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Correlation of the time course of development and decay of tolerance to morphine with alterations in sodium pump protein isoform abundance

Peng Li, Hercules T. Maguma, Kathleen Thayne, Barbara Davis, David A. Taylor *

Department of Pharmacology and Toxicology, The Brody School of Medicine at East Carolina University, The Brody Medical Sciences Building, 600 Moye Boulevard, Greenville, NC 27834, United States

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ABSTRACT

Since the heterologous tolerance that develops after chronic morphine administration has been proposed to be an adaptive process, it follows that the time course of the change in the cellular components should coincide with the time course of the altered responsiveness. This study correlated the time course over which heterologous tolerance develops with changes in the abundance of selected proteins in the guinea-pig longitudinal muscle/myenteric plexus (LM/MP) preparation. Tissues were obtained at various times following a single surgical implantation procedure and heterologous tolerance confirmed by a significant reduction in the sensitivity of the LM/MP to inhibition of neurogenic twitches by morphine, DAMGO, and 2-CADO. Tolerance developed with a delayed onset (significant 2–5-fold reduction in sensitivity by day 4 after pellet implantation) that reached a maximum by 7 days (4–8-fold reduction in responsiveness) that was maintained through 14 days with normal sensitivity spontaneously returning by 21 days post-implantation. Dot blot analysis was used to examine the abundance of the alpha$_1$ and alpha$_3$ subunit isoforms of the Na^+/K^+ ATPase and beta-actin over the same time course. The results showed significant decreases in abundance of the alpha$_3$ subunit at 4, 7, and 10 days following pellet implantation but no change in beta-actin or the alpha$_1$ subunit at any time period. These data support the idea that heterologous tolerance following chronic morphine exposure results from a cellular adaptive change that may involve a change in the abundance of the alpha$_3$ subunit isoform of the Na^+/K^+ ATPase.

1. Introduction

Tolerance and dependence are phenomena associated with long-term opioid use. While a number of cellular mechanism(s) and/or proteins have been suggested to be fundamentally involved in the phenomena, no clear singular entity has been identified as being responsible and it is generally agreed that both result from some form of cellular adaptation to acute chronic mu-opioid receptor activation [1,2,3,4,5]. Tolerance is a complicated phenomenon often studied at the cellular and molecular level using *in vitro* tissues from chronically treated animals. Chronic treatment with morphine has been accomplished using two primary procedures: pellet implantation and subcutaneous (s.c.) injection [6,7,8]. However, no studies have evaluated whether tolerance produced by pellet implantation differs from that produced by other methods of administration in the same species. Thus, one goal was to determine if the characteristics of tolerance were impacted by the method of chronic drug exposure.

The foundation of cellular adaptation suggests that neurons have the ability to undergo long-term compensatory changes that develop over time in response to the acute actions of an agonist. In order to fully define a cellular entity as part of an adaptive change responsible for the development of tolerance, certain criteria should be met [1,9]. These criteria were originally suggested to be as follows: "*The proposed cellular change must: 1) be induced by experimental procedures identical to those that induce tolerance and/or dependence; 2) follow a similar time course as the tolerance and/or dependence in that tissue; 3) quantitatively account for the tolerance and/or dependence; 4) account for the qualitative characteristics of tolerance and/or dependence; and 5) occur in the very cells upon which the opioid is acting*".

Opioid tolerance in the guinea-pig ileum (LM/MP) is accompanied by the development of non-specific supersensitivity to excitatory agonists [7,10] and non-specific sub-sensitivity to inhibitory agonists [11,12] utilizing different receptor and signaling pathways and has been termed "heterologous". The non-

Abbreviations: (Na^+-K^+ ATPase), Na^+-K^+ adenosine triphosphatase [EC 3.6.1.37]; (DAMGO), Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol; (2-CADO), 2-chloroadenosine; (LM/MP), longitudinal muscle/myenteric plexus.

* Corresponding author. Tel.: +1 252 744 2734; fax: +1 252 744 3203.
E-mail address: taylorda@ecu.edu (D.A. Taylor).

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specific super- and sub-sensitivity correlate with the observation that chronic opioid exposure leads to membrane depolarization in myenteric 'S' neurons without changes to either the threshold for action potential generation or the magnitude of the hyperpolarizing effect of morphine and 2-CADO [13,14]. These electrophysiological changes coincide with the decrease in the electrogenic contribution of the Na^+, K^+ pump to cellular membrane potential (15) and lead to an increased cellular excitability thus requiring lower concentrations of excitatory agonists to generate action potentials and higher concentrations of inhibitory agonists to reach the same level of inhibition. This membrane depolarization and decrease in Na^+/K^+ ATPase activity [15] is associated with a reduction in the abundance of the $alpha_3$ subunit isoform [16]. The $alpha_3$ subunit is the isoform that is specifically associated with neurons in the myenteric plexus [16]. Thus, the evidence suggests that in this model the decrease in pump activity, membrane depolarization, and heterologous tolerance may represent the same phenomenon [11,13,14,15]. Therefore, the only criterion not yet tested is the time course over which these changes occur.

The hypotheses of the present experiment were that (1) single pellet implantation procedure produces tolerance that is qualitatively and quantitatively similar to other methods of chronic morphine administration and, therefore, can be employed to define the time course over which tolerance develops and decays; (2) that heterologous tolerance that develops following chronic morphine exposure exhibits a delay in onset and a spontaneous, slow return to normal responsiveness; and (3) that down-regulation of the $alpha_3$ subunit isoform but not other proteins follows a time course of decay and return similar to that of the decrease in responsiveness. Since the reduced abundance of the $alpha_3$ subunit isoform of the protein appeared to coincide with a decrease in sodium pump activity that produces the membrane depolarization observed after 7 days of chronic exposure, this change in cell function may represent a compensatory alteration that accounts for characteristics of tolerance observed in the guinea-pig ileum.

2. Methods

2.1. Subjects

Adult albino guinea-pigs of either sex weighing 150–500 g (Charles River Laboratories, Inc., Wilmington, MA) were housed two per cage with free access to food and water. Animals were acclimated to the animal facility for 1-week prior to initiation of the treatment. All experimental procedures employing animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Brody School of Medicine at East Carolina University and were conducted in accordance with the guidelines for the humane use of animals in research (NIH "Guide for the Care and Use of Laboratory Animals" [Publication 80-23, revised 1996]). Every effort was made to reduce the use of animals to the minimum number required to achieve sufficient statistical power.

2.2. Drugs and chemicals

Morphine and placebo pellets were obtained from K. H. Davis (Research Triangle Institute, Research Triangle Park, NC) through NIDA. Each morphine pellet contained 75 mg morphine whereas a similar amount of lactose replaced morphine in the placebo pellets. Ketamine/xylazine (85 mg/ml ketamine; 15 mg/ml xylazine) was provided by the Department of Comparative Medicine at the Brody School of Medicine at East Carolina University. Morphine (morphine sulfate salt pentahydrate), DAMGO (Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol) and 2-CADO (2-chloroadenosine) were obtained from the Sigma Chemical Company (St. Louis, MO).

