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Dynorphin A and cAMP-dependent protein kinase independently regulate neuronal calcium currents

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ABSTRACT **The κ-selective opioid peptide dynorphin A (DYN) inhibits neuronal adenylate cyclase activity and reduces neuronal voltage-dependent calcium currents. It is not yet known, however, whether the regulation of calcium channel activity is dependent on or independent of the adenylate cyclase/cAMP system. We used the whole-cell variation of the patch clamp technique to show that DYN reversibly reduced, in a naloxone-sensitive manner, calcium currents in acutely dissociated rat nodose ganglion neurons. DYN slowed the rate of current activation and had a greater effect on currents evoked from relatively negative holding potentials. These actions were mimicked by guanosine 5′-[γ-thio]triphosphate, which activates GTP-binding proteins (G proteins), and were blocked by pretreatment with pertussis toxin, which inactivates G_i- and G_o-type G proteins. In contrast, calcium currents recorded in the presence of the catalytic subunit of the cAMP-dependent protein kinase (AK-C), included in the recording pipette, increased in magnitude throughout the recording. DYN was applied to neurons before and after the effect of AK-C became apparent; the reduction of calcium currents by DYN was greater in the presence of AK-C than in its absence. We conclude that the acute reduction of neuronal calcium currents by DYN occurred by means of activation of pertussis toxin-sensitive G_i- or G_o-type G proteins. The persistence of the action of DYN in the presence of AK-C indicates, however, that this effect was independent of a reduction of the activity of the adenylate cyclase/cAMP system and suggests in addition that phosphorylated channels may be preferentially inhibited by DYN.**

The κ-selective opioid peptide dynorphin A (DYN) reduces neuronal calcium currents (1–3) and inhibits neuronal adenylate cyclase activity (4, 5), but the pathway by which it alters channel activity is not known. Several neurotransmitters reduce voltage-dependent neuronal calcium currents by means of GTP-binding proteins (G proteins) (for example, see refs. 6–9), effects that can be mimicked by guanosine 5′-[γ-thio]triphosphate (GTP[γS]) (6), an activator of G proteins (10). In addition, pertussis toxin (PTX), which inactivates G_i- and G_o-type G proteins, can block neurotransmitter action on calcium currents (6–9). The G protein subtypes that couple neurotransmitter receptors to calcium channels have been identified by using specific antibodies to block a neurotransmitter response or exogenous G proteins to restore a response previously blocked with PTX (8, 11, 12). The coupling of κ-opioid receptors to calcium channels may thus require G proteins, but this has not yet been demonstrated directly.

Furthermore, the identity of the G protein-dependent pathway(s) that mediates neurotransmitter effects on calcium channels is not entirely known. Neurotransmitter-activated G proteins might affect calcium channels directly, as G_s does in heart (13), or indirectly, by activating a second messenger system. For example, elevated intracellular levels of cAMP or increased activity of the cAMP-dependent protein kinase (AK) increases calcium channel activity in heart cells, invertebrate neurons, and clonal pituitary cells (14–16).

Taken together, the results of these studies suggest that DYN might reduce calcium currents by inhibition of adenylate cyclase, a reduction of intracellular cAMP levels, and a reduction in the activity of AK. It is also possible that DYN-induced changes in calcium currents could be dependent on G proteins but independent of the adenylate cyclase/cAMP system. To distinguish between these possibilities, we examined the effects of DYN on calcium currents in acutely dissociated rat nodose ganglion neurons by using the whole-cell variation of the patch clamp technique. The effects of DYN were tested after PTX pretreatment and in the presence of GTP[γS] to assess the role of G proteins in the coupling of κ receptors to calcium channels. In addition, we determined the effect of DYN in the absence and presence of the catalytic subunit of AK (AK-C). We reasoned that if a reduction in adenylate cyclase activity were the sole mechanism for the DYN-induced reduction in calcium current, then the effect of DYN would not be apparent in the presence of exogenous AK-C.

