Br. J. Pharmac. (1971), **43**, 604–611.

Effects of isoxazolium cations on some isolated muscle preparations

T. W. MITTAG* AND ANNE TORMAY

Department of Pharmacology, New York Medical College, 1249 Fifth Avenue, New York, N.Y. 10029, USA

Summary

1. The quaternary ammonium compound, N,*t*-butyl-5-methyl isoxazolium perchlorate (BIP), an anionic group reagent, initially causes contractions of the rat phrenic-nerve diaphragm, guinea-pig ileum and rabbit aortic strip preparations *in vitro*.

2. In addition, the drug produces an irreversible block of indirectly elicited twitch responses in the diaphragm and of contractions induced by acetylcholine, methylfurmethide, dimethyl-phenylpiperazinium, 5-hydroxytryptamine (5-HT), histamine, angiotensin and pilocarpine in the ileum, while direct electrical stimulation of the diaphragm and contractions of the ileum to Ba and K ions are relatively unaffected.

3. BIP is also an irreversible inhibitor of acetylcholinesterase but not of butyrylcholinesterase.

4. On rabbit aortic strip preparations, responses to histamine, noradrenaline and 5-HT were differentially sensitive to irreversible blockade by BIP.

5. Diphenhydramine, used in conditions which gave complete protection of the histamine response to irreversible block by dibenamine, did not protect against the blocking action of BIP but increased the blockade.

6. These results suggest that BIP reacts covalently with anionic groups which mediate receptor initiated stimuli. The isoxazolium group may be useful in conferring irreversible properties by its substitution in drug molecules for the pyrrole or pyrrolidine group.

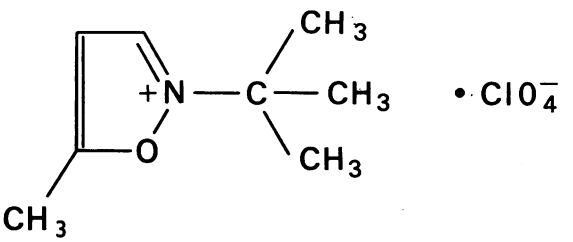
Introduction

Drugs with a covalent labelling moiety are potentially useful therapeutic agents because of their persistence. In addition, specific, site directed drug reagents show promise for obtaining information about the molecular structure of pharmacological receptor sites. For this purpose, drugs containing aziridinium (Gill & Rang, 1966), diazonium (Changeux, Podleski & Wofsy, 1967), β-halo-ethylamine (Graham, 1962; Furchgott, 1966), or other alkylating moieties (Silman & Karlin, 1969) have been investigated as labelling agents and several have proved useful for the quantitation of both adrenergic and cholinergic receptors (May, Moran, Kimelberg & Triggle, 1967 ; Moran & Triggle, 1970). However, the use of some of these compounds for

*Present address: Department of Pharmacology, Mount Sinai School of Medicine, Fifth Avenue and 100th St., New York, N.Y. 10029.

identification and isolation of receptor proteins has been complicated by their considerable non-specific reactivity (Takagi, Akao & Takahashi, 1965; Moran, May, Kimelberg & Triggle, 1967). Similarly, the therapeutic uses of these drugs is limited by non-specific toxicity due to their alkylating activity.

The potential use of the isoxazolium group as a suitable covalent labelling moiety has been explored by examining the pharmacological action of N,*t*-butyl-5-methyl isoxazolium perchlorate (BIP), on the rabbit aortic strip, guinea-pig ileum and rat



phrenic nerve-diaphragm preparations. This reactive moiety could prove useful as a replacement for pyrrole or pyrrolidine groups in drug molecules and, thereby, confer prolonged time of action.

Isoxazolium cation reagents were developed by Woodward *et al.* (1966) as coupling agents for peptide synthesis. These workers utilized the reaction of such compounds with nucleophiles, for example carboxylate anions, to produce enol esters. Subsequently, it was shown that these reagents in aqueous solution can react with carboxyl groups of macromolecules (Bodlaender, Feinstein & Shaw, 1968). Reaction of the isoxazolium ring with anionic groups of receptors could prolong the action of drugs containing this group. The group reacts by esterification and might, therefore, show less non-specific toxicity in comparison to alkylating agents. Since the reagents are quaternary ammonium ions, they would have limited access to the interior of cells and may possibly be selective for cholinergic systems.