2.3. Procedures

2.3.1. Morphine pellet implantation

Guinea-pigs were anesthetized with ketamine/xylazine, administered i.p. at 0.07–0.1 ml/100 g of body weight and one morphine- or lactose-containing pellet per 100 gm of body weight was implanted subcutaneously in the flank. The animals were allowed to recover then returned to the animal facility until the day of the experiment. For the multiple pellet implantation procedure, additional surgeries were performed at 48 and 96 h after the initial surgery with morphine doses of 75 mg per 100 g body weight plus an additional 75 mg and 150 mg for the second and third surgeries, respectively.

2.3.2. Subcutaneous morphine injection

Parenteral administration of morphine was accomplished using two different schedules. The first was based upon a procedure employed by Mizutani et al., [8] to induce dependence in guinea-pigs which employed twice daily subcutaneous (s.c.) injections of morphine (10:00 a.m. and 10:00 p.m.) at doses that ascended from 10 to 100 mg/kg in the following sequence: day 1 (10 and 20 mg/kg); day 2 (40 and 60 mg/kg; day 3 (80 and 100 mg/kg); day 4 (100 mg/kg each injection). Tissues were obtained from animals exposed to morphine in this way at 9:00–11:00 a.m. (approximately 12 h after the last injection) on the 5th day after the initiation of treatment. A second, less aggressive, form of treatment was employed to compare the effects of chronic parenteral exposure over a time period similar to that studied after pellet implantation. This procedure also employed twice daily (10:00 a.m. and 10:00 p.m.) s.c. injections of ascending doses of morphine ranging from 10 to 80 mg/kg that escalated over a more gradual schedule of 10 mg/kg for day 1, 20 mg/kg for days 2 and 3, 40 mg/kg for days 4, 5, 6 and 80 mg/kg for day 7. As with the 4-day treatment procedure, the guinea-pigs were euthanized and tissues obtained approximately 12 h after the final injection.

2.3.3. Longitudinal smooth muscle-myenteric plexus (LM/MP) preparations

Preparations of LM/MP were obtained from placebo and treated guinea-pigs at various times after pellet implantation and the isolated LM/MP prepared as previously described [11,12]. Briefly, animals were euthanized by decapitation following isoflurane anesthesia and approximately 10 cm of ileum proximal to the ileo-caecal junction discarded and the LM/MP preparations obtained from the adjacent 10 cm of ileum. After removal, the ileum was not bathed in opioid to prevent development of acute tolerance due exposure to high concentrations of agonists over a short period of time (see discussion in Johnson and Fleming [17]). The segments of ileum were threaded onto a glass rod and the longitudinal smooth muscle gently teased from the circular muscle using cotton balls soaked in physiological saline. The resulting sheet of LM/MP was tied at each end with fine thread and passed through platinum-ring electrodes (approximately 3 mm in diameter) and placed in a 10 ml organ bath. Tissues were attached to a Grass FT.03 force transducer for recording isometric tension using an initial tension of 1 g. Isometric contractions were displayed and recorded on a computer through a 4 channel PowerLab system (AD Instruments, Colorado Springs, CO) consisting of a 4 channel A/D converter interface [Quad bridge] (AD Instruments, Colorado Springs, CO) and appropriate software (AD Instruments, PowerLab/Chart5). Neurogenic contractions were elicited via square wave electrical pulses (0.9 ms wide) using supramaximal voltage (50 V) generated by a Grass S48 stimulator and delivered to the platinum-ring electrodes at 10 s intervals through a Med Lab Attenuator and Stimu-splitter (Med-Lab Instruments, Loveland, CO). Tissues were maintained at 37 °C in physiological salt solution consisting of (in

mM): NaCl (117); KCl (4.7); $CaCl_2$ (2.5); KH_2PO_4 (1.2); $MgSO_4$ (1.2); $NaHCO_3$ (25) and glucose (11.5) and continuously bubbled with a mixture of 95% O_2/5% CO_2. Following an equilibration period of approximately 1 h, tissues were exposed to cumulatively increasing concentrations of inhibitory drugs (final bath concentrations of agonist ranging from 1 nM to 10 μM) and the effect of each concentration of each agonist on the amplitude of the neurogenic twitches determined and calculated as percent inhibition. Computer assisted analysis of each concentration–response curve using SigmaPlot® software (Systat Software, San Jose, CA) was employed to determine the concentration of agonist required to reduce the amplitude of the neurogenic contractions to 50% of the initial value (IC_{50}). Geometric mean IC_{50} values were calculated and used for comparison among treatment groups for each agonist as previously described [11].

2.3.4. Quantitative analysis of protein abundance

The LM/MP tissues used for these studies were obtained and snap frozen in liquid nitrogen. Tissues were stored at −80 °C until homogenization at which time the tissues were thawed, weighed and placed in ice-cold protease inhibitor buffer (PIB: [0.25 M sucrose, 10.0 mM EDTA, 4.08 mM phenylmethylsulfonyl fluoride {PMSF}, 1 mM 4-aminobenzamidine and 1 mg/ml bacitracin]) at a volume of 1 ml per 100 mg tissue wet weight and homogenized to completion using a ground glass homogenizer powered with PowerGen 125 (Fisher Scientific). The homogenate was transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 5 s and the supernatant then transferred to a fresh microcentrifuge tube and either used immediately or aliquoted and stored at −80 °C until it was used for analysis.

Spectrophotometric protein determination was made based on methods previously described [18]. A standard curve was generated with dilutions of bovine serum albumin (Sigma Chemical Company, St. Louis, MO). Tissue homogenates or standards (5 μl) were added to phosphate buffered saline solution (995 μl) and absorbance measured in duplicate using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) at wavelengths of 224 and 233 nm. The change in absorbance between the two wavelengths was calculated and the protein concentrations of the samples were determined based on the standard curve using the computer program Prism (GraphPad Software, Inc., San Diego, CA).

Preliminary Western blot experiments were performed to verify the specificity of the primary antibodies employed and to determine the appropriate range of protein concentrations of the LM/MP to employ in the dot blot experiments. The procedure for Western blotting was similar to that previously described by our laboratory for this tissue [16]. Homogenates ranging from 10 to 50 μg/ml of protein were loaded on to 10% precast Tris–HCl ReadyGels (Bio-Rad Laboratories, Inc., Hercules, CA) and size fractionated via electrophoresis at 110 V using a Mini-PROTEAN II Cell (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were then transferred to nitrocellulose membranes presoaked in transfer buffer using a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA). The membrane was removed from the gel and allowed to dry completely. The dried membrane was prehybridized in 3.0% blocker grade non-fat dried milk in phosphate buffered saline–0.1% Tween-20 (PBS-T: 137 mM NaCl; 2.7 mM KCl; 10.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4)/0.1% Tween) for 4 h. The membrane was washed with PBS-T and incubated overnight with the appropriate dilution of primary antibody. Primary antibodies against the α subunits of the Na^+/K^+ ATPase were obtained from Affinity Bioreagents, Inc. (Golden, CO) and antibodies to beta-actin were obtained from Cell Signaling Technology, Inc. (Danvers, MA). After incubation with the primary antibody, blots were washed with PBS-T and incubated with the appropriate secondary antibody (Goat anti-mouse or goat anti-rabbit HRP conjugated) obtained from Bio-Rad Laboratories, Inc. (Hercules, CA) for 1 h. The final blot was washed in PBS-T to remove any excess secondary antibody before detection. Detection of the immobilized antigens/antibodies was accomplished using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) and the VersaDoc imaging system (Bio-Rad Laboratories, Inc., Hercules, CA) for enhanced chemiluminescence. The results of these studies revealed that the antibodies employed exhibited a very high specificity for the proteins being examined and that appropriate dilutions could be obtained without pooling tissues suggesting that their use in the dot blot analysis would be appropriate.