MATERIALS AND METHODS

Preparation of Acutely Dissociated Neurons. Nodose ganglion neurons were prepared from 6- to 10-day-old rats, using a procedure similar to that already described (17). Nodose ganglion neurons were isolated after enzymatic treatment and trituration and were plated in minimum essential medium (GIBCO) supplemented with $NaHCO_3$ (16.5 mM), glucose (28.2 mM), nerve growth factor (10 ng/ml; Boehringer Mannheim), penicillin (50 units/ml), streptomycin (50 mg/ml), and fetal calf serum (10%; GIBCO). Cultures were incubated at 37°C in a 93% air/7% CO_2 atmosphere and used for recordings within 1–24 hr.

Preparation of Solutions. DYN (Peninsula Laboratories) was dissolved in distilled water and stored frozen as a lyophilized powder. Just before the experiment, DYN was reconstituted in bath solution (see below) containing 0.1% bovine serum albumin (Sigma) at a final concentration of 3 μM. Naloxone (Sigma) was dissolved in bath solution, also at a concentration of 3 μM. Purified AK-C was prepared within 24 hr of the experiment as described (18) and stored at 4°C as a 1 mg/ml stock. AK-C was diluted into the recording pipette solution (see below) to a final concentration of 50 μg/ml. This solution, stored on ice, retained full activity for several hours (assayed as described in ref. 19). PTX (Sigma) was prepared as described (17).

Whole-Cell Patch Clamp Recordings. Voltage clamp recordings were obtained using the whole-cell variation of the

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Abbreviations: DYN, dynorphin A; AK, cAMP-dependent protein kinase; AK-C, AK catalytic subunit; PTX, pertussis toxin; G protein, GTP-binding protein; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

patch clamp technique. Cells were bathed in a solution of 67 mM choline chloride, 100 mM tetraethylammonium chloride, 5.6 mM glucose, 5.3 mM KCl, 5.0 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 10 mM Hepes (pH 7.3–7.4, 310–330 milliosmolar; all reagents from Sigma). Glass recording patch pipettes (Fisher brand microhematocrit tubes) with resistances of 0.5–1.25 MΩ were filled with recording solution consisting of 140 mM CsCl, 10 mM Hepes, 10 mM EGTA, 5 mM ATP (magnesium salt), and 0.1 mM GTP (lithium salt) or 0.1 mM GTP[γS] (lithium salt) (all reagents from Sigma). The pH (7.3–7.4) was adjusted with 1 M CsOH after the addition of ATP, and the osmolality was 10–15% below that of the bath solution (280–300 milliosmolar).

Recordings were made at room temperature by using an Axopatch 1-B patch clamp amplifier (Axon Instruments, Burlingame, CA). Pipette and whole-cell capacitance and series resistance were corrected by compensation circuitry on the patch clamp amplifier. Typically, initial input resistances were 500 MΩ–1 GΩ, and the series resistance was 1–4 MΩ. Voltage step commands were generated, and currents were digitized (5 kHz), stored, and analyzed by a microcomputer (IBM AT or equivalent) using the program pClamp (Axon Instruments). The current traces were filtered with a Bessel filter at 10 kHz (−3 decibels).

DYN and naloxone were applied to the cell by using pressure ejection (6–10 kPa) from blunt-tipped glass micropipettes positioned ≈50 μm from the cell. Applications of DYN or naloxone were 2–5 s in duration, just before currents were evoked. Neither diluent nor naloxone (usually applied before DYN) had any effect on evoked currents. The pressure ejection technique allowed application of compounds to individual neurons but did not allow determination of the exact concentration of test compounds at the cell under study, a concentration that could be less than the concentration in the pressure-ejection micropipette. The pipettes were removed from the bath when not in use.

DYN is a κ-selective opioid agonist that can bind to μ or δ receptors *in vitro* at concentrations in the micromolar range (20). In our preparation DYN reduced calcium currents at concentrations as low as 300 nM (data not shown). In addition, we have shown that μ- and δ-selective agonists increased potassium conductance of dorsal root ganglion neurons (21), an effect also noted in locus coeruleus and myenteric neurons (22, 23). DYN, in contrast, reduced calcium conductance in dorsal root ganglion neurons (1, 2, 21, 24) and in myenteric (25) and spinal cord neurons (26). In the present experiments, there was no effect of μ- and δ-receptor ligands, morphiceptin, and [2-D-penicillamine, 5-D-penicillamine]enkephalin, respectively, on calcium currents in neurons responsive to DYN (unpublished observations). We have assumed, therefore, that the effects of DYN on nodose ganglion neurons were likely mediated by κ receptors.