Methods

Rat phrenic nerve diaphragm

Left hemidiaphragms of male Sprague Dawley rats (150–200 g) with attached costal margin and mounted in electrode holders modified after Bülbring (1946) were incubated in Tyrode's solution, 30°C, and aerated with oxygen containing 5% CO_2. Surface enzyme assays and drug treatments were carried out essentially as described by Ehrenpreis, Mittag & Patrick (1970), while the phrenic nerve was continuously stimulated with supramaximal square wave pulses (0·2 ms) at a frequency of 0·2 Hz. The muscle was stimulated directly with supramaximal square wave pulses (30–50 V, 0·2 ms duration, 0·2 Hz) applied to platinum electrodes inserted 1 cm apart into the muscle. Isometric contractions were recorded from a baseline tension of 5 g with a Grass FTO3 force displacement transducer and a Grass Model 7 recorder.

Enzyme assays

Surface acetylcholinesterase (AChE) activity was determined with 10^{-6}M acetyl-l-^{14}C-choline (ACh) or acetyl-l-^{14}C-β methyl choline (MeCh) as substrate in Tyrode's

acetate solution bathing the diaphragm. Radioactive acetic acid was separated from unhydrolysed choline substrate on cation exchange columns (BioRex 40, Na^+ form, 200–400 mesh), collected in Bray's solution and radioactivity determined by liquid scintillation counting (Ehrenpreis *et al.*, 1970; Mittag, Ehrenpreis & Hehir, 1971).

Guinea-pig ileum

Ileum from 250–500 g male guinea-pigs killed by cervical fracture was used. Starting 5 cm from the ileocaecal junction, the ileum was washed thoroughly in gassed (O_2 containing 5% CO_2) Krebs solution, 37°C and cleaned by stripping off the mesenteric membrane. A length of tissue of about 1 cm was mounted in the tissue bath at 37°C and isometric contractions recorded from a baseline tension of 1 g. Control log dose-response curves were obtained by the cumulative addition of appropriate concentrations of agonists at intervals of 3–5 min, followed by a wash period of 20 minutes. After treatment of the preparation with BIP, the cumulative log dose-response curve was obtained again in some experiments. With the ileum this was much flatter than initially so in most experiments the only doses tested were those which were 10 or 100 times the dose which had initially produced half the maximum response obtainable.

Aortic strip

Spiral strips, cut from the aorta of albino male New Zealand rabbits, weighing 2–3 kg, were set up as described by Furchgott (1960). Control cumulative log dose-response curves were obtained, as with the guinea-pig ileum, and again after the preparation had been treated with BIP. If the graphs of log dose-response appeared to be linear, by eye, the method of least squares was applied in order to obtain the slope of the regression line which was the best fit.

Protection experiments with diphenhydramine were carried out as described by Furchgott (1954).

Treatment with N,t-butyl-5-methyl isoxazolium perchlorate

The drug (Aldrich Chemical Co.) was made up fresh shortly before use in Krebs or Tyrode's solution (pH 7·4) and applied to the tissue at a final concentration of 120 μg/ml (5×10^{-4}M) for a specified time at the appropriate temperature. Preparations were thoroughly washed with five changes of physiological salt solution over 30 min after BIP treatment before retesting with agonists.

Drugs were obtained from the following sources: Acetylcholine bromide, K.K. Chemical Co., Plainview, N.Y.; angiotensin amide, phentolamine, Ciba Pharmaceutical Co.; 2-brom-D-lysergic acid, Sandoz Pharmaceutical Co.; dibenamine hydrochloride, Smith, Kline and French Pharmaceutical Co.; diphenhydramine, Parke-Davis Pharmaceutical Co.; DMPP (1,1-dimethyl-4-phenyl piperazinium iodide), BIP (N,*t*-butyl-5-methyl isoxazolium perchlorate); Aldrich Chemical Co., Cedar Knolls, N.J.; histamine dihydrochloride, Sigma Chemical Co., St. Louis, Mo.; methylfurmethide, gift of E. J. Ariens, Nijmegen; L-(-)noradrenaline, Regis Chemical Co., Chicago, Ill.; pilocarpine hydrochloride, Merck and Co., Inc., Rahway, N.J.; 5-HT (serotonin creatinine sulphate), Nutritional Biochemicals Corp., Cleveland, Ohio.

