0026-895X/94/050793-06$03.00/0

MOLECULAR PHARMACOLOGY, 46:793–798, 1994.

ACCELERATED COMMUNICATION

Nucleotide-Independent Modulation of Ca^{2+}-Dependent K^+ Channel Current by a μ-Type Opioid Receptor

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Received July 7, 1994; Accepted August 25, 1994

SUMMARY

Physiological responses to opiates and opioid peptides are transduced via receptors coupled to G proteins. The effectors for these G proteins are often ion channels or second messenger systems that modulate channel activity. In cultured bovine adrenal medullary chromaffin cells (BAMCCs), the activity of a calcium-dependent, voltage-sensitive, potassium (BK) channel is robustly potentiated by a μ-type opioid receptor, an effect consistent with the inhibitory role of opioids versus neural excitability. Patch-clamp electrophysiology was used to investigate coupling between the μ receptor and BK channel, leading to rather surprising results. Potentiation of BK channel activity by the μ-selective agonist [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (10 nM) was unaffected by all attempts to disrupt or alter G protein function, including incubation of cells with pertussis toxin (PTX) and inclusion of guanosine 5′-*O*-(2-thio)diphosphate (GDPβS) or guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) in intracellular recording solutions. However, dopamine D_2 receptor potentiation of BK current in these same cells was affected by PTX, GDPβS, and GTPγS in predictable fashion. Thus, PTX and GDPβS inhibited dopamine potentiation of BK current, and GTPγS prolonged reversal of dopamine action. These results suggest that the BAMCC BK channel is not coupled to the μ receptor via a GTP-dependent mechanism, whereas in the same cells the dopamine D_2 receptor modulates BK channel activity in a conventional GTP-dependent manner. In addition, replacement of both ATP and GTP with nonhydrolyzable analogs also failed to affect either potentiation or recovery of BK channel activity in response to [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin. These results indicate that in BAMCCs the μ-opioid receptor modulates BK channel activity independently of either G proteins or phosphorylation-dependent processes.

Opiates and endogenous opioid peptides act via a group of G protein-coupled receptors (μ, κ, and δ) to participate in a wide variety of centrally and peripherally mediated physiological events, such as control of nociception, regulation of cardiovascular output, coordination of gastrointesinal motility, and modulation of respiration (for reviews, see Refs. 1–3). In nervous tissue a major action of opioid peptides is the inhibition of neuroexcitability via modulation of Na^+, K^+, and Ca^{2+} channels. However, the exact nature of opioid receptor-ion channel interactions underlying these effects is incompletely understood (4–10). We are studying the mechanisms that couple opioid receptors to ion channels in cultured BAMCCs. BAMCCs represent a useful model for studying opioid-mediated changes in neuroexcitability, in that they receive neuronal inputs that are in part opioid peptidergic, they co-secrete opioid peptides and catecholamines, and their secretory output is modulated by exogenous opiates and endogenous opioid peptides (11–13). Moreover, we have previously shown that a potentially significant physiological action of opioids in BAMCCs is the potentiation of a voltage-sensitive BK current that should suppress secretion by shortening action potential duration (14, 15).

Here we provide evidence that a μ-type opioid receptor increases the activity of BK channels in BAMCCs via a mechanism that is independent of G proteins, protein kinases, or protein phosphatases. Treatment with PTX failed to disrupt coupling of the μ receptor to the BK channel. Furthermore, this coupling persisted despite dialysis of the cell interior with the nonhydrolyzable GDP and ATP analogs GDPβS and AMP-PNP, respectively. GDPβS and AMP-PNP were able, however, to disrupt potentiation of BK current by 100 nM dopamine acting via a D_2 receptor. Dopamine potentiation of whole-cell

This work was supported by a grant-in-aid (S.G.R.) and a predoctoral fellowship (W.A.T.) from the American Heart Association, Indiana Affiliate.

