in plasma membrane permeability to trypan blue. The depolarization was dependent upon both the concentration of, and time of exposure to, phenothiazines, was more pronounced in injured cells, and appeared immediately upon exposure to the drug.

Acknowledgements—Dr. Scott is the recipient of an NIH New Investigator Research Award CA33570. This work was also supported by NIH Ischemia SCOR HL-56212, NIH Grant AM-27401, and by a grant from R. J. R. Nabisco.

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Biochemical Pharmacology, Vol. 37, No. 19, pp. 3787–3790, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Irreversible inhibition by tyrosine-directed alkylating reagents of muscarinic cholinergic receptors in membranes from rat forebrain and heart

(*Received* 16 *November* 1987; *accepted* 7 *March* 1988)

Muscarinic cholinergic receptors (mAChRs) are composed of several types. The genes of rat cerebral M_1 and cardiac M_2 receptors have been recently cloned and sequenced [1, 2]. Other potential mAChR types have been suggested based on biochemical and functional studies as well as by gene cloning studies [2]. A major characteristic of the mAChR types is their different affinities for selective muscarinic ligands such as pirenzepine (PZ) [3] and AF-DX 116 [4]. Whether or not this difference reflects a variation of the amino acid compositions at the ligand binding site of the different receptor types is a field of active investigation. In the present study, two tyrosine-directed reagents were used to modify the mAChRs from rat forebrain and heart. The results suggest that the existence of a tyrosyl residue is essential for ligand binding to both rat forebrain (mostly M_1) and cardiac (mostly M_2) muscarinic receptors.

Methods and results

Male Sprague–Dawley rats (200–300 g) were killed by decapitation. The forebrains and whole hearts were dissected and homogenized in 20 vol. of ice-cold 50 mM sodium/potassium phosphate buffer (pH 7.4) with a Polytron homogenizer. The homogenates were centrifuged at 1000 *g* for 5 min. The supernatant fractions were recentrifuged at 40,000 *g* for 20 min. The pellets were suspended in the same buffer supplemented with 25 mM $MgCl_2$ and with either 4-fluorosulfonyl-1-hydroxy-2-naphthoic acid (FSNA, Aldrich Chemical Co. Inc.) or *p*-nitrobenzenesulfonyl fluoride (pNBSF, Pierce Chemical Co.) dissolved in absolute alcohol (control samples received an equal amount of alcohol). The final concentration of alcohol in homogenates was 2%. The homogenates were incubated at room temperature for 15 min. In the protection experiments the homogenates were preincubated with muscarinic drugs for 1 hr. The reaction was stopped by immersion of the tubes in an ice-water bath followed by centrifugation. The resuspension–centrifugation process was repeated three times. The final pellets were resuspended in 50 mM sodium/potassium phosphate buffer. An aliquot of the homogenate was incubated with [^{3}H](−)quinuclidinyl benzilate ([^{3}H](−)QNB; 33.2 Ci/mmol, New England Nuclear) at 25° for 2 hr, in the presence or absence of atropine (1 μM) for the determination of specific binding. Bound and free radioligands were separated by rapid filtration through GF/B filters followed by four rinses using 3 ml of ice-cold buffer. The radioactivity

on the filters was then extracted and counted using a liquid scintillation spectrophotometer at an efficiency of 42%.

