REVIEW

Drug Receptor Mechanisms in Smooth Muscle: β-Chloroethylamine-Sensitive and -Resistant Receptor Mechanisms

Issei Takayanagi, Katsuo Koike, Mitsutoshi Satoh and Ayako Okayasu

Department of Chemical Pharmacology, Toho University School of Pharmaceutical Sciences, Chiba 274, Japan

Received August 20, 1996

ABSTRACT—Both α_1-adrenoceptors and M_3-cholinoceptors can be divided into two subtypes discriminated by the β-chloroethylamines, chloroethylclonidine and propylbenzilylcholine mustard (PrBCM), only in the presence of GTP. The full agonists interact with both subtypes to induce responses. The partial agonists activate one of them to induce responses but behave as competitive antagonists when they interact with the other. The responses mediated through the receptors that are activated by the partial agonists are resistant to myosin light chain kinase inhibitors, while the response through the activation of the other receptors are suppressed by the inhibitors. The receptor stimulations through α_{1A}-adrenoceptor and PrBCM-sensitive M_3-cholinoceptor subtypes mainly activate the myosin light chain-phosphorylation-independent pathway mediated through protein kinase C and low molecular weight GTP-binding protein, whereas the stimulations through α_{1B}-adrenoceptors and the PrBCM-phosphorylation-dependent pathway are directly related to Ca^{2+}/calmodulin.

Keywords: Drug receptor mechanism, β-Chloroethylamine, M_3-Cholinoceptor, α_1-Adrenoceptor, Signal transduction

I. Introduction

The effects of most drugs result from their interaction with macromolecular components of an organism. Many drugs interact with pharmacological receptors and are often particularly selective, because receptors are specialized to recognize and respond to individual signaling molecules with great selectivity. Drugs that bind to receptors and mimic the effects of the endogenous regulatory compounds are termed agonists, and there are full and partial agonists.

There is one concept that full and partial agonists bind to the same receptor site but do not produce equal maximum effects. The intrinsic activity of a full agonist can be set equal to 1 and that of a partial agonist to a value between 0 and 1. It is assumed that since occupation of all receptors produces a maximum response less than that to the full agonist, any reduction of receptor numbers by an irreversible antagonist would cause an immediate reduction of the maximum response to the partial agonist, suggesting that there is no spare receptor for the partial agonist. The irreversible antagonist causes a parallel shift

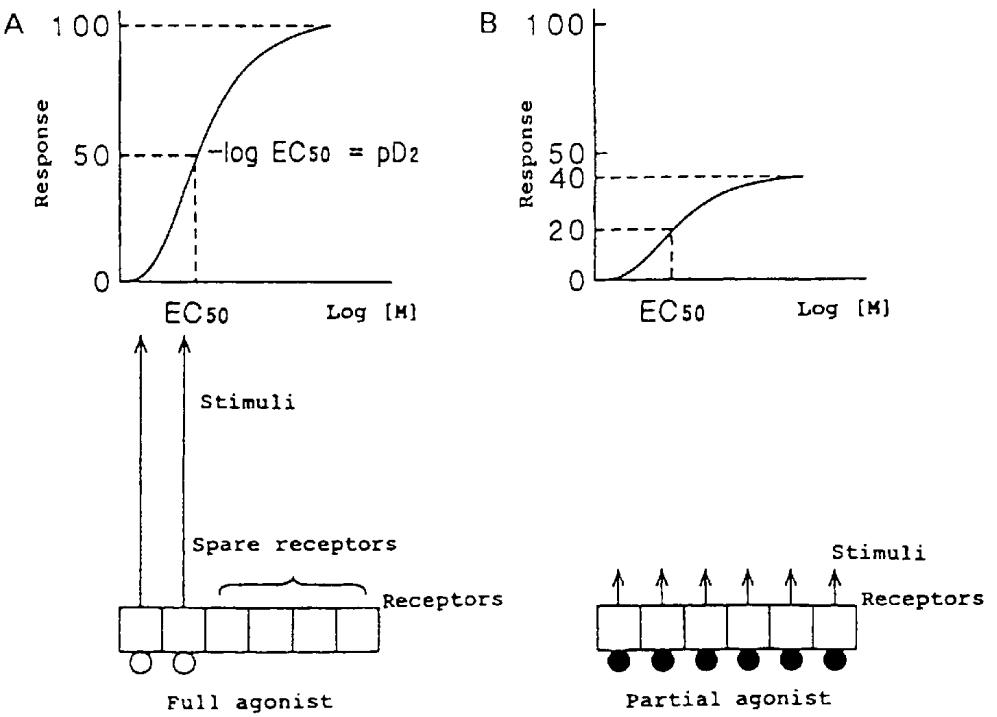

Fig. 1. Classical drug receptor mechanisms. Upper figures: concentration-response curves for a full agonist (A) and partial agonist (B), of which the maximum contraction is 40%. Lower figures: interactions of the full agonist (A) and partial agonist (B) with receptors.

in the concentration-response curve of the full agonist without a reduction of the maximum response, suggesting that the full agonist has spare receptors (Fig. 1) (1–3). A fractional effect is then equal to the product of fractional occupancy of the receptor and the fractional efficacy. These correction factors are dependent both on the molecular properties of receptors and on the concentrations of agonists and interactions of transducer and effector proteins.

