κ - and μ -Opioids Reverse the Somatostatin Inhibition of Ca^{2+} Currents in Ciliary and Dorsal Root Ganglion Neurons

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Neuromodulators, including transmitters and peptides, modify neuronal excitability. In most neurons, multiple neuromodulator receptors are present on a single cell. Previous work has demonstrated either occlusive or additive effects when two neuromodulators that target the same ion channel are applied together. In this study, we characterize the modulation of Ca^{2+} and K^+ channels in embryonic chick ciliary ganglion neurons by somatostatin (Som) and opioids, including the effects of these neuromodulators when applied in combination. We report a modulation of calcium current by κ - or μ -opioids that can prevent Som effects when applied before Som and can replace Som effects when applied after Som. We term these effects demodulation because they do not have the characteristics of simple occlusion but rather represent a dominant effect of opioid-mediated modulation of calcium channels over Som-mediated modulation. These opioid effects persist in the pres-

ence of kinase and phosphatase inhibitors, as well as after alteration of the intracellular Ca^{2+} concentration. Furthermore, they are present in both whole-cell and perforated-patch recording configurations. These effects of opioids on Som-mediated modulation do not seem to be mediated by a general uncoupling of Som receptors from G-protein-coupled signaling systems because K^+ current modulation by Som can persist in the presence of opioids. Demodulation by opioids was also observed in dorsal root ganglion neurons on the modulation of calcium current by GABA and norepinephrine (NE). In both preparations, this demodulatory interaction occurred between voltage-independent (opioids) and voltage-dependent (Som, GABA, and NE) modulatory pathways.

Key words: Ca^{2+} channel; modulation; demodulation; somatostatin; GABA; NE; opioid peptides

The functional significance of multiple receptors in neurons is not well understood, especially in the case of transmitters and neuromodulators acting on the same effector (Surprenant et al., 1990; Cox and Dunlap, 1992; Vaughan, 1998). For example, in rat superior cervical ganglion neurons, activation of at least six distinct receptors inhibits the same Ca^{2+} channel (Bean, 1989; Beech et al., 1992; Shapiro and Hille, 1993). The concurrent application of two modulators, each of which alone inhibits Ca^{2+} channels, can produce a larger inhibition of the current than when either transmitter is presented separately (Elmslie, 1992; Diverse-Pierluissi and Dunlap, 1995). Alternatively, the response of one has been shown to be occluded by the activation of the other (Surprenant et al., 1990; Diverse-Pierluissi and Dunlap, 1993; Ehrlich and Elmslie, 1995). What is the biological relevance of two receptors in the same cell, especially when they both elicit a similar modulation of Ca^{2+} entry?

We have demonstrated previously that somatostatin (Som) and opioids reduce an inward Ca^{2+} current in dissociated ciliary ganglion (CG) cells (Polo-Parada and Pilar, 1998a). These inhibitory effects result from neurotransmitters binding to G-protein-coupled receptors. In this paper we establish that opioid peptides prevent the inhibitory effect of Som on Ca^{2+} currents when the opioids are applied before Som and that they replace the

voltage-dependent Som-mediated modulation with the voltage-independent opioid-mediated modulation when applied after Som.

Opioid and Som receptors are expressed on CG cell somas and terminals, and both of these peptides are endogenous to these neurons (Gray et al., 1989; De Stefano et al., 1993). Additionally, endogenous Som has been shown to be released by KCl depolarization as well as to inhibit the release of ACh, the primary neurotransmitter of ciliary neurons (Gray et al., 1992). This complement of receptors and active endogenous neuropeptides suggests a potential role for these neuromodulators in the control of transmitter release. In this report we characterize the interactions between Som and opioids on the modulation of Ca^{2+} influx and K^+ efflux. Additionally, we extend our observations to GABA- and norepinephrine (NE)-mediated modulation of Ca^{2+} current in dorsal root ganglion (DRG) neurons. We suggest that demodulation occurs downstream of receptor occupation and propose possible mechanisms of action based on an interaction of voltage-dependent and -independent pathways. Demodulation is thus a novel form of neuromodulator interactions.

Parts of this paper have been published previously (Polo-Parada and Pilar, 1998a,b).

MATERIALS AND METHODS

Isolation of ciliary and dorsal root ganglion neurons. Ciliary ganglia from White Leghorn chick 16 d (St 40) embryos were dissected out in oxygenated Tyrode's solution (in mM, 128 NaCl, 3 KCl, 1 MgCl_2 , 3 CaCl_2 , 27 NaHCO_3 , 10 HEPES, and 10 glucose, pH 7.3) and incubated in 0.08% trypsin (Life Technologies, Gaithersburg, MD) in Tyrode's solution for 30 min at 37°C. Thereafter trypsin was removed and inhibited by washing the cells three times with M-199 (Sigma, St. Louis, MO) and 10% heat-inactivated horse serum (Life Technologies). Neurons were dissociated mechanically by gentle trituration in M-199 and 10% chicken extract and centrifuged for 5 min at 100 $\times$ g. The pelleted cells were

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Figure 1. Effects of the sequential application of Som and κ -agonist on $I_{\text{Ca}^{2+}}$. In this and subsequent figures (see Figs. 2, 6), the data are presented using the same layout. **A**, Representative samples are shown of the Ca^{2+} macroscopic currents that have been used to graph in **B** the time course of the Ca^{2+} current changes from the same neuron every 10 sec. The numbers in parentheses in **B** correspond to the labeled dots from which the Ca^{2+} current traces in **A** were selected. **B**, Diary plot of the changes in $I_{\text{Ca}^{2+}}$ produced by the application of the different drugs is graphed as the percent reduction of the normalized peak control Ca^{2+} current. Horizontal bars denote the initial time and duration of compound applications. **C**, Summaries of $I_{\text{Ca}^{2+}}$ inhibition are shown. The numbers in parentheses on the bars indicate the number of observations. **A**, In this figure, Ca^{2+} currents elicited by step depolarizations are shown. **a**, Four selected traces (*tr*) are superimposed. *tr* 5, The Ca^{2+} control current; *tr* 10, a Ca^{2+} current during κ -agonist application (5 μM); *tr* 15, a current during the demodulation of Som by κ -agonist; *tr* 16, the full Som response after the washout of κ -agonist. **b**, Four superimposed exemplar traces from **B** recorded during the second drug application are shown. *tr* 24, The Ca^{2+} current control; *tr* 25, a current after the application of Som; *tr* 30, a current during the demodulation of Som by κ -agonist; *tr* 33, a current after the washout of κ -agonist during sustained application of Som. **B**, When the κ -agonist was applied first, there was a 13% inhibition of the $I_{\text{Ca}^{2+}}$ (*tr* 8–10) with no change in current kinetics (*Aa*, *tr* 10), and the inhibition by Som was occluded (*tr* 11–15). After the κ -agonist washout, the continuous presence of Som reduced the peak $I_{\text{Ca}^{2+}}$ by 52% (*tr* 16), and the current displayed KS (*Aa*, *tr* 16). In the second application of Som maintained for 100 sec, the Ca^{2+} current was reduced by 40% (*tr* 25). Sixty seconds after the application of the κ -agonist, the inhibition induced by Som is reversed (demodulated) (*tr* 28–32). **C**, The quantification of these data illustrates that the Som-mediated Ca^{2+} current inhibition during demodulation is reduced to the level of the inhibition of κ -opioid. CdCl added to the superfusion blocked all current.

resuspended and plated onto 35 mm tissue culture dishes (coated with 1% poly-L-lysine or poly-L-ornithine solution in Na^+ borate, dried overnight, and washed in distilled water).

Chick embryo extract was prepared by extruding 9-d-old embryos through a syringe, adding an equal volume of Tyrode's solution, and rotating the tubes for 30 min. Extract was obtained after centrifugation at 50,000 rpm for 2 hr at 4°C. The supernatant was aliquoted and stored at -20°C for later use as a culture media supplement.

DRG neurons were isolated from the thoracic and lumbar ganglia from 14 d chick embryos (St 38). Ganglia were incubated in Ca^{2+} - and Mg^{2+} -free Tyrode's solution (in mM, 128 NaCl, 3 KCl, 27 NaHCO_3 , 10 HEPES, and 10 glucose, pH 7.3) for 30 min at 37°C in an incubator with 5% CO_2 . Trypsin was omitted for the dissociation of DRG neurons. Tyrode's solution was replaced by M-199 and 10% fetal bovine serum (FBS), and neurons were dissociated by trituration and plated onto 35 mm culture dishes.