Results

Treatment of the guinea-pig ileum preparation with BIP (120 μg/ml, Krebs solution, pH 7·4, 37°C) for 15 min consistently produced a complex response (Fig. 1). Immediately after application of the drug, one to three brief, sharp contractures occurred over 1 minute. Thereafter, there was a gradual increase in tone, reaching a maximum after 3 or 4 min and then gradually fading until the baseline tension was reached again within about 10 minutes. From 10–15 min after the addition of the compound, irregular, sharp contractures began to occur with a frequency of between one and three per min (Fig. 1). This sequence of effects was unaltered by pretreatment of the preparation with either atropine or diphenhydramine. Irregular, sharp contractures persisted after washout of the drug, but they gradually declined in intensity and frequency and disappeared after 20–25 minutes.

Thirty minutes after treatment of the ileum with BIP, responses to acetylcholine, methylfurmethide or dimethylphenylpiperazinium were abolished; occasionally slight signs of recovery were observed 90 min after washout of the drug. Contractions to histamine, angiotensin, serotonin and pilocarpine were similarly inhibited by the treatment, but this also markedly reduced the slope of their log dose-response curves. The extent of the antagonism could only be assessed roughly by testing responses to doses 10 and 100 times those producing half the maximal response initially. The responses to K^+ and Ba^{++}, however, were much less affected, being about half their initial size. This is consistent with the idea that half the effects of these ions are ganglionic in origin (Paton & Aboo Zar, 1968) and the remainder are due to direct effects, which are unaffected by BIP.

On the rabbit aortic strip preparation, a single treatment with BIP for 15 min caused a contraction which faded spontaneously in the presence of the drug. The log dose-response curves to noradrenaline, 5-hydroxytryptamine and histamine were moved to the right but their slope was not significantly altered, so the log dose-ratios were calculated and are shown in Table 1. The blocking agent affected the

TABLE 1. *Log dose-ratios showing decreased sensitivity of rabbit aortic strip to agonists as a result of irreversible blockade by BIP treatment* (2×10^{-4} M, *15 min, Krebs solution, pH* 7·4, 37° *C*)

Agonist conditions	Log-dose-ratio (mean)
(−) Noradrenaline	1·87 (n=4)
5-HT	0·68 (n=4)
Histamine	0·43 (n=8)
Histamine (BIP treatment in presence of diphenhydramine, 5×10^{-5} M)	0·80 (n=6)

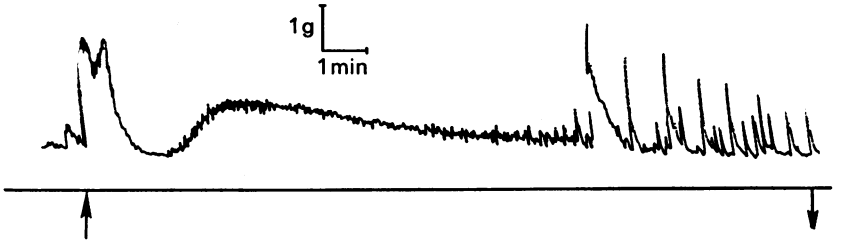


FIG. 1. Effects of N,*t*-butyl-5-methyl isoxazolium perchlorate (5×10^{-4}M) on the guinea-pig ileum at 37° C. The compound was allowed to act for 15 min (between the arrows) and the responses were recorded isometrically.

agonists to different extents, noradrenaline most and histamine least. Further treatment of the preparation with BIP decreased the slope of the log dose-response curves and with noradrenaline the effect depended on the length of time the compound was applied, as well as on its concentration. To determine whether BIP reacted at receptor sites, protection experiments were devised using reversible antagonists, phentolamine, brom-LSD and diphenhydramine, as protecting agents. Phentolamine and brom-LSD did not meet requirements for their use as reversible protecting agents in this case since, when applied for the necessary time interval of 15 min at doses required to provide significant receptor occupancy, they were not fully reversible, even after washing for 90 minutes. Diphenhydramine (5×10^{-5}M), however, was reversible and completely protected the histamine response against irreversible block by dibenamine (Furchgott, 1954). The preparation returned to the control histamine response 1 h after washing out the drug mixture. In contrast to dibenamine, the irreversible block by BIP was enhanced considerably, rather than prevented, by the presence of diphenhydramine (Table 1). Enhanced block of 5-HT responses was also observed when BIP was applied together with concentrations of brom-LSD sufficient to give partial protection.