Incubation of membranes with either FSNA or pNBSF resulted in a concentration-dependent decrease of specific [^{3}H](−)QNB (1 nM) binding in both forebrain and heart tissues (Fig. 1). The apparent EC_{50} values were about 0.9 mM for FSNA and 2.4 mM for pNBSF in both tissues. Saturation studies with [^{3}H](−)QNB showed a decrease in B_{max} values but not K_d values after alkylation of membrane proteins with FSNA in both tissues (Table 1). FSNA (at a concentration of 2 mM) also inhibited [^{3}H]PZ (10 nM) binding by 62% in the cerebral cortex and [^{3}H]AF-DX 116 (10 nM) binding by 61% in the heart. Table 2 shows the relative potency of muscarinic drugs in protecting mAChRs from inactivation by 2 mM FSNA. In preliminary experiments, it was found that maximum protection of the receptor was obtained using 1 μM atropine or 100 mM carbachol with IC_{50} values of 0.05 μM and 5 mM respectively. Therefore, a concentration of 1 μM was used for muscarinic antagonists to compare their potencies in protecting mAChRs. The order of potency for antagonist was atropine > PZ > AF-DX 116 in both tissues. The antagonists did not show any statistical difference in their potency between the forebrain and heart. The muscarinic agonist carbachol (100 mM) produced full protection in each tissue. The addition of 100 μM Gpp(NH)p into the incubation mixture did not change the potency of carbachol.

Table 1. Effect of FSNA treatment on the binding parameters of [^{3}H](−)QNB to rat forebrain and cardiac membranes

Tissue	FSNA (mM)	B_{max}* (fmol/mg protein)	K_d* (pM)
Forebrain	0	1210 (942–1450)	27.2 (10.8–53.4)
	0.8	949† (747–1230)	31.1 (17.3–58.0)
	1.6	566†‡ (257–880)	35.1 (24.6–52.2)
Heart	0	240 (233–249)	50.3 (43.5–59.2)
	0.8	187§ (186–219)	50.3 (54.6–59.8)
	1.6	152†‡ (142–157)	46.4 (39.3–56.3)

Data are presented as the arithmetic means of B_{max} values and the geometric means of K_d values from four experiments done in duplicate. The range of data is given in the parentheses.

* One-way ANOVA test showed significant differences among the three groups of the B_{max} values in the forebrain ($P < 0.05$) and the heart ($P < 0.01$), but not among the groups of K_d values.

†–§ The following statistical analyses were done using the paired Student's t-test: †$P < 0.01$ vs control, ‡$P < 0.01$ vs 0.8 mM group, and §$P < 0.05$ vs control.

Discussion

Both FSNA and pNBSF have been reported to react preferentially with the tyrosyl residue [5, 6]. pNBSF was found to react only with the tyrosyl residue [5], while FSNA had weak reactions with cysteinyl residues [6]. It is unlikely that the irreversible inactivation of mAChRs observed in the present study was the result of labelling cysteinyl residue(s), inasmuch as the treatment of mAChRs with sulfhydryl reagents did not alter significantly [^{3}H](−)QNB binding in the rat cerebral cortex [7].

Tyrosine has been proven to be present at the ligand binding site of the β-adrenergic [6] and α_2-adrenergic receptors [8] as well as at the active site of several enzymes. However, the role of the tyrosyl residue in the mAChRs is not clear. Our present study showed that binding of the non-selective muscarinic antagonist [^{3}H](−)QNB to muscarinic receptors in rat forebrain and heart involved the tyrosyl residue(s). The tyrosyl residue(s) labeled by these two alkylating reagents was most likely located at or near the ligand binding site of both receptors, since the alkylation of the tyrosyl residue(s) caused a loss of binding sites for