Some neurons were pretreated with PTX (150–200 ng/ml) for 18–24 hr before use, as described (17). The recording pipette solution also contained PTX (150 ng/ml) when recording from pretreated neurons.

For all recordings in the presence of AK-C, the tip (0.5–1 mm) of the recording pipette was filled with standard pipette solution and the pipette was "back-filled" with the experimental solution. For the experiments illustrated in Fig. 3, the tip was filled to 2 mm with standard solution, thereby delaying the onset of the actions of AK-C. The effects of AK-C on calcium currents were reduced by boiling or eliminated by incubation of AK-C with a specific peptide inhibitor (27).

Analysis of Current Components. Leak current was estimated as the inverse of the current evoked with hyperpolarizing voltage commands of equal magnitude to the depolarizing commands used to evoke the inward currents. This current was digitally subtracted from the relevant inward current to obtain the calcium current. Leak currents were unaffected by DYN, AK-C, or naloxone.

Three calcium current components were present in nodose ganglion neurons (17), similar to the T, N, and L currents described in dorsal root ganglion neurons (2, 28). The T current was evoked at clamp potentials (V_c) at or positive to −50 mV, whereas at V_c positive to −20 mV the N and L current components were evoked. In order to distinguish between N and L components, we evoked currents at 1-min intervals after patch rupture, alternating between holding potentials (V_h) of −80 and −40 mV. We used the current evoked from V_h = −40 mV as an estimate of the L current component, and the additional more rapidly inactivating current component evoked from V_h = −80 mV as an estimate of the N current component. The L current component was slightly underestimated, and the N current component was somewhat overestimated by using this protocol because there was steady-state inactivation of the L current component at V_h = −40 mV. The results are described in terms of the evoked currents unless we refer to a particular current component. The T/N/L nomenclature is used, with the caveat that the whole-cell technique does not allow unequivocal identification of currents carried by channel subtypes.

Statistical Comparisons. Statistical comparisons were made by using the Student two-tailed *t* test.

RESULTS

In the first series of experiments, we compared the effects of DYN and AK-C on the calcium current components of nodose ganglion neurons (Fig. 1). Stabilization of the currents occurred within the first 5 min after patch rupture. Thereafter, T currents remained constant (not shown; see refs. 17 and 27), but currents containing the N and L components declined slowly to about 60% of their maximal value by 20 min after patch rupture (Fig. 1*A*, compare early and late currents). Application of 3 μM DYN had no effect on T currents (data not shown; see below) but reduced calcium currents consisting of the N and L components (Fig. 1*A*). DYN reduced currents in 22 out of 25 neurons, with a mean reduction of peak current (I_p), evoked from V_h = −80 mV, of 43 ± 3% (mean ± SEM; $P < 0.05$). DYN also slowed the rate of current activation. For example, the time to I_p in currents evoked from V_h = −80 mV increased from 10–15 ms to 30–80 ms in currents evoked in the presence of DYN. Calcium currents returned to control values within 2–5 min after DYN application.

All effects of DYN were reduced or prevented by prior application of 3 μM naloxone (data not shown). In 1 out of 5 neurons, naloxone reduced the effect of DYN 60%, and in the remaining 4 neurons, naloxone application resulted in a >90% block.

The reduction of calcium currents by DYN was voltage-dependent in two respects. First, the action of DYN was greater on the current evoked from V_h = −80 mV than on the current evoked from V_h = −40 mV. This was seen most easily by comparing the I_p values of currents evoked from the two V_h; in the presence of DYN, this difference was virtually eliminated. Second, the effect of DYN was not present at all V_c. We evoked a series of currents from V_h = −90 mV at V_c ranging from −120 to +80 mV and used I_p values to construct current-voltage plots (Fig. 1*C*). The T current component, evoked at V_c between −50 and −20 mV, was unaffected by DYN, whereas currents evoked at V_c between −10 and +40 mV were reduced by DYN. Outward currents, evoked at V_c positive to +50 mV, were also unaffected by DYN.