Application of BIP (120 μg!ml) to the diaphragm preparation was sometimes followed by a transient potentiation of the first one or two of the indirectly elicited

TABLE 2. *Irreversible inhibition of AChE* (10^{-6} M, *ACh or MeCh as substrate*) *and BuChE* (5×10^{-6}M, *BuCh as substrate*) *by BIP treatment* (5×10^{-4} M, 30° *C*, 15 *min, Tyrode's solution, pH* 7·4) *of the rat phrenic nerve-diaphragm preparation*

Substrate	Stimulation rate (Hz)	% Enzyme inhibition
ACh	0	55
ACH	0	67
MeCH	0	65
ACh	0·2	40
ACh	0·2	41
ACh	0·84	40
MeCh	0·84	43
MeCh	0·84	57
MeCh	0·2	42
BuCh	0	<10
BuCh	0	<10
BuCh	0	<10

The phrenic nerve was stimulated supramaximally at the rates indicated during the treatment with BIP.

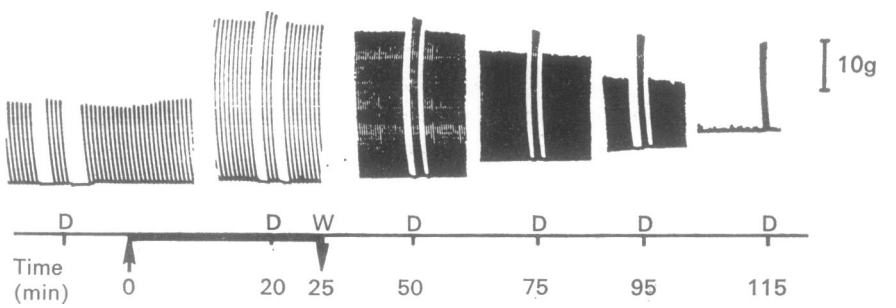


FIG. 2. Effects of N,*t*-butyl-5-methyl isoxazolium perchlorate (5×10^{-4}M) at 30° C, applied between the arrows, on the indirectly and directly (D) elicited twitch response (0·2 mg duration, 0·2 Hz) of the rat phrenic nerve-diaphragm preparation. W=wash.

twitch responses. This initial response was followed by a gradual increase of both the direct and indirect twitch responses by +35 to +95% (mean +60%, sixteen experiments) after treatment for 15 min (Fig. 2). After washout of the drug the twitch response remained augmented and declined very slowly over 90 min or longer.

Cholinesterase assays were carried out on the intact tissues showing a potentiated twitch response, immediately after washout of the drug (Mittag *et al.*, 1971), and showed a 45% inhibition of acetylcholine and acetyl-β-methylcholine hydrolysis but no significant inhibition of butyrylcholine hydrolysis. However, without electrical stimulation during drug treatment, the inhibition of true cholinesterase (AChE) was found to be greater, that is 62% (Table 2). The inhibition of acetylcholinesterase could not be reversed by treatment with the organophosphate reactivator 2-pyridine aldoxime methiodide (2×10^{-3}M for 10 min).

On the phrenic nerve preparation, an analogue of the isoxazolium drug, Woodward's reagent K (Woodward & Olofson, 1966) at a higher concentration (10^{-3}M) gave a much smaller degree of potentiation (20–30%) which passed off spontaneously within about 30 minutes.

A longer treatment with BIP (25 min) followed by washout, resulted in a progressive and irreversible blockade of transmission until complete failure of the twitch response occurred 60–90 min after washout (Fig. 2). A gradually increasing contracture of the diaphragm up to a tension of 10 g accompanied the decline of the twitch height. After complete failure of the indirect twitch response, direct stimulation of the muscle still gave a response, but this was somewhat reduced from the original control contraction to direct stimulation (Fig. 2).

