ACTIONS OF MEMBRANE-ACTIVE DRUGS ON AGONIST OCCUPATION AND FUNCTIONAL STATE OF NICOTINIC ACETYLCHOLINE RECEPTORS*

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ABSTRACT The activities of 3 inhibitors of the nicotinic Ach receptor were examined for their capacity to affect agonist-elicited permeability and agonist occupation of the receptor in intact BC3H-1 cells. Nicardipine, dansylpropranolol and propranolol all inhibited receptor function in a manner which was noncompetitive with agonist. Each enhanced the affinity of carbachol for the receptor as do the local anesthetics which promote receptor desensitization. Comparisons of the agonist binding functions with the state functions for desensitization of the permeability response in the presence of the 3 inhibitors showed that at least part of the non-competitive action arised from the enhancement of receptor desensitization. For nicardipine, the block of agonist-elicited Na^+ and Ca^{++} permeabilities occured over identical concentration ranges which indicates that the antagonism is not ion-specific.

KEY WORDS nicotinic receptors; BC3H-1 cell line; carbachol; nicardipine; propranolol; dansylpropranolol

It has become possible to measure simultaneously ligand occupation and permeability response of nicotinic acetylcholine receptor in intact cells[1,2,3] and membrane vesicles[4]. Initial studies have delineated the binding requirements for activation[5], competitive antagonism[6] and desensitization[2] of the receptor. One can also examine the effect of noncompetitive inhibitors on agonist occupation and the functinal responses by this approach[7,8].

Clones of BC3H-1 muscle cell[9] enriched in nicotinic acetylcholine and adrenergic receptors can be separately isolated and studied[9,10]. With a monolayer of cells equivalent drug exposure to each receptor is achieved and the large internal volume of an intact cell permits one to measure initial rate of influx of cations into the cell upon agonist stimulation. Thus the molecular actions of drugs on the receptors can be examined on intact cells avoiding the complexicities of an entire synapse.

Propranolol inhibits junctional transmission at concentrations approaching those achieved during therapy[11]. Dansylpropranolol possesses useful spectroscopic properties as a fluorecent probe. Nicardipine is known to block Ca^{++} influx through voltage-sensitive, Ca^{++}-selective channels[14] but it may also inhibit receptor-operated channels. This study employed BC3H-1 cells to examine the influence of propranolol, dansylpropranolol and nicardipine on the nicotinic receptor.

EXPERIMENTAL PROCEDURES

Materials Dansylpropranolol was obtained from Molecular Probes Inc. Plano TX, USA. Propranolol and carbachol (carbamylcholine) were products of Sigma Chemical Co, Nicardipine was kindly provided by Syntex, *d*-Tubocurarine was a gift of Eli Lilly Co, ^{22}Na and ^{45}Ca were obtained carrier free from New England Nuclear, Boston MA, USA. The BC3H-1 cell line was kindly provided by

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Drs Patrick and Boulter of Salk Institute, La Jolla CA, USA and carried in our laboratory for past 8 yr. Dulbecco's modified Eagle's medium and Ham's nutrient mixture, F-12, were obtained in powdered form from Gibco, Grand Island NY, USA. Fetal calf serum and horse serum were from Gibco. [^{125}I]monoiodo-cobra α-toxin was prepared and separated from the noniodinated and diiodinated species by isoelectric focusing[12].

Cell Culture Stock cultures of BC3H-1 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a humidified atmosphere of 12% CO_2 + 88% air at 37℃, and maintained in the log phase of growth by subculture every 5 d. Experimental cultures were seeded from stock cultures[2]. Optimal growth conditions were achieved with a medium containing a 3:1 mixture of Dulbecco's modified Eagle's and Ham's F-12 media supplemented with 8% fetal calf serum and 2% horse serum. The resulting differentiated cells adhered tightly to the dish and produced highly uniform quantities of surface cobra α-toxin binding sites (1.5-2.0 pmol/dish of 35 mm diameter).