AK-C (50 μg/ml), included in the recording pipette, also had no effect on T currents (data not shown; see ref. 27). Currents containing the N and L components, recorded in the

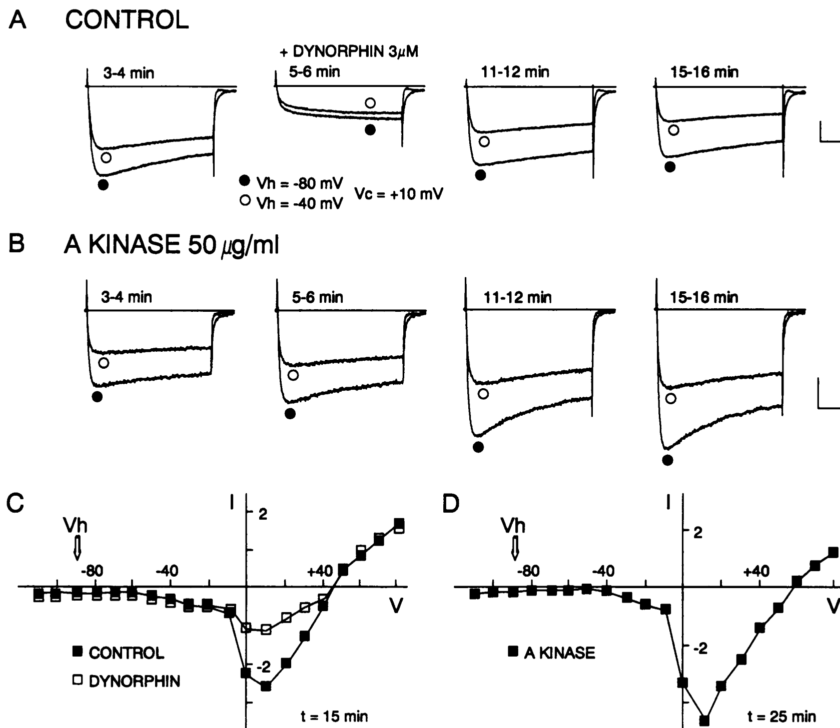

FIG. 1. DYN and AK-C effects on calcium currents in nodose ganglion neurons. (*A*) Currents were evoked at V_c = +10 mV from V_h of −80 (●) and −40 mV (○) (the position of the symbol indicates I_p) in the absence and presence of 3 μM DYN. (*B*) Currents recorded, in a different neuron, as in *A* except that the recording pipette contained AK-C (50 μg/ml). Calibration bars: 1 nA vertical; 20 ms horizontal. (*C* and *D*) Peak current–voltage plots derived from control currents recorded in the absence and presence of 3 μM DYN (*C*) or from currents recorded in the presence of AK-C (*D*).

presence of AK-C, were similar to control currents in the first 5 min of the recording but increased in magnitude thereafter (Fig. 1*B*). This effect of AK-C was maximal 12–15 min after patch rupture. In one experiment, for example, I_p values of currents evoked from V_h = −80 mV increased 33 ± 8% over initial I_p values by 15 min (n = 9 neurons, $P < 0.05$).

AK-C also had a greater effect on the additional current component evoked from V_h = −80 mV compared to that evoked from V_h = −40 mV. Compared to the 33% increase in I_p of currents evoked from V_h = −80 mV, the I_p of currents evoked from V_h = −40 mV increased only 17 ± 18% over 15 min (different than control values, $P < 0.05$). In addition, the rate of current inactivation was more rapid in the presence of AK-C, particularly in those currents evoked from the more negative V_h. The voltage range of current activation was similar to that in control neurons (compare Fig. 1 *C* and *D*).

We next tested the effect of DYN on currents recorded in the presence of PTX or GTP[γS] (Fig. 2). First, we tested the effect of the peptide on currents in neurons that had been pretreated with PTX (150–200 ng/ml) for 18–24 hr. Whereas 88% of control neurons (22 out of 25) responded to DYN, only 20% of PTX-treated neurons (2 out of 10) responded to DYN with an average current reduction of 14% (Fig. 2 *A* and *B*). Thus, the effect of DYN was blocked by PTX, an inhibitor of G_i- and G_o-type G proteins. Inclusion in the recording pipette of GTP[γS], an activator of G proteins, mimicked the DYN effect (compare Fig. 2 *A* and *C*) within the first minutes after patch rupture (see ref. 17). The reduction of calcium currents by GTP[γS] was irreversible, however, and application of DYN in the presence of GTP[γS] was without effect. Taken together, these results indicate that the reduction of calcium currents by DYN was mediated by activation of G_i- and/or G_o-type G proteins.

