Cyclic AMP-Dependent Regulation of the Number of [3H]Batrachotoxinin Benzoate Binding Sites on Rat Cardiac Myocytes*

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We sought to assess the effect of an increase in cAMP on sodium channels on adult rat cardiac ventricular myocytes. Sodium channels were studied with the use of the radiolabeled sodium channel-specific toxin [3H]batrachotoxinin benzoate ([3H]BTXB). Forskolin, isoproterenol, prostaglandin E1, cholera toxin, and pertussis toxin each increased cAMP levels and decreased the number of [3H]BTXB binding sites without changing the affinity of [3H]BTXB for the sodium channel. The cAMP analog 8-bromo-cyclic AMP (8-Br-cAMP) reduced the number of [3H]BTXB binding sites by half. The costs 19 fmol/10⁶ cells to 11 fmol/10⁶ cells. [3H]BTXB binding site down-regulation was reversible, cAMP dose-dependent, and time-dependent. To test the hypothesis that the cAMP effect was mediated by cAMP-dependent phosphorylation, we determined the effect of 8-Br-cAMP on [3H]BTXB binding after preincubation of myocytes with N-(2-(methylamino)ethyl)-5-isouquinolinesulfonamide dihydrochloride (H8), a protein kinase A inhibitor. H8 inhibited 70% of the decrease in the number of [3H]BTXB binding sites induced by 8-Br-cAMP. Thus increases in intracellular cAMP in cardiac myocytes reversibly induced a decrease in the number of [3H]BTXB binding sites via cAMP-dependent protein phosphorylation, possibly of the sodium channel.

There is accumulating evidence that the cardiac sodium channel may be regulated by a cyclic adenosine 3',5'-monophosphate (cAMP)-dependent mechanism. For example, Ono and colleagues (1) have shown that isoproterenol alters the gating mechanism of the sodium channel in a CAMP-dependent, G protein-dependent fashion. In earlier work, Catterall and colleagues (2) showed that the overall numbers of skeletal muscle sodium channels are responsive to the frequency at which they are electrically active and to interventions which alter intracellular levels of calcium and CAMP. This suggests that both the overall number of sodium channels and their gating mechanism(s) might be responsive to CAMP.

We have developed a biochemical approach to this problem with the use of a radioligand assay for the cardiac sodium channel. The radiolabeled sodium channel-specific toxin [3H] batrachotoxinin benzoate ([3H]BTXB) is used to measure sodium channel numbers on freshly isolated cardiac myocytes (6, 7). The [3H]BTXB binding site has many features characteristic of the cardiac sodium channel (5) and has been shown to be associated with the receptor for class I antiarrhythmic drugs (8).

The purposes of this work were to determine whether cAMP regulated [3H]BTXB binding to myocytes and to determine whether G proteins and protein kinase A might be involved in this regulation. This was done by assessing the effect of a variety of hormones and toxins on the binding of [3H]BTXB to freshly isolated cardiac myocytes.

MATERIALS AND METHODS

Myocyte Preparation—Cardiac ventricular myocytes were isolated from adult male Sprague-Dawley rat ventricles (200–250 g) by collagenase dispersion using the method of Kryski et al. (6). This method routinely yielded about 60 mg (dry weight) of myocytes, which corresponds to 1.2 × 10⁶ cells (6, 7). The cells were 85–90% viable rod-shaped cells which excluded trypan blue and were tolerant of 1 mM calcium.

