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Functional Coupling of Presynaptic $GABA_B$ Receptors with Voltage-Gated Ca^{2+} Channel: Regulation by Protein Kinases A and C in Cultured Spinal Cord Neurons

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SUMMARY

Depolarization-induced ^{86}Rb efflux, an index of K^+ efflux, was developed by using mammalian cultured spinal cord neurons to study the effect of gamma aminobutyric acid ($GABA_B$) receptor activation on Ca^{2+}-activated K^+-channels. The Ca^{2+}-activated ^{86}Rb efflux was obtained by using two methods. The first method utilized depolarizing concentrations of KCl (100 mM) to study the voltage-gated Ca^{2+} channel activation, whereas in the second method, calcium ionophore A 23187 was used to get the voltage-independent Ca^{2+}-activated ^{86}Rb efflux. The $GABA_B$ receptor agonist baclofen inhibited the efflux induced by depolarization but not by A 23187, whereas tricyclic antidepressant desipramine inhibited the efflux induced by both depolarization and A 23187. These results suggest that the $GABA_B$ receptor activation inhibits ^{86}Rb efflux by inhibiting the voltage-gated Ca^{2+} channels. Moreover, forskolin and the analogs of cAMP antagonized the action of baclofen, suggesting that the $GABA_B$ receptors are negatively coupled to adenylate cyclase. Furthermore, protein kinase C activators antagonized this action of baclofen, while the antagonists of protein kinase C reversed their action on baclofen. In addition, the inactive forskolin, 1,9-dideoxy forskolin, and the inactive phorbol analog, phorbol 12,13-didecanoate, did not influence the action of baclofen. Thus, it is suggested that the $GABA_B$ receptor activation inhibited the voltage-gated Ca^{2+} influx and that this action is under modulatory control by kinases A and C.

$GABA_B$ receptors are activated by baclofen as well as by $GABA_B$, and their responses are selectively antagonized by phaclofen (1, 2). $GABA_B$ receptors are located both presynaptically and postsynaptically. The presynaptic $GABA_B$ receptors are apparently coupled to voltage-gated Ca^{2+} channels (3, 4, 5), and postsynaptic $GABA_B$ receptors mediate slow inhibitory postsynaptic potential responses, which involves opening of K^+ channels (for reviews, see 1, 6). Three types of neuronal Ca^{2+} channels have been characterized, and in some cases they may coexist in the same neuron (for a review, see Ref. 5). We have recently shown with primary cultured spinal cord neurons that the stimulation of $GABA_B$ receptors inhibited voltage-gated Ca^{2+}-activated ^{86}Rb efflux (7). It has also been reported that the inhibition of voltage-dependent Ca^{2+} conductance by $GABA_B$ receptor activation leads to a presynaptic inhibitory action on neurotransmitter release (4, 8, 9). Moreover, G_i/G_o proteins have been reported to be involved in the action of the $GABA_B$ receptors, as pertussis toxin, which inactivates the G proteins (for a review, see Ref. 10), blocked the effect of $GABA_B$ receptor agonists like baclofen in several preparations (7, 9, 11, 12).

Our preliminary studies have shown that activation of adenylate cyclase with forskolin, leading to an accumulation of cAMP, antagonized the $GABA_B$ receptor-mediated inhibition of Ca^{2+}-activated ^{86}Rb efflux (7). It has been suggested that the stimulation of cAMP by treatment with cAMP derivatives or with agents which elevate cAMP levels might lead to an activation of PKA (for a review, see Ref. 13). Several lines of evidence have suggested that the $GABA_B$ receptors are negatively coupled with adenylate cyclase, leading to an inhibition of cAMP synthesis and, thus, the activation of PKA (12–15).

In addition, PKC has also been implicated in the action of $GABA_B$ receptor stimulation. Activation of PKC by phorbol esters has been shown to block both the presynaptic (7) and postsynaptic (16, 17) responses induced by baclofen. All these lines of evidence regarding $GABA_B$ receptor pharmacology have been described in our previously preliminary report (7), in which we developed a functional assay for measuring $GABA_B$ receptor responses *in vitro* by using an ^{86}Rb efflux assay in primary-cultured spinal cord neurons. This is an indirect assay in which depolarization leads to Ca^{2+} influx through voltage-gated, channels, which then activate a K^+ channel, as measured by ^{86}Rb efflux. The rationale for using ^{86}Rb as a substitute for

ABBREVIATIONS: $GABA_B$, gamma aminobutyric acid; PKA, protein kinase A; PKC, protein kinase C; MEM, minimum essential medium; FUDR, 5-fluoro-2′-deoxyuridine; TEA, tetraethyl ammonium chloride; TBA, tetrabutyl ammonium chloride; PDBu, phorbol 12,13-dibutyrate; PDDc, phorbol 12,13-didecanoate; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; PTX, pertussis toxin; HBr, hydrogen bromide.

K^+ has already been well justified (18). As an extension of our previous report, we have attempted to see if the activation of the $GABA_B$ receptors involves the voltage-gated Ca^{2+} channel by using depolarization-induced and calcium-channel ionophore (A 23187)-induced ^{86}Rb efflux. Furthermore, we have characterized $GABA_B$ receptor activation by using various cAMP analogs, activators, and antagonists of PKC in primary-cultured spinal cord neurons. Finally, since tricyclic antidepressants also reduce the inward Ca^{2+} current in several preparations (19, 20), and $GABA_B$ receptors have been reported to be altered following the treatment with the antidepressants (21, 22), we have compared the effect of desipramine with that of baclofen.