ABBREVIATIONS: BAMCC, bovine adrenal medullary chromaffin cell; AMP-PNP, adenylylimidodiphosphate; BK channel, big conductance, calcium-dependent, potassium channel; DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; PTX, pertussis toxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

BK current was used to verify the efficacy of our efforts to disrupt G protein-coupled processes in BAMCCs. Our findings indicate that the potentiation of BK channel activity by opioids is independent of GTP- or ATP-dependent processes. This suggests the existence of an alternative to the G protein-dependent mechanisms by which opioid receptors, and other members of the seven-transmembrane domain superfamily of receptors, modulate the activity of ion channels.

Materials and Methods

Preparation and maintenance of BAMCC cultures. Primary cultures of adult BAMCCs were obtained via collagenase digestion of fresh adrenal glands [by a method modified from that of Greenberg and Zinder (16)]. After digestion and wash, the crude cell population was resuspended in culture medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin; all from GIBCO). Nonchromaffin cells were removed by incubating the suspension in 100-mm tissue culture plates in a 95% air/5% CO_2 atmosphere at 37° for 4–5 hr. By that time, nonchromaffin cells had adhered to the plastic tissue culture plates and the poorly adherent BAMCCs could be aspirated along with the culture medium (17). BAMCCs were then seeded onto 35-mm tissue culture plates coated with 5 μg/cm^2 type 1 rat tail collagen (Collaborative Biomedical Products). More than 85% of the cells in these cultures stained positively with neutral red (0.3 mg/ml, for 15 min at 37°), indicating that they contained catecholamines and were most likely BAMCCs (18). Conversely, the majority of the cells adhering to plastic tissue culture plates did not stain with neutral red, and their morphologies were clearly different from those of the neutral red-positive cells plated on collagen. Culture medium was completely changed 24 hr after initial seeding and every 72 hr thereafter. Cultures were maintained in a 95% air/5% CO_2 atmosphere at 37° and were used for experiments at 1–8 days after plating.

Recording procedure and measurement of BK currents. Whole-cell and single-channel currents were recorded using an Axopatch 200A patch-clamp amplifier (Axon instruments), filtered at 3 kHz with an eight-pole Bessel filter (Frequency Devices), and digitized at the appropriate frequency. Standard $P/4$ leak subtraction was used on all whole-cell recordings. Data acquisition and analysis were performed with Pulse (Instrutech Corp., Great Neck, NY) and TAC (HEKA Elektronik, Göttingen, Germany) software, running on a Macintosh Centris 650 computer.

Recording solutions. For patch-clamp experiments the external solution contained 143 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25 mM $CdCl_2$ (to block inward Ca^{2+} current), and 10 mM HEPES, pH 7.4, and the internal solution contained 150 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and either 0.3 μM or 1 μM free Ca^{2+} (1 mM EGTA plus either 0.70 or 0.89 mM $CaCl_2$, pH 7.4). Unless otherwise indicated, all patch pipette solutions contained 1 mM ATP and 0.5 mM GTP. Patch pipettes had resistances of 4–7 MΩ, and cells and patches were allowed to dialyze for at least 2–4 min before the beginning of data acquisition. Solutions of test compounds were made up at the appropriate concentration, on the day of the experiment, from either powder or 1–100 mM frozen (−30°) stock aliquots. Compounds were applied to cells and excised patches via blunt-tipped micropipettes (10–15-μm i.d.).

Materials. SCH23390, spiperone, and PTX were from Research Biochemicals International. Rat tail collagen was obtained from Collaborative Biomedical Products. Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, and streptomycin were obtained from GIBCO. All other compounds were obtained from Sigma Chemical Co.

Data analysis and presentation. Increases in current are reported as percentage increase over control current. All measurements are given as mean ± standard error. Increases in current during experimental perturbations of signal transduction mechanisms were statistically compared with control increases by a paired t test at a 0.05 level of significance.