[3H]BTXB Equilibrium Binding Assays—Equilibrium binding assays were performed as described previously (6, 8). Myocytes (6 × 10⁶ cells/assay) in 50 μl of incubation buffer (minimal essential medium with 50 μM CaCl₂ and 1% dialyzed bovine serum albumin) were incubated with [3H]BTXB at 37°C for 55 min at various concentrations of [3H]BTXB and detergents, and various concentrations of [3H]BTXB for 55 min at 37°C in a total volume of 65 μl. Tetrodotoxin was added to prevent depolarization induced by sodium influx without tetrodotoxin, no specific binding is observed (5, 9). Assays were done in parallel with tubes containing 0.4 mM aconitine to define the nonspecific binding. Reactions were stopped by adding 10 ml of Krebs-Henseleit-bovine serum albumin buffer (127 mM NaCl, 2.33 mM KCl, 1.3 mM KH₂PO₄, 1.25 mM KHPO₄, 1.25 mM MgSO₄, 26 mM NaHCO₃, 10 mM glucose, 50 μM CaCl₂, and 1% bovine albumin) that was equilibrated with 95% O₂, 5% CO₂ incubated at 37°C for 1 min, filtered through a Whatman GF/C 24-μm filter, and washed four times with 5 ml of rinse buffer (25 mM Tris HCl, pH 7.4, 130 mM NaCl, 5.5 mM glucose, and 50 μM CaCl₂). The filters were dried and counted in Econofluor scintillation fluid. The retained radioactivity represents [3H]BTXB bound to myocytes.

The rationale for the incubation and filtration conditions have previously been described (5). The conditions provide a maximal reduction in background and scatter with a minimal reduction in specific binding. The total wash time is 45 s. Initial control experiments showed that under these conditions less than 10% of the specifically bound [3H]BTXB dissociated from the complexes. Under these reaction conditions about 60–75% of total radioactivity retained on the filters is bound specifically to the [3H]BTXB binding site.

1. The abbreviations used are: [3H]BTXB, [3H]batrachotoxinin benzoate; PGE₁, prostaglandin E₁; CTX, cholera toxin; PTX, pertussis toxin; 8-Br-cAMP, 8-bromo-cAMP; H8, N-(2-methylamino)ethyl)-5-isouquinolinesulfonamide dihydrochloride; IBMX, isobutylmethylxanthine.

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Intracellular Cyclic AMP Levels—Cardiac myocytes (4 x 10^6 cells/ml) were incubated with or without agents which stimulate cAMP production for 30 min. Each tube was then treated with 5% perchloric acid. The samples were centrifuged at 12,000 X g for 10 min, and the pellet was discarded. Therefore, 75 μl of 5 M NaHCO₃ were added to 800 μl of the supernatant fluid. After 15 min on ice the samples were centrifuged for 10 min at 12,000 X g. Cyclic AMP levels were measured by a radioimmunological method using a cAMP assay kit.

Drugs and Chemicals—[3H]BTXB (specific radioactivity 56.8 Ci/mmol) was from Du Pont-New England Nuclear. The cAMP assay kit was from Amersham Canada (Oakville, Ontario). PGE₁, cholera toxin (CTX), pertussis toxin (PTX), 8-Br-cAMP, isoproterenol, forskolin, and isobutylmethylxanthine (IBMX) were from Sigma. H8, which is N-(2-(methylamino)ethyl)-5-isoquinolinesulfonamide dihydrochloride, was from Seikagaku America, Inc. (St. Petersburg, FL).

RESULTS

Increases in cAMP Levels—Agents were studied which were thought likely to increase intracellular cAMP levels by a variety of modes of action. These agents included isoproterenol (a β-adrenoceptor agonist) (10), prostaglandin E₁ (10), pertussis toxin (a G₁ blocker) (11), cholera toxin (a G₂ activated state blocker) (12–14), forskolin (an adenylylcyclase activator) (15) and IBMX (a cAMP-dependent phosphodiesterase inhibitor) (16). Fig. 1 illustrates a typical experiment in which isoproterenol, PGE₁, and forskolin increased cAMP levels in a dose-dependent fashion. All of the agents which were tested increased intracellular CAMP levels in rat cardiac myocytes (Table I). Forskolin was the most potent, increasing the cAMP level at least 10-fold. Isoproterenol, PTX, CTX, and PGE₁ also increased cAMP levels 6-8-fold.