Materials and Methods

Preparation of cell cultures. Spinal cords were dissected from 13–14 day-old C57BL/6J mouse embryos, as described previously (7, 23). The spinal cords were then minced with iridectomy scissors and the tissue was taken up in 1.5 ml of nutrient medium (MEM 10/10), pH 7.4, which contained 80% Eagle's MEM, glucose (33.3 mM), $NaHCO_3$ (44 mM), 10% heat-inactivated (56° for 30 min) horse serum, and 10% fetal bovine serum, and transferred to a sterile 15-ml centrifuge tube. The tissue fragments were subjected to dissociation by trituration until a supernatant volume of 0.75 ml/spinal cord was attained. Dissociated cells were plated on poly-L-lysine-coated sterile 25-mm (diameter) round coverslips by adding 0.5 ml of the suspension to dishes containing 1 ml of MEM 10/10 which had been preincubated with 95% air and 5% CO_2 for at least 1 hr at 37°C.

The plated cultures were incubated, and the growth medium (MEM 10/10) was replaced with 1 ml of the medium containing 10% heat-inactivated serum (MEM 10) on days two and five. On day seven, one-half ml of the medium was again replaced with MEM 10. A mixture of sterile FUDR plus uridine (2 mg of FUDR per ml and 5 mg of uridine per ml at a final concentration of 10 μg/ml) was added on day two in order to inhibit the growth of nonneuronal cells.

Efflux studies. Our initial studies with PKA and PKC activators were performed at 37°C as well as at room temperature. As there was no significant difference between the two, all the studies were conducted at room temperature.

All the efflux studies were conducted on 8-day-old intact primary-cultured spinal cord neurons at room temperature. On day seven, the coverslips with cells were incubated overnight with 2 μCi of ^{86}Rb per ml in the tissue culture medium. All the coverslips including the controls (5 and 100 mM KCl) were preincubated with the activators and antagonists of protein kinases for 20 min in wash buffer prior to the studies on the day of the assay. At the 16th min of preincubation, baclofen was added to the petri dish containing the coverslips (with cells) and the various other test compounds, so that the cells were exposed to baclofen for 4 min and to the compounds influencing the protein kinases for 20 min. In the experiments with baclofen or desipramine alone, the cells were preincubated with it for 4 min. In studies involving A 23187, the coverslips were incubated with 20 μM of the ionophore and then given a 4-min exposure to baclofen or desipramine prior to efflux determination.

The experiment was started by washing the coverslips four times with 2 ml each of wash buffer (in mM: NaCl 145, KCl 5, $MgCl_2$ 2, RbCl 0.1, HEPES 10, glucose 10, adjusted to pH 7.4 with Tris-base) kept in four separate petri dishes in order to remove the excess of ^{86}Rb. The nondepolarizing buffer contained (in mM: NaCl 145, KCl 5, $CaCl_2$ 1.8, RbCl 0.1, HEPES 10, and glucose 10) and adjusted to pH 7.4 with Tris-

TABLE 1

Effects of baclofen and desipramine on the ^{86}Rb efflux induced with 100 mM KCl

Values represent means ± standard deviations of four experiments. Each experiment utilized one coverslip from which duplicate samples were taken for efflux determination. The % ^{86}Rb efflux was calculated as shown in Materials and Methods. Efflux (cpm) represents the counts obtained from 2 ml of assay solution. The cpm_t (total count) includes the efflux cpm as well as the counts in the coverslip at the end of the experiment. Thus, cpm_t minus efflux cpm would be the counts remaining in the coverslip at the end of the test period. The cpm_t also indicates the counts present in the coverslip at the end of the pretreatment time, i.e., the ^{86}Rb available for release before the efflux determination. In addition to the effect of the test drugs, the ^{86}Rb efflux (cpm), is also proportional to cpm_t. Furthermore, all of the above factors, like efflux and cpm_t, ultimately depend on the number of cells present in each coverslip, which varies from coverslip to coverslip and also with various batches of cultures.

Treatment	^{86}Rb efflux	cpm_t	^{86}Rb efflux
	cpm		%
5 mM KCl	2,328 ± 232	37,154 ± 461	6.3 ± 0.6
5 mM KCl + 10^{-4} M (−)baclofen	1,969 ± 362	28,210 ± 3,261	7.2 ± 2.1
5 mM KCl + 10^{-4} M desipramine	2,935 ± 728	38,277 ± 1,148	7.6 ± 1.7
100 mM KCl	6,250 ± 270	34,443 ± 292	18.5 ± 0.8
100 mM KCl + 10^{-4} M (−)baclofen	4,976 ± 326	34,671 ± 313	14.4 ± 0.8[a]
100 mM KCl + 10^{-4} M desipramine	3,205 ± 189	38,383 ± 4,336	8.4 ± 9.8[b]

[a] $p < 0.01$, compared with 100 mM KCl.
[b] $p < 0.0001$, compared with 100 mM KCl.