Discussion

The isoxazolium cation, BIP, has several pharmacological actions on muscular preparations. Initially, it acts as a stimulant of both smooth and striated muscle. This direct agonist response may be due to an initial occupancy of anionic receptor sites which then become progressively blocked by a reaction with the isoxazolium ring.

The irreversible inhibition of acetylcholinesterase by the drug is also an anionic site-directed reaction. The mechanism of inhibition is, thus, different from that of organophosphate anticholinesterases, and similar to the alkylation of the enzyme by aziridinium ions (Belleau & Tani, 1966; Purdie & McIvor, 1966). BIP also inhibited purified acetylcholinesterase from electric eel to the same extent as was found for the enzyme in intact diaphragm preparations. There is some indication that there was less inhibition of the enzyme in preparations which were stimulated electrically during drug treatment (Table 2), suggesting that acetylcholine liberated by nerve stimulation partially protected the enzyme against the effects of the inhibitor. Butyrylcholinesterase was not inhibited. This has no anionic group in the active centre (Augustinsson, 1966), so this result supports the idea that BIP affects an anionic group in acetylcholinesterase.

The potentiated twitch response of the diaphragm might be attributable to the inhibitory effect of BIP on acetylcholinesterase but direct electrical responses were similarly potentiated. Possibly BIP potentiates initially by enhancing calcium release

from the sarcoplasmic reticulum and later causes contraction by preventing calcium sequestration. In its irreversible blocking action on muscular tissues, the isoxazolium ion did not show the anticipated selectivity for cholinergic receptors, but appears to react with a site or sites implicated in a wide variety of receptor initiated responses. However, contractions initiated by depolarization not involving receptors (by K^+, Ba^{++} or electrical stimulation) were relatively unaffected. Results of the experiments on the aortic strip showed that the site of blockade by BIP is different from the sites where the antagonists diphenhydramine and brom-LSD reversibly antagonize histamine and 5-HT, respectively. The reaction of BIP is, however, modified when these antagonists bind to their receptor sites, since the block by BIP is significantly increased in their presence. The enhanced block persisted up to 3 h, despite continuous washing. However, it is possible that the increased block is the combined residual blocking action of the reversible antagonist together with BIP. Accordingly, experimental conditions were carefully established to ensure that the sensitivity of the preparation recovered completely after one hour of washing out the reversible antagonist.

The results on smooth muscles indicate that receptor initiated responses to muscarinic agents, biogenic amines, ganglionic stimulants and peptides all involve similar anionic groups which are proximal to the level at which K^+ and Ba^{++} induce contractions. The site of action may be in the cell membrane and one of the steps in excitation-contraction coupling. Another possibility is that the initiation of a response by an agonist-receptor interaction requires free anionic groups in the different receptor macromolecules corresponding to each agonist. These different receptor anions may react at different rates with BIP giving different degrees of blockade to the various agonists as observed in differential blockade of agonists on the aortic strip. In either case, it would appear that agonist stimuli are mediated by a similar chemical group which is possibly a carboxylate or phosphate anion. The isoxazolium group is, thus, shown to be capable of reacting with chemical groups in tissues and modifying their pharmacological response. Incorporation of this moiety into a carrier drug molecule can be expected to increase both potency and specificity of action. This work also illustrates the need for careful interpretation of the specificity of receptor-directed drug reagents because there may be important sites adjacent to the receptor, which are also susceptible to chemical attack by irreversible drugs.

This investigation was supported by US Public Health Service Grant NS-08474. Miss Katherine Blaser provided excellent technical assistance.

REFERENCES

AUGUSTINSSON, K. B. (1966). The nature of an "anionic" site in butyrylcholinesterase compared with that of a similar site in acetylcholinesterase. *Biochim. Biophys. Acta*, **128**, 351–362.

BELLEAU, B. & TANI, H. (1966). A novel irreversible inhibitor of acetylcholinesterase specifically directed at the anionic binding site: structure-activity relationships. *Mol. Pharmac.*, **2**, 411–422.