Effect of cAMP on the Number of [3H]BTXB Binding Sites—The Kᵦ and the Bₓₒₓₓ of [3H]BTXB binding were assessed during treatments which increased intracellular cAMP levels. Fig. 2 illustrates typical experiments in which treatment with forskolin (2A), IBMX (2B), and CTX and PTX (2C) all resulted in decreases in the Bₓₒₓₓ of [3H] BTXB without causing significant changes in Kᵦ. The most prominent effects were observed with forskolin and CTX. Forskolin at a concentration of 200 μM reduced the Bₓₒₓₓ from 20 to 12 fmol/10⁵ cells without a significant effect on Kᵦ. Table II summarizes the mean effects of these interventions on the Bₓₒₓₓ and Kᵦ of [3H]BTXB binding. Regardless of which agent was used to increase cAMP similar results were obtained: a significant decrease in the Bₓₒₓₓ of [3H]BTXB binding without a change in Kᵦ.

These results suggested that increases in intracellular cAMP might be responsible for the reduction in the Bₓₒₓₓ of [3H]BTXB binding. To test this we determined the effect of the cAMP analog 8-Br-cAMP on [3H]BTXB binding. Fig. 2D shows that 8-Br-cAMP also caused a significant reduction in the Bₓₒₓₓ of [3H]BTXB binding without altering the Kᵦ. The mean results of seven experiments are presented in Table II. The addition of 10 mM 8-Br-cAMP reduced the Bₓₒₓₓ of [3H] BTXB binding from 18 ± 5 fmol/10⁵ cells to 10 ± 4 fmol/10⁵ cells (p < 0.002).

Concentration Dependence of cAMP Effects—The relationship between intracellular cAMP levels and sodium channel number was explored by determining the simultaneous effect of various concentrations of forskolin on cAMP levels and on [3H]BTXB binding. Fig. 3A shows that forskolin increased

![Fig. 1. Concentration dependence of increases in intracellular cAMP by forskolin, PGE₁, and isoproterenol results from a typical experiment.](image1)

![Fig. 2. Effect of various agents which increase intracellular cAMP level on [3H]BTXB binding sites. Results are from a typical experiment.](image2)
diminish [3H]BTXB binding was assessed by first allowing
of forskolin or 8-Br-cAMP, [3H]BTXB binding decreased
CAMP, 200
concentration increased from
0.001). Thus increased cAMP levels apparently can
destabilize previously formed [3H]BTXB-channel complexes
over a period of 5–60 min.

Reversibility of the cAMP Effect on [3H]BTXB Binding—
To determine whether the reduction in sodium channel
number by cAMP was reversible we assessed whether [3H]BTXB
binding returned to pretreatment levels when forskolin was
removed. Cardiac myocytes were first incubated for 30 min in
the presence or absence of forskolin. The cell suspension
was washed extensively, then incubated without forskolin for 90 min.
At that time the [3H]BTXB binding assay was performed as described under “Materials
and Methods.” The original control cells (which had not been exposed to
forskolin) were also incubated for 90 min in the absence of forskolin.
The data are expressed in Scatchard rearrangements of [3H]BTXB
binding curves and the lines plotted by linear regression analysis.

FIG. 3. [3H]BTXB binding decrease correlates with intracellular cAMP levels. Rat cardiac myocytes were preincubated for 30 min in the presence or absence of various concentrations of forskolin. Specific [3H]BTXB binding (fmol/10⁶ cells) (O) and cAMP level (pmol/10⁶ cells) (□) were determined as described under “Materials and Methods” and plotted in panel A. The percentage of [3H]BTXB specific binding compared with control cells is plotted as a function of cAMP levels in panel B. The line is derived by linear regression analysis (r = 0.97). The results are the mean of two experiments, and each experimental point was done in duplicate.

cAMP levels and decreased [3H]BTXB binding in a concentration-dependent fashion with a maximal effect at 200 μM forskolin. In the presence of 200 μM forskolin intracellular cAMP concentration increased from 4 to 117 pmol/10⁶ cells, and [3H]BTXB binding was reduced by 55%. Fig. 3B demonstrates a strong linear correlation between the reduction of [3H]BTXB binding and cAMP concentration (r = 0.97, p < 0.001).