Results and Discussion

Opiate potentiation of BK current is stereoselective. Based on work by Marty and Neher (19), we have established recording conditions for isolating the BAMCC BK current from other outward currents and for studying its modulation by opioids (14). BK current is identified by its resistance to voltage-dependent inactivation when the cell is held at 0 mV, its strong voltage sensitivity (revealed by outwardy rectifying whole-cell currents in response to ramp commands from 0 to 100 mV), its dependence on intracellular calcium, and its sensitivity to block by charybdotoxin. Other outward currents are inactivated by the prolonged holds at 0 mV used for this study. Indeed, the only significant outward current that can be activated when the holding potential is set to 0 mV is Ca^{2+} and voltage sensitive as well as charybdotoxin blockable, consistent with the BK identification. Whole-cell current isolation is further substantiated by the lack of contaminating currents in single-channel BK records obtained with either depolarizing voltage ramps from 0 mV or constant depolarizations (Ref. 14 and this study). Thus, under the recording conditions used here, BK current was the only outward current likely to be modulated by applied opioid peptides and other ligands. Through the use of selective opioid agonists and antagonists, the receptor mediating the opioid potentiation of the BK current was previously shown to be of the μ type (14). This initial determination that the effect of opioids on BK current is mediated by an opioid receptor is supported by the differential effects of the stereoisomers levorphanol and dextrorphan on whole-cell BK current (Fig. 1a). Overall, levorphanol increased BK current by 109 ± 7% (seven cells), whereas 10 nM and 10 μM dextrorphan increased current by 9 ± 1% (three cells) and 38 ± 11% (four cells), respectively.

The μ receptor and BK channel are tightly coupled. Whole-cell BK currents are potentiated in a reversible and repeatable manner by the μ-opioid receptor agonist DAGO, as is BK channel activity in outside-out patches, suggesting tight receptor channel coupling (14). Additional evidence of tight coupling is demonstrated in Fig. 1b, which shows that extracellular application of DAGO did not affect BK channel activity in isolated cell-attached patches (six cells). This indicates that the μ receptor does not act via a freely diffusible second messenger. After completion of the cell-attached recording, whole-cell access was established (in three of the six cells) and the whole-cell BK current was shown to be responsive to DAGO (Fig. 1b, *right inset*). Thus, the failure of DAGO to elicit potentiation of BK channel activity in the cell-attached configuration was not due to the cell being nonresponsive to DAGO.

The μ receptor does not require GTP to potentiate BK current. The close association of the BK channel with the μ receptor and the lack of freely diffusible second messenger involvement is analogous to other K^+ channel-receptor associations, particularly those with opioid receptors, for which coupling is typically via PTX-sensitive G proteins (5–8, 20, 21). We therefore attempted to uncouple the μ-opioid receptor and the BK channel by disrupting G protein-driven signaling processes in BAMCCs. To verify the disruption of G protein function, another G protein-coupled receptor that would affect whole-cell BK current in BAMCCs was sought. Dopamine (100 nM) was found to reversibly potentiate whole-cell BK current by 84 ± 6% (18 cells) (Fig. 2a1) but did not affect BK channel activity in outside-out patches (five patches) (data not shown). Based on the use of the selective antagonists spiperone (1 μM)