TABLE 2

Effects of baclofen on the ^{86}Rb efflux induced by A 23187

Coverslips were incubated with or without A 23187 (20 μM) for 4 min prior to the efflux determination, as described in Materials and Methods. Each experiment utilized one coverslip from which duplicate samples were taken for efflux determination. Values represent mean ± standard deviation. Numbers in parentheses represent the number of experiments.

Treatment	^{86}Rb efflux
	%
5 mM KCl	11.82 ± 2.65 (7)
5 mM KCl + A 23187	23.95 ± 2.98[a] (4)
5 mM KCl + A 23187 + 10^{-4} M (−)baclofen	25.89 ± 3.61[a] (3)
5 mM KCl + A 23187 + 10^{-4} M desipramine	11.41 ± 1.84[b] (5)

[a] $p < 0.001$, compared with 5 mM KCl.
[b] $p < 0.001$, compared with 5 mM KCl + A 23187.

TABLE 3

Effects of activators of protein kinase A and their inactive congener on the baclofen-induced inhibition of ^{86}Rb efflux

Depolarization-induced ^{86}Rb efflux was obtained with 100 mM KCl for 30 sec, as described in Materials and Methods. The coverslips were treated with or without the respective compounds for 20 min before starting the efflux studies. Forskolin and the cAMP analogs did not influence the basal efflux (data not shown). After this pretreatment, the ^{86}Rb available for release was between 10,000 and 20,000 counts, as described in Table 1. In the experiments conducted at 37° forskolin antagonized the effect of baclofen and the % ^{86}Rb efflux was 22.46 ± 1.50 (n = 4). Values represent mean ± standard deviation. Each experiment utilized one coverslip from which duplicate samples were taken for efflux determination. Numbers in parentheses represent the number of experiments.

Treatment	^{86}Rb efflux	
	Without baclofen	With 10^{-4} M (−)baclofen
	%	
5 mM KCl	7.36 ± 0.93 (5)	7.49 ± 1.01 (6)
100 mM KCl	23.57 ± 1.55 (4)	17.93 ± 2.13[a] (4)
100 mM KCl + 10^{-5} M forskolin	22.29 ± 1.87 (4)	21.98 ± 1.37[b] (4)
100 mM KCl + 2×10^{-3} M 8-Bromo cAMP	20.75 ± 1.02 (4)	20.74 ± 1.41[c] (6)
100 mM KCl + 10^{-3} M dibutyryl cAMP	22.19 ± 3.31 (4)	21.62 ± 0.97[b] (4)
100 mM KCl + 10^{-5} M 1,9-dideoxy Forskolin	21.94 ± 0.58 (4)	17.42 ± 1.13[d] (6)

[a] $p < 0.001$, compared with 100 mM KCl.
[b] $p < 0.01$, compared with 100 mM KCl and baclofen.
[c] $p < 0.05$, compared with 100 mM KCl and baclofen.
[d] Not significant, compared with 100 mM KCl and baclofen.

TABLE 4

Effects of activators of protein kinase C and their inactive congener on the (−)baclofen-induced inhibition of ^{86}Rb efflux

Coverslips were treated with or without the compounds influencing protein kinase C for 20 min, as described in Materials and Methods. The ^{86}Rb available for release was between 10,000 and 20,000 counts at this time. KCl (100 mM) was used to depolarize the cells, as described in the text. The PKC activators did not influence the basal efflux obtained with 5 mM KCl. PDBu, at 37° antagonized the effect of baclofen and the % ^{86}Rb efflux was 24.04 ± 0.56 (n = 4). Each experiment utilized one coverslip from which duplicate samples were taken for efflux determination. Values are expressed as mean ± standard deviation. The numbers in parentheses represent the number of experiments.

Treatment	^{86}Rb efflux	
	Without baclofen	With 10^{-4} M baclofen
	%	
5 mM KCl	7.27 ± 1.39 (4)	8.01 ± 1.47 (6)
100 mM KCl	25.92 ± 1.53 (5)	18.77 ± 0.81[a] (4)
100 mM KCl + 10^{-6} M PDBu	24.41 ± 1.78 (4)	26.21 ± 2.18[b] (6)
100 mM KCl + 10^{-6} M PMA	26.30 ± 1.58 (4)	23.32 ± 2.09[c] (5)
100 mM KCl + 10^{-6} M dioctanoylglycerol	26.24 ± 1.60 (4)	26.59 ± 1.59[b] (4)
100 mM KCl + 10^{-6} M PDDc	24.41 ± 2.09 (4)	17.66 ± 1.67 (5)

[a] $p < 0.001$, compared with 100 mM KCl.
[b] $p < 0.001$, compared with 100 mM KCl and baclofen.
[c] $p < 0.01$, compared with 100 mM KCl and baclofen.

TABLE 5

Effects of activators and antagonists of protein kinase C on (−)baclofen-induced inhibition of ^{86}Rb efflux

All the coverslips including the control (5 and 100 mM KCl) were treated with or without the activators and antagonists of protein kinase C for 20 min before the efflux measurement, at which time, the ^{86}Rb available for release was in the range of 10,000 and 20,000. Both polymyxin B and staurosporine did not influence the basal efflux seen with 5 mM KCl or the effect of PMA on 100 mM KCl-induced ^{86}Rb efflux. Values are expressed as mean ± standard deviation. Each experiment utilized one coverslip from which duplicate samples were taken for efflux determination. The numbers in parentheses represent the number of experiments.