Ciliary and dorsal root ganglion neurons were maintained at 37°C in M-199 plus either 10% embryo extract (CG neurons) or 10% FBS (DRG neurons) in a tissue culture incubator with 5% CO_2 1–4 hr after plating, at which time media was exchanged with the bath solution. At this time, neurons were devoid of processes, and soma diameters ranged from 10 to 20 μm .

Patch-clamp recording. Recordings were made with both the standard (ruptured membrane patch) whole-cell and the perforated-patch-clamp configuration at room temperature (22 – 24°C). Pipettes for whole cell

(0.8 – 2 M Ω) and perforated (2 – 4.5 M Ω) patch were pulled from N51A glass (Garner Glass, Claremont, CA), coated with Sylgard (Dow Corning, Midland, MI), and fire-polished. For CG neurons recorded using the whole-cell configuration, access resistance averaged 1.91 ± 0.3 M Ω ($n = 314$), and membrane capacitance averaged 24.5 ± 0.6 pF ($n = 314$). For DRG neurons, access resistance averaged 1.3 ± 0.4 M Ω ($n = 211$), and membrane capacitance averaged 28.8 ± 0.7 pF ($n = 211$). For perforated-patch recordings, series resistance averaged 16.2 ± 0.6 M Ω ($n = 74$), and membrane capacitance averaged 25.1 ± 0.2 pF ($n = 74$).

Capacitive transients and series resistance were electronically compensated (75–90%) using an EPC-7 patch amplifier (Medical System). Currents were activated, acquired, and leak-subtracted using a hyperpolarizing P/4 protocol using pClamp 6.0 (Axon Instruments, Foster City, CA) running on a Pentium processor-based computer (Dell 200 Pro). Currents were four-pole Bessel-filtered at 5 kHz and digitized at 20 kHz. All values expressed are mean $\pm$ SEM (n = number of observations).

Measurement of the effects of neuromodulators on Ca^{2+} current. Inhibition of Ca^{2+} current was measured at an isochronal point where control Ca^{2+} currents reached a peak. To quantify current inactivation or the presence of kinetic slowing during the step depolarization, we calculated the ratio between the current amplitude measured at the end of the test pulse and the current amplitude measured where control Ca^{2+} current peaked ($I_{\text{end}}/I_{\text{peak}}$). For example, for the trace shown in Figure 1Aa, the effects of Som (*tr* 16) on the current at the end of the test pulse are larger than at the beginning. In this example the kinetic slowing (KS) ratio was

calculated to be 1.18. This ratio represents an 18% increase in the amplitude of the current at the end of the step depolarization with respect to the initial peak of the control Ca^{2+} current. In contrast for the data shown in Figure 1*Aa*, *tr* 5, the ratio KS was calculated to be 0.94, which represents a 6% decrease of the current by the end of the test pulse. Essentially, a ratio >1 represents an increase of current, meaning that the inhibited current is activating more slowly than is the control (kinetic slowing), a ratio <1 indicates that the current inactivated during the voltage step (inactivation), and a ratio = 1 indicates no change in the kinetics of the Ca^{2+} current during the step depolarization.

Prepulse facilitation was determined measuring the $I_{\text{Ca}^{2+}}$ elicited by a 50 msec step depolarization from -80 to 0 mV, an interval of 250 msec separating the pre- from the postpulse. Twenty-five milliseconds before the postpulse, a 25 msec conditioning depolarization to $+80$ mV was applied. Ratios were calculated by dividing the postpulse Ca^{2+} currents by the initial prepulse peak Ca^{2+} current recorded before and after the $+80$ mV conditioning pulse.

Solutions. The bathing solution in most experiments was (in mM): 150 TEA-Cl, 10 HEPES, 10 glucose, 1 MgCl_2 , and 2–10 CaCl_2 , pH 7.3 (adjusted with TEA-OH), with osmolarity = 320 ± 5 mOsm/l. TEA-Cl replaced external Na^+ to prevent contamination of the Ca^{2+} current with potassium currents. Tetrodotoxin (TTX; $1 \mu\text{M}$) was added to all external solutions to block sodium currents. For whole-cell recordings, the pipette solution contained (in mM): 50 *N*-methyl-D-glucamine-Cl (NMG-Cl), 30 CsCl, 30 TEA-Cl, 5 EGTA-NMG-Cl, 10 HEPES, 4 MgCl_2 , 4 creatine phosphate, 4 ATP-Na, 0.2 Na_2GTP , 0.0025 leupeptin, and 0.0025 creatine kinase, pH 7.3 (adjusted with CsOH), with osmolarity = 305 ± 5 mOsm/l. In some experiments, BaCl_2 was exchanged for CaCl_2 . To isolate the inward current through Ca^{2+} channels using the perforated-patch configuration, we used a similar bath solution but filled the pipette in a two-step manner. The tip was dipped for 5 sec in a solution containing (in mM): 75 CsSO_4 , 55 CsCl, 8 MgCl_2 , 10 HEPES, and 10 CaCl_2 , pH 7.3 (adjusted with CsOH). The remainder of the pipette was backfilled with the same solution plus 400 $\mu\text{g/ml}$ amphotericin B (Rae et al., 1991); 10–15 min after seal formation, amphotericin B gradually increased the patch membrane permeability, inducing a low-resistance pathway with an access resistance ranging from 8 to 15 M Ω . Under these conditions, Ca^{2+} current recordings lasted up to 1 hr. To record K^+ currents, the bathing solution contained (in mM): 140 NaCl, 20 HEPES, 10 glucose, 2 CaCl_2 , 0.03 CdCl, 1 MgCl , and 2 μM TTX, with pH adjusted to 7.2 with NaOH. Osmolarity was 320 ± 5 mOsm/l. The pipette solution contained (in mM): 150 KCl, 10 HEPES, 10 EGTA-NMG-Cl, 4 MgCl , 4 Na-ATP, and 1 leupeptin. Osmolarity was 300 ± 5 mOsm/l. The sodium salt of okadaic acid (OKA) was applied intracellularly by dilution into the intracellular recording solution and delivered to the cell via passive diffusion from the whole-cell patch pipette. Substitution of external solutions was accomplished using a manually operated multibore perfusion system ending in a single 200- μm -diameter tip, which was placed 25–50 μm from the neuron under study. By the use of this system, drug applications reached the cells in <1 sec. Usually one neuron was studied from each dish to avoid long-term effects of drugs or changes in effects because of repeated exposure. For each experiment the modulation of Ca^{2+} current by Som was initially measured in a few cells and used as an indicator of the reproducibility of the results. If the values were in the range of the Som-mediated Ca^{2+} current inhibition measured in all previous neurons, the experiment was continued.

Pharmacological agents. Stock solutions of the following drugs at the indicated concentrations were made in distilled water and kept at 20°C until the day of use: somatostatin (1 mM), ATP and GTP (60 mM), leupeptin (20 mM), TTX (1 mM), U-50488 (a κ -agonist peptide; 1 mM), the DAMGO (a μ -agonist peptide; 2 mM), *nor*-binaltorphimine (a μ -antagonist; *nor*-BNI; 1 mM), baclofen (a GABA_B agonist; 10 mM), and OKA (0.2 mM). A stock solution of NE (10 mM) was prepared the day of the experiment. Stock solutions of amphotericin B (400 $\mu\text{g/ml}$) and staurosporine (0.1 mM) were made in DMSO. Bathing solutions were made fresh daily by diluting an aliquot of the frozen stock into the saline bath. Special precautions were taken with staurosporine and OKA solutions because of their light sensitivity. The opioid peptides, OKA, and staurosporine were purchased from Research Biochemicals (Natick, MA). The rest of the drugs and 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA), TEA-Cl, HEPES, creatine phosphate, and all salts were purchased from Sigma.