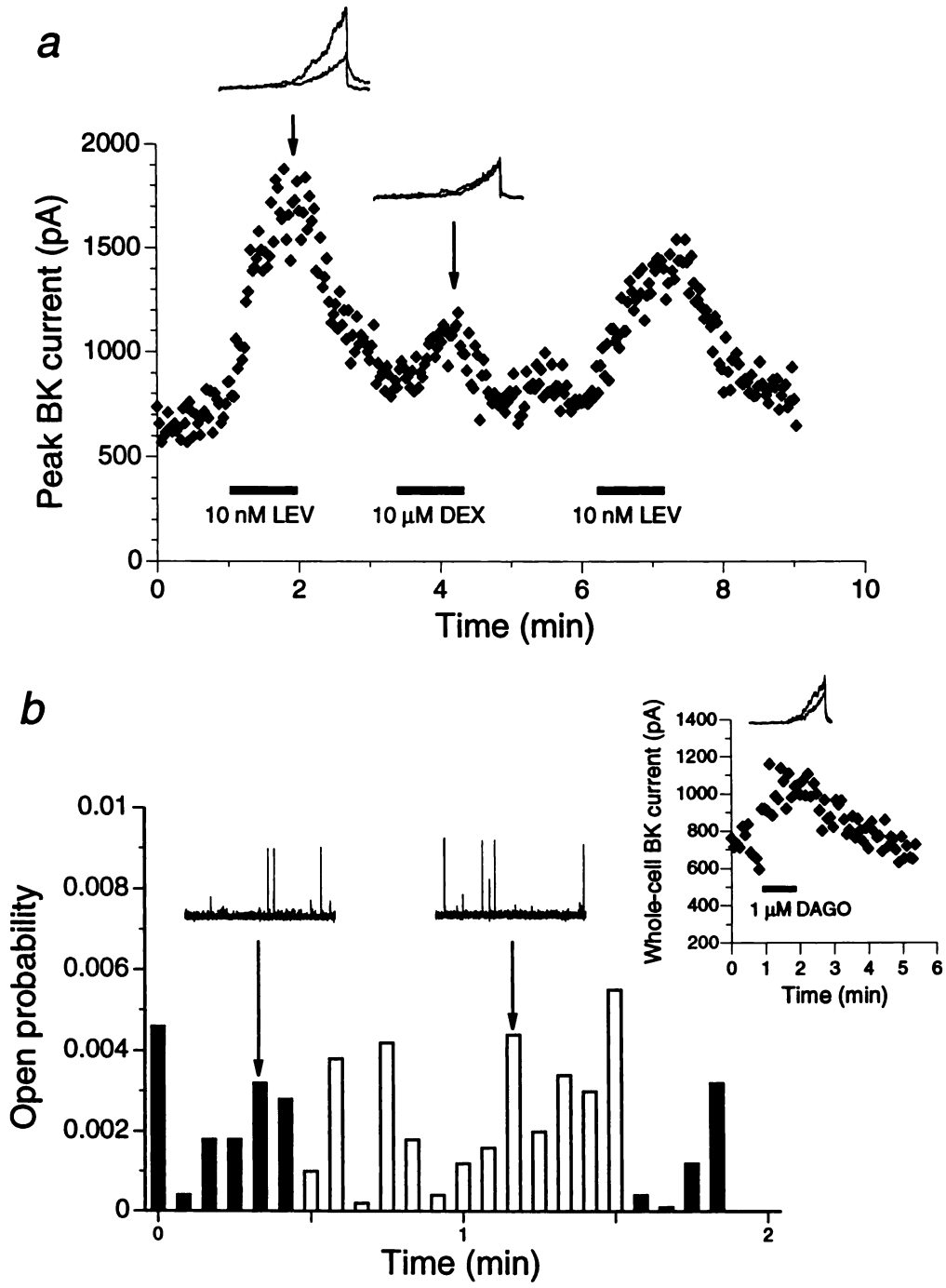
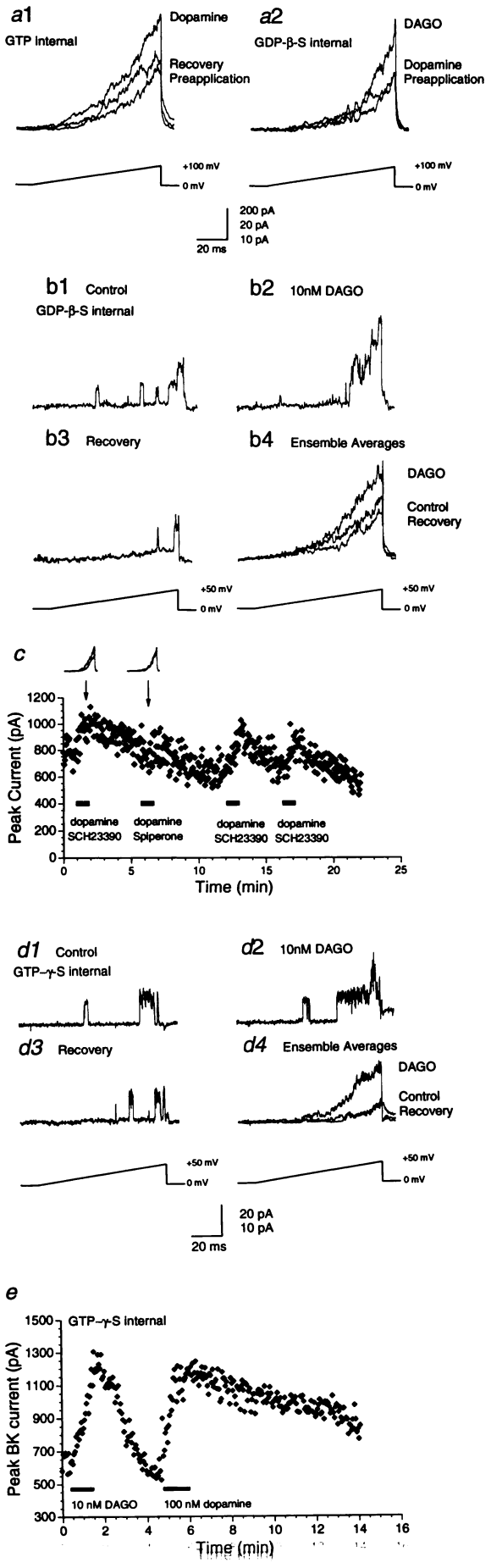