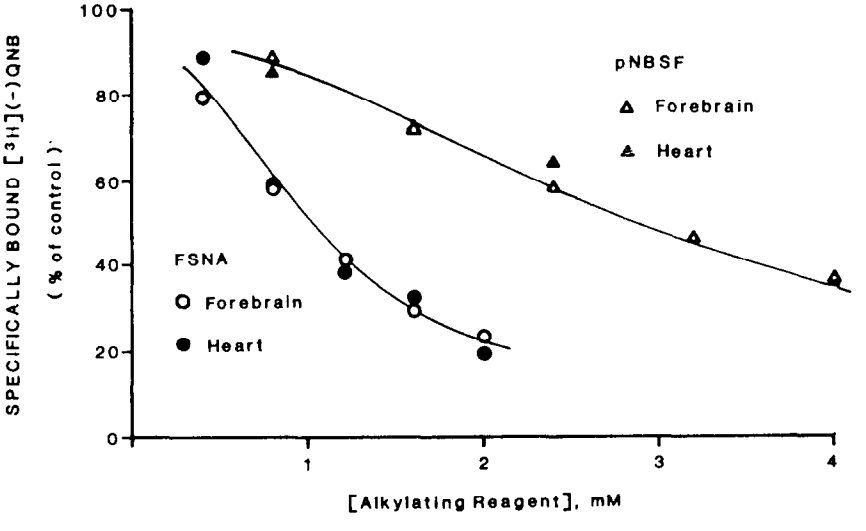

Fig. 1. Concentration-dependent decrease of specific [^{3}H](−)QNB binding to mAChR in membranes from rat forebrain and heart after treatment with FSNA or pNBSF. The ordinate is the specific binding of [^{3}H](−)QNB (1 nM) expressed as percentage of the controls (1030 ± 64 fmol/mg protein for the forebrain and 223 ± 9 fmol/mg protein for the heart). The abscissa is the concentration of the alkylating reagents. Data shown are the means of three experiments done in triplicate.

Table 2. Protection by muscarinic drugs of [^{3}H](−)QNB binding sites from inactivation by 2 mM FSNA

Drug	Concentration (mM)	Forebrain				Heart			
		Specific binding*		% of Control†	Percentage protected‡	Specific binding*		% of Control†	Percentage protected‡
		−FSNA	+FSNA			−FSNA	+FSNA		
None		1026 ± 62	398 ± 39§	39 ± 3‖		245 ± 17	114 ± 6§	46 ± 4‖	
Atropine	0.001	1066 ± 19	889 ± 96¶	78 ± 8**‖	72 ± 14‖	254 ± 22	216 ± 30	85 ± 5**‖	71 ± 8‖
Pirenzepine	0.001	1067 ± 107	836 ± 17¶	73 ± 6**‖	67 ± 15‖	234 ± 16	194 ± 21	83 ± 4**‖	68 ± 6‖
AF-DX 116	0.001	1084 ± 72	499 ± 81§	46 ± 8‖	16 ± 13‖	261 ± 28	142 ± 30§	55 ± 9‖	14 ± 5‖
Carbachol	100	749 ± 85††	672 ± 15	92 ± 10**	86 ± 16	174 ± 11**	180 ± 25	103 ± 8**	106 ± 15
Carbachol (+Gpp(NH)p)	100 (0.1)	828 ± 106	751 ± 66	95 ± 18**	93 ± 31	182 ± 39	180 ± 23	103 ± 12**	99 ± 24

* Specific binding of [^{3}H](−)QNB is presented as fmol/mg protein.

† The percentage of control was calculated using the corresponding specific binding of [^{3}H](−)QNB in the membranes preincubated with drugs in the absence of FSNA as 100%. See Methods and Results for details.

‡ The percentage of sites protected was calculated by the following equation: $[(B\text{-}A)/(100\text{-}A)] \times 100\%$, where A is the percentage of control without preincubation with drug, and B is the percentage of control with drug preincubation.

Values are presented as means ± SEM of at least three independent experiments done in triplicate. One-way ANOVA test indicates significant ($P < 0.05$) variance within each column. The following statistics were done using Student's t-test: §$P < 0.01$, vs group without FSNA treatment (−FSNA); ‖$P < 0.01$, vs 100%; ¶$P < 0.05$, vs group without FSNA treatment (−FSNA); **$P < 0.01$, vs group without drug preincubation (none); and ††$P < 0.05$, vs group without drug preincubation (none).

[^{3}H](−)QNB rather than an alteration of receptor affinity. Moreover, the specific radioligand binding was conserved by including muscarinic drugs in the reaction. Nonetheless, an allosteric mechanism cannot be excluded from the present data.