RESULTS

Inhibition of calcium currents by Som and opioid peptides

Ca^{2+} currents ($I_{\text{Ca}^{2+}}$) were isolated with techniques similar to those described previously (Meriney et al., 1994). Figure 1*Aa*, *tr* 5, shows peak inward $I_{\text{Ca}^{2+}}$ elicited in dissociated ciliary ganglion cells by a 50 msec step depolarization from a holding potential of -80 to 0 mV, using the whole-cell variant of the patch-clamp technique in the presence of 5 mM external Ca^{2+} . We determined from I - V plots that this depolarization step elicited maximal current. Both large and small cells from St 40 ciliary ganglia were used in this study. As we have demonstrated previously, application of 100 nM Som produced a $40.1 \pm 1.6\%$ ($n = 157$) block (Fig. 1*C*) of peak $I_{\text{Ca}^{2+}}$ in 151 of 154 cells sampled. Furthermore, the modulated current exhibited slowing activation kinetics (Fig. 1*Aa*, *tr* 16, *b*, *tr* 25) (Bean, 1989; Meriney et al., 1994). Similarly, application of opioid agonists selective for κ and μ receptors also inhibited $I_{\text{Ca}^{2+}}$, as has been described in other systems (Schroeder et al., 1991; Seward et al., 1991). The effects of U-50488 (a κ -agonist) at 5 μM , blocking $I_{\text{Ca}^{2+}}$ by $20.1 \pm 1.7\%$ ($n = 71$), and DAMGO (a μ -agonist) at 5 μM , inhibiting $I_{\text{Ca}^{2+}}$ by $18.7 \pm 2\%$ ($n = 57$), are shown in Figures 1*C* and 2*C*, respectively. κ - and μ -agonists did not change the kinetics of the $I_{\text{Ca}^{2+}}$ steady-state inhibition (Figs. 1*Aa*, *tr* 10, 2*Aa*, *tr* 9, *b*, *tr* 26). These concentrations of opioid agonists produced maximal inhibition (Polo-Parada and Pilar, 1998a). We have determined previously that 100 nM Som was a saturating dose (Meriney et al., 1994; White et al., 1997).

Addition of CdCl (100 μM) to the bath solution abolished the inward current by $99.1 \pm 0.5\%$ ($n = 21$; Fig. 1*C*), demonstrating that we have isolated the Ca^{2+} current. Furthermore, in ciliary neurons from St 40 embryos, Som has been shown to interact only with N-type Ca^{2+} channels, which represent 70% of the total Ca^{2+} current in St 40 ganglia (White et al., 1997). In other experiments we also determined that opioids (κ and μ) predominantly inhibited the N-type Ca^{2+} current in these cells (data not shown).

Sequential application of Som and opioids: demodulation of Som inhibition

Dunlap and collaborators (Diverse-Pierluissi and Dunlap, 1993; Diverse-Pierluissi et al., 1995) showed that when two modulators (NE and GABA) converged on the same Ca^{2+} channel, either the combined response of their concurrent application was additive or one modulator occluded the effect of the other (see also Elmslie, 1992). In the next series of experiments we characterized Som- and opioid (κ and μ)-induced responses when drugs were sequentially applied to the same cell. The second drug was applied 15–30 sec after the first in the continued presence of the first compound. In this paper the term sequential application will be used in this context. In Figures 1 and 2 the basic findings of this work are illustrated, namely that κ - and μ -opioids prevent or relieve the Som-mediated Ca^{2+} current inhibition. For example, in Figure 1*Aa*, there are four superimposed records obtained from the same cell; *tr* 5 is a control Ca^{2+} current, *tr* 10 and 16 are currents after the application of κ -agonist and Som alone, and *tr* 15 shows the current after the sequential application of Som and U-50488 (a κ -agonist). The Ca^{2+} current inhibition produced by Som is characterized by KS (Fig. 1*Aa*, *tr* 16, *b*, *tr* 25, 33), unlike the inhibition produced by the κ -agonist, which does not alter the current kinetics (Fig. 1*Aa*, *tr* 10). The inhibition elicited by Som was larger than that elicited by the κ - and μ -opioids (Figs. 1, 2). In

Figure 2. Effects of the sequential application of Som and μ -agonist on $I_{\text{Ca}^{2+}}$. This composite figure is similar to Figure 1, except that the μ -agonist (DAMGO) was used to demodulate the Som-mediated Ca^{2+} current inhibition. *A, B*, Illustrated here is a robust demodulation of the Som-mediated inhibition (*tr 9, 26*) despite the low level of Ca^{2+} current blocked by the opioid peptide (*A, B tr 9, 20*). *C*, The Som-mediated inhibition was reversed to the level of the opioid-mediated inhibition.

Figure 1*Ba*, the time course of the drug application seen in Figure 1*Aa* is plotted. Figure 1*Ab* illustrates the records of a second sequential compound application, but in this case the κ -agonist was applied in the middle of a prolonged application of Som. Relief of Som inhibition also occurred in this instance. This figure also illustrates that, despite the occurrence of desensitization to Som during repeated applications, the κ -induced relief of inhibition is clearly observed, decreasing the Som-mediated inhibition from 44 to 11%. This relief of Som inhibition by κ -opioids was seen in 24 of 25 CG neurons tested (Fig. 1*C*). The sequential application of Som and the κ -agonist blocks $I_{\text{Ca}^{2+}}$ by $19.5 \pm 2\%$ ($n = 24$).

Similar effects on the release of Som Ca^{2+} current inhibition were seen after the application of the μ -agonist (DAMGO) (Fig. 2). The range of inhibition varied between 3 and 24%. The exemplar records of Figure 2*A, a* and *b*, illustrate that relief of the Som inhibition was present even when the μ -opioid Ca^{2+} current inhibitory response was small. In Figure 2*A* the superimposed records shown were obtained from a cell before (control; Fig. 2*Aa*, *tr 4*) and after the sequential application of Som and the μ -agonist (Fig. 2*Aa*, *tr 9*). During the inhibition produced by Som, the Ca^{2+} current showed KS (Fig. 2*Aa*, *tr 5, b, tr 28*) unlike the inhibition produced by the μ -agonist that did not alter the current kinetics (Fig. 2*Aa*, *tr 9, b, tr 26*). In the initial application of 100 nM Som, a 60% inhibition of the $I_{\text{Ca}^{2+}}$ was induced (Fig. 2*Ba*) that was almost completely released (to $\sim 4\%$) 10 sec after the application of 5 μM μ -agonist. In the subsequent application the peptides were applied in reverse order (Fig. 2*Bb*). Application of the μ -agonist first elicited a small inhibition of $I_{\text{Ca}^{2+}}$ and also occluded the inhibition normally produced by Som. This occlu-

sion was released after the washout of the μ -agonist. Figure 2*C* is the summary of these measurements. Som coapplied with the μ -agonist blocked $17 \pm 1.5\%$ ($n = 6$) of the Som-mediated inhibition, similar to the effect of the μ -agonist alone ($18.7 \pm 2\%$; $n = 57$).

Comparable results were obtained with κ - or μ -agonist concentrations from 50 pM to 5 μM . In contrast, if the two different opioids were applied concurrently, the combined response was additive, or one opioid occluded the effect of the other (data not shown). These results suggested that μ - and κ -opioids selectively release the Som-mediated inhibition. However, because the Ca^{2+} inhibition by μ -opioid was more variable, the majority of measurements were done with the κ -agonist. The prevention or release of Som inhibition of $I_{\text{Ca}^{2+}}$ was rapid and characterized by substitution of the inhibition of the Som-mediated modulation with μ and κ calcium current modulation (Figs. 1*C*, 2*C*; see also Figs. 6*C*, 9*A*).

Thus, in summary, we observed two types of responses. In one, opioids, when applied first, prevented the effect of Som; this is reminiscent of similar occlusion effects of combined drug application seen by Dunlap and collaborators (Diverse-Pierluissi and Dunlap, 1993; Diverse-Pierluissi et al., 1995). In the other response, the initial effect of Som, distinguished by a potent inhibition of Ca^{2+} channels, was replaced or substituted by the Ca^{2+} current inhibition mediated by opioids. This current is characterized by a smaller amplitude and the absence of KS. We will call both types of interaction demodulation. This demodulation observed in ciliary neurons was similar with sequential, repetitive application of both peptides and was quickly reversible. Thus, it appears to be operating as a switch. The occlusion of the response

of Som by opioids also indicates that the same sets of channels are affected by both receptors.

Is demodulation an artifact of the recording technique?

Because the effects just described were observed in dialyzed cells, we next used the perforated-patch (PP) technique to keep the cells metabolically intact. The same drug application protocol used with the whole-cell recordings was used to ascertain whether the observed effects were caused by an artificial removal of cytoplasmic soluble components. Demodulation responses recorded with the perforated patch ($n = 7$) were similar to those recorded with the whole cell. The only difference with respect to the whole-cell recordings was the disappearance of kinetic slowing in the Som response. This was expected, because this characteristic, which probably requires protein phosphorylation, is abrogated when the perforated patch is used (Meriney et al., 1994). The demodulation observed with perforated patch was comparable with the one recorded with the whole-cell technique. However, the percentage inhibition of the Som Ca^{2+} current recorded with PP was smaller because of the disappearance of KS. The percentage of $I_{\text{Ca}^{2+}}$ inhibition was $33.2 \pm 2\%$ ($n = 32$) after Som application, $19.9 \pm 2\%$ ($n = 7$) after the κ -agonist application, and $19.5 \pm 2\%$ ($n = 7$) in the presence of both Som and the κ -agonist.