Treatment	^{86}Rb efflux	
	Without baclofen	With 10^{-4} M (−)baclofen
	%	
5 mM KCl	7.20 ± 0.45 (4)	7.49 ± 1.21 (4)
100 mM KCl	22.26 ± 0.55 (4)	17.53 ± 0.89[a] (4)
100 mM KCl + 10^{-6} M PMA	21.04 ± 2.31 (4)	21.21 ± 1.92[b] (4)
100 mM KCl + 10^{-6} M PMA + 5×10^{-6} M polymyxin B	18.49 ± 0.80[c] (4)	13.15 ± 2.13[d] (6)
100 mM KCl + 10^{-6} M PMA + 10^{-8} M staurosporine	19.63 ± 1.42 (4)	13.37 ± 2.25[d] (6)

[a] $p < 0.001$, compared with 100 mM KCl.
[b] $p < 0.01$, compared with 100 mM KCl and baclofen.
[c] $p < 0.01$, compared with 100 mM KCl.
[d] $p < 0.001$, compared with baclofen and PMA.

base. The depolarizing buffer contained 100 mM KCl instead of an equal amount of NaCl. Both the nondepolarizing and depolarizing buffers contained 2 mM ouabain to inhibit the Na^+/K^+ ATPase. The efflux was initiated by incubating the coverslips for a period of 30 sec in petri dishes with 2 ml of the respective assay solutions in which the test compounds were present.

After the incubation, efflux was terminated by rapid transfer and immersion of the coverslips for 10 sec in 1000 ml of a continuously stirred ice-cold stop solution. This type of washing procedure has been employed in our studies, as it showed minimum variation compared with draining the coverslip in tissue paper. The stop solution contained (in mM: TEA 145, TBA 1, RbCl 5, $MgCl_2$, 5, $NiCl_2$ 10, HEPES 20) and was adjusted to pH 7.4 with Tris-base. Following 10 sec of immersion in the stop solution, each coverslip was drained on tissue paper and transferred to a scintillation vial containing 1.5 ml of 0.2 N NaOH. This was neutralized with 0.3 ml of 1 N HCl, mixed with 15 ml of hydrofluor, and counted by liquid scintillation.

In order to measure the efflux, 100-μl duplicate samples were taken from each petri dish and transferred to bio-vials. To this, 3 ml of hydrofluor was added, mixed well, and counted by liquid scintillation. The results of all efflux assays were corrected for the background counts per minute present at time zero. The % ^{86}Rb efflux was calculated as follows:

$$\%^{86}\text{Rb efflux} = \frac{\text{cpm}}{\text{cpm}_t} \times 100$$

where cpm_t refers to the count present in coverslips and the counts per minute found at 30 sec. The results were analyzed by one-way analysis of variance.

Materials. ^{86}Rb was purchased from Du Pont (Boston, MA). Baclofen isomer was a gift from CIBA-GEIGY (Basel, Switzerland). A 23187, polymyxin B sulfate, ouabain, desipramine HCl, TEA, TBA, poly-L-lysine HBr, FUDR, forskolin, PDBu, PMA, PDDc, polymyxin B sulfate, 8-bromo cAMP, and dibutyryl cAMP were purchased from Sigma (St. Louis, MO). Uridine and 1,9-dideoxy forskolin were obtained from Cal-Biochem (La Jolla, CA), and staurosporin and dioctanoylglycerol were purchased from Boehringer Mannheim (Mannheim, Federal Republic of Germany).

Baclofen, desipramine HCl, dibutyryl cAMP, polymyxin B sulfate, and PDBu were dissolved in buffer, whereas 8-bromo cAMP was dissolved in acidic (pH 4) buffer. All the other chemicals were dissolved in DMSO.

Results

Experiments utilizing calcium ionophore A 23187. In the initial studies, the Ca^{2+}-activated ^{86}Rb efflux was measured under two experimental conditions. A high concentration (100 mM) of KCl was used to depolarize the cells in order to get the voltage-dependent Ca^{2+}-activated ^{86}Rb efflux. The second method utilized the calcium ionophore A 23187 to develop an efflux pattern which was activated by Ca^{2+} but which was unrelated to voltage-gated Ca^{2+} channels. Both of these methods produced a significant increase in ^{86}Rb efflux over the basal efflux values. $GABA_B$ agonist baclofen inhibited the depolarization-induced ^{86}Rb efflux (Table 1) without affecting the ^{86}Rb efflux produced with A 23187 (Table 2). In contrast, the antidepressant desipramine antagonized the ^{86}Rb efflux induced by both KCl (Table 1) and calcium ionophore A 23187 (Table 2). These results suggest that baclofen but not desipramine inhibited the ^{86}Rb efflux by inhibiting voltage-gated Ca^{2+} channels. (−)Baclofen and desipramine did not affect the basal efflux. All further experiments with baclofen were performed with KCl-induced Ca^{2+}-activated ^{86}Rb efflux.

