IC_{50} level but no change in the maximum inhibition. After chronic exposure to morphine, the inhibitory action of acutely applied morphine (1 μM) was also significantly reduced ($p < 0.05$), and increasing the concentration of acutely administered morphine to 10 μM elicited no greater significant inhibition of peak I_{Ca} (Fig. 1B). In two cells, further increasing the concentration of morphine to 100 μM inhibited peak I_{Ca} by only 30%. It was not viable to use higher concentrations of morphine. Thus, the maximum inhibition elicited by morphine appears to be reduced, whereas that of DAMGO is not. This indicates that morphine has a lower efficacy than DAMGO and that tolerance results from a functional reduction in the μ-opioid receptor reserve, probably by disruption of the receptor-effector pathway. Chronic exposure to morphine also induced tolerance to the inhibitory effect of morphine on the time to peak I_{Ca}, whereas the action of DAMGO was unaffected (Table 1).

Effect of NA on I_{Ca}. We next investigated whether morphine-induced tolerance was specific for the μ-opioid receptor,

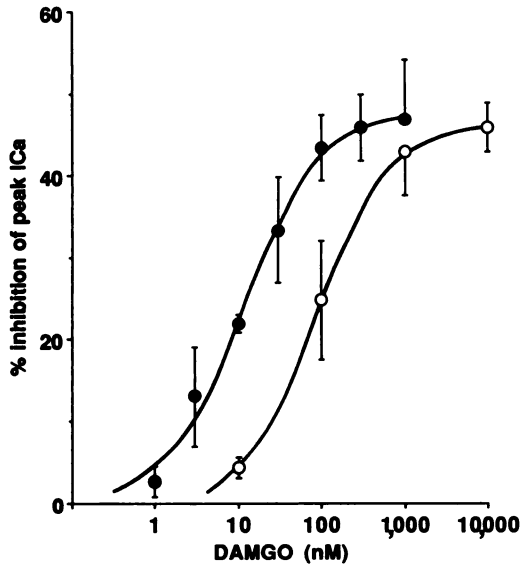

Fig. 2. Inhibition of I_{Ca} by DAMGO. Concentration-response relationships for DAMGO-induced inhibition of I_{Ca} in morphine-naive cells (●) and in cells chronically exposed to morphine (1 μM) for 3–7 days (○). The plots were obtained by pooling data from a number of cells (control, $n = 3$–14; tolerant, $n = 3$–5), and each *point* represents the mean ± standard error. *Solid lines*, best fit of the data to the Hill equation (control, Hill slope = 0.92, correlation coefficient > 0.99, IC_{50} = 11.8 nM; tolerant, Hill slope = 1.03, correlation coefficient > 0.99, IC_{50} = 85.5 nM). The control data are compiled from those of Ref. 9 and more recently obtained results.

TABLE 1

Modulation by opioids of time to peak I_{Ca}

Values are the mean ± standard error of data obtained from the number of cells shown in parentheses.

	Time to peak I_{Ca}
	msec
Untreated cells	
Control	13.8 ± 0.9 ($n = 12$)
+Morphine (1 μM)	25.5 ± 1.9 ($n = 6$)
+DAMGO (1 μM)	26.4 ± 2.0 ($n = 6$)
Morphine-tolerant cells	
Control	13.4 ± 0.7 ($n = 11$)
+Morphine (1 μM)	15.3 ± 1.5 ($n = 6$)
+DAMGO (10 μM)	26.3 ± 4.2 ($n = 5$)

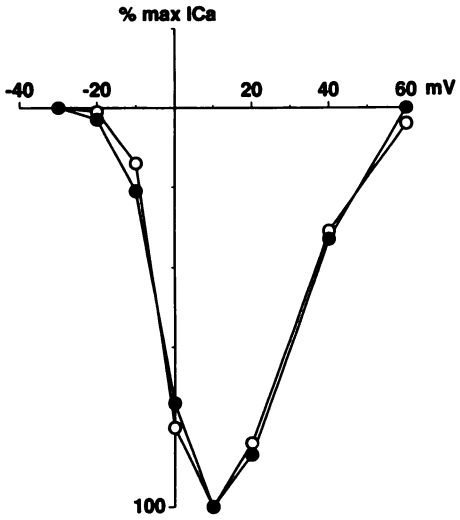

Fig. 3. Chronic exposure to morphine has no effect on the current-voltage relationship for I_{Ca}. The current-voltage relationships for I_{Ca} obtained from a morphine-naive cell (●) and a cell chronically exposed to morphine (○) are shown superimposed. The relationships were obtained by stepping from a holding potential of −90 mV to potentials between −30 mV and +60 mV, for 500 msec. The peak current elicited at each stepping potential is plotted as a percentage of the maximum peak current evoked by stepping to +10 mV, allowing direct comparison between the two cells. Similar results were obtained in a total of five cells.

i.e., homologous, or whether the inhibitory actions of other receptor types on I_{Ca} were also depressed, i.e., tolerance was heterologous. To do so we used NA as the test ligand, first in morphine-naive cells and then in cells chronically exposed to morphine. Functional α_2-adrenoceptors are present in SH-SY5Y cells (17, 18).