Because the recording configuration did not alter the demodulation, the standard whole-cell recording was used in all of the following experiments to simplify the experimental design and analysis.

Involvement of G-proteins in the inhibition of the $I_{\text{Ca}^{2+}}$

It is known that μ and κ receptors are integral membrane proteins coupled to heterotrimeric GTP-binding proteins. We demonstrated the involvement of G-proteins in the peptide-signaling pathways using pertussis toxin (PTX) as a reagent that specifically reduces the ability of G_o and G_i to be activated. Incubation of CG neurons for 5 hr with 200 ng/ml PTX substantially decreased Som-mediated inhibition of $I_{\text{Ca}^{2+}}$ from $40.1 \pm 1.6\%$ ($n = 157$) to $7.2 \pm 0.8\%$ ($n = 12$). We have demonstrated previously that PTX applied for longer times (6–8 hr) virtually eliminates the Som inhibition (Meriney et al., 1994). Incubation of the CG cells with the same dose of PTX and the same incubation time diminished the κ -agonist Ca^{2+} current inhibition from $20.1 \pm 1.7\%$ ($n = 71$) to $4.7 \pm 0.9\%$ ($n = 12$) and that of the μ -agonist from $18.7 \pm 2\%$ ($n = 57$) to $8 \pm 1.2\%$ ($n = 12$). In similar studies, the overnight application of PTX resulted in a large decrease in the opioids' Ca^{2+} inhibition (Wilding et al., 1995). Thus, it is likely that incubating the CG cells with PTX for longer times could result in a greater inhibition. However our recording conditions limited the application time to 5 hr. These experiments indicate that most of the observed inhibition of $I_{\text{Ca}^{2+}}$ is mediated by a G_o/G_i protein (Hille, 1994). We have not sought to determine the identity of the particular G-protein subtype that couples each receptor to the Ca^{2+} channel.

Som and the μ - and κ -opioids block Ca^{2+} currents via different G-protein pathways

It is known that G-protein interactions with Ca^{2+} channels are either voltage dependent or voltage independent (Beech et al., 1992; for review, see Dolphin, 1998). One method of determining voltage dependence is the use of a strong depolarizing conditioning prepulse to relieve the G-protein-mediated Ca^{2+} channel inhibition. Facilitation results from the relief of the Ca^{2+} channel

inhibition by the conditioning pulse and is measured as the ratio of currents elicited by the same test pulse before and after this conditioning step (Jones and Elmslie, 1997). Facilitation is assumed to occur as a result of the unbinding of G-protein subunits from the Ca^{2+} channel (Bean, 1989; Ikeda, 1991; Elmslie, 1992; Boland and Bean, 1993; Swartz et al., 1993; Luebke and Dunlap, 1994; Jones and Elmslie, 1997). In cases in which no voltage dependence occurs, a conditioning pulse was unable to relieve the Ca^{2+} current inhibition (Diverse-Pierluissi and Dunlap, 1993; Shapiro and Hille, 1993; Luebke and Dunlap, 1994). Therefore, we used prepulse facilitation to evaluate the voltage dependence of the binding of G-proteins with the N-type Ca^{2+} channel caused by either Som or opioid peptides.

The kinetics and voltage dependence of the Som inhibition in CG neurons were similar to those reported previously by others for peripheral neurons (Ikeda and Schofield, 1989). In contrast, there was no facilitation of the inhibition induced by the conditioning depolarization pulse after application of κ - or μ -agonists (Fig. 3A). Pooled results are shown in Figure 3A from control neurons (0.95 ± 0.009 ; $n = 147$) and from cells after application of different drugs. Som exhibited a mean facilitation ratio of 1.14 ± 0.02 ($n = 80$), whereas the μ - and κ -agonists showed no facilitation [1.01 ± 0.03 ($n = 6$) and 0.94 ± 0.01 ($n = 25$), respectively]. We therefore concluded that, in CG neurons, the interaction of the G-protein activated by Som with the Ca^{2+} channel was voltage dependent, whereas the G-protein interaction with the Ca^{2+} channel caused by μ - and κ -opioids was voltage independent.

This systematic difference observed in facilitation, between Som on the one hand and μ - and κ -opioid peptides on the other hand, reinforces the hypothesis that these compounds are acting via different G-protein heterotrimers. It also demonstrates the existence of at least two pathways that modulate the Ca^{2+} current, as has been shown previously in sympathetic and DRG cells (Diverse-Pierluissi et al., 1995; Ehrlich and Elmslie, 1995).

Demodulation replaces a voltage-dependent form of inhibition with a voltage-independent inhibition

A pulse-facilitation protocol was used during the sequential application of Som and opioids to examine the characteristics of modulated current before and after demodulation. Figure 3C illustrates the demodulation effect of the κ -agonist on the Som-mediated inhibition. In Figure 3B, three selected traces before application of Som (control Ca^{2+} current, *tr 1*), during the Som-mediated inhibition (*tr 5*), and 10 sec after κ -agonist-induced demodulation of the Som-mediated inhibition (*tr 8*) were superimposed. The effects of a conditioning prepulse are shown in Figure 3B, *right*. During the Som application, the conditioning depolarization relieved the inhibition by 20%, and kinetic slowing was eliminated (Fig. 3B, *right dashed trace*). In contrast the demodulation of Som by the κ -agonist U-50488 (Fig. 3B, *dotted trace*) was not affected by the conditioning depolarization (there are no differences between *right* and *left dotted traces*). The time course of the Som prepulse facilitation is graphed in Figure 3D during the κ -agonist demodulation. The facilitation reached a maximum value during the Som application and thereafter gradually decreased in the continued presence of the κ -agonist until the response was no longer facilitated. In the pooled values shown in Figure 3A, the facilitation ratio was altered from 0.95 ± 0.01 ($n = 25$) before peptide application, to 1.21 ± 0.05 ($n = 24$) after Som application, and back to 0.99 ± 0.003 ($n = 24$) after demodulation by the κ -agonist. Thus, facilitation was present during

Figure 3. Prepulse facilitation of Ca^{2+} current inhibition during application of Som and opioid peptides and during demodulation of the $I_{\text{Ca}^{2+}}$ inhibition by the κ -agonist. **A**, Pooled results of the maximal ratio of prepulse facilitation. The Som ratio of 1.2 represents a robust measure of voltage-dependent inhibition. With the same prepulse depolarization, the ratios during the application of opioids (5 μM κ -agonist U-50488 and 5 μM μ -agonist DAMGO) are ~ 1.0 (a measure of voltage-independent inhibition). During the coapplication of Som and κ -agonist, the facilitation ratio is similar to that of the ratio of κ -agonist alone. **B**, Three superimposed exemplar traces of $I_{\text{Ca}^{2+}}$ from the same cell plotted in **C**. Bottom (solid trace), Control Ca^{2+} current; top (dashed trace), maximal effect of Som inhibition; middle (dotted trace), current after demodulation of the Som Ca^{2+} current inhibition by the κ -agonist, before and after the prepulse depolarization (left, right traces, respectively). Som $I_{\text{Ca}^{2+}}$ showed a partial relief of inhibition (facilitation) and the disappearance of KS (dashed trace, left). **C**, Sequential application of Som and κ -agonist. **D**, Time course of prepulse facilitation changes during demodulation of Som by a κ -agonist plotted in **C**. During demodulation of the Som current by the κ -agonist, facilitation gradually disappeared. Demodulation replaces a voltage-dependent inhibition with a voltage-independent inhibition.

Som-mediated inhibition but not after demodulation of the Som-mediated inhibition by opioid peptides. Therefore, demodulation replaces a voltage-dependent modulation of Ca^{2+} current with a voltage-independent modulation.

Are soluble cytoplasmic components involved in demodulation? Role of phosphorylation and cytosolic Ca^{2+}

To assess the participation of protein phosphorylation in the demodulation of Som-mediated modulation, we used Na-OKA (1 μM added to the pipette solution) to prevent protein phosphatase activity (PP1 and PP2A) and to increase the general level of phosphorylation. If changes in phosphorylation were involved in this phenomenon, inhibiting phosphatases would be expected to prevent or at least modify demodulation. In the presence of OKA, the Som-mediated modulation (Fig. 4A, *tr 10*) was demodulated to κ -agonist-mediated modulation (Fig. 4A, *tr 15*) as described in Figure 1 above. These traces were selected from the diary plot of the percentage block of Ca^{2+} current inhibition illustrated in Figure 4B. The bar graph (Fig. 4C) summarizes the observations showing that demodulation in the presence of the phosphatase inhibitor was not different from that observed in control cells.