The dot blot procedure was modified based on the slot blot technique previously described for quantitative protein abundance comparison [16,19]. Serial dilutions of protein samples (three consecutive 50% serial dilutions were made with initial protein concentration of 10 μg/200 μl for beta-actin, the $alpha_1$ subunit isoform of the Na^+/K^+ ATPase, and 30 μg/200 μl for the $alpha_3$ subunit isoform of the Na^+/K^+ ATPase) from each of the designated time periods of the time course experiment (1, 4, 7, 10, 14, and 21 days) were directly loaded as duplicates onto a nitrocellulose membrane by vacuum filtration using a 96 well Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). After the vacuum filtration, the membrane was allowed time to dry completely and the dried membrane prehybridized with 3.0% blocker grade non-fat dried milk in PBS-T, incubated in primary antibody and secondary antibody in the same manner as that previously described for western blotting and imaged using the Versadoc Imaging System. The averaged densitometric value from the row expected to possess the lowest concentration of the protein of interest was used to normalize the densitometric value of the other wells on the same membrane to produce a value defined as a relative densitometric unit (RDU). As predicted the RDU values decline with lower total protein amounts and RDU versus protein concentration curves were generated for each of the designated time periods of the time course experiment for both morphine and placebo treatments. The RDU value for the average protein concentration was calculated for each curve (similar to estimating the IC_{50} on a concentration–response curve) and used as an estimate of protein abundance for statistical comparison between morphine and placebo treatments.

2.3.5. Data analysis

Statistical differences between treatment groups were determined using one-way ANOVA followed by Tukey's post hoc test. Sample groups that failed either the normality test or the equal variance test were analyzed using one-way ANOVA on ranks followed by Dunn's post hoc test. Mean ratio of IC_{50} values were determined by calculating the ratio of each individual IC_{50} value to the mean IC_{50} value for placebo treated animals. The mean ratio values obtained were compared using Student's "*t*" test. The numerical differences were considered to be statistically significant when $p \leq 0.05$.

3. Results

3.1. Impact of method of treatment

Since chronic treatment with morphine is associated with a reduced rate of weight gain, the effect of treatment on animal weight was monitored through the course of the different treatment procedures by comparing the weight of the animal at the time of the experiment to the weight at the time of initiation of the treatment (Fig. 1). The rate of weight gain was not significantly different among any of the animal groups treated with either

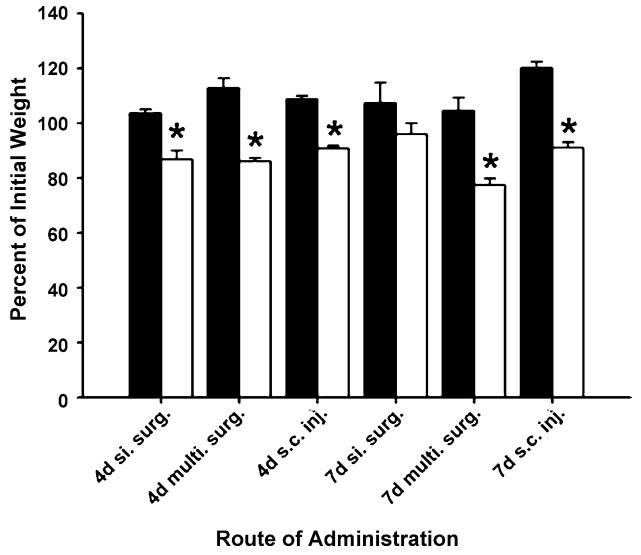

Fig. 1. The effect of different methods of chronic morphine treatment on body weight. The final weight of the guinea-pigs was determined on the day of experiment and is presented as a percentage of the animal's initial weight at the beginning of the treatment. Guinea-pigs received either placebo (■) or morphine (□) treatment by single or multiple surgical pellet implantations or by twice daily subcutaneous injection. Statistically significant differences are identified with *($p \leq 0.05$). The *N* values for the experimental sets are between 4 and 9.

placebo pellet implantation or by injection and ranged between 5% and 20% increases over their initial weight. In contrast, animals treated with morphine displayed a significantly lower rate of weight gain which actually led to significant reductions from their initial weights for all groups except animals exposed to morphine via single pellet implantation and tested 7 days after the surgery. As shown in Fig. 1, animals exposed to morphine via multiple escalating implantations lost the largest percentage of their initial body weight (>23%). However, animals surgically implanted only once lost only approximately 5% of their body weight at 7 days (Fig. 1) while guinea-pigs exposed to morphine via either parenteral administration method lost nearly 10% of their initial body weight (Fig. 1).

Exposure to morphine for 4 days following a single surgical implantation procedure produced a 2–3-fold rightward shift of the concentration–response curve of either DAMGO or 2-CADO (Table 1) that modestly increased to 4–8-fold to both agonists after 7 days (Table 1). The multiple surgery pellet implantation protocol produced a 2–3-fold reduction in responsiveness at 7 days (Table 1). However, after 4 days (Table 1) and within 24 h after the second surgical implantation procedure, the rightward shift of the concentration–response curve for DAMGO was somewhat greater (6-fold) than the shift observed to the effects of 2-CADO (2–3-fold) but was not statistically significant. Chronic exposure by subcutaneous injection produced a 2–3-fold reduction in responsiveness of LM/MP preparations to both agonists after 4 days of treatment that increased to 3–4-fold after 7 days of treatment (Table 1). While chronic exposure did lead to significant alterations in the geometric mean IC_{50} values, there were no significant changes in the calculated mean ratio of IC_{50} values among all of the treatment paradigms (Table 1). Thus, despite using different methods and time periods to chronically expose guinea-pigs to morphine, LM/MP preparations obtained from the animals displayed a significant and quantitatively similar reduction in responsiveness to the inhibitory effects of both DAMGO and 2-CADO based upon the ratio of the geometric mean IC_{50} values (Table 1). Therefore, the data suggest that the nature of tolerance produced by these different methods of chronic exposure is quantitatively and qualitatively similar indicating that evaluation of the time course could be effectively performed by following the characteristics of tolerance development over time after a single pellet implantation procedure.