NA (10 nM to 10 μM) inhibited peak I_{Ca} in a concentration-dependent manner, and this action was antagonized by the α_2-adrenoceptor antagonist idazoxan (1 μM) (Fig. 4). When the data were fitted to the Hill equation, the IC_{50} for NA was 50.0 nM and the Hill slope was not significantly different from unity. The inhibition elicited by a maximally effective concentration of NA (10 μM) (45 ± 5%; $n = 3$) was not significantly different from that induced by DAMGO. Also, like the opioid agonists, NA (1 μM) substantially increased the time taken by I_{Ca} to reach a peak (23.5 ± 4.9 msec; $n = 4$).

These results show that NA and μ-opioid agonists both inhibit peak I_{Ca} by almost half. In five cells in which NA and DAMGO were coapplied, their effects were found to be non-additive. Fig. 5 shows a plot of peak I_{Ca} versus time in one of two control cells where DAMGO was administered first. A maximally effective concentration of DAMGO (1 μM) inhibited I_{Ca} by 57%, and this effect was maintained for the duration of administration. Subsequently, coadministration of a maximally effective concentration of NA (10 μM) evoked no further inhibition. The same lack of additivity was seen if the order of agonist administration was reversed. In an additional three cells, initial administration of NA (10 μM) inhibited peak I_{Ca} by 50 ± 10% and coadministration of NA (10 μM) and DAMGO (1 μM) inhibited the current by 51 ± 11%. Therefore, NA and DAMGO appear to inhibit the same population of calcium channels in SH-SY5Y cells.

Finally, we examined the effect of NA on I_{Ca} in morphine-treated cells. Fig. 4 shows that chronic exposure to morphine had no effect on the inhibitory action of NA (10 nM to 10 μM). Both the IC_{50} and the maximum inhibition were unchanged. Thus, morphine-induced tolerance appears to be specific or homologous for μ-opioid receptors in SH-SY5H cells and does not show cross-tolerance with the α_2-adrenoceptor.

Discussion

The results of the present study show that morphine, like the selective μ-opioid receptor agonist DAMGO, acutely inhib-