Experiments with cAMP analogs. Table 3 shows the effects of various compounds influencing the cAMP on Ca^{2+}-induced ^{86}Rb efflux. These analogs per se did not affect the basal efflux (data not shown) or the depolarization-induced efflux (Table 3). However, forskolin, an activator of adenylate cyclase, reversed the inhibitory effect of baclofen on ^{86}Rb efflux. Similarly, the analogs of cAMP, 8-bromo cAMP and dibutyryl cAMP, also antagonized the baclofen-induced inhibition of ^{86}Rb efflux baclofen (Table 3). In contrast, the inactive congener of forskolin, i.e., 1,9-dideoxy forskolin, failed to block the action of baclofen.

Experiments with the activators of PKC. The effects of activators of PKC on Ca^{2+}-activated ^{86}Rb efflux are summarized in Table 4. The activators of PKC did not alter the basal (data not shown) or the K^+-stimulated ^{86}Rb efflux (Table 4). However, the activators of PKC, like PDBu (10^{-6} M) and PMA (10^{-6} M), reversed the inhibitory effect of baclofen on Ca^{2+}-

activated ^{86}Rb efflux. Further, it is known that PKC requires the presence of diacylglycerol, a second messenger produced by the breakdown of membrane phospholipids, for its full activation (24). In this study, dioctanoylglycerol, a synthetic diacylglycerol, also antagonized the action of baclofen (Table 4). In contrast, an inactive phorbol ester, PDDc, did not antagonize the action of baclofen (Table 4).

Experiments with the antagonists of PKC. The effects of staurosporin and polymyxin B, the antagonists of PKC, on the blockade of the $GABA_B$ receptor response produced by PKC activators were examined. In this regard, PMA was chosen as the prototype PKC activator to antagonize the effect of baclofen. Table 5 shows that PMA, at a concentration of 10^{-6} M, reversed the inhibition of baclofen on ^{86}Rb efflux, without altering the depolarization-induced efflux. Both staurosporine (10^{-8} M) and polymyxin B (5×10^{-6} M) reversed this action of PMA and, in addition, potentiated the action of baclofen (Table 5). These PKC antagonists per se did not influence the basal efflux or the effect of PMA on KCl-induced efflux (data not shown). However, both polymyxin B and staurosporine inhibited the KCl-induced efflux, although the effect of staurosporine was not statistically significant.

Discussion

Protein kinases are a diverse family of enzymes that participate in transmembrane signaling (25). PKA is dependent on cyclic nucleotides, whereas PKC is dependent upon calcium and phospholipids (for a review, see Ref. 13). The effects of a wide variety of neurotransmitters, hormones, growth factors, and many other biologically active substances are known to be mediated by these protein kinases (for reviews, see Ref. 25 and 26).

Recent studies have shown that the $GABA_B$ receptor stimulation inhibits Ca^{2+} uptake and the subsequent neurotransmitter release (3, 4, 27, 28) and Ca^{2+}-activated K^+ efflux (7). Moreover, $GABA_B$ receptors are negatively coupled to adenylate cyclase and involve G-protein mechanisms in this action (12, 14, 17, 29). In our previous study, we showed that baclofen inhibited Ca^{2+}-activated K^+ efflux in a concentration-dependent manner, and with properties which are consistent with $GABA_B$ receptor pharmacology (7). This effect was blocked by pertussis toxin (7), a substance which inactivates G-proteins by ADP-ribosylation (10). Furthermore, forskolin and PDBu, the activators of adenylate cyclase and PKC respectively, also blocked baclofen-induced inhibition of ^{86}Rb efflux (7). This previously described assay apparently measures presynaptic $GABA_B$ receptors and is distinct from postsynaptic $GABA_B$ receptors which involve opening of K^+ channels (6, 12, 17).

In this investigations, ^{86}Rb efflux, an index of K^+ efflux, was induced by depolarizing the cells with either high KCl (100 mM) or calcium ionophore A 23187. Even though both of these methods produced apparently similar and significant efflux, a difference exists in the nature of Ca^{2+} translocation. In the case of KCl-induced depolarization, the efflux is dependent on voltage-gated Ca^{2+} channels. But on the other hand, with the ionophore A 23187, the efflux of ^{86}Rb does not depend on the voltage-gated Ca^{2+} channels. In this study, baclofen was selective in inhibiting the efflux induced by depolarization but not that induced with A 23187. This suggests that the $GABA_B$ receptor-stimulated inhibition of the K^+ efflux mechanism is linked with the voltage-gated Ca^{2+} channels. $GABA_B$ receptor activation has been reported to inhibit either the L type or all three (T, N, and L) types of Ca^{2+} channels (5). Besides $GABA_B$ receptor activation, Ca^{2+}-activated ^{86}Rb efflux was also inhibited by tricyclic antidepressants but not by monoamine oxidase inhibitors.[1] In this study, we found that tricyclic antidepressants like desipramine inhibited the efflux observed with both high KCl and A 23187. These results suggest that the tricyclic antidepressants inhibit the efflux which occurs at a stage subsequent to the voltage-gated Ca^{2+} channels. Thus, $GABA_B$ receptors differ from antidepressants in the way of expressing their action mainly through the voltage-gated Ca^{2+} channels. Furthermore, the effect of desipramine on ^{86}Rb efflux is not sensitive to $GABA_B$ antagonists, phaclofen, or the activators of PKA or PKC,[2] suggesting a mechanism different from that of baclofen.