Fig. 1. The μ-type opioid receptor in BAMCCs is stereoselective and closely associated with the BK channel. a, The opioid receptor agonist levorphanol (*LEV*) is far more potent than the stereoisomer dextrorphan (*DEX*) in potentiating whole-cell BK current. Cells were held at 0 mV and whole-cell currents were recorded using a voltage ramp from 0 to +100 mV over 100 msec. *Insets*, sample current records are from just before drug application and during maximal response to the drug. Drugs were extracellularly applied via pressure ejection from micropipettes (*black lines*, applications). b, Cell-attached single-channel recordings are suggestive of close association between the μ receptor and BK channel. In a cell-attached patch recording, application of DAGO (*open columns*) to the extrapatch membrane does not alter the open probability of BK channel(s) isolated in the patch pipette. *Black columns* represent periods when cells were bathed in external recording solution only. *Left insets*, single-channel records are 5 sec of continuous recording from the indicated bins. The recording pipette was filled with normal recording solution and the membrane potential was held +150 mV, relative to rest (pipette potential, −150 mV). Single-channel amplitudes were approximately 18 pA. After completion of the cell-attached recording, whole-cell access was established (in three of six cells) and the whole-cell BK current was shown to be responsive to DAGO (*right inset*).

and SCH23390 (1 μM), the dopamine potentiation of whole-cell BK current appears to be mediated by a D_2 type receptor (Fig. 2c), possibly the same receptor shown by Sontag *et al.* (22) to inhibit Ca^{2+} currents in BAMCCs via a PTX-sensitive mechanism. PTX treatment inhibited dopamine potentiation of whole-cell BK current (5 ± 5%, six cells, compared with 84 ± 6%, 18 cells, for nontreated cells) (Fig. 3a) but did not significantly affect potentiation of whole-cell BK current by subsequent application of 10 nM DAGO (78 ± 5%, six cells, compared with 85 ± 5%, 37 cells, for nontreated cells) (Fig. 3a).

It was possible that the G protein mediating the opioid effect was insensitive to PTX. To test general G protein involvement,