Because the application of OKA had no effect on demodulation, it was important to have positive controls for the effect of this phosphatase inhibitor. Two effects were seen; in the control Ca^{2+} current, there was an increased inactivation of the $I_{\text{Ca}^{2+}}$ measured by the ratio ($I_{\text{end}}/I_{\text{peak}}$, see Materials and Methods; Fig. 4D) from control Ca^{2+} current (0.83 ± 0.008 ; $n = 45$) to that from control and OKA current (0.77 ± 0.01 ; $n = 39$). This effect of OKA was originally described by Werz et al. (1993) after the application of OKA. A second effect was the disappearance of KS

with a consequent decrease in the Som inhibition in the presence of OKA, in comparison with the control (Fig. 4C). Som blocked $41.2 \pm 1.8\%$ ($n = 45$), and Som plus OKA blocked $28.8 \pm 3\%$ ($n = 18$; Fig. 4C). This is because we made the measurements of the Som and OKA current at the peak of the control Ca^{2+} current, when KS is absent in the modulated current.

In previous studies (Meriney et al., 1994), we have found that KS present in whole-cell recordings was not observed in PP, unless a protein kinase inhibitor was added to the bath solution. The increased level of phosphorylation present in the intact cell prevents the appearance of KS. Figure 4A, *tr 10*, shows that the KS produced by Som in whole-cell recording was abolished when OKA was added to the pipette. This probably occurred because after the inhibition of phosphatases by OKA, the endogenous kinases that remained attached to the membrane were more effective. The presence of KS under control conditions (no OKA) increased the Ca^{2+} current ratio ($I_{\text{end}}/I_{\text{peak}}$) from the Som and OKA current (0.9 ± 0.002 ; $n = 18$) to that from Som (1.03 ± 0.01 ; $n = 45$; Fig. 4D). There were other effects of OKA on changes in Ca^{2+} current inactivation. In Figure 4D it is seen that the kinetics of the Ca^{2+} current in the presence of the κ -agonist was larger in the presence of OKA (κ -agonist and OKA, 0.86 ± 0.002 ; $n = 9$) than in the control (κ -agonist, 0.77 ± 0.01 ; $n = 46$). It should be noticed that the ratio after demodulation of Som by κ -agonist remains the same (Som and κ -agonist, 0.83 ± 0.01 ; $n = 24$; Som plus κ -agonist and OKA, 0.86 ± 0.02 ; $n = 12$). These changes in Ca^{2+} current kinetics indicate that OKA was effective on CG in our experimental conditions.

In other neurons, protein kinases (PKs) have been shown to modulate G-protein interactions with $I_{\text{Ca}^{2+}}$ channels (Diverse-

Figure 4. Changes in phosphorylation levels by a phosphatase inhibitor do not play a role in the demodulation of $I_{\text{Ca}^{2+}}$ by Som and opioids. *A*, Addition of $1 \mu\text{M}$ OKA to the patch pipette during the demodulation of Som before (*tr* 5) and after application of Som (*tr* 10) and of Som and the κ -agonist U-50488 (*tr* 15). Recordings were initiated 10 min after the membrane was ruptured. *B*, Som induction of a 39% block of $I_{\text{Ca}^{2+}}$ that was demodulated by the κ -agonist to 25%. *C*, Bar graph of the comparison of Ca^{2+} current inhibition between OKA-treated (cross-hatched bars) and untreated neurons (open bars). *D*, Positive controls for OKA dialysis. OKA decreased the KS, which resulted in a percentage decrease of Som inhibition, with respect to the untreated cells (*C*, two left-hand bars). There is a decrease in the ratio of the current time course ($I_{\text{end}}/I_{\text{peak}}$) with Som (less KS) and an increase in the ratio during the application of the κ -agonist (more inactivation). During demodulation there was no significant change in the $I_{\text{end}}/I_{\text{peak}}$ ratio because the joint application of Som and κ -agonist would cancel out each other's effects. $*p < 0.005$, using one-way ANOVA.

Pierluissi and Dunlap, 1993, 1995; Shapiro and Hille, 1993; Swartz, 1993; Swartz et al., 1993; Meriney et al., 1994; Boehm et al., 1996; Diverse-Pierluissi et al., 1997). Staurosporine, a broad-spectrum inhibitor of PKs, was used to determine the involvement of PKs in the modulation of $I_{\text{Ca}^{2+}}$ channels by opioids or in the demodulation of Som-mediated modulation. Figure 5*A* illustrates selected recordings and Figure 5*B* shows the time course of changes in Ca^{2+} currents from the same CG neuron. Adding staurosporine ($1 \mu\text{M}$) to the bath solution for 15–18 min before the recording did not modify the amplitude or kinetics of the $I_{\text{Ca}^{2+}}$ block induced by opioids or Som, nor did it alter the demodulation of Som-mediated effects (Fig. 5*C*).

As a positive control for the action of staurosporine, we applied this PK inhibitor to block the effect of OKA described above. Staurosporine ($1 \mu\text{M}$) was applied extracellularly 10–18 min before the initiation of internal perfusion with $1 \mu\text{M}$ OKA (Fig. 5*D*). We hypothesized that OKA modifies the Ca^{2+} currents because there is an active protein kinase, whose activity is opposed by an active protein phosphatase (see above). When the phosphatase is inhibited by OKA, an endogenous kinase phosphorylates proteins. Figure 5*D**b* shows that OKA modifies the control Ca^{2+} current time course and increases its inactivation as well as reduces KS in the modulated current (Fig. 5*D**a*). These OKA actions are reversed by staurosporine (Fig. 5*D**c*). After staurosporine, the KS is restored, and the rate of inactivation is decreased similar to that in control cells (Fig. 5*D**a*). Figure 5*D**d* shows the average of these changes as ratios ($I_{\text{end}}/I_{\text{peak}}$) of the

Som and opioid-modulated Ca^{2+} current (see Materials and Methods). These results indicate that staurosporine is active in our experimental conditions, most probably by inhibiting cGMP-dependent protein kinase (Meriney et al., 1994), responsible for the changes in Ca^{2+} current kinetics by OKA.

Finally, we tested for the involvement of Ca^{2+} as a second messenger by measuring demodulation of Ca^{2+} current when neurons were dialyzed with 10 mM BAPTA instead of 5 mM EGTA (Beech et al., 1991). Shown in Figure 6 are selected records of applications of Som followed by κ -agonist (*Aa*) and after these drugs were applied in the reverse order (*Ab*). Figure 6*B* shows the time course of changes in Ca^{2+} current. Som-mediated inhibition was decreased by $5 \mu\text{M}$ κ -agonist, which also occluded the Som-induced Ca^{2+} current inhibition during the second trial. In this plot κ -agonist desensitization can be seen during the repetitive application of this drug [from $\sim 18\%$ (*tr* 12) to $\sim 10\%$ (*tr* 30)].

Figure 6*C* is a bar graph of the summary data from these experiments demonstrating that opioid peptide-mediated demodulation of Som effects is present despite the inclusion of 10 mM BAPTA in the pipette solution. Demodulation was of equal magnitude in cells recorded with or without BAPTA in the pipette. Thus demodulation seems independent of intracellular enzymes that require Ca^{2+} (Kennedy, 1989).

In summary, the three experiments described in this section suggest that many cytoplasmic components including serine/threonine protein phosphorylation and intracellular Ca^{2+} are un-

Figure 5. Demodulation during decreased phosphorylation because of the addition of staurosporine to the superfusion solution. Staurosporine did not modify the κ -agonist demodulation of Ca^{2+} channels by Som. *A*, Three selected traces are superimposed. Tr 6, Control; tr 8, after the Som inhibition; tr 11, during the demodulation of Som by κ -agonist in the presence of staurosporine, added to the bath solution 15 min before recordings. *B*, Temporal course of demodulation in the presence of staurosporine is shown. Som induced a 45% inhibition of $I_{\text{Ca}^{2+}}$, which is reversed to 12% by the coapplication of κ -agonist. *C*, Staurosporine superfusion of 10–20 min did not modify the demodulation of Som by κ -agonist of the average of Ca^{2+} current block that is similar to the control average of Figure 1*C*. *Da–c*, Positive control of the effects of staurosporine is shown. After dialysis of Na-OKA in the cell, there is decreased KS in the Ca^{2+} current Som-mediated inhibition (compare *b*, top tr, with control, *a*, top tr). The addition of staurosporine to the bath restored the KS induced by Som (*c*), overriding the effects of OKA. *d*, Average $I_{\text{end}}/I_{\text{peak}}$ ratios of $I_{\text{Ca}^{2+}}$ modulated by Som in the presence and absence of OKA and OKA plus staurosporine are shown. During OKA dialysis, ratios are statistically different from those of control and of OKA plus staurosporine (* $p < 0.005$, using one-way ANOVA).

likely to be involved in the demodulation of Som effects by opioids.