3.2. Time course of sensitivity changes following implantation

The time course over which heterologous tolerance develops to the inhibitory effects of the agonists following a single surgical pellet implantation was determined using LM/MP preparations obtained at various times following implantation. The onset of tolerance was delayed to all agonists as there was no reduced responsiveness observed to the inhibitory effects of morphine (Table 2; Fig. 2), DAMGO (Table 2; Fig. 2), and 2-CADO (Table 2; Fig. 2) 24 h after the pellet implantation procedure. However, by 4 days after the pellet implantation procedure, a significant rightward shift of the concentration–response curve with no reduction in maximum response was observed to all agonists (Table 2; Fig. 3). The magnitude of the reduction in responsiveness at 4 days ranged between 2 and 5-fold rightward shifts of the concentration–response curves that were not statistically significantly different among the agonists (Table 2). The development of tolerance to all agonists reached its maximum 4–8-fold rightward shift by 7 days after pellet implantation and that same relative magnitude of shift in the concentration–response curves was observed at both 10 and 14 days (Table 2; Fig. 4) following a single surgical pellet implantation procedure. The reduction in responsiveness to all agonists disappeared by 21 days following the pellet implantation procedure (Table 2; Fig. 5) with nearly full recovery of sensitivity to all agonists in the tissues. Based upon the geometric mean IC_{50} values (Table 2; Fig. 6), the time course for the development of tolerance following chronic morphine

Table 1
Geometric mean IC_{50} values for agonist-induced inhibition of neurogenic twitches in LM/MP preparations from animals treated with morphine by different routes of administration.

Treatment	Agonist					
	DAMGO		Mean ratio of IC_{50}	CADO		Mean ratio of IC_{50}
	Placebo	Morphine		Placebo	Morphine	
4-day single surgery	7.59 (±0.13)	7.22* (±0.11)	2.3 (±1.1)	7.12 (±0.06)	6.61* (±0.12)	3.2 (±1.0)
4-day multiple surgery	7.59 (±0.14)	6.81* (±0.18)	6.0 (±4.7)	6.98 (±0.08)	6.56* (±0.13)	2.6 (±1.1)
4-day s.c. injection	7.47 (±0.14)	7.13* (±0.09)	2.2 (±0.6)	7.00 (±0.11)	6.55* (±0.09)	2.9 (±1.1)
7-day single surgery	7.52 (±0.09)	6.93* (±0.11)	3.9 (±1.6)	7.13 (±0.05)	6.24* (±0.07)	7.6 (±2.4)
7-day multiple surgery	7.15 (±0.10)	6.72* (±0.09)	2.6 (±0.9)	6.91 (±0.08)	6.64* (±0.09)	1.9 (±0.7)
7-day s.c. injection	7.16 (±0.09)	6.61* (±0.13)	3.5 (±1.4)	7.22 (±0.09)	6.65* (±0.07)	3.7 (±0.8)

Geometric mean IC_{50} values are displayed as the $-\log M$ (±S.E.M.) value of the concentration of agonist that reduced neurogenic twitch amplitude to 50% of its original value. Statistically significant differences versus placebo are identified by *($p \leq 0.05$). None of the DAMGO mean ratio of IC_{50} values differed significantly compared to those for CADO. The *N* values for the experimental sets are between 4 and 9.

Table 2
Geometric mean IC_{50} values for the inhibitory effect of Morphine, DAMGO and 2-Chloroadenosine at various times following a single pellet implantation procedure.

Days after implantation procedure	Agonist								
	Morphine IC_{50} Values			DAMGO IC_{50} values			2-CADO IC_{50} values		
	Placebo	Morphine	Mean ratio	Placebo	Morphine	Mean ratio	Placebo	Morphine	Mean ratio
1	7.32 (±0.07)	7.21 (±0.13)	1.3 (±0.8)	7.57 (±0.16)	7.51 (±0.14)	1.1 (±1.0)	7.04 (±0.05)	6.96 (±0.11)	1.2 (±0.8)
4	7.24 (±0.17)	6.59* (±0.12)	4.5 (±1.4)	7.59 (±0.13)	7.22* (±0.11)	2.3 (±1.1)	7.12 (±0.06)	6.61* (±0.12)	3.2 (±1.1)
7	7.28 (±0.07)	6.54* (±0.11)	5.5 (±4.4)	7.52 (±0.09)	6.93* (±0.11)	3.9 (±1.6)	7.13 (±0.05)	6.24* (±0.07)	7.6 (±2.4)
10	7.24 (±0.12)	6.57* (±0.08)	4.7 (±1.2)	7.64 (±0.10)	6.99* (±0.10)	4.4 (±2.2)	6.96 (±0.10)	6.21* (±0.09)	5.7 (±1.6)
14	7.31 (±0.07)	6.55* (±0.08)	5.7 (±1.1)	7.45 (±0.09)	6.91* (±0.07)	3.5 (±0.6)	6.96 (±0.06)	6.13* (±0.08)	6.7 (±2.0)
21	7.32 (±0.20)	7.08 (±0.08)	1.7 (±0.5)	7.27 (±0.10)	7.08 (±0.12)	1.6 (±0.8)	6.97 (±0.09)	6.83 (±0.10)	1.4 (±0.7)

The geometric mean IC_{50} values for agonists (the agonist concentration required to reduce the amplitude of the neurogenic twitch of the LM/MP to 50% of its initial value) are displayed as $-\log M$ (± S.E.M.). Statistical significance between morphine and placebo treatments are identified by *($p \leq 0.05$). The *N* values for the experimental sets are between 4 and 12.

exposure via a single pellet implantation not only suggests that tolerance following a single exposure develops slowly (4-day delay) and reaches a maximum level by 7 days after implantation but also that the level of tolerance is maintained for a period of over 10 days (from days 4 to 14) which is consistent with development of heterologous tolerance in the guinea-pig LM/MP being a gradual adaptive process (Fig. 6). The data also suggest that the changes that account for the development of tolerance will spontaneously revert; further supporting the idea that the underlying mechanism involves some adaptive change in cell function.

3.3. Time course of changes in sodium pump isoforms

Another series of experiments used dot blot analysis to compare the abundance of three proteins (the $alpha_1$ and $alpha_3$ subunit isoforms of the Na^+/K^+ ATPase, and beta-actin) in homogenates of the guinea-pig LM/MP tissues obtained from the same animals employed in the functional responsiveness studies. As expected, there was no significant change in abundance of either the $alpha_1$ subunit isoform of the Na^+/K^+ ATPase (Fig. 7A) or beta-actin (Fig. 7B) at any time period after chronic morphine exposure. In contrast, a significant decrease in the abundance of the $alpha_3$ subunit isoform of the Na^+/K^+ ATPase was observed at 4, 7, and 10 days but not at 1, 14 or 21 days after pellet implantation (Fig. 7C) as illustrated in the typical dot blot provided (Fig. 7D). Previous studies have shown that chronic morphine exposure does not alter the $alpha_1$ subunit isoform abundance at 7 days after implantation [16]. Since the bulk of the contents of the tissue homogenate is smooth muscle and the $alpha_1$ subunit isoform is predominately expressed in muscle [16] the abundance of this protein should approximate the total protein concentration which did not change significantly. The idea that the significant reduction in the $alpha_3$ subunit occurred without a significant change in total protein is further reinforced by the observation that beta-actin abundance was also not altered at any time period after morphine pellet implantation (Fig. 7B). Thus, it appears that the time course for the decline in the abundance of the $alpha_3$ subunit isoform of the Na^+/K^+ ATPase closely mimics (with exception at 14 days after pellet implantation) the time course for the development of functional tolerance observed in the LM/MP while other proteins whose abundance was measure remained unaltered.