Time Dependence of the Effect of cAMP on [3H]BTXB Binding—The time dependence of the ability of cAMP to diminish [3H]BTXB binding was assessed by first allowing [3H]BTXB binding to reach equilibrium, then serially measuring [3H]BTXB binding to myocytes after the addition of forskolin or 8-Br-cAMP. Cardiac myocytes were preincubated with [3H]BTXB for 55 min, and then either 10 mM 8-Br-cAMP, 200 μM forskolin, or incubation buffer alone was added. Fig. 4 shows that control [3H]BTXB binding was constant over time after equilibrium. However in the presence of forskolin or 8-Br-cAMP, [3H]BTXB binding decreased rapidly and progressively. After 10 min of incubation with forskolin [3H]BTXB binding had already decreased by 40%.
Cyclic AMP-dependent Reduction of Sodium Channel Number

**FIG. 6. Effect of kinase inhibitor H8 on concentration-dependent effect of 8-Br-cAMP on \([H]BTXB binding.\)** Rat cardiac myocytes were preincubated in the absence (○) or in the presence (●) of 50 µM of H8 for 60 min at 37°C. Myocytes were then incubated with various concentrations of 8-Br-cAMP. \([H]BTXB binding was assessed as described under “Materials and Methods.” The percentage of \([H]BTXB specific binding compared with control cells (no H8) is plotted as function of 8-Br-cAMP concentration. The curves represent the mean values of three separate experiments ± S.D. * p < 0.01; ** p < 0.005 by ANOVA test.

cAMP is quickly reversed as cAMP levels return to normal.

**DISCUSSION**

The principal findings of this study are that, in freshly isolated cardiac myocytes, 1) agents which increase intracellular cAMP level by a variety of mechanisms decreased the number of \([H]BTXB binding sites; 2) the cAMP analog 8-Br-cAMP decreased binding site number; 3) the sodium channel number was closely, linearly and inversely correlated with the intracellular cAMP concentration; 4) the effects of cAMP were time-dependent and reversible; and 5) inhibition of protein kinase A blocked this cAMP effect.

There is an accumulating body of data which attests to the importance of neuromodulation of sodium channel activity. Both Ono et al. (1) and Schubert et al. (18) have shown that isoproterenol inhibits voltage-dependent gating of the cardiac sodium channel. These adrenergic effects were mimicked by 8-Br-cAMP and forskolin. Thus cAMP appears to be an important link between adrenergic stimulation and changes in sodium channel activity, suggesting that sympathetic neurotransmitters interact with the sodium channel via cAMP.

Our data confirm and extend the work of Ono et al. (1) and Schubert et al. (18). Data from this study show that not only does isoproterenol decrease sodium channel number, but cAMP is a critical link in this interaction. Moreover, this effect is not limited to the adrenergic receptor but can be activated by PGE, In addition, we have demonstrated that both the adenylylclase inhibitory protein G, and the adenylylclase activating protein G, also alter sodium channel B, likely through a cAMP-mediated event. Indeed, the ability of pertussis toxin to decrease sodium channel B, by inhibiting a G, protein is a novel observation. Finally, we have shown for the first time that inhibition of protein kinase A blocks the cAMP-mediated decrease in sodium channel B.

Thus, the sodium channel may be modulated not only by the adrenergic system but also by other receptors which interact with G proteins; cAMP and protein kinase A appear to be in the final common pathway for this modulation of the cardiac sodium channel.

Previous studies have shown that the sodium channel has four phosphorylation sites (19). As well, forskolin and 8-Br-cAMP cause phosphorylation of the sodium channel (20). Thus protein kinase-mediated phosphorylation of the sodium channel may mediate the change in B, observed in this study and possibly mediate the decrease in sodium current seen in voltage clamp studies as well. Although a change in overall sodium channel number appears to be produced by phosphorylation of the sodium channel, it is possible that phosphorylation of the channel may alter channel conformation and thereby allosterically decrease access to radioligand binding sites. Reductions in the B, of radioligand binding are uncommon but acknowledged effects of allostERIC inhibitors (21-25). In particular, lidocaine inhibits \([H]BTXB binding by decreasing \([H]BTXB B, yet is an allosteric inhibitor of the sodium channel (26).