Demodulation is not the result of a competition between Som and opioids for the same receptor

Where could the demodulation described above be taking place? The most parsimonious explanation would be that demodulation occurs at the receptor level, where opioids might act as Som receptor antagonists. However this is not the case as shown by the data presented in Figure 7. The specific κ -antagonist nor-BNI (5 μM) prevented the demodulation of Som effects by the κ -agonist U-50488 but not the modulation of the $I_{\text{Ca}^{2+}}$ by Som. Figure 7*Aa–c* shows selected traces from the experiment graphed in Figure 7*B*. Notice that the KS induced by Som (Fig. 7*Aa*, tr 6) disappears in the presence of the κ -agonist (tr 11) but not in the presence of nor-BNI (Fig. 7*Ab*, tr 31). nor-BNI itself has no effect on the Som-mediated Ca^{2+} current inhibition. In Figure 7*B*, demodulation of Som effects by the κ -agonist was observed before the addition of nor-BNI into the superfusion fluid (Fig. 7*B*, compare tr 6 with tr 11). However, when nor-BNI was added to the perfusion fluid, the κ -agonist did not have any effect on the Ca^{2+} current (Fig. 7*B*, tr 30), and when Som was applied (in the continued presence of κ -agonist), demodulation was not observed

(Fig. 7*B*, second application of Som, tr 31). In this case, Som inhibited the $I_{\text{Ca}^{2+}}$, and this modulation was not affected by the κ -agonist. The bar graph is a summary of these measurements (Fig. 7*C*). These observations point out that the demodulation of Som is not attributable to a competition between opioids and Som for the receptor.

Is opioid receptor occupation preventing Som receptors from activating G-proteins?

Evidence against this interpretation was provided by the effect of Som and opioids on K^{+} currents in these same neurons. In these experiments, K^{+} currents were elicited by a 50 msec step depolarization from a holding potential of -80 to $+40$ mV (Fig. 8*A,B*). It is known that in other neurons, Som and μ -opioid receptor occupation also modulates K^{+} currents (North, 1986, 1989). This is the case in CG cells as well. The effects of Som and the κ -agonist on K^{+} currents were similar in amplitude and time course; both reduced the K^{+} currents (Fig. 8). The K^{+} currents recorded were a mixed contribution from several types of K^{+} currents that included $I_{\text{K}^{+}}$, $I_{\text{K}^{+}\text{A}}$, and $I_{\text{K}^{+}\text{IR}}$ (Dryer et al., 1991). However, because we used Cd ions in the bath solution and EGTA was added to the pipette, the activation of $I_{\text{K}^{+}\text{(Ca)}}$ (Wisgirda and Dryer, 1993) was prevented. Demodulation could have

Figure 6. Cytosolic Ca^{2+} does not play a role in $I_{\text{Ca}^{2+}}$ demodulation. Chelating global Ca^{2+} does not modify demodulation. BAPTA (10 mM) was dialyzed into the neurons. *Aa*, Superimposed traces from *B*. *Tr* 5, Control; *tr* 7, after the application of Som; *tr* 12, during demodulation of Som by κ -agonist. *b*, Traces from the second drug application. *B*, A flow chart of the changes in $I_{\text{Ca}^{2+}}$ recorded 4–5 min after the neuronal membrane was ruptured. Initially Som inhibits 45% of the $I_{\text{Ca}^{2+}}$, which is reduced by κ -agonist to 20%. In the second trial, κ -agonist blocks the current to 13% (*tr* 20). Sequential application of Som did not change the amplitude of the $I_{\text{Ca}^{2+}}$ block (*tr* 22), and after κ -agonist washout, Som blocked 45% of the Ca^{2+} current (*tr* 23). Reintroduction of the κ -agonist demodulated the Som response (*tr* 30). *C*, $I_{\text{Ca}^{2+}}$ of Som-mediated inhibition, of κ -agonist alone, and after demodulation in the presence (solid bars) and absence (open bars) of BAPTA. The amplitudes of $I_{\text{Ca}^{2+}}$ modulation and demodulation by peptides in the presence and absence of BAPTA are not different.

a differential effect on different K^+ channel subtypes. For this reason, we measured the inhibition during both the transient (open bars in Fig. 8*Ac,Bc*) and sustained (cross-hatched bars in Fig. 8*Ac,Bc*) components of the K^+ currents (Fig. 8*Ac,Bc*).

With sequential application of Som followed by Som and the κ -agonist, we observed an additive block of both peak and sustained K^+ current (Fig. 8*A*). When the κ -agonist was applied first, there was an apparent occlusion of the Som-mediated response of the transient K^+ current but an additive effect of the κ -agonist and Som on the sustained K^+ current (Fig. 8*B*).

Specifically, opioids did not demodulate the effects of Som on K^+ currents (Fig. 8*A,B*) as they did on the Ca^{2+} channels. Instead, both peptides produced a reduction in the total I_{K^+} that was additive when Som and κ -opioid were applied concurrently. If the opioids were acting by interfering with the Som receptor, we would have expected that both peptides would affect $I_{\text{Ca}^{2+}}$ and I_{K^+} currents in a similar way (i.e., both being additive or both producing demodulation). Because the sustained component of the K^+ current is modulated by Som, in the presence of κ -agonist, this argues that the Som receptor remains coupled to G-proteins. These results do not support the interpretation that the activation of κ - and μ -opioid receptors alters the Som receptor so it cannot any longer activate G-proteins.

Is demodulation confined to ciliary ganglion neurons?

To establish whether demodulation was unique to CG neurons, we investigated whether other neurons showed a similar response pattern. In the DRG of the chick, the modulation of Ca^{2+} currents by GABA and NE has been extensively studied and provides useful background for our work. Dunlap and collaborators (Diverse-Pierluissi and Dunlap, 1993, 1995) reported in several seminal papers that the inhibition of Ca^{2+} currents by GABA and NE was mediated via two distinct PTX-sensitive G-protein-mediated pathways. GABA- and NE-mediated inhibition of Ca^{2+} current has been shown to be characterized by modulated current with KS (Diverse-Pierluissi et al., 1995; see also Hille, 1994).

We corroborated and extended these findings to include the effects of Som, NE, baclofen, and opioid peptides on DRG neurons (Fig. 9*A*). The three transmitter receptor agonists that showed KS [Som, baclofen (an agonist of the GABA_B receptor), and NE] blocked Ca^{2+} current to a similar extent (Fig. 9*A*), whereas the opioid peptides (which did not display KS) blocked a smaller percentage of total Ca^{2+} current (Fig. 9*A*). These results are reminiscent of our findings in CG neurons and previous studies in DRG neurons (Wilding et al., 1995).

Prepulse facilitation was also studied in DRG neurons to de-

Figure 7. The κ -agonist is not an antagonist of the Som receptor because the block of the κ receptor does not prevent Som modulation. *Aa*, The recordings are superimposed traces of $I_{\text{Ca}^{2+}}$ before (*tr* 5), during the application of Som (*tr* 6), and during demodulation of Som by κ -agonist (*tr* 11). *b*, The same drug applications were repeated in the presence of nor-BNI, a selective κ -antagonist, and no effects were seen in the peak $I_{\text{Ca}^{2+}}$ or in the Som inhibition (*tr* 26, 30, 31). *c*, Recordings are during the partial washout of nor-BNI (*tr* 49, 52, 57). *B*, The time course of Ca^{2+} inhibition changed during drug applications. Initially, Som produced a 45% block, which was demodulated by the addition of κ -agonist, maintaining a 10% inhibition. During continued Som application and after the κ -agonist washout, the Som inhibition was less, probably because of desensitization; it reached 30%. Reintroduction of κ -agonist maintains the Som Ca^{2+} current inhibition at 10%. After the cell's superfusion with nor-BNI, the control $I_{\text{Ca}^{2+}}$ (*tr* 26) is unchanged. In the presence of nor-BNI, the response of the κ -agonist was blocked (*tr* 30), but the application of Som induced a 40% Ca^{2+} current inhibition (*tr* 31). Desensitization is evident during the prolonged Som application, because the $I_{\text{Ca}^{2+}}$ block decreased to 25% in 2 min. However, the Som inhibition was not affected by κ -agonist U-50488. After the κ -antagonist nor-BNI was partially washed out, the κ -agonist U-50488 inhibited the $I_{\text{Ca}^{2+}}$ and demodulated the Som effects (*tr* 52, 57). *C*, Quantitative summary of results of the sequential drug application with and without nor-BNI is shown.

termine the voltage dependence of the pathways mediating inhibition of Ca^{2+} current produced by Som, GABA, NE, and the opioids. Figure 9*B* presents the summary data for these experiments and demonstrates that the inhibition mediated by Som, baclofen, and NE is voltage dependent, whereas the inhibition produced by the κ - and μ -opioids is voltage independent.