4. Discussion

The development and characteristics of tolerance were compared using different methods of chronic morphine treatment (1) the extensively used pellet implantation method (single and multiple surgeries); and (2) two parenteral protocols that included a 4-day schedule shown to induce dependence in the guinea-pig [8] and a 7-day schedule that corresponded more closely to the time frame of the implantation studies. However, the characteristics of tolerance were similar regardless of the method of exposure (Table 1). Furthermore, the data suggest that a more accurate evaluation of the decay component of tolerance can be accomplished using the injection schedule compared to pellet implantation since the latter provides an opportunity to stop treatment and avoid the other complicating components such as the inflammatory process and additional surgeries associated with removing implanted pellets. Significant weight loss was observed in morphine-treated guinea-pigs versus controls regardless of route of administration except in animals sacrificed 7 days after a single implantation procedure. Thus, the results confirmed our hypothesis that qualitatively and quantitatively similar heterologous (to non-opioid agonists like 2-CADO) and homologous (to opioid agonists like morphine and DAMGO) tolerance develops regardless of the method of administration and suggested that the least physiological insult to the animal occurs following a single pellet implantation procedure in animals permitted to recover for more than 4 days.

The LM/MP has been used by many investigators as a reliable model system to evaluate the characteristics [6,7,11] and cellular mechanisms of tolerance [13,14,15,20,21]. The fact that the tolerance that develops after chronic opioid exposure extends to many unrelated agonists has been demonstrated in the early efforts to describe the utility of this model system to study the functional changes that occur after chronic exposure either *in vivo* or *in vitro* [6,7,10,22,23]. Many studies have been conducted in tissues obtained 7 days after a single implantation procedure but few studies have purposefully addressed the time course over which heterologous tolerance develops. The results of our experiments evaluating the time course confirmed earlier work that no significant reduction in tissue responsiveness is present 24 h after pellet implantation but that tolerance appeared to all agonists and became maximal between 4 and 7 days [6,12,17] and lasted for between 14 and 21 days. These data verify that the 7-day time period is an appropriate time period to investigate potential mechanisms since this time period displayed maximal changes in responsiveness to all agonists. However, the data provide the first longitudinal evaluation of the time course over which tolerance develops and decays as an effort to establish a framework for determining the potential contribution of many other proteins to the phenomenon.

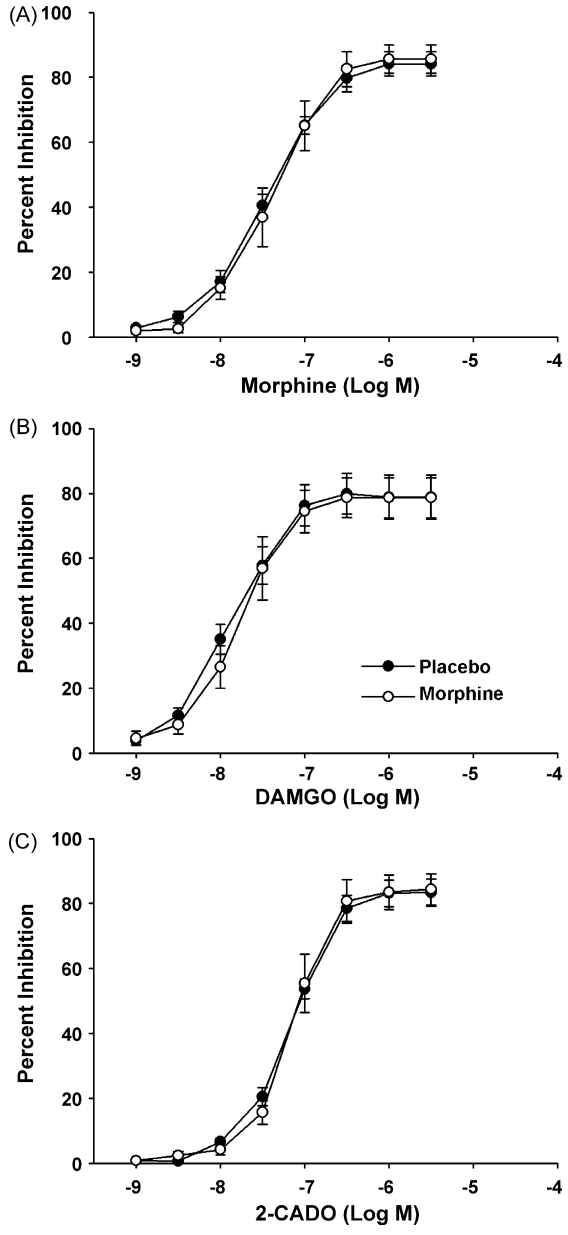

Fig. 2. Concentration–response curves for the inhibitory effects of (A) morphine, (B) DAMGO, and (C) 2-CADO on the neurogenic twitches of the guinea-pig LM/MP 1-day following surgical pellet implantation. Tissues were obtained from guinea-pigs treated with single surgical implantation of either morphine (○) or placebo (●) pellets on day 0.