Finally, as a direct test of the hypothesis that the action of DYN on calcium currents was dependent on the activity of the adenylate cyclase/cAMP system, we applied DYN in the presence of AK-C. We reasoned that if the DYN-induced reduction of calcium currents required G protein-mediated inhibition of adenylate cyclase activity, an effect of DYN would not be apparent in the presence of exogenous AK-C. The effect of DYN on calcium currents was determined twice in each neuron, before and after the effect of AK-C was apparent (Fig. 3). The initial application of DYN reduced I_p 46 ± 8% (V_h = −80 mV; n = 7 neurons, $P < 0.05$). AK-C increased I_p 32 ± 9% ($P < 0.05$), and when DYN was reapplied, the reduction in current was *greater* than the initial

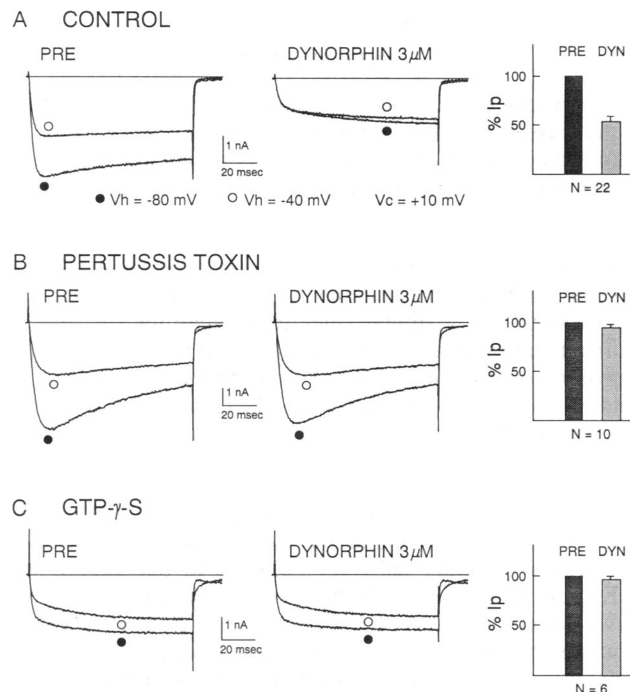

FIG. 2. DYN effects on calcium currents recorded from neurons pretreated with PTX or recorded in the presence of GTP[γS]. Currents recorded alternately from V_h of −80 (●) and −40 mV (○) in the absence (PRE) and presence of 3 μM DYN. The bar graphs show the responses of the stated number of neurons to DYN as a percent of the PRE I_p value (V_h = −80 mV). The height of each bar is the mean ± SEM (error bars shown in one direction only). Currents recorded from control neurons (*A*), from neurons pretreated with PTX (*B*), and from neurons in the presence of GTP[γS] (*C*) are shown.