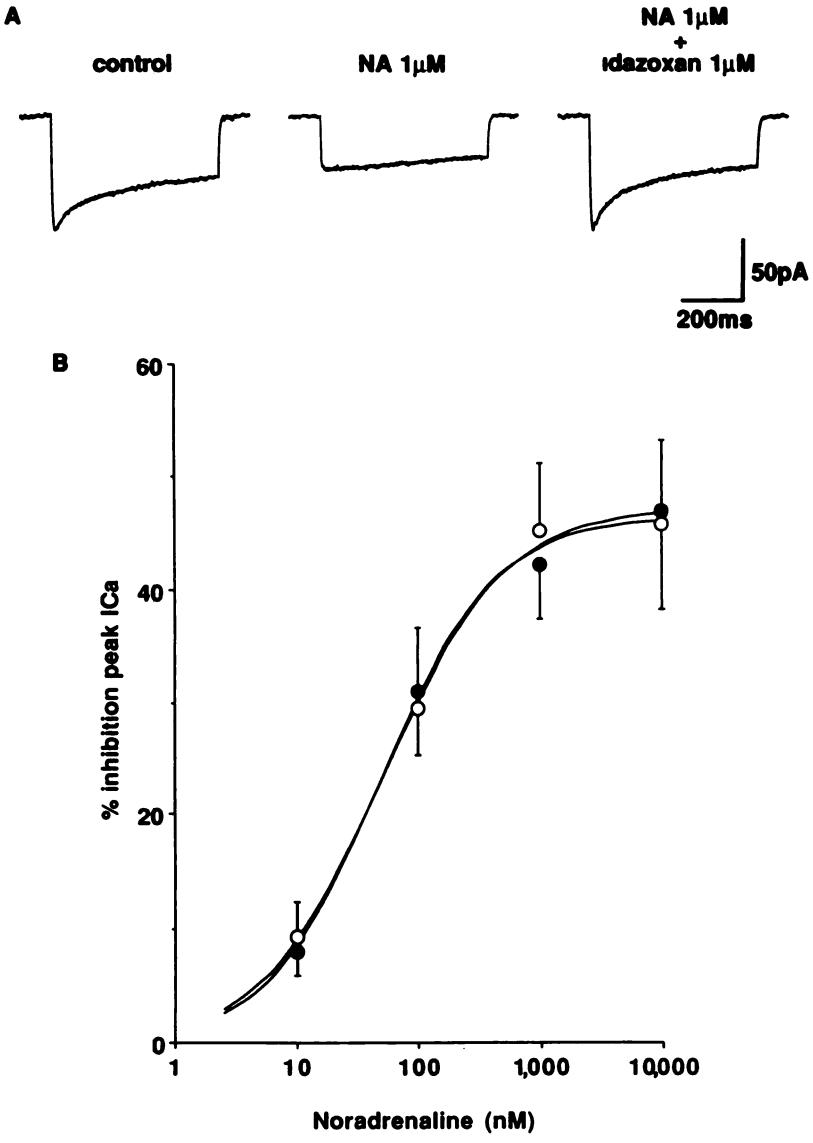

Fig. 4. Inhibition of I_{Ca} by NA. A, Averaged whole-cell I_{Ca} evoked in a single morphine-naive cell. I_{Ca} was evoked by stepping from a holding potential of −90 mV to +10 mV for 500 msec every 20 sec and was greatly inhibited by NA (1 μM). This effect was reversed by idazoxan (1 μM). B, Concentration-response relationship for inhibition of I_{Ca} by NA (10 nM to 10 μM) in morphine-naive cells (●) and cells chronically exposed to morphine (1 μM) (○). *Solid lines*, best fit of the results obtained from a number of cells (n = 3–5) to the Hill equation (control, Hill slope = 0.94, correlation coefficient > 0.99, IC_{50} = 50.0 nM; tolerant, Hill slope = 0.88, correlation coefficient > 0.99, IC_{50} = 51.4 nM). Each *point* represents the mean; *vertical bars*, standard errors.

its, in a naloxone-sensitive manner, the N-type I_{Ca} present in the human SH-SY5Y neuroblastoma cell line. Chronic exposure to morphine induced tolerance to these acute actions. Although the concentration-inhibition curve for DAMGO was shifted to the right, the maximum response to DAMGO was unchanged. In contrast, the maximum inhibition by morphine appeared to be reduced. This suggests that, although morphine and DAMGO both act as full agonists in control cells, morphine has a lower efficacy than DAMGO. Thus, in tolerant cells, where the spare receptor reserve has been reduced, the maximum inhibition in response to morphine, but not DAMGO, is depressed. Similar results have been reported for μ-opioid agonist-induced inhibition of adenylate cyclase in SH-SY5Y cells (4, 7) and activation of a potassium conductance in locus coeruleus neurons (19). Morphine has previously been shown to be of low efficacy, by study of the antagonist activity of morphine at the μ receptor in the rat vas deferens, a preparation with a very low μ receptor reserve (20).

How then does tolerance occur? This study shows that opioid tolerance can occur *in vitro* at the level of a single cell receiving no neuronal input. In contrast, induction of morphine tolerance *in vivo* may require on-going excitatory amino acid-mediated synaptic transmission (1). This difference may be explained if the excitatory neuronal input is involved in the learning processes that are important in the development of tolerance *in vivo*.

Inhibition of I_{Ca} in SH-SY5Y cells by μ-opioid receptors is mediated via a PTX-sensitive G protein (9). The identity of the G protein(s) is not yet known, but both G_{i1} and G_o are present in these cells (21). μ Agonists also inhibit adenylate cyclase via a PTX-sensitive G protein in SH-SY5Y cells (4), but the resultant decrease in intracellular cyclic AMP levels does not appear to lead to the decrease in I_{Ca} (12). Also, μ-opioid agonists do not increase the turnover of phosphatidylinositol in SH-SY5Y cells (22) or change intracellular cyclic GMP levels in the parent SK-N-SH cell line (23). Although a novel change in arachidonic acid metabolism cannot as yet be ruled out, the simplest intracellular mechanism that can be evoked to link μ-opioid receptor activation to calcium channel inhibition is a direct interaction between the activated G protein α subunit and the calcium channel.

Where then in this model does tolerance occur? The results reported here indicate no change in the biophysical properties of I_{Ca} after chronic exposure to morphine. Because NA and DAMGO appear to affect the same set of calcium channels and NA was able to inhibit I_{Ca} equally well in control and morphine-treated cells, there appears to be no change in the ability of activated α subunits of the G proteins to interact with the calcium channel. Finally, radioligand binding studies have shown no change in either B_{max} or K_D values for μ-opioid receptor ligands after chronic exposure of SH-SY5Y cells to morphine (7). Thus, the most likely explanation for morphine-induced tolerance in SH-SY5Y cells is a change in the coupling between the μ-opioid receptor and the G protein, rather than receptor down-regulation or a change in a subsequent component of the effector pathway.