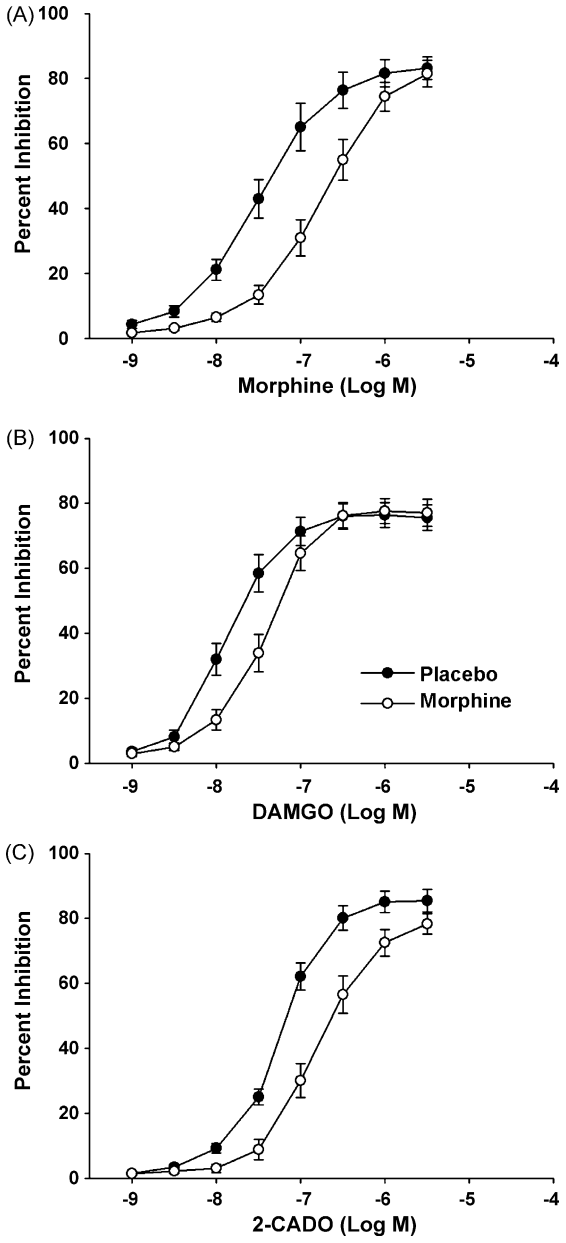

Fig. 3. Concentration–response curves for the inhibitory effects of (A) morphine, (B) DAMGO, and (C) 2-CADO on the neurogenic twitches of the guinea-pig LM/MP 4 days following surgical pellet implantation. Tissues were obtained from guinea-pigs treated with single surgical implantation of either morphine (○) or placebo (●) pellets on day 0.

The results reaffirmed the hypothesis that tolerance is an adaptive response since it developed following a defined latency to onset and maximum, and spontaneously reversed. Evidence that changes in basic cellular physiology may be an underlying mechanism of tolerance stem from the development of reduced responsiveness to both opioids (e.g., morphine and DAMGO) and 2-CADO that followed a similar time course of onset and decay. Since neurogenic twitch inhibition by 2-CADO involves hyperpolarization of myenteric 'S' neurons without an alteration in input resistance [14], these data support the idea that the mechanism by which cellular adaptation develops must occur beyond the mu-opioid receptor. The adaptive nature of tolerance is also supported by the relationship between the pharmacokinetics of implanted morphine pellets and the time course for tolerance development. Pellet-implanted rats showed a peak in morphine plasma concentration within 4–6 h after implantation which correlates to an initial "dumping" phase [24] when as much as 25% of the total morphine is absorbed within the first 24 h [6]. The maximum antinociceptive effect of morphine occurred during this peak concentration and gradually disappeared after about 36 h [24] during the secondary phase when plasma concentrations declined. In the present study, no tolerance was observed 24 h after implantation (a time when plasma morphine concentration should peak) but heterologous tolerance was observed at day 4 (a time period that surpassed the 36 h time frame over which effective antinociceptive concentrations of morphine were observed). Furthermore, tolerance lasted beyond day 14 which far exceeds the time period during which significant residual morphine should have been present. While plasma levels of morphine should clearly be below the level of detection at this time, the possibility that other factors may contribute to the maintenance of tolerance cannot be ruled out. For example, it is possible that inflammatory processes triggered by the presence of the pellets

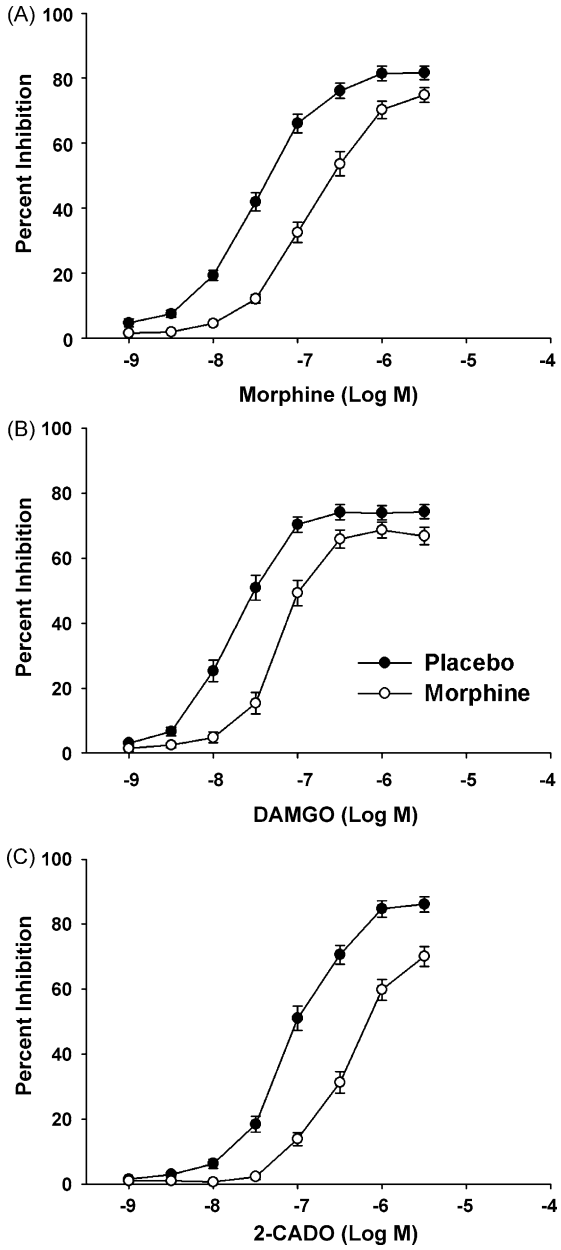

Fig. 4. Concentration–response curves for the inhibitory effects of (A) morphine, (B) DAMGO, and (C) 2-CADO on the neurogenic twitches of the guinea-pig LM/MP 14 days following surgical pellet implantation. Tissues were obtained from guinea-pigs treated with single surgical implantation of either morphine (○) or placebo (●) pellets on day 0.