Kinetics of α-Toxin Binding and Competition with Cholinergic Ligands Experiments were performed at 21℃. A depolarizing K^+-HEPES buffer containing 140 m*M* KCl, 5.4 m*M* NaCl, 1.8 m*M* $CaCl_2$, 1.7 m*M* $MgSO_4$, 1 m*M* Na_2HPO_4, 5.5 m*M* glucose, 25 m*M* HEPES, 0.006% bovine serum albumun, adjusted to pH 7.4 was used for both α-toxin-binding measurements and permeability estimates for $^{22}Na^+$ and $^{45}Ca^{++}$[2]. Identical cultures were rinsed free of growth medium and incubated in the presence or absence of a specified concentration of cholinergic ligand for 30 to 60 min prior to the competition assay, which was initiated by replacing this solution with an identical solution containing a stoichiometric excess of [^{125}I] monoiodo-cobra α-toxin (typically 10 to 20 n*M*). After a 60 s interval, free radioligand was thoroughly removed by four 3-ml washes. The rate constant for toxin association, K_T, was determined from the resulting specific binding and initial free toxin and receptor concentrations according to the bimolecular rate equation[2].

Measurement of Permeability to $^{22}Na^+$ and $^{45}Ca^{++}$ $^{22}Na^+$ or $^{45}Ca^{++}$ influx was measured under conditions similar to those described above for α-toxin binding. After equilibration of the cultures in the depolarizing K^+-HEPES buffer, carbachol at the specified concentration and tracer quantities of either $^{22}Na^+$ or $^{45}Ca^{++}$ were added and influx of the radioisotope was measured over a 15 s and a 30 s interval, respectively. The permeability increase to sodium or calcium ions, K_G, was determined according to Equation [1], from the specific uptake, X, measured during the interval, t, and the equilibrium uptake into the freely exchangeable intracellular volume, ∞:

$$\ln[\infty/(\infty - X)] = K_G t \qquad (1)$$

Lower basal rates of $^{45}Ca^{++}$ influx were achieved when $^{45}Ca^{++}$ was added to cells bathed in buffer rather than adding a $^{45}Ca^{++}$ containing solution to cells where the buffer had been immediately aspirated. In the latter case calcium may stick to cell surface not in contact with the buffer.

RESULTS AND DISCUSSION

Agonist-Elicited Cation Permeability in BC3H-1 Cells In BC3H-1 cells, we observed that carbachol stimulated the unidirectional

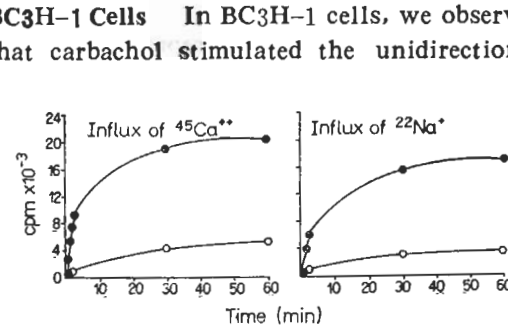

Fig 1. Carbachol-elicited cation permeability in BC3H-1 cells. Cells were washed with K^+-HEPES buffer and then equilibrated for 30 min in the same buffer at 21°C. Then tracer $^{22}Na^+$(18.5 kBq/ml)or $^{45}Ca^{++}$(37 kBq/ml)with or without carbachol(0.3 m*M*) was added and the reaction was stopped at various time thereafter. Control (○); 0.3 m*M* carbachol (●). Cellular volume = 8-10 μl/mg protein.

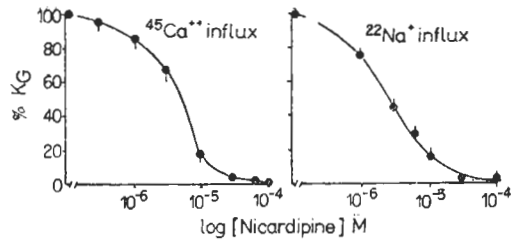

Fig 2. Inhibition of carbachol-elicited Na^+ permeability and Ca^{++} permeability by nicardipine. Cells were washed and equilibrated as in Fig 1, then changed to the same buffer containing nicardipine and equilibrated for another 30 min. Carbachol (0.3 m*M*)and $^{22}Na^+$ (18.5 kBq/ml) or $^{45}Ca^{++}$ (37 kBq/ml) were added while maintaining the same nicardipine concentration. The reaction was stopped after 15 s($^{22}Na^+$)or 30 s($^{45}Ca^{++}$).