Forskolin, which activates the catalytic subunit of adenylate cyclase (30) leading to an accumulation of cAMP, antagonized the action of baclofen. In contrast, the inactive analog of forskolin, 1,9-dideoxy forskolin, was ineffective, suggesting selectivity of action. Furthermore, the membrane-permeant analogs of cAMP, 8-bromo cAMP, and dibutyryl cAMP also reversed the action of baclofen. These results suggest an involvement of adenylate cyclase inhibition in the $GABA_B$ receptor-mediated events. Since PKA is considered an intracellular receptor for cAMP (31, 32), these results suggest that the presynaptic $GABA_B$ receptors are negatively coupled to adenylate cyclase. It has previously been reported that the vast majority of Ca^{2+} current-inhibiting agonists (including GABA via $GABA_B$ receptors) also inhibit adenylate cyclase (33). The exact mechanism by which activation of PKA modulates the effect on the $GABA_B$ receptors is not clear. However, it may be noted that forskolin and phorbol ester reduce the same K^+ conductance in mouse neurons in culture (34). Moreover, cAMP, or the catalytic subunit of PKA, modulates both the voltage-sensitive and calcium-activated potassium conductances in a number of invertebrate neurons (35, 36). Further, the $GABA_B$ channel activities may also be modulated by G-proteins, since the voltage-dependent Ca^{2+} channel inhibition seen with $GABA_B$ receptor activation was blocked by pertussis toxin (7, 9), and they are also activated by cAMP-dependent protein phosphorylation (37). Coupling of adenylate cyclase to $GABA_B$ receptors via pertussis toxin-sensitive G-proteins in bovine cerebral cortex has also been demonstrated (16). Thus, all these lines of evidence suggest that both adenylate cyclase and voltage-gated Ca^{2+} channels are regulated by presynaptic $GABA_B$ receptors. In contrast, postsynaptic $GABA_B$ receptors do not appear to be dependent on changes in cAMP, however, they are coupled to G-proteins (6, 12, 17).

Recently, it has been suggested that PKC is involved in synaptic transmission and in mediating the actions of neurotransmitters, for example, inhibition of calcium-dependent potassium conductance (38). It has been shown that the $GABA_B$ receptor action, both presynaptic and postsynaptic, was blocked by phorbol esters (7, 17). In this study, two active phorbol esters (PDBu and PMA) reversed the inhibition of baclofen on Ca^{2+}-activated K^+ efflux. Further, dioctanoylglycerol, one of the membrane-permeable synthetic diacylglycerols, also antagonized the action of baclofen on ^{86}Rb efflux. PKC is activated

[1] G. Kamatchi and M. Ticku, unpublished observations.
[2] Unpublished observations.

by dioctanoylglycerol, as it is known that diacylglycerol, one of the earliest products of the inositol phospholipid hydrolysis, is essential for the activity of PKC. Further, support for the involvement of PKC is demonstrated by the fact that the antagonists of PKC, polymyxin B, and staurosporine (39, 40), blocked the effect of PMA over that of baclofen. Polymyxin B per se inhibited the KCl-induced efflux, as it is known to block Ca^{2+} channels (41), but did not interfere with the effect of PMA on K^+-stimulated efflux. On the contrary, both polymyxin B and staurosporine potentiated the effect of baclofen, in addition to the reversal of the effect of PMA on baclofen-induced inhibition of the efflux. Thus, the potentiation of the effect of baclofen may be due to the influence of these agents on Ca^{2+} channels (41). Moreover, PDDc, an inactive phorbol analog, did not antagonize the action of baclofen. These lines of evidence suggest that the $GABA_B$ receptor activation is blocked by the activators of PKC. Although the activation of $GABA_B$ receptors is not known to influence the PKC directly, antagonism of the action of baclofen by the activators of PKC could be mediated through the G-protein mechanism, since PKC has been shown to phosphorylate and inactivate certain PTX-sensitive G-proteins (42). It is also possible that the PKC activators antagonized the $GABA_B$ receptor activation by interfering with the Ca^{2+} channels, although both an increase (43) and a decrease (44) in Ca^{2+} channel current have been reported with PKC.

Finally, our observations lead to the suggestion that the activation of the $GABA_B$ receptors is modulated by both PKA and PKC systems. Thus, the antagonism of the action of baclofen by cAMP analogs shows the inverse relationship of the $GABA_B$ receptors with adenylate cyclase. Similarly, the functional link between PKC and the $GABA_B$ receptors in this action may be related to the ability of activators of PKC to phosphorylate and inactivate the requisite G-proteins or Ca^{2+} channels or both. The observation that PKA and PKC have similar effects in modulating presynaptic $GABA_B$ receptors is not unique, since in several other systems, these two kinases exhibit similar unidirectional effects (25, 45). Furthermore, both PKA and PKC have been reported to phosphorylate the same proteins in many instances (46). In summary, the presynaptic $GABA_B$ receptor mechanisms may involve the interplay of G-protein, PKA, and PKC systems.