A change in μ-opioid receptor/G protein coupling has also been suggested to underlie morphine tolerance seen in other studies. Chronic exposure to morphine leads to tolerance to μ-opioid agonist-induced activation of an inwardly rectifying potassium current in the noradrenergic neurons of the rat locus coeruleus but has no effect on α_2-induced activation of the same current (19). In the mouse 7315c tumor cell line, μ-opioid agonists inhibit adenylate cyclase, and incubation with morphine for 5 hr induces tolerance to this action (24). Binding studies show that this is associated with a decrease in receptor affinity but no change in μ-opioid receptor number, implying an impaired μ-opioid receptor/G protein interaction. Longer exposure to morphine did, however, cause receptor down-regulation.

Tolerance at the μ-opioid receptor may be due to a change in the receptor itself, and this would confer specificity or homology. In rat striatal membranes, phosphorylation of the μ-opioid receptor by cyclic AMP-activated protein kinase prevents the receptor from activating associated G proteins (25). Note, however, that if phosphorylation of the μ-opioid receptor does occur in the intact cell then the role of cyclic AMP-dependent protein kinase in this reaction is unclear, because μ-opioids inhibit rather than activate adenylate cyclase. Also, the tolerance seen in the present study is unlikely to be due to phosphorylation via protein kinase C, because differentiation of SH-SY5Y cells with a phorbol ester, phorbol 12-myristate

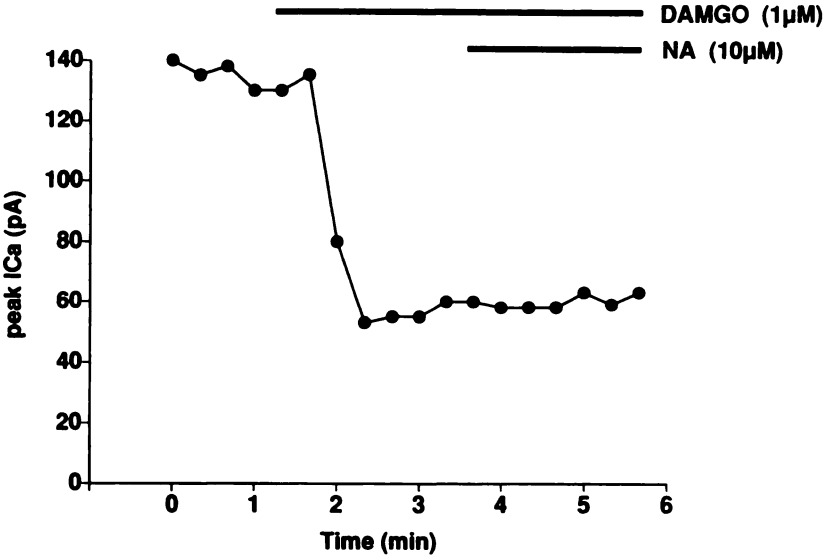

Fig. 5. The actions of DAMGO and NA are not additive. Plot of peak I_{Ca} amplitude versus time in a morphine-naive cell. I_{Ca} was evoked by stepping from a holding potential of −90 mV to +10 mV for 500msec every 20 sec. DAMGO (1 μM) and NA (10 μM) were applied where indicated by the *horizontal bars*.

13-acetate, did not depress the maximum inhibitory response of morphine on peak I_{Ca}.[4] Desensitization of the β-adrenoceptor has been shown to be due to phosphorylation by protein kinase A and β-adrenergic receptor kinase. The former is associated with heterologous desensitization and the latter with homologous desensitization (26). The role in morphine tolerance of a kinase analogous to β-adrenergic receptor kinase and active on the μ-opioid receptor remains to be elucidated.

Tolerance at the δ-opioid receptor has been extensively studied in the neuroblastoma-glioma NG108-15 hybrid cell line. In these cells, the δ-opioid receptor mediates inhibition of adenylate cyclase via G_i (27), as well as inhibition of I_{Ca} (28). Chronic exposure to the opioid etorphine induces homologous tolerance to the inhibition of adenylate cyclase, which is associated with a change in the coupling between the δ-opioid receptor and G_i, rather than a change in the number of δ-opioid receptors (29). Preincubation of cell membranes with an acid phosphatase mimics tolerance and potentiates δ-agonist-induced desensitization but has no effect on the number of δ-opioid receptors (30). This suggests that, in contrast to the β-adrenoceptor and perhaps the μ-opioid receptor, desensitization of the δ-opioid receptor is associated with dephosphorylation. Thus, the mechanism underlying homologous desensitization may vary depending on the receptor system under study.

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