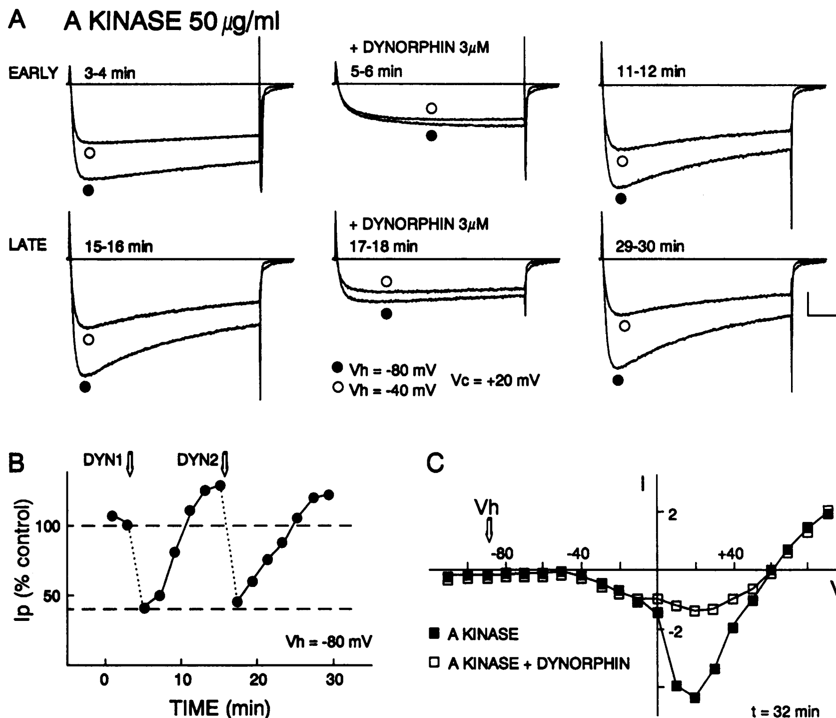

FIG. 3. DYN-induced reduction of calcium current in the presence of AK-C. (*A*) Currents evoked alternately from $V_h = -80$ (●) and -40 mV (○). The tip of the recording pipette contained the standard solution, and the body of the pipette contained standard solution plus AK-C (50 μg/ml). DYN was applied before (EARLY) and after (LATE) the effect of AK-C became apparent. Calibration bars: 1 nA vertical; 20 ms horizontal. (*B*) I_p ($V_h = -80$ mV) values of currents in *A* plotted as a function of time. DYN applications are indicated by arrows, and initial I_p values in the absence and presence of DYN are indicated by the horizontal dashed lines. (*C*) Peak current–voltage relation of currents recorded in the presence of AK-C, before (■) and after (□) the application of DYN.

reduction (Fig. 3*A*), averaging 168 ± 30% of the initial effect ($n = 7$ neurons, $P < 0.05$). The time course of a typical experiment is illustrated in Fig. 3*B*, which shows the I_p values ($V_h = -80$ mV) obtained throughout the recording. Current–voltage plots showed that the effect of DYN was evident over a similar range of V_c in the presence of AK-C as in its absence in all six neurons tested (Fig. 3*C*).

DISCUSSION

The present results show that DYN reduced neuronal calcium currents by a G protein-dependent mechanism. The experiments with AK-C confirm that phosphorylation is an important regulatory mechanism of calcium channel activity in neurons but that this pathway is probably not involved in mediating the acute effect of DYN on calcium channels.

An interesting finding of the present experiments is that, although DYN decreased and AK-C increased calcium currents, both had a similar selectivity regarding the affected current components. Neither DYN nor AK-C affected the T current component, whereas both primarily affected the additional current component evoked from $V_h = -80$ mV compared to $V_h = -40$ mV. This is consistent with our previous finding that DYN selectively reduced the N calcium current component of cultured dorsal root ganglion neurons (2). AK (or agents that increase intracellular cAMP levels) increase L-type currents in myocytes, invertebrate neurons, and clonal pituitary cells (14–16), and in our experiments AK-C did increase slightly the current evoked from $V_h = -40$ mV, which probably consisted largely of the L current component. AK-C had a greater effect on currents evoked from more negative potentials, however, which suggests that AK-C may be a potent regulator of the N current component as well. The more rapid inactivation of the whole-cell current in the presence of AK-C supports this view. Additional analysis using curve-fitting routines showed that AK-C had a greater effect on the N current component than on the L current component (27). Single-channel analysis will be required to confirm this hypothesis, but the present data are entirely consistent with the conclusion that DYN and AK-C regulate the N current component primarily. The selective regulation of calcium current components by DYN or AK-C may be of physiological importance, particularly if different channel types control specific neuronal processes such as neurotransmitter release (29–31).

PTX blocked the actions of DYN, indicating that a G_i- or G_o-type protein was required for its effects on neuronal calcium currents. Attali *et al.* (3) have shown that the inhibition by κ-opioid agonists of K^+-evoked calcium influx in cultured neurons is partially reversed by PTX. Hescheler *et al.* (32) have reported that [D-Ala[2], D-Leu[5]]enkephalin (DADLE), a μ- or δ-selective opioid agonist, reduced calcium currents in a neuroblastoma × glioma cell line, an effect mediated by G_o. In most cases, μ- or δ-opioid ligands couple to potassium channels (21–23), so it is not clear if the mechanism by which DADLE reduces calcium currents in neuroblastoma cells occurs in other neuronal preparations. These data are consistent with ours in a general sense, however, in that we show the effect of DYN on calcium currents clearly requires a G protein.