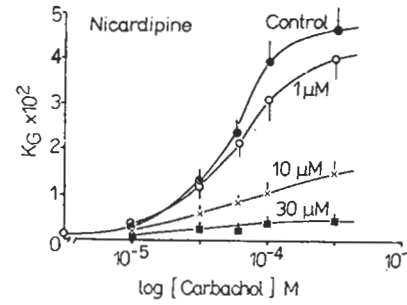

Fig 3. Nicardipine inhibition on carbachol-elicited Na permeability. The reaction was carried out as in Fig 2. Cultures were equilibrated with nicardipine for 20 min. $^{22}Na^+$influx was measured in the presence of the specified concentrations of nicardipine plus increasing concentrations of carbachol.

influx of $^{45}Ca^{++}$ and $^{22}Na^+$(Fig 1). If both $^{45}Ca^{++}$ and $^{22}Na^+$ traverse the receptor-linked channel by a common mechanism, then we would expect antagonists to affect the agonist-elicited permeability of Na^+ and that of Ca^{++} to an equivalent extent. In Fig 2, the influx of Ca^{++} and that of Na^+ were inhibited at similar concentrations of nicardipine, suggesting a common mode of inhibition for the 2 cations.

The competitive inhibitor *d*-tubercurarine showed equivalent antagonism of carbachol-elicited Na^+ influx and Ca^{++} influx. The action of nicardipine appeared reversible as exaustive washing of the cells allowed the nicardipine effect to disappear.

Mode of Nicardipine Inhibition on Agonist-Elicited Permeability We found that over a range of concentrations nicardipine inhibited the nicotinic receptor in a noncompetitive fashion (Fig 3). Increasing concentrations of nicardipine depressed the maximal Na^+ permeability elicited by agonist without appreciably increasing the agonist concentration required for half-maximal activation.

Influence of Nicardipine on Receptor Desensitization Desensitization of the nicotinic receptor is a consequence of a relatively slow formation of a state which is refractory to activation but possesses an enhanced affinity for the agonist[13]. Since the low densities of receptor on the cell surface proclude measuring agonist occupation directly, the use of a competition assay for the determination of agonist occupation was validated[2]. An enhanced capacity of agonists to inhibit α-toxin binding is a consequence of an increase in affinity for the agonist rather than an alternation in α-toxin binding[12]. If nicardi pine were to spromote desensitization it would enhance the affinity of carbachol for the receptor. As shown in Fig 4 A, nicardipine caused a ubstantial enhancement in the capacity of carbachol to inhibit the initial rate of α-toxin binding, indicating the affinity of carbachol for the receptor was enhanced. Nicardipine alone also slightly reduced α-toxin binding. However, this effect was small compared to the enhancement of carbachol affinity.

The noncompetitive action of nicardipine may also be analyzed in terms of its capacity to affect the state function for desensitization (Fig 4 B). Here, the receptors on the intact cells were exposed to increasing concentrations of carbachol in the presence or absence of a specified concentration of nicardipine, and then the influx of $^{22}Na^+$ elicited by carbachol plus the same concentration of nicardipine employed in the prior exposure was examined. We found that prior exposure to carbachol alone showed that high concentrations reduced the

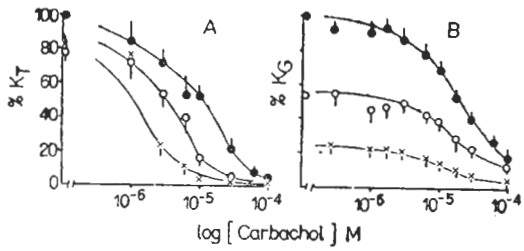

Fig 4. Influence of nicardipine on the binding function for carbachol and the state function for carbachol desensitization. Cells were washed and equilibrated as in previous figures, and then exposed to carbachol and nicardipine for 30 min, as initial exposure. Panel A: Binding function for carbachol. Initial rate of α-toxin binding were measured by adding [^{125}I]monoiodocobra α-toxin 20 n*M* at the same carbachol and nicardipine concentrations as used in initial exposure over a 60-s interval. Panel B: State function for desensitization. Influx of $^{22}Na^+$ was elicited by carbachol 0.3m*M* plus the nicardipine concentration employed in initial exposure and measured over a 15-s interval. Control (●); nicardipine 3.0 μ*M* (○); nicardipine 10 μ*M* (×).