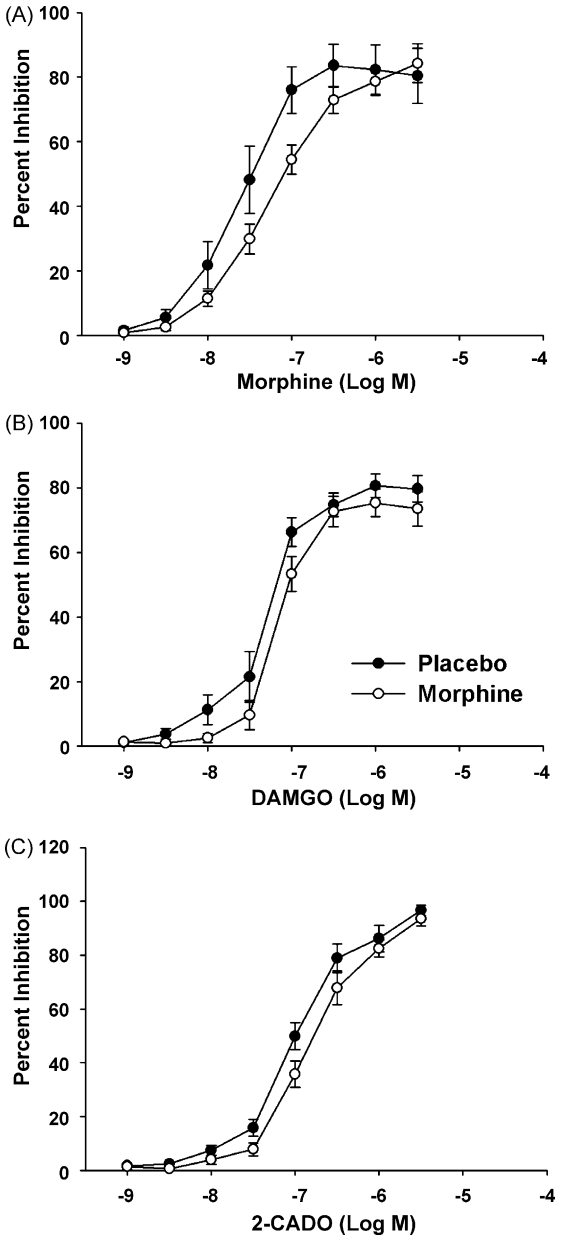

Fig. 5. Concentration–response curves for the inhibitory effects of (A) morphine, (B) DAMGO, and (C) 2-CADO on the neurogenic twitches of the guinea-pig LM/MP 21 days following surgical pellet implantation. Tissues were obtained from guinea-pigs treated with single surgical implantation of either morphine (○) or placebo (●) pellets on day 0.

could activate different signaling pathways that modify in cell function in a way that permits the reduced responsiveness to remain. Such a potential contributor could now be evaluated effectively using an injection schedule since the data indicate that the characteristics of tolerance in this model system appear to be comparable using this method of administration which should be less likely to provoke inflammatory cascades. Nevertheless, the results do indicate that quantitatively and qualitatively similar heterologous tolerance develops, is maintained and spontaneously reverses at times during which levels of agonist would be expected to be below effective levels which further supports the idea that adaptive changes in cell function underlie the development of tolerance.

The time course study is the first directed effort to develop a longitudinal model that would leverage the fact that this is a compensatory process to make it possible to begin identifying the time-based cellular changes associated with development of tolerance during and after chronic opioid exposure. The cellular effectors and characteristics that have been proposed as potential contributors to tolerance in this tissue include G-proteins [4,21], the adenylyl cyclase system [25,26], protein kinase A (PKA) [27], protein kinase C (PKC) [20,28], ERK1/2 phosphorylation [29], membrane potential [13,14], and the abundance of the alpha$_3$ subunit of the Na^+/K^+ ATPase [16,30]. Most of these changes were investigated at a single time period after exposure (i.e. 7 days) which coincided with the maximum magnitude of loss of responsiveness; thus, the time course provides important novel information related to the ability to define changes in cell signaling pathways and molecules that are integral to the adaptation process as opposed to those that are coincident to the process since the

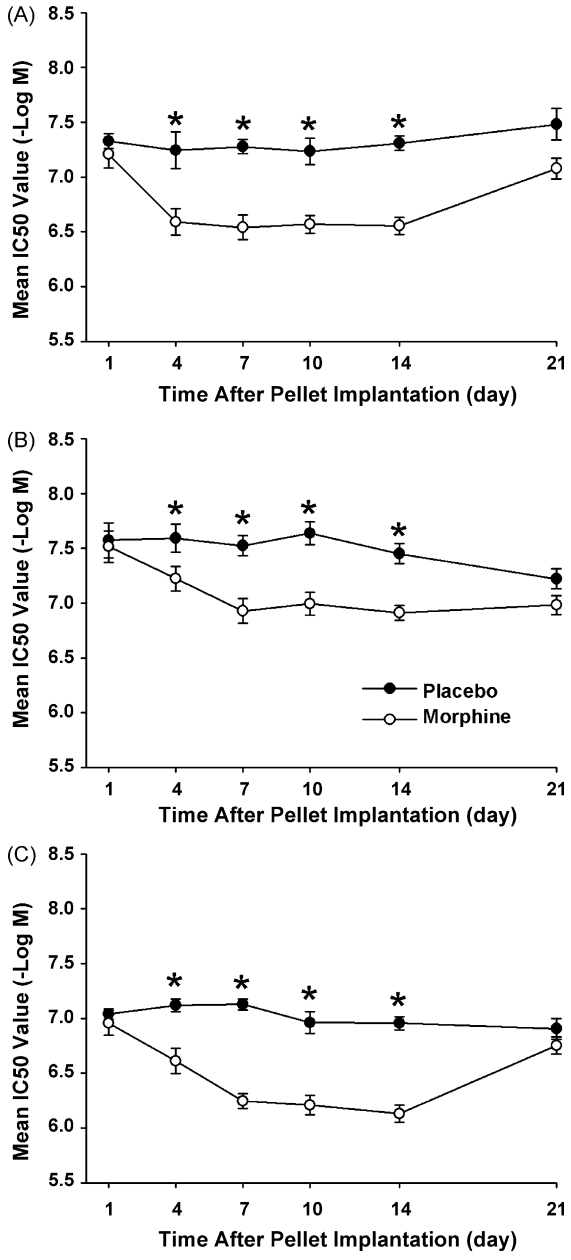

Fig. 6. The time course for the change in geometric mean IC_{50} values for inhibition of neurogenic twitches in the guinea-pig LM/MP produced by (A) morphine, (B) DAMGO, and (C) CADO (see Table 2). Guinea-pigs received either morphine (○) or placebo (●) treatment via a single surgical pellet implantation procedure on day 0. Statistically significant differences between IC_{50} values are identified with *($p \leq 0.05$).

contributing effectors should change over a time course similar to the reduction of functional responsiveness.

The protein analysis confirmed earlier studies demonstrating a decrease in the abundance of the $alpha_3$ subunit isoform 7 days after implantation [16,30]. However, the earlier studies only examined one time period (i.e. 7 days) after implantation. This downregulation has been suggested to play a critical role in the decreasing activity of the sodium pump which leads to membrane depolarization and the appearance of heterologous tolerance in the guinea-pig ileum that was observed 7 days after implantation [16]. The results also demonstrated that abundance of this protein declined and resolved over a time course analogous to the development and disappearance of heterologous tolerance. Unlike the $alpha_3$ subunit isoform, the abundance of the $alpha_1$ subunit isoform and beta-actin was unaffected by chronic morphine exposure. These data confirmed earlier studies indicating that levels of the $alpha_1$ subunit isoform were unchanged 7 days after implantation [16] and also extended that result to include all other time periods over which tissues were evaluated. The data also demonstrated that opioid exposure does not appear to produce a significant change in overall protein expression since the levels of beta-actin and the $alpha_1$ subunit were not substantially modified during the time course. A similar observation regarding the development of "heterologous tolerance" and concurrent depolarization due to an apparent reduction in the $alpha_3$ subunit isoform has been reported for neurons of the nucleus tractus solitarius (nTS) [31] and the nucleus locus ceruleus (LC) of the guinea-pig [32]. Thus, it appears that tolerance can develop in more than one neuron in a circuit if opioid receptors are present and activated and that the specificity of the change in responsiveness can be used as a gauge to predict the nature of the cellular changes involved. Chronic depression of cellular activity in both smooth muscle and neurons leads to compensatory changes in responsiveness characterized by supersensitivity to excitatory agonists and subsensitivity to inhibitory agonists [33]. Therefore, neurons in the CNS as well as the myenteric plexus should exhibit comparable generalized changes in cell excitability that may possess a similar underlying basis. The modulation of protein transcription and translation is well recognized as a mechanism of compensation and the data provided from these studies suggests that such changes in protein abundance can be directed toward proteins that cause generalized changes in cell excitability in neurons of the myenteric plexus as well as neurons in the CNS.