The present data do not allow us to deduce the exact mechanism by which G proteins may alter calcium channel function or the pathway by which this regulation is effected. A possible mechanism may be postulated on the basis of the following two observations: first, that DYN significantly slowed calcium current activation and second, that its effects were not evident on outward currents evoked at very positive V_c. These findings confirm those of Bean (24), who proposed that the effects of certain neurotransmitters (including DYN) on neuronal calcium currents are due to the conversion of the target channel from its normal ("willing") state to one less responsive to changes in membrane potential ("reluctant"). This model predicts that strongly depolarizing commands can overcome the neurotransmitter effect. The present results are consistent with the proposed model and support the idea that this type of change in channel activity is due to the action of an activated G protein. Since GTP[γS] would be expected to activate all endogenous G proteins but mimics the effect of DYN, then there may be only a limited number of G protein subtypes (G_i or G_o) that regulate neuronal calcium channels or there may be a particular subtype that has a "dominant" effect on channel gating.

The acute reduction of neuronal calcium currents by DYN occurred by a G protein-coupled pathway independent of

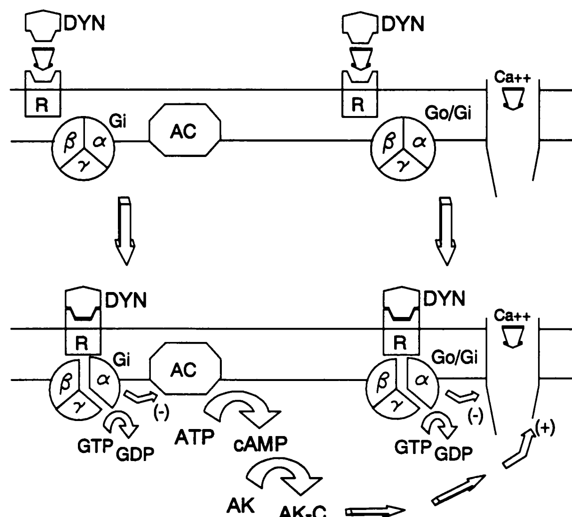

FIG. 4. Scheme showing two pathways by which dynorphin A may affect calcium channel activity. The α subunit of the respective G protein is assumed to be the coupling moiety. Channel activity is enhanced by AK and reduced by DYN. R, κ receptor; α, β, and γ, G protein subunits; AC, adenylate cyclase.

inhibition of the adenylate cyclase/cAMP/AK system. This effect may be mediated by a direct action of G proteins, but the present data cannot exclude the involvement of other second messenger systems. It is clear, however, that AK was an important regulator of calcium currents. Current rundown was reversed by AK-C, and in its presence, the effect of DYN was enhanced. Phosphorylation of calcium channels (33–35) or associated proteins might be, therefore, an important mechanism to maintain calcium channels in an activatable state (16) that is sensitive to the actions of neurotransmitters. Nevertheless, the effect of DYN (and of G proteins) was "dominant" over the effect of AK-C, suggesting that phosphorylation and G proteins have mutually dependent but separate regulatory actions on calcium channels.

Given these different regulatory roles of G proteins and AK-C, it is important to note that the present experiments do not eliminate the possibility that opioid peptides may affect calcium channel activity by means of the adenylate cyclase/ cAMP system but show only that the *acute* effect of DYN did not require this pathway. In fact, recent work has shown that μ and δ receptors as well as κ receptors may inhibit neuronal adenylate cyclase activity (4, 5, 36–39), which could, in turn, result in a reduction of calcium channel activity. This hypothesis could account for both *acute* (AK-independent) and *long-term* (AK-dependent) modulation of calcium channel activity by κ-opioid peptides, by means of different G protein-dependent pathways (Fig. 4). Such a scheme may also account for development of desensitization or tolerance to opiates (for example, see refs. 5, 38, and 39). The end result, a decrease in calcium influx, would influence a wide variety of calcium-dependent intraneuronal processes and may be the basis for the inhibition of neurotransmitter release by opioid peptides (25, 40, 41). If DYN and other neurotransmitters regulate ion channel activity by means of independent pathways, the result would be greater flexibility of signal transduction.

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