we used nonhydrolyzable GTP and GDP analogs. The GDP analog GDPβS (0.5 mM) was substituted for GTP in the recording pipette solution to block any G protein-mediated effect. Fig. 2a2 shows a representative experiment for six cells challenged with dopamine followed by DAGO; the cells failed to respond to the application of dopamine but did respond to a subsequent application of DAGO. Overall, these cells and others showed that GDPβS blocked dopamine augmentation of whole-cell BK current (4 ± 4%, nine cells, compared with 84 ± 6%, 18 cells, with GTP) (Figs. 2a2 and 3a) but had no effect on DAGO potentiation (71 ± 5%, 12 cells, compared with 85 ± 5%, 37 cells, with GTP) (Figs. 2a2 and 3a). When GTP was replaced by GDPβS in the patch pipette solution for outside-out patch recording, DAGO still produced reversible and repeatable increases in BK channel activity (Figs. 2b and 3b). This experiment was not repeated with dopamine, because dopamine fails to affect BK channel activity in excised membrane patches. To further test the involvement of GTP-dependent processes in the μ receptor potentiation of whole-cell BK current, the nonmetabolizable GTP analog GTPγS was substituted for GTP in the pipette solution, to make any G protein-mediated effect poorly reversible. Fig. 2, d and e, shows that, despite the presence of GTPγS, BK potentiation by DAGO recovered readily in both whole-cell and outside-out patch configurations. DAGO-responsive, GTPγS-treated cells that were subsequently exposed to dopamine showed incomplete (~50%) recovery even after 8 min (Fig. 2e) (compare this with the 2–3 min for full recovery observed under control conditions in Fig. 2c). Taken together, these results strongly indicate that, whereas the BK channel in BAMCCs is coupled to a dopamine receptor via a GTP-dependent, PTX-sensitive

Fig. 2. The dopamine D_2 receptor couples to the BK channel via a GTP-dependent mechanism but the μ-opioid receptor does not. a1, Dopamine at 100 nM potentiates whole-cell BK current in a reversible manner. Whole-cell recordings of BK current in response to a voltage ramp (0 to +100 mV in 100 msec) are shown. *Vertical scale bar*, 200 pA. a2, Substitution of 0.5 mM GDPβS for GTP in the whole-cell pipette solution blocks potentiation of whole-cell BK current by 100 nM dopamine but does not affect BK potentiation by a subsequent application of 10 nM DAGO. This experiment was replicated in six cells. *Vertical scale bar*, 200 pA. b, DAGO potentiates BK channel activity despite the presence of 0.5 mM GDPβS in the patch pipette solution. An outside-out patch was held at 0 mV and the channel activity was measured with 100-msec ramps to +50 mV. DAGO potentiated single-channel activity despite the presence of GDPβS. Digital averages of 25 consecutive traces under each condition clearly show the reversibility of the DAGO effect (b4). This experiment was replicated in six patches. *Vertical scale bar*, 10 pA for ensemble averages and 20 pA for the single-channel traces. c, Dopamine modulates BK current via a D_2 receptor. Dopamine (100 nM) potentiation of whole-cell BK current persisted in the presence of the D_1 receptor antagonist SCH23390 (1 μM) but was inhibited by the D_2 antagonist spiperone (1 μM) (*black lines*, applications). *Insets*, current records are from just before application and during maximal response to the drug. This experiment was replicated in four cells. d, BK channel activity shows recovery from DAGO potentiation despite the presence of 0.5 mM GTPγS in the patch pipette solution. An outside-out patch was held at 0 mV and the channel activity was measured with 100-msec ramps to +50 mV. DAGO potentiation of single-channel activity was reversible despite the presence of GTPγS. Digital averages of 25 consecutive traces under each condition clearly show the reversibility of the DAGO effect (d4). This experiment was replicated in eight patches. *Vertical scale bar*, 10 pA for ensemble averages and 20 pA for the single-channel traces. e, With GTPγS in the patch pipette, DAGO potentiation of whole-cell BK current remains readily reversible (compare with Fig. 1a, *insets*), whereas dopamine potentiation reverses poorly. This experiment was replicated in four cells.