Sequential application of Som, NE, GABA, and opioid peptides in dorsal root ganglion neurons

Having established that DRG neurons expressed the same types of responses to transmitters that were involved in demodulation in the CG, it was of interest to see whether demodulation was also present in DRG neurons. Demodulation of Som-mediated Ca^{2+} current inhibition by opioids was present in both large and small neurons (Fig. 9*A*). However it did not occur in all cells tested. In 23 of 39 cells, Som demodulation did not occur despite the fact

that Som and opioids independently inhibited Ca^{2+} currents. When demodulation did occur, Som current inhibition was demodulated by the κ -agonist to $16.4 \pm 2.5\%$ ($n = 10$) and by the μ -agonist to $13.6 \pm 2\%$ ($n = 6$).

We then sought to determine whether GABA-mediated inhibition of Ca^{2+} current was also demodulated by opioids. Figure 9*C* shows responses to 50 μM baclofen (an agonist of GABA_B receptor) that inhibits Ca^{2+} current by $\sim 60\%$ with KS (*tr* 8) and to the μ -agonist without KS (*tr* 17) that blocks 20% of the Ca^{2+} current. The inhibition of Ca^{2+} current mediated by baclofen (*tr* 26) is replaced by the inhibition mediated by the μ -agonist (*tr* 31).

The demodulation by opioids of GABA-mediated inhibition was observed in all neurons investigated. The κ -agonist reversed baclofen-mediated inhibition of $I_{\text{Ca}^{2+}}$ to $16.32 \pm 3.3\%$ ($n = 12$), similar to the inhibition mediated by κ -agonist alone (Fig. 9*A*).

Figure 8. Additive effects of the sequential application of Som and opioid on the transient and sustained components of I_{K^+} . *Aa, Bb*, The effects of Som and κ -agonist (U-50488) on the K^+ currents. The outward control currents (*tr 6, 4*) were inhibited by the drug application (*a, tr 9, 12; b, tr 7, 10*). *Aa*, Time course of the transient component of K^+ current inhibition during the sequential activation of Som and κ -agonist. *Ba*, Time course of the transient (solid circles) and sustained (open squares) component of the I_{K^+} during the sequential application of κ -agonist and Som. The application of Som resulted in a 20% decrease in the transient component of I_{K^+} (*A*). The concurrent application of κ -agonist further decreased to 35%. When the peptides were applied sequentially in the reverse order (*B*), a smaller increase in inhibition on the K^+ current was observed on the transient component of I_{K^+} . However, on the sustained component of the I_{K^+} , a clear additive effect was seen. *Ac, Bc*, Quantitative summaries of Som and κ current inhibition of the transient (open bars) and sustained (cross-hatched bars) outward components of K^+ current (* $p < 0.005$, one-way ANOVA).

Demodulation was also present after the sequential application of baclofen and the μ -agonist. Baclofen-mediated $I_{Ca^{2+}}$ inhibition was decreased to $14.23 \pm 2\%$ ($n = 6$), which is the level of μ -agonist Ca^{2+} inhibition alone (Fig. 9*A*). NE ($50 \mu\text{M}$) inhibited Ca^{2+} current in 12 of 21 (57%) neurons randomly selected. In only 5 of the 12 cells examined (41%), the κ -agonist was found to demodulate the NE-mediated Ca^{2+} current inhibition to $14.8 \pm 2\%$. In the graph in Figure 9*A*, bars only include measurements on neurons in which demodulation was present.

Because DRG neurons are a mixed cell population, it would be of interest to determine whether the presence of demodulation correlates with a distinct sensory modality.

During demodulation in the DRG, there is also a change from voltage-dependent to voltage-independent Ca^{2+} channel inhibition

A protocol similar to that used in CG neurons was used in DRG neurons. A prepulse facilitation (as in Fig. 6) was used during the sequential application of baclofen and opioids (Fig. 10*Aa, b*). In Figure 10*Aa*, three traces were superimposed, showing the current before application of baclofen (*tr 5*, control Ca^{2+} current), during the baclofen inhibition (*tr 17*), and 10 sec after demodulation of the baclofen response by κ -agonist (*tr 15*). Furthermore, the effect of a prepulse conditioning depolarization is shown (Fig. 10*Aa*, right traces). During the baclofen application, a conditioning depolarization facilitated the response by $\sim 20\%$, and KS was eliminated (Fig. 10*Aa*, right *tr 17*). In contrast the demodulation

of Som-mediated inhibition by the κ -agonist U-50488 (Fig. 10*Aa*, right *tr 15*) was not affected by the conditioning prepulse. Figure 10*Ab* plots the time course of the sequential application of κ -agonist and baclofen. Initially, the κ -agonist decreased the Ca^{2+} inhibition by 9%, a level that was maintained during the sequential application of baclofen. After the opioid washout, baclofen rapidly blocked 75% of $I_{Ca^{2+}}$. A second application of baclofen also blocked 75% of the Ca^{2+} current, but this inhibition was reduced to 9% in the presence of the κ -agonist. During the demodulation of baclofen-mediated inhibition by the κ -agonist, the Ca^{2+} inhibition reached the level of the opioid inhibition. Figure 10*Ac* plots facilitation ratios during the sequential application of the transmitters shown in Figure 10*Ab*. When demodulation was achieved, there was a change in ratio from 1 (κ -agonist and baclofen) to 1.9 (baclofen alone).

Thus facilitation was present during baclofen inhibition but not after demodulation of the baclofen inhibition by opioid peptides. Therefore, demodulation in DRG neurons is characterized by a replacement of voltage-dependent with voltage-independent inhibition. This behavior is similar to the demodulation of Som-mediated inhibition described in the CG.

DISCUSSION

This work demonstrates that two modulators, Som and opioids, act on the same Ca^{2+} channel effector, presumably using different pathways to transduce the receptor activation in CG cells.

Figure 9. The demodulation of Som, baclofen, and NE by opioid peptides (μ and κ) is also present in DRG neurons. *A*, Pooled results of the percentage inhibition of the $I_{\text{Ca}^{2+}}$ in DRG by Som, NE, baclofen, and opioids (κ and μ) before and after demodulation. *B*, Bar graph of $I_{\text{Ca}^{2+}}$ prepulse facilitation ratios. *C*, Two sets of three selected traces from *D* superimposed. *Top*, Tr 4 is the control current. Application of 50 μM baclofen (a GABA $_B$ -agonist) reduced $I_{\text{Ca}^{2+}}$ by 62%, displaying KS (tr 8). The μ -agonist (DAMGO) inhibited $I_{\text{Ca}^{2+}}$ by 18% (tr 17). *Bottom*, The effects of the sequential activation of baclofen and the μ -agonist receptors are shown in tr 31. The μ -agonist reduced the initial block of 61% on $I_{\text{Ca}^{2+}}$ (tr 26) to 18% (tr 31). *D*, Diary plot of $I_{\text{Ca}^{2+}}$ changes induced by baclofen and the μ -agonist applied individually, followed by the sequential application of both drugs.

Both pathways appear to act without the requirement for serine/threonine phosphorylation or intracellular Ca^{2+} . Furthermore, the sequential activation of an opioid prevents or reverses the effect of Som, a phenomenon we call demodulation. Other neurons such as DRG cells also have a complement of peptide and opioid receptors in the same cell. Dunlap and coworkers (Diverse-Pierluissi and Dunlap, 1993, 1995; Diverse-Pierluissi et al., 1995, 1996) have shown previously in DRG neurons the existence of parallel cascades converging on the same Ca^{2+} channel effector.

In most instances when more than one modulator acts on Ca^{2+} channels, there is converging inhibition on the Ca^{2+} current such that one transmitter occludes the effect of the other. In this paper we have demonstrated a different kind of interaction. Each modulator activates different parallel pathways via different G-protein subunits that converge on the same effector: the N-type Ca^{2+} channel. This results in the replacement of Som, NE, and GABA Ca^{2+} current modulation by μ - and κ -mediated inhibition.