It is not surprising that the concentration–inactivation curves of [^{3}H](−)QNB binding in both the forebrain and heart were virtually the same. It has been noted that among the 16 and 14 tyrosyl residues in the primary structures of rat brain M_1 and heart M_2 receptors, respectively, 13 of them are aligned in the same positions of the receptor proteins [2]. Therefore, the tyrosyl residue(s) involved in the ligand binding may have a similar topographical localization in both the forebrain and cardiac receptors. Interestingly, the apparent EC_{50} values for FSNA and pNBSF at the muscarinic receptors are similar to those reported for the β-adrenergic receptor [6]. A structural homology between muscarinic receptors, β-adrenergic receptors [9] and α_2-adrenergic receptors [10] also has been noted. The existence of tyrosyl residue(s) in the binding site of mAChR, β- and α_2-adrenergic receptors suggest a degree of homology in the ligand binding sites among the members of the opsin family.

The M_1 selective antagonist PZ and the M_2 selective antagonist AF-DX 116 failed to show any preference in protecting [^{3}H](−)QNB binding sites between the forebrain and heart. Therefore, the tyrosyl residue labeled by FSNA and PMSF may not be a determinant for muscarinic selectivity of the cerebral cortical M_1 and cardiac M_2 receptors. However, a different tyrosyl residue(s) may be involved in the binding of the receptor selective ligands. For example, nitration of tyrosyl residues with tetranitromethanol increases the affinity of the muscarinic agonist carbachol without altering the binding of antagonists in the rat brain [11].

In summary, the present study revealed that [^{3}H](−)QNB binding was irreversibly inactivated by alkylation of the tyrosyl residue(s) in mAChRs from both rat forebrain and heart. The inactivation of mAChRs was prevented by muscarinic drugs, suggesting a possible involvement of the tyrosyl residue(s) in the binding site of the muscarinic receptors.

Acknowledgements—This study was supported by a grant from the American Heart Association, Arizona Affiliate, and in part by USPHS Grants DK-36289 and HL-20984. We thank Pam Davis for her secretarial services.

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Biochemical Pharmacology, Vol. 37, No. 19, pp. 3790–3793, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Mediation of norepinephrine effects on free cytosolic calcium in rat parotid acinar cells by α_1 adrenergic receptors

(*Received* 10 *February* 1988; *accepted* 28 *April* 1988)

Stimulation of sympathetic nerves or injection of norepinephrine (NE*) produces a pronounced increase in parotid salivary amylase secretion and weakly stimulates salivary fluid secretion, relative to parasympathetic nerve stimulation [1, 2]. The contributions of different adrenergic receptors to these effects have been studied extensively *in vitro*. Adrenergic effects on amylase exocytosis are primarily mediated via β receptors and involve a cyclic AMP (cAMP) dependent pathway [2]. However, a small activation of protein exocytosis is provided by α_1 receptor agonists, similar to that observed with muscarinic agonists and substance P, and apparently results from protein kinase C activation [3]. NE effects on parotid salivary fluid secretion appear absolutely dependent on an increase in the intracellular free calcium concentration (Ca_i^{2+}) in these cells, which stimulates vectorial ion movements [4, 5]. Although initial studies of Ca^{2+}-dependent ion movements in parotid and submandibular cells suggested that the α_2 receptor was primarily responsible for adrenergic increases in salivary fluid secretion [6, 7], more extensive characterization of ion fluxes in salivary glands has established the α_1 receptor as the primary adrenergic mediator [8–10]. Moreover, mobilization of Ca^{2+} via phospholipase C-activated phosphatidylinositide (PI) turnover also is mediated by α_1 (but not α_2 or β) adrenergic receptors in parotid cells [11, 12].