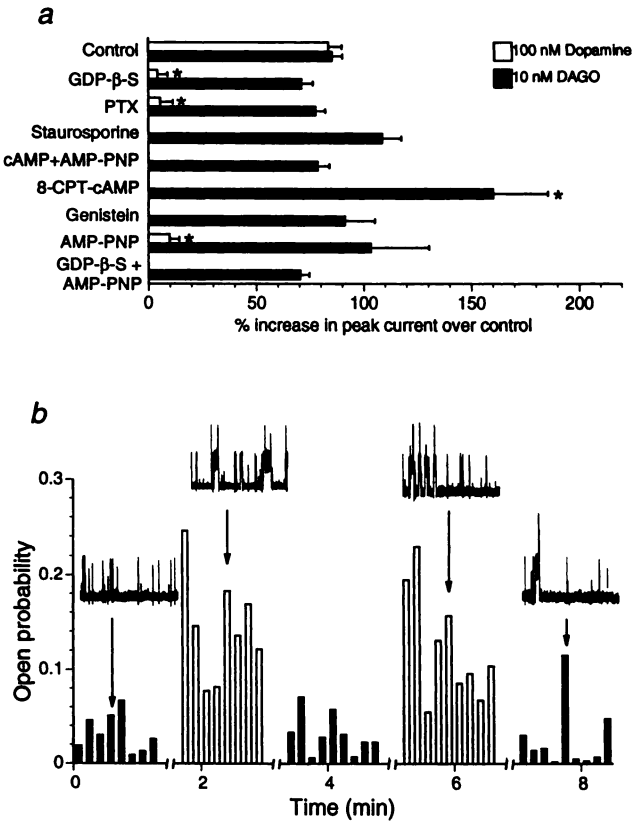


Fig. 3. Coupling between the BK channel and the μ-opioid receptor does not require a G protein or kinase. a, Perturbations directed at G protein or kinase function fail to affect the whole-cell BK current response to the μ agonist DAGO (10 nM) (■) but do uncouple dopamine modulation (□). *Bars*, mean percentage increase of whole-cell peak current over preapplication peak current; *error bars*, standard error. *, Statistically significant differences, compared with control. All bars represent at least four experiments. Staurosporine (200 nM) and genistein (50–500 μM) were included in the patch pipette solution. GTP was replaced with 0.5 mM GDPβS, and ATP was replaced with 1 mM AMP-PNP in the patch pipette solution. Cells were incubated with 300 ng/ml PTX for 8–14 hr at 37°. *8-CPT-cAMP*, 8-(4-chlorophenylthio)-cAMP. b, DAGO potentiates BK channel activity in outside-out patches even in the presence of 1 mM AMP-PNP and 0.5 mM GDPβS in the patch pipette solution. The patch was held at +50 mV and DAGO was applied via a micropipette (□). *Insets*, single-channel records are 5 sec of continuous recording from the indicated 5-sec bins. Single-channel amplitudes were approximately 13 pA. The breaks on the *x*-axis represent intervals of 1–2 min each. Drug application actually begins and ends during the breaks. This experiment was repeated in eight cells.

mechanism, the μ-opioid receptor does not require a GTP-dependent mechanism to potentiate BK channel activity.

The μ receptor does not require ATP to potentiate BK current. The experiments described above show that the mechanism coupling the BK channel to the μ-opioid receptor does not require GTP; however, they do not definitively rule out the possibility that a closely associated kinase or phosphatase could directly modulate BK channel activity via phosphorylation or dephosphorylation (23–28). A possible direct role for protein kinase A or protein kinase G was discounted when extracellular addition of the membrane-permeable cyclic nucleotide analogs 8-(4-chlorophenylthio)-cAMP (100 μM) and 8-bromo-cGMP (100 μM) was found not to affect whole-cell BK current or BK single-channel activity in outside-out patches (12 and five cells, respectively) (data not shown). These results indicated that the opioid potentiation of BK current did not involve an increase in cAMP or cGMP levels.

A well known result of μ-opioid receptor activation is inhibition of adenylyl cyclase; thus, an opioid-induced decrease in cAMP was considered a possible modulatory mechanism for BK current. To occlude the effects of any decrease in cAMP, 100 μM 8-(4-chlorophenylthio)-cAMP was included in the patch pipette solution. This treatment failed to diminish the effect of DAGO on whole-cell BK current and actually potentiated it by ~125%, compared with the control DAGO response (four cells) (Fig. 3a). These data indicate that, whereas cAMP or cAMP-dependent processes may modulate the signal transduction mechanism, opioid receptor potentiation of the channel does not require cAMP as an intermediate.