permeability to 18% of the control value; half-maximal desensitization occured at 30 μ*M* carbachol. When the prior exposure included nicardipine plus carbachol, ion permeability was reduced more over the range of carbachol concentrations used. The concentrations of carbachol promoting desensitization were lowered in the presence of nicardipine, consistent with nicardipine's influence on the binding function. These data indicate that at least part of the influence of nicardipine is to promote agonist-induced desensitization.

Inhibition of Agonist-Elicited Permeability by Propranolol and Dansylpropranolol Both propranolol and dansylpropranolol also inhibited the permeability increase elicited by carbachol noncompetitively with propranolol being only slightly more potent (Fig 5). Both of them enhanced the binding of carbachol at equilibrium (Figs 6 A and 7 A), and slightly lowered the concentration dependence for carbachol-induced desensitization (Figs 6 B and 7 B). Thus their behavior was consistant with promoting formation of a desensitized state

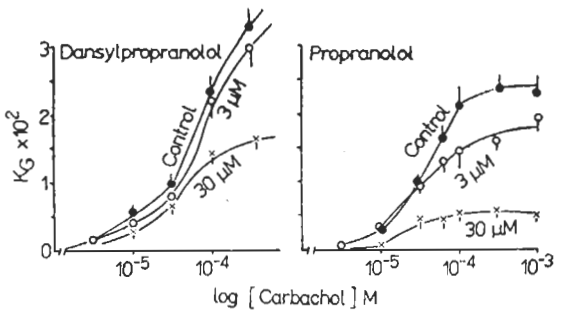

Fig 5. Inhibition of carbachol-elicited Na^+ influx in the presence of propranolol and dansyl propranolol. Conditions and procedures were identical to Fig 3.

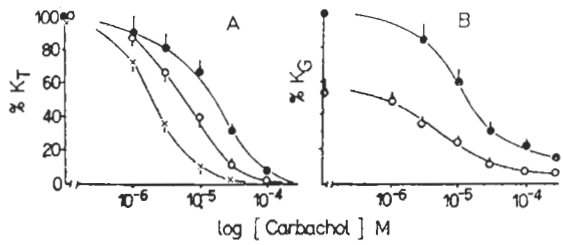

Fig 6. Influence of dansylpropranolol on binding function for carbachol(A)and state function for carbachol desensitization (B). Conditions and procedures were identical to Fig 4. Control (●); dansylpropranolol 30 μ*M* (○); 60 μM(×).

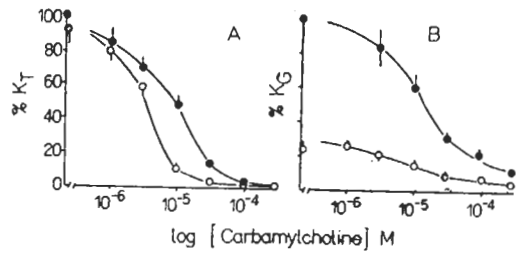

Fig 7. Influence of propranolol on binding function for carbachol (A) and state function for carbachol desensitization (B). Conditions and procedures were identical to Fig 4. Control (●); propranolol 30 μ*M*(○).

with a higher affinity for agonist.

In the BC3H-1 cells it has been possible to demonstrate that the kinetics of conversion in state and its subsequent reversion correlate

with the onset and recovery of desensitization [2,7]. An overall scheme which satisfactorily described the data is shown below:

$$\begin{array}{ccccc} & & K_R & & K_R \\ 2A + R & \rightleftarrows & AR + A & \rightleftarrows & A_2R \\ M\uparrow\downarrow & & \uparrow\downarrow & & \uparrow\downarrow \\ & & K_{R'} & & K_{R'} \\ 2A + R' & \rightleftarrows & AR' + A & \rightleftarrows & A_2R' \end{array} \qquad (2)$$

where $M = R'/R$.