The Ca^{2+} current inhibition in DRG neurons by GABA and NE is the result of the activation of two signaling cascades. In one cascade GABA and NE are directly coupled to a voltage-dependent pathway that displays KS. The other pathway is volt-

age independent. Furthermore it has been shown that a protein tyrosine kinase is implicated in the GABA inhibition and protein kinase C is involved in the NE modulation (Diverse-Pierluissi et al., 1997). However, with the activation conditions used in this study, both NE- and GABA-induced Ca^{2+} current inhibition showed KS and facilitation. The Som-mediated inhibition in CG is also voltage dependent and displays KS (Meriney et al., 1994).

During demodulation, the inhibition of Ca^{2+} currents by these peptides (Som, NE, and GABA) is replaced by opioid inhibition. In support of this contention, we found that activation of Som, NE, and GABA results in KS and facilitation in contrast to the opioids (κ and μ). During demodulation, KS and facilitation disappear, and the level of the Ca^{2+} current block is the same as that induced by the opioids (κ or μ) alone. Therefore, there is a predominance of the modulation of Ca^{2+} channels by opioids. This demodulation is specific for the actions of μ - and κ -opioids. Although opioids themselves only have a modest effect on inhibiting the calcium current in CG neurons (see Fig. 2), their effects on demodulation are quite robust. This then might be their principal role in these neurons.

Despite differences in the protein kinases involved in the inhibition of individual Ca^{2+} channels by NE, Som, and GABA,

Figure 10. In DRG neurons, there is a change of Ca^{2+} inhibition from voltage dependence to independence during demodulation. *Aa*, Exemplar paired records from the same cell during demodulation (*left tr*) and prepulse depolarization (*right tr*) after the application of baclofen and the κ -agonist alone and after sequential application of the κ -agonist and baclofen. *b*, The chart of Ca^{2+} current inhibition during the drug application of baclofen and κ -agonist alone and after their sequential application. *c*, The values of prepulse facilitation during the demodulation responses illustrated in *b*. There is a 100% change of facilitation between baclofen application alone and during demodulation. *B*, Summary of prepulse facilitation experiments shown in *Aa* (*right tr*) and *c*. The larger ratio (postpulse/prepulse) was seen for baclofen, whereas for baclofen and κ -agonist and for κ -agonist alone there was no facilitation.

demodulation by opioids in all three cases is present in both the CG and DRG. It thus seems that there is an underlying commonality in the demodulation of the three transmitters, which display KS and facilitation and suggest that the phenomenon may be generalized to other neurons with a similar complement of transmitters.

The demodulation we have described is reminiscent of the effect of cholecystokinin octapeptide in occluding the effect of the opioid agonist ohmefentanyl in dissociated rat DRG neurons (Liu et al., 1995).

At what level does the demodulation of Som by opioid peptides occur?

In the cascade initiated by an agonist interacting with a seven-transmembrane receptor, intracellular signaling begins with the activation of a heterotrimer G-protein that causes the G-protein to dissociate into a $G\alpha$ -subunit and $G\beta\gamma$ -dimer, each of which can modulate the ion channel. The demodulation we have observed could be taking place at various points along the transduction pathway. We have however ruled out the participation of the signaling complex at the most proximal level, competition for occupancy of the receptor itself. We reached this conclusion because opioid peptides were not antagonists of Som for its receptor occupation, as was demonstrated in Figure 7.

Another possible locus could be the intracellular domain of the Som receptor at the binding site with G-proteins, where μ - and κ -opioids would directly alter the receptor and thus block activa-

tion of the G-protein. However, because chick CG appears to have only one type of Som receptor (Klann et al., 1998), which inhibits both Ca^{2+} and K^{+} currents, we would have expected that κ and μ peptides would produce demodulation of both Ca^{2+} and K^{+} currents. However, as shown in Figure 8, this did not occur; instead there was an additive effect of Som and opioids on the K^{+} current. This shows that the Som receptor is available for continued binding and G-protein activation during demodulation.

The G-protein-binding site of the receptor also seems to be important for short-term desensitization (Dohlman et al., 1991) that involves phosphorylation of the receptor's intracellular binding domain. One form of desensitization, known as heterologous, can proceed in the absence of agonist receptor binding (Freedman and Lefkowitz, 1996). During this process there is a diminution over the course of several minutes of a cell's response to diverse agonists after exposure to a specific agonist, and this is mediated by second messengers. If a mechanism comparable with heterologous desensitization was involved in demodulation, we would have expected its disappearance or an altered time course after the application of protein kinase inhibitors. In contrast, demodulation occurred in seconds and was still present when phosphorylation was inhibited, even during desensitization. For these reasons, we propose that demodulation probably takes place downstream of the receptor, within the G-protein subunits or at the N-type Ca^{2+} channel-binding site.

Possible mechanism of demodulation

Demodulation is probably the replacement of the Som inhibition by the opioid inhibition mediated by different signaling cascades. It is likely that this interaction of the signaling pathways occurs at the binding site of G-protein subunits with the Ca^{2+} channel. A consensus has emerged that $\beta\gamma$ -subunits directly interact with the α_1 -subunits of the Ca^{2+} channel (Herlitze et al., 1996; Ikeda, 1996). KS has been shown to result from the interaction of a specific $\beta\gamma$ -subunit with the Ca^{2+} channel (Ikeda and Schofield, 1989; Dolphin, 1998). In the present study, we have used KS and voltage-dependent facilitation as a signature of the Som pathway. However, it is not well understood which α - or $\beta\gamma$ -subunits or intracellular pathways are responsible for the steady-state inhibition that is a signature of the μ - and κ -opioid pathway (i.e., no KS and no voltage-dependent facilitation) (Diverse-Pierluissi et al., 1997; Delmas et al., 1998). Consequently, different $\beta\gamma$ - or α -subunits must be interacting with the channel to account for the differences observed in KS and voltage-dependent facilitation of the Som compared with the opioid pathways. Also the opioid subunits may have a higher affinity for the Ca^{2+} channel-binding sites than the Som.

Demodulation could be caused by a competition for the Ca^{2+} channel-binding site by different G-protein subunits activated by Som and opioids. Alternatively, the G-protein subunits activated by opioids could have different binding sites on the Ca^{2+} channel than do the $\beta\gamma$ -subunits activated by Som and thereby cause a conformational change in the Ca^{2+} channel. This would prevent or abolish the Som effect without affecting the binding of the $\beta\gamma_{\text{Som}}$ -subunit, for example, by inducing a change in the channel's voltage-dependent properties. Indeed, there is evidence that the pore properties of N-type Ca^{2+} channel are affected by G-protein stimulation (Kuo and Bean, 1993).

A facilitating component of the competition of the G-protein subunits for the binding site may be the recently discovered regulators of G-protein signaling (RGS) proteins (Berman and Gilman, 1998) that interact with G-protein α -subunits (Arshavsky and Pugh, 1998). The RGS proteins are strong stimulants of the GTPase activity of G-proteins (especially G_i and G_o) and result in an excess of inactivated GDP and a speeding up in the reassociation of G-protein subunits (Doupnik et al., 1997), facilitating G-protein subunit exchange. We speculate that there may be cross talk between specific opioid RGS to the Som G-protein. This could result in an increase of Som G-protein subunit reassociation, preventing their interaction with the Ca^{2+} channel and facilitating the opioid subunit binding to the channel. However, at present too little is known about the regulation of these RGS proteins (Berman and Gilman, 1998) to speculate further on their role in demodulation.

Physiological relevance of demodulation

The present findings are interesting because they have demonstrated a new mechanism of opioid action and have shown that sequential activation of two peptides can modify membrane excitability and thus transmitter release. This mechanism could have broad physiological relevance in that it shows how signaling via G-proteins can influence a cell's behavior in a variable way, depending on the recent past history of neuronal activation.

It is well accepted that Ca^{2+} influx triggers neurotransmitter release. Electrical activity starts the physiological response, but the pathways mediated by G-proteins provide a degree of flexibility in the regulation of this release (Diverse-Pierluissi and Dunlap, 1995). We have shown previously that Som and enkepha-

lins depress ACh release mediated by K^+ depolarization of choroid nerve terminals innervating vascular smooth muscle (Meriney and Pilar, 1987; Gray et al., 1989). Therefore if the opioids demodulate Som, as we show in this work, this novel mechanism could be useful for fine control of transmitter release.

The specific role of modulation in DRG neurons is less clear, although GABA and NE similar to Som in ciliary neurons could also attenuate the synaptic response by inhibiting Ca^{2+} current. The fact that opioids also demodulated the inhibition of Ca^{2+} currents by GABA and NE and the possibility that such modulation could occur at their central synapses in the spinal cord suggest the widespread importance of this mechanism.

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