BODLAENDER, P., FEINSTEIN, G. & SHAW, E. (1968). Isoxazolium salts for carboxyl-group modification in proteins. *Fedn. Proc.*, **27**, 291, Abstract No. 425.

BÜLBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br. J. Pharmac. Chemother.*, **1**, 38–61.

CHANGEUX, J. P., PODLESKI, T. R. & WOFSY, L. (1967). Affinity labelling of the acetylcholine-receptor. *Proc. Natn. Acad. Sci., U.S.A.*, **58**, 2063–2070.

EHRENPREIS, S., MITTAG, T. W. & PATRICK, P. (1970). Radiometric assay of cholinesterases in intact tissues in the nanomolar concentration range of acetylcholine. *Biochem. Pharmac.*, **19**, 2165–2169.

FURCHGOTT, R. F. (1954). Dibenamine blockade in strips of rabbit aorta and its use in differentiating receptors. *J. Pharm. exp. Therap.*, **111**, 265–284.

FURCHGOTT, R. F. (1960). Spiral-cut strip of rabbit aorta for *in vitro* studies of responses of arterial smooth muscle. In: *Methods in Medical Research*, ed. Bruner, H. D., Vol. **8**, pp. 177–186.

FURCHGOTT, R. F. (1966). The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. *Adv. Drug Res.*, **3**, 21–55.

GILL, E. W. & RANG, H. P. (1966). An alkylating derivative of benzilylcholine with specific and long-lasting parasympatholytic activity. *Mol. Pharmac.*, **2**, 284–297.

GRAHAM, J. D. P. (1962). 2-Halogenoalkylamines. *Progr. med. Chem.*, **2**, 132–175.

MAY, M., MORAN, J. F., KIMELBERG, H. & TRIGGLE, D. J. (1967). Studies on the noradrenaline α-receptor. II. Analysis of the "spare-receptor" hypothesis and estimation of the concentration of α-receptors in rabbit aorta. *Mol. Pharmac.*, **3**, 28–36.

MITTAG, T. W., EHRENPREIS, S. E. & HEHIR, R. M. (1971). Functional acetylcholinesterase of rat diaphragm muscle. *Biochem. Pharmac.*, in the Press.

MORAN, J. F., MAY, M., KIMELBERG, H. & TRIGGLE, D. J. (1967). Studies on the noradrenaline α-receptor. I. Techniques of receptor isolation. The distribution and specificity of action of N-(2-Bromoethyl)-N-ethyl-1-naphthyl-methylamine, a competitive antagonist of noradrenaline. *Mol. Pharmac.*, **3**, 15–27.

MORAN, J. F. & TRIGGLE, D. J. (1970). Approaches to the quantitation and isolation of pharmacological receptors. In: *Fundamental Concepts in Drug-Receptor Interactions*, Danielli, J. F., Moran, J. F. & Triggle, D. J., pp. 133–176. New York: Academic Press.

PATON, W. D. M. & ABOO ZAR, M. (1968). The origin of acetylcholine released from guinea pig intestine and longitudinal muscle strips. *J. Physiol., Lond.*, **194**, 13–33.

PURDIE, J. E. & MCIVOR, R. A. (1966). Modification of the esteratic activity of acetylcholinesterase by alkylation with 1,1-dimethyl-2-phenylaziridinium ion. *Biochim. Biophys. Acta.*, **128**, 590–593.

SILMAN, I. & KARLIN, A. (1969). Acetylcholine receptor: covalent attachment of depolarizing groups at the active site. *Science, N.Y.*, **164**, 1420–1421.

TAKAGI, K., AKAO, M. & TAKAHASHI, A. (1965). Tritium-labelled acetylcholine receptor in the smooth muscle of the small intestine of the dog. *Life Sci.*, **4**, 2165–2169.

WOODWARD, R. B. & OLOFSON, R. A. (1966). The reaction of isoxazolium salts with nucleophiles. *Tetrahedron*, Suppl. No. 7, 415–440.

WOODWARD, R. B., OLOFSON, R. A. & MAYER, H. (1966). A useful synthesis of peptides. *Tetrahedron*, Suppl. No. **8**, pt. 1, 321–346.

(*Received March* 29, 1971)