We addressed the possibility that a kinase or other ATP-dependent mechanism was directly involved in the modulation of the BK current by the μ-opioid receptor (27). To test this hypothesis we dialyzed the interior of BAMCCs with serine/threonine and tyrosine kinase inhibitors (200 nM staurosporine and 50–500 μM genistein) or replaced ATP with the nonhydrolyzable ATP analog AMP-PNP. None of these treatments significantly affected the DAGO potentiation of BK current or its recovery (six cells each) (Fig. 3a). These results strongly suggest that ATP does not play a direct role in the modulation of the BK channel by the μ-type opioid receptor.

ATP included in the recording solutions does not provide an alternative GTP source for G proteins. In atrial myocytes, endogenous enzymes have been shown to convert exogenous ATP to GTP, thus providing, in the absence of added GTP, a nucleotide source for ion channel modulation by muscarinic receptors (29). To eliminate the possibility that a similar process occurred in the single-nucleotide substitution experiments shown above, we substituted both GDPβS and AMP-PNP for GTP and ATP in the patch pipette solution. Under these conditions the effect of DAGO on whole-cell BK current was not significantly different from that in control experiments with ATP and GTP (seven cells) (Fig. 3a). In outside-out patches with AMP-PNP and GDPβS in the patch pipette solution, the potentiation of BK channels by applied DAGO was reversible and repeatable to the same extent as it was under control conditions (eight patches) (Fig. 3b). This response to DAGO lasted as long as the patch was maintained (up to 20 min). The reversibility and repeatability of the BK response in the presence of AMP-PNP argue against a direct role for phosphatases or kinases in the coupling of the μ receptor to the BK channel. Furthermore, these results indicate that the μ-opioid receptor in BAMCCs is coupled to a BK-type channel via a mechanism that does not require a G protein.

Coupling between the BAMCC μ receptor and BK channel represents an alternative mechanism by which G protein-coupled receptors can mediate signal transduction. This is, to our knowledge, the first time that a member of the opioid receptor family has been shown to exert its effect via a G protein-independent mechanism. However, there have been suggestions of G protein-independent signaling activated by other members of the seven-transmembrane domain receptor superfamily. Experiments with muscarinic m3/m2 receptor chimeras showed that the m3 ligand binding domain was still able to activate transmembrane Ca^{2+} flux when its third intracellular loop was replaced with the third intracellular loop of the m2 receptor (which does not activate transmembrane Ca^{2+}

flux) (30). These results raise the possibility that the third intracellular loop (the primary determinant of receptor-G protein coupling) may not be the only transducer of seven-transmembrane domain receptor-mediated signaling. Muscarinic activation of an inwardly rectifying whole-cell K^+ current in bovine aortic endothelial cells has also been reported to be resistant to removal of GTP and was not affected by the presence of GTPγS (31). In addition, cloning and expression studies have identified somatostatin and 5-hydroxytryptamine receptors whose high affinity ligand binding is not altered by the presence or absence of nonhydrolyzable GTP analogs, suggesting that these receptors may not necessarily couple to G proteins (32–34) (however, see also Refs. 35–38). Alternatively, it could be that the opioid receptor mediating potentiation of the BAMCC BK channel is not a member of the classical μ/δ/κ-opioid receptor family and thus has some atypical signaling properties. This seems unlikely, because the receptor mediating this effect demonstrates stereoselectivity and agonist and antagonist selectivity similar to those of the cloned μ receptor (Fig. 1a) (14, 39, 40). Rather, our results demonstrate that a member of the opioid receptor family may have signaling functions independent of G protein-dependent processes, and this may represent the first clear example of an alternative mechanism by which seven-transmembrane domain receptors can transduce extracellular signals.

Acknowledgments

We thank Deann Woodward for her technical assistance and Drs. J. Strong, S. Rossie, and K. Robinson for critical review.

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