An examination of individual steps yield values of $K_R = 6 \times 10^{-5}$; $K_{R'} = 2 \times 10^{-7}$; and $M = 10^{-4}$[7]. Thus in the absence of ligand the R state prevails and the conversion in state is driven by the 300-fold higher affinity of the R′ state for agonists. Many local anesthetics and certain peptide antibiotics enhance the overall affinity for agonists and their behavior appears to result from an increase in the value of M[7].

The 3 ligands examined in this study also inhibit the receptor noncompetitively and appear to promote desensitization. However, in contrast to the local anesthetics and histrionicotoxin described above, enhanced desensitization alone can not fully account for the observed degree of noncompetitive block. The 3 drugs depress receptor responsiveness to a greater extent than anticipated from their effects on the concentration dependence for carbachol desensitization of the receptor(Figs 4 B, 6 B, 7 B).Under these circumstances the noncompetitive inhibition of the receptor appears to be occuring by multiple mechanisms.

Nicardipine can affect the function of receptor-operated ion channels other than the voltage-sensitive, Ca^{++}-selective channels. However inhibition of the receptor-operated ion channel occured in a higher concentration range as the antagonism of Ca^{++}-selective channels occured in the submicromolar range[14]. In brief, the effectiveness of nicardipine in blocking ion permeability in the BC3 H-1 system is not ion-specific and appears to arise from multiple mode of noncompetitive inhibition.

Dansylpropranolol and propranolol were chosen for comparative study since propranolol has local anesthetic properties in addition to its β-adrenergic antagonizing action[11]. The dansyl analogue should prove useful in identifying the site of local anesthetic action on the receptor using fluorescent energy transfer techniques. We found that propranolol and dansylpropranolol exert their effects in part by enhancing receptor desensitization, an action similar to other local anesthetics[7].

REFERENCES

1 Catterall WA. *J Biol Chem* 1975; 250 : 1776
2 Sine S, Taylor P. *ibid* 1979; 254 : 3315
3 Taylor P, Brown RD, Johnson DA. The linkage between ligand occupation and response of the nicotinic acetylcholine receptor. In: Kleinzeller A, Martin BR, eds. *Current topics in membranes and transport*; vol 18. NY: Academic Press, 1983 : 407
4 Neubig RR, Cohen JB. *Biochemistry* 1980; 19 : 2770
5 Sine S, Taylor P. *J Biol Chem* 1980; 255 : 10144
6 Sine S, Taylor P. *ibid* 1981; 256 : 6692
7 Sine S, Taylor P. *ibid* 1982; 257 : 8106
8 Brown RD, Taylor P. *Mol Pharmacol* 1983; 23 : 8
9 Schubert D, Harris AJ, Devine CE, Heinemann S. *J Cell Biol* 1974; 61 : 398
10 Mauger JP, Moura AM, Worcel M. *Br J Pharmacol* 1978; 64 : 29
11 Argor Z, Mastaglia FL. *N Engl J Med* 1979; 301 : 409
12 Weiland G, Georgia B, Lappi S, Chignell CF, Taylor P. *J Biol Chem* 1977; 252 : 7648
13 Katz B, Thesleff S. *J Physiol (Lond)* 1957; 138 : 63
14 Terai M, Takenaka T, Maeno H. *Biochem Pharmacol* 1981; 30 : 375

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膜活性药物对N-胆碱受体的激动剂亲和力和功能状态的作用

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提要　硝苯吡啶胺、心得安、甲酰萘磺酰心得安均以非竞争型式抑制 BC_3H-1 细胞系细胞表面N-胆碱受体的功能，使受体激动剂引起的 Na^+ 和 Ca^{++} 通透性减低。三药均能增加N-胆碱受体对氨甲酰胆碱的亲和力，能促进受体转变为去敏感状态。硝苯吡啶胺对N-胆碱受体激动所致阳离子通透性的抑制作用没有离子特异性。

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关键词　烟碱受体；BC_3H-1 细胞株；硝苯吡啶胺；心得安；甲酰萘磺酰心得安；氨甲酰胆碱

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