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References

1. Bowery, N. G., D. R. Hill, A. L. Hudson, and G. W. Price. $GABA_B$ receptors in *GABA and Benzodiazepine Receptors, vol. 1* (R. F. Squires, ed.). CRC Press, Inc., Boca Raton, FL, 107–121 (1988).
2. Kerr, D. I. B., J. Ong, R. H. Prager, B. D. Gynther, and D. R. Curtis. Phaclofen, a peripheral and central baclofen antagonist. *Brain Res.* **405**:150–154 (1987).
3. Dolphin, A. C., and R. H. Scott. Inhibition of calcium currents in cultured rat dorsal root ganglion neurons by (−)baclofen. *Br. J. Pharmacol.* **88**:213–220 (1986).
4. Xing-Zu Zhu, and D.-M. Chuang. Modulation of calcium uptake and D-aspartate release by $GABA_B$ receptors in cultured cerebellar granule cells. *Eur. J. Pharmacol.* **141**:401–408 (1987).
5. Tsien, R. W., D. Lipcombe, D. V. Madison, K. R. Bley, and A. P. Fox. Multiple types of neuronal calcium channels and their selective modulation. *TINS* **11**:431–438 (1988).
6. Nicoll, R. A. The coupling of neurotransmitter receptors to ion channels in the brain. *Science (Wash. D.C.)* **241**:545–551 (1988).
7. Kamatchi, G. L., and M. K. Ticku. $GABA_B$ receptor activation inhibits Ca^{2+}-activated ^{86}Rb-efflux in cultured spinal cord neurons via G-protein mechanism. *Brain Res.* **506**:181–186 (1990).
8. Dunlap, K. Two types of γ-aminobutyric acid receptor on embryonic sensory neurones. *Br. J. Pharmacol.* **74**:579–585 (1981).
9. Holz, G. G., S. G. Rane, and K. Dunlap. GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature (Lond.)* **319**:670–672 (1986).
10. Ui, M. Islet-activating protein, pertussis toxin: a probe for functions of the inhibitory guanine nucleotide regulating component of adenylate cyclase. *Trends Pharmacol. Sci.* **5**:277–279 (1984).
11. Asano, T., M. Ui, and N. Ogasawara. Prevention of the agonist binding to γ-aminobutyric acid B receptors by guanine nucleotides and islet-activating protein, pertussis toxin, in bovine cerebral cortex. *J. Biol. Chem.* **260**:12653–12658 (1985).
12. Andrade, R., R. C. Malenka, and R. A. Nicoll. A G-protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. *Science (Washington DC)* **234**:1261–1265 (1986).
13. Nairn, A. C., H. C. Hemmings, Jr., and P. Greengard. Protein kinases in the brain. *Annu. Rev. Biochem.* **54**:931–976 (1985).
14. Wojcik, W. J., and N. H. Neff. γ-Aminobutyric acid B receptors are negatively coupled to adenylate cyclase in brain and in the cerebellum. These receptors may be associated with granule cells. *Mol. Pharmacol.* **25**:24–28 (1984).
15. Xu, J., and W. J. Wojcik. Gamma-aminobutyric acid B receptor-mediated inhibition of adenylate cyclase in cultured cerebellar granule cells: blockade by islet activating proteins. *J. Pharmacol. Exp. Ther.* **239**:568–573 (1986).
16. Nishikawa, M., and K. Kuriyama. Functional coupling of cerebral gamma aminobutyric acid ($GABA_B$) receptor with adenylate cyclase system: effect of phaclofen. *Neurochem. Int.* **14**:85–90 (1989).
17. Dutar, P., and R. A. Nicoll. Pre- and postsynaptic $GABA_B$ receptors in the hippocampus have different pharmacological properties. *Neuron* **1**:585–591 (1988).
18. Bartschat, D. R., and M. P. Blaustein. Calcium-activated potassium channels in isolated presynaptic nerve terminals from rat brain. *J. Physiol. (Lond.)* **361**:441–457 (1985).
19. Aronstain, R. S. and W. Moss. Tricyclic antidepressant inhibition of depolarization-induced uptake of calcium by synaptosomes from rat brain. *Biochem. Pharmacol.* **34**:902–904 (1985).
20. Isenberg, A., and J. Tamargo. Effect of imipramine on calcium and potassium currents in isolated bovine ventricular myocytes. *Eur. J. Pharmacol.* **108**:121–131 (1985).
21. Pilc, A., and K. A. Lloyd. Chronic antidepressants and $GABA_B$ receptors: a GABA hypothesis of antidepressant drug action. *Life Sci.* **35**:2149–2154 (1984).
22. Suzdak, P. D., and G. Gianutsos. Parallel changes in the sensitivity of γ-aminobutyric acid and noradrenergic receptors following chronic administration of antidepressant and GABAergic drugs. *Neuropharmacol.* **24**:217–222 (1985).
23. Ransom, B. R., E. Neale, M. Henkart, P. N. Bullock, and P. A. Nelson. Mouse spinal cord in-cell culture. I. Morphological and intrinsic neuronal electrophysiological properties. *J. Neurophysiol.* **40**:1132–1150 (1977).
24. Kishimoto, A., Y. Takai, T. Mori, U. Kikkawa, and Y. Nishizuka. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* **255**:2273–2276 (1980).
25. Kikkawa, U., and Y. Nishizuka. The role of protein kinase C in transmembrane signalling. *Annu. Rev. Cell. Biol.* **2**:149–178 (1986).
26. Limbird, L. E. Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB J.* **2**:2686–2695 (1988).
27. Collins, G. G. S., J. Anson, and E. P. Kelly. Baclofen: effects on evoked field potentials and amino acid neurotransmitter release in the rat olfactory cortex slice. *Brain Res.* **238**:371–378 (1982).
28. Kato, K., M. Goto, and H. Fukuda. Baclofen: inhibition of the release of L-[^{3}H]glutamate and L-[^{3}H]aspartate from rat whole brain synaptosomes. *Gen. Pharmacol.* **13**:445–447 (1982).
29. Dutar, P., and R. A. Nicoll. A physiological role for $GABA_B$ receptors in the central nervous system. *Nature (Lond.)* **332**:156–158 (1988).
30. Takeda, J., K. Adachi, K. M. Halprin, S. Itami, V. Levine, and C. Woodyard. Forskolin activates adenylate cyclase activity and inhibits mitosis in vitro in pig epidermis. *J. Invest. Dermatol.* **81**:236–240 (1983).
31. Walter, U., I. Uno, A. Y.-C. Liu, and P. Greengard. Identification, characterization and quantitative measurement of cyclic AMP receptor proteins in cytosol of various tissues using a photoaffinity ligand. *J. Biol. Chem.* **252**:6494–6500 (1977).
32. Glass, D. B., and E. G. Krebs. Protein phosphorylation catalyzed by cyclic AMP-dependent and cyclic GMP-dependent protein kinases. *Annu. Rev. Pharmacol. Toxicol.* **20**:363–388 (1980).
33. Jakobs, K. H., K. Aktories, and G. Schultz. Inhibition of adenylate cyclase by hormones and neurotransmitters. *Adv. Cyclic Nucleotide Protein Phosphoarylation Res.* **14**:173–187 (1981).
34. Grega, D. S., M. A. Werz, and R. L. Macdonald. Forskolin and phorbol esters reduce the same potassium conductance of mouse neurons in culture. *Science (Wash. D.C.)* **235**:345–348 (1987).
35. Klein, M., and E. R. Kandel. Mechanism of calcium current modulation underlying presynaptic facilitation and behavioral sensitization in aplysia. *Proc. Natl. Acad. Sci. USA* **77**:6912–6916 (1980).
36. De Peyer, J. E., A. B. Cachelin, I. B. Levitan, and H. Reuter. Ca^{2+}-activated