Interestingly, the abundance of the $alpha_3$ subunit returned to control levels by day 14 post-implantation even though significant functional tolerance was still observed. Two possible explanations could account for this observation. The Na^+/K^+ ATPase consists of an alpha and a beta subunit, with the former responsible for the catalytic activities and the latter facilitating formation of the heterodimer and transport of the enzyme to the plasma membrane [34]. It is possible that the $alpha_3$ subunit is expressed at normal levels but the protein is not fully functional because morphine exposure also affected beta subunit abundance which was not evaluated in these studies. An alternative explanation is that protein abundance was restored to normal levels but Na^+/K^+ ATPase enzyme activity was inhibited by other cellular processes; chronic opioid administration leads to PKC activation [20,35,36] which phosphorylates several other intracellular proteins. Phosphorylation modulates the expression and activity of many proteins and PKA and PKC have both been reported to directly inhibit Na^+/K^+ ATPase through phosphorylation of the alpha subunit [37]. Prolonged opioid exposure leads to the phenomenon of "adenylyl cyclase superactivation" where cAMP levels and PKA activity are substantially increased due to a shift in mu-opioid receptor coupling from $G_{\alpha i}$-dependent to $G_{\alpha s}$-dependent signaling [4,5,21,25]. Thus, the increased activity of intracellular kinases could phosphorylate the Na^+/K^+ ATPase either directly or indirectly leading to reduced activity but normal levels.

In summary, the data in the present study provide important evidence related to the cellular mechanism underlying the development of tolerance in the guinea-pig LM/MP. The results indicate that the method of exposure does not influence the heterologous nature of tolerance. The results also suggest that, using the LM/MP as a model system, one time pellet implantation can produce qualitatively and quantitatively comparable heterologous tolerance as other methods of exposure while appearing to cause less change in body weight. Our results support the concept that tolerance following chronic morphine exposure results from cellular adaptive changes and provide the first longitudinal analysis of tolerance in this animal and tissue. The heterologous

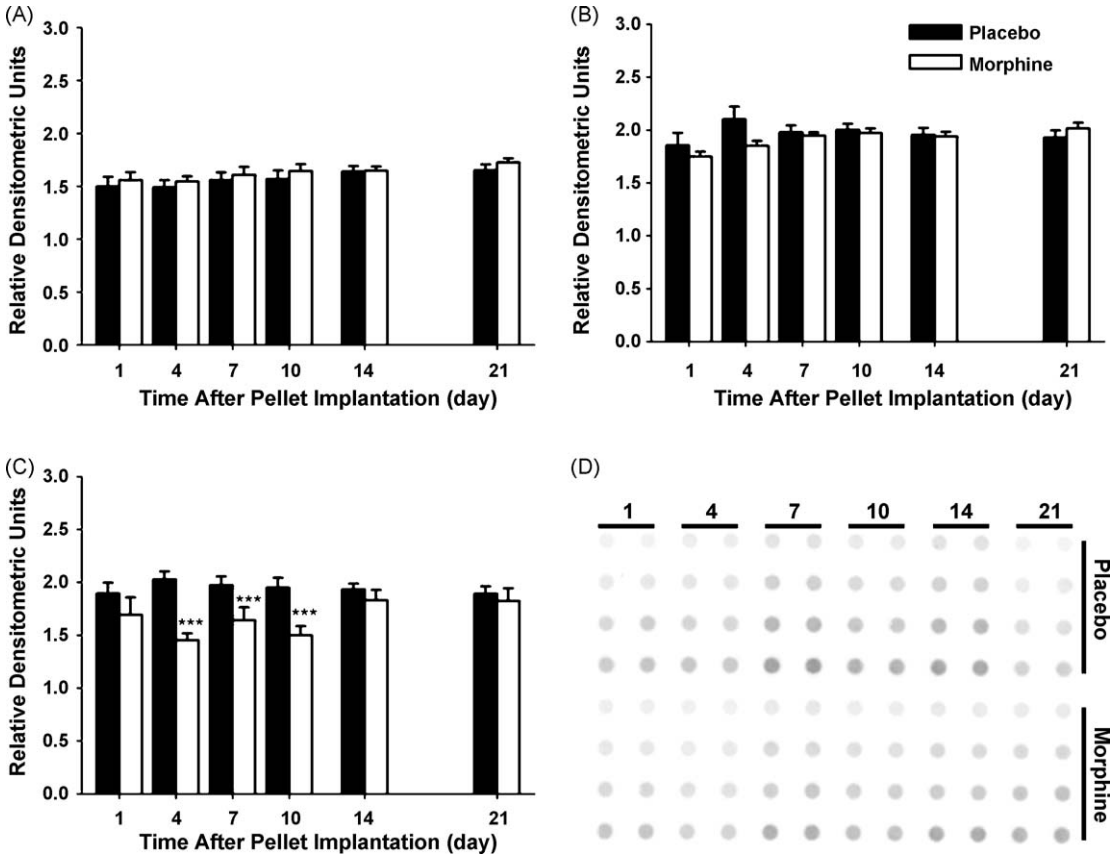

Fig. 7. Analysis of ECL dot blot studies using relative densitometric units. Dot blot analysis was performed using tissue homogenates obtained from guinea-pigs implanted once with either morphine (□) or placebo (■) pellets. (A) Analysis of the alpha$_1$ subunit isoform of the Na^+/K^+ ATPase. (B) Analysis of β-actin. (C) Analysis of the alpha$_3$ subunit isoform of the Na^+/K^+ ATPase. (D) A typical dot blot of homogenates from tissues obtained at different time periods following implantation using an antibody to the alpha$_3$ subunit isoform. Statistical analysis was performed using a one-way ANOVA followed by either Tukey's or Dunn's post hoc test when appropriate. Statistically significant differences are identified with ***($p \leq 0.05$). $N = 4$ for all experimental groups.

tolerance and the non-specific supersensitivity are consistent with the proposed cellular mechanism of down-regulation of the alpha$_3$ subunit isoform leading to decreased Na^+/K^+ ATPase activity and the subsequent depolarization of the resting membrane potential. The reduction in the abundance of this protein induced by pellet implantation can quantitatively and qualitatively explain the development of tolerance (the development of both super- and sub-sensitivity would require a change in cellular physiology) that occurred in the very cells that morphine acted upon [13,14,15], and, based upon the results of the present study, appears to follow a similar time course as the development of functional tolerance. Therefore, the present experiments provide strong evidence for the involvement of the alpha$_3$ subunit isoform of the Na^+/K^+ ATPase in the expression of heterologous tolerance in the guinea-pig ileum.

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