Thus at this time we cannot distinguish between these two hypotheses.

In conclusion, an increase in intracellular cAMP concentration induced a significant decrease in B, of \([H]BTXB binding sites on rat cardiac myocytes without changing the Kd of \([H]BTXB. The cAMP-dependent effect on \([H]BTXB binding was reversible, dose-dependent, and time-dependent. Finally H8, an inhibitor of cAMP-dependent protein kinase A, inhibited the effect of cAMP on sodium channel B, suggesting that channel phosphorylation is involved in the decrease in the number of sodium channel binding sites.

**REFERENCES**

1. Ono, K., Kiyosue, T., and Arita, M. (1989) Am. J. Physiol. 256, C1181-C1197.
2. Sherman, S. J., Chirvia, J., and Catterall, W. A. (1985) J. Neurosci. 5, 1570-1576.
3. Catterall, W. A., Morrow, C. S., Daly, J. W., and Brown, G. B. (1981) J. Biol. Chem. 256, 8922-8927.
4. Creveling, C. R., McNesl, E. T., Daly, J. W., and Brown, G. B. (1983) Mol. Pharmacol. 23, 350-358.
5. Sheldon, R. S., Cannon, N. J., and Duff, H. J. (1986) Mol. Pharmacol. 30, 617-623.
6. Kryski, A., Kennio, K. A., and Severson, D. L. (1985) Am. J. Physiol. 248, H208-H210.
7. Farmer, B. B., Mancina, M., Williams, E. S., and Watanabe, M. (1983) Life Sci. 33, 1-18.
8. Sheldon, R. S., Cannon, N. J., and Duff, H. J. (1987) Circ. Res. 61, 492-497.
9. Postma, S. W., and Catterall, W. A. (1984) Mol. Pharmacol. 25, 248-254.
10. Hayes, J. S., Brunton, L. L., and Mayer, S. E. (1980) J. Biol. Chem. 255, 5113-5119.
11. Katada, K., Oinuma, M., and U, M. (1986) J. Biol. Chem. 261, 5215-5221.
12. Casel, D., and Selinger, Z. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3307-3311.
13. Moss, J., and Vaughan, M. (1979) Annu. Rev. Biochem. 48, 581-600.
14. Gilman, A. G. (1984) Cell 36, 577-579
15. Seamon, K. B., Padgett, W., and Daly, J. W. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3363-3367
16. Parsons, W. J., Ramkumar, V., and Stiles, G. L. (1986) Mol. Pharmacol. 34, 37-41
17. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) Biochemistry 23, 5036-5041
18. Schubert, B., VanDongen, A. M. J., Kirsh, G. E., and Brown, A. M. (1989) Science 245, 516-519
19. Costa, M. R. C., Casnellie, J. E., and Catterall, W. A. (1989) J. Biol. Chem. 257, 7913-7921
20. Yang, J., and Burchi, R. (1990) J. Neurochem. 54, 954-962
21. DeLean, A., Stadel, J. M., and Lefkowitz, R. J. (1980) J. Biol. Chem. 255, 7108-7117
22. Sibley, D. R., DeLean, A., and Creese, I. (1982) J. Biol. Chem. 257, 6351-6361
23. Pedigo, N. W., Yamamura, H. I., and Nelson, D. L. (1981) J. Neurochem. 36, 220-226
24. Rothman, R. B., and Westfall, T. C. (1982) Mol. Pharmacol. 21, 538-547
25. Rothman, R. B., and Westfall, T. C. (1982) Mol. Pharmacol. 21, 548-557
26. Hill, R. J., Duff, H. J., and Sheldon, R. S. (1989) Mol. Pharmacol. 36, 150-159