K^+-conductance in internally perfused snail neurons is enhanced by protein phosphorylation. *Proc. Natl. Acad. Sci. USA* **79**:4207–4211 (1982).
37. Gray, R., and D. Johnston. Noradrenaline and β-adrenoceptor agonists increase activity of voltage-dependent calcium channels in hippocampal neurons. *Nature (Lond.)* **327**:620–622 (1987).
38. Baraban, J. M., S. H. Snyder, and B. E. Alger. Protein kinase C regulates ionic conductance in hippocampal neurons: electrophysiological effects of phorbol ester. *Proc. Natl. Acad. Sci. USA* **82**:2538–2542 (1985).
39. Mazzei, G. J., N. Katoh, and J. F. Kuo. Polymyxin B is a more selective inhibitor for phospholipid-sensitive Ca^{2+}-dependent protein kinase than for calmodulin-sensitive Ca^{2+}-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **109**:1129–1133 (1982).
40. Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita. Staurosporine, a potent inhibitor of phospholipid/Ca^{2+}-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**:397–402 (1986).
41. Knaus, H. G., J. Striessnig, A. Koza, and H. Glossman. Neurotoxic aminoglycoside antibiotics are potent inhibitors of [^{125}I]-omega-conotoxin GVIA binding to guinea pig cerebral cortex membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **336**:583–586 (1987).
42. Jakobs, K. H., S. Bauer, and Y. Watanabe. Modulation by adenylate cyclase of human platelets by phorbol ester-impairment of the hormone-sensitive inhibitory pathway. *Eur. J. Biochem.* **151**:425–430 (1985).
43. De Riemer, S. A., J. A. Strong, K. A. Albert, P. Greengard, and L. K. Kaczmarek. Enhancement of calcium current in aplysia neurons by phorbol ester and protein kinase C. *Nature (Lond.)* **313**:313–316 (1985).
44. Rane, S. G., and K. Dunlap. Kinase C activator 1,2-oleoylacetylglycerol attenuates voltage-dependent calcium current in sensory neurons. *Proc. Natl. Acad. Sci. USA* **83**:184–188 (1986).
45. Nordstedt, C., and B. B. Fredholm. Phorbol-12,13-dibutyrate enhances the cyclic AMP accumulation in rat hippocampal slices induced by adenosine analogues. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **335**:136–142 (1987).
46. Kishimoto, A., K. Nishiyama, H. Nakanishi, Y. Uratsuji, and H. Nomura. Studies on the phosphorylation of mylin basic protein by protein kinase C and adenosine-3′,5′-monophosphate dependent protein kinase. *J. Biol. Chem.* **260**:12492–12499 (1985).

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