The development of fluorescent Ca_i^{2+}-indicator dyes has allowed direct examination of the effects of the different adrenergic receptors on Ca_i^{2+} in dispersed parotid cell or acini preparations. Initial studies with quin 2 provided evidence for an α receptor-mediated increase in Ca_i^{2+} but there were conflicting reports as to the effects of β agonists [13–15]. Recently Nauntofte and Dissing have re-examined the contribution of the different parotid adrenergic receptor types to the increase in Ca_i^{2+} using the more sensitive indicator Fura 2 [16]. They found that the β agonist isoproterenol (ISO) is only 10% as effective as epinephrine and that the selective α_1 agonist phenylephrine (PE) is only 60% as effective. There was no additivity between ISO and PE effects on parotid Ca_i^{2+}, and these authors concluded that ISO was acting through α_1 receptors. The difference between epinephrine and PE effects was ascribed to additional α_2 effects of epinephrine [16]. An alternative explanation for the latter finding is that PE is a partial agonist at parotid α_1 receptors. In the present study, we examined this possibility and closely studied the effects of various adrenergic agonists and antagonists on Ca_i^{2+}.

* Abbreviations used: NE, norepinephrine; cAMP, cyclic AMP; Ca_i^{2+}, intracellular free calcium concentration; ISO, isoproterenol; PE, phenylephrine; Met, Methoxamine; Yoh, yohimbine; Cor, corynanthine; Aten, atenolol; Phent, phentolamine; and Clon, clonidine.

Materials and methods

Prazosin, phentolamine, methoxamine, clonidine, and atenolol were gifts from Pfizer (New York, NY), Ciba Geigy (Summit, NJ), Burroughs-Wellcome (Research Triangle Park, NC), Boehringer Ingelheim (Ridgefield, CT) and Stuart Pharmaceuticals (Wilmington, DE) respectively. NE, PE, ISO, propranolol, corynanthine and yohimbine were from Sigma (St. Louis, MO). All drugs were dissolved in distilled water (except prazosin which was dissolved in ethanol). In some experiments, NE and ISO were added with equimolar ascorbate. Male Sprague–Dawley rats (200–350 g) were obtained from Charles River Laboratories (Kingston, NY). Fura 2 acetoxymethylester was purchased from Molecular Probes (Eugene, OR) and [^{3}H]inositol (15 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO).

Rat parotid cells were prepared and Fura 2 studies performed as previously described [17]. If necessary, Ca_i^{2+} measurements were corrected for drug fluorescence or quenching. Cells were loaded with [^{3}H]inositol as reported [11, 17], but accumulation of total [^{3}H]inositol phosphates rather than of [^{3}H]inositol trisphosphate was measured.

Results and discussion

NE consistently produced a small elevation of Ca_i^{2+} in rat parotid acinar cells. The adrenergic effect on Ca_i^{2+} in this system and the effect on PI turnover appeared to be mediated solely by the α_1 adrenergic receptor, as follows: (1) the α_1-selective antagonist prazosin potently blocked NE effects on Ca_i^{2+} as well as on [^{3}H]inositol phosphate accumulation (Table 1 and Fig. 1C), (2) the beta blockers atenolol (10^{-5} M) and propranolol (10^{-6} M) had no effect on NE stimulation of Ca_i^{2+} or PI turnover (Fig. 1D and Table 1), and (3) yohimbine, an antagonist used at an α_2-selective concentration (10^{-6} M) also was without effect on NE actions (Fig. 1D and Table 1). Corynanthine, a structural analog which lacks the α_2 selectivity of yohimbine [18], was about 10-fold more potent than yohimbine in blocking NE effects and about 1000-fold less potent than prazosin (Table 1 and Fig. 1A, B and C). Importantly, no α_2 or β effects of NE on Ca_i^{2+} were apparent even under conditions where the α_1 receptor was mostly blocked (Fig. 1B and C).

In agreement with the findings of Nauntofte and Dissing [16], the selective α_1 agonist PE was much less effective than NE in elevating Ca_i^{2+} (Fig. 1D and E) and also as a stimulus for PI turnover (Table 1). However, this reflects the partial agonist property of PE in parotid cells, rather than an additional α_2 effect of NE. At a concentration supramaximal for effects on Ca_i^{2+} (300 μM), PE increased Ca_i^{2+} only 40% as effectively as NE (Fig. 1D and E and Table 1). PE also partially reversed NE effects on Ca_i^{2+} (Fig. 1 and Table 1), as would be expected of a partial