Presented here is an overview of the modification of the classical drug receptor mechanisms by investigating the mechanisms of the two subtypes of both M_3-cholinoceptor and α_1-adrenoceptor that are discriminated by the β-chloroethylamines: propylbenzilylcholine mustard (PrBCM, *N*-2′-chloroethyl-*N*-propyl-2-aminoethyl benzylate) or chloroethylclonidine (CEC).

II. β-Chloroethylamines and the mechanisms of M_3-muscarinic cholinoceptors and α_1-adrenoceptors

II-1. M_3-Cholinoceptor

II-1-1. Effects of treatment with PrBCM

The longitudinal smooth muscle of guinea pig ileum and the guinea pig taenia caecum are contracted by M_3-receptor activation (4).

Carbachol, butyltrimethylammonium (Fig. 2), propionylcholine and butyrylcholine behaved as full agonists on M_3-receptors in the longitudinal muscle of guinea pig ileum. They were progressively inhibited by 10- to 50-min treatment with PrBCM (3×10^{-6} M), and the results were in good agreement with those obtained with guinea pig taenia caecum (5, 6). The 50-min treatment of the longitudinal muscle strip with PrBCM irreversibly inhibited the concentration-response curves of the full agonists. These facts confirmed the results of Young et al. (7) who reported that PrBCM irreversibly antagonized the contractile response of the intestinal muscle to muscarinic agonists. More prolonged (90 min) treatment with PrBCM had no further significant inhibitory effect on the concentration-response curves of full agonists (Fig. 2). The results suggest that there are two subtypes of M_3-cholinoceptors, PrBCM-sensitive and -resistant cholinoceptors.

Pilocarpine, heptyltrimethylammonium, benzoylcholine (Fig. 3), hexyltrimethylammonium, pentanoylcholine and hexanoylcholine acted as partial agonists. Their concentration-response curves were completely inhibited by the 50-min treatment with PrBCM.

The full agonists elicit a stimulus through an interaction of two subtypes of M_3-cholinoceptors, as the limiting inhibitory effect of PrBCM was observed on the concen-

tration-response curves of the full agonists. The response to the partial agonists disappeared as a result of the 50-min treatment with PrBCM. These facts suggest that the partial agonists produce contraction by activating PrBCM-sensitive cholinoceptors. However, the fact that in the strips treated with PrBCM, pilocarpine shifted the concentration-response curve of carbachol in a parallel fashion suggests that the interaction of pilocarpine with PrBCM-resistant receptors does not induce contraction but acts as a muscarinic antagonist (Fig. 4). The full agonists contract the intestinal smooth muscle through the interaction of two cholinoceptors, PrBCM-sensitive and -resistant ones, while the partial agonists produce the contraction through the activation of the PrBCM-sensitive ones (Fig. 5) (8).

Dörje et al. (9) determined the affinity profiles of several selective muscarinic antagonists at five cloned human muscarinic receptors (m1–m5) stably expressed in Chinese hamster ovary cells (CHO-K1). At least four subtypes of muscarinic receptors were pharmacologically characterized and are designated as M_1, M_2, M_3 and M_4 according to their affinity for selective antagonists (4, 10–12). Comparison of the location, antagonist binding properties and functional coupling of the native muscarinic M_1–M_4 receptors with those of the expressed m1–m4 proteins showed a good correlation. Therefore, the subtype of PrBCM-sensitive and -resistant muscarinic cholinoceptors in the longitudinal smooth muscle of guinea pig ileum was examined using four selective muscarinic antagonists: pirenzepine (M_1 [m1]-

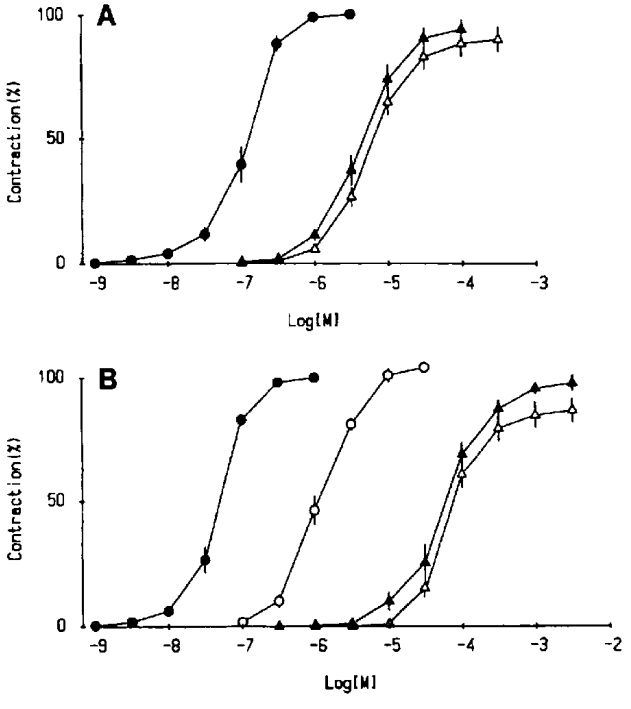

Fig. 2. Irreversible inhibitory effects of 50- and 90-min treatments with PrBCM (3×10^{-6} M) on concentration-response curves of full agonists, carbachol (A) and butyltrimethylammonium (B). Ordinate: contraction (%), which is expressed as a percent of the contractile response to carbachol (10^{-6} M). Abscissa: logarithm of drug concentration (M). Each value is expressed as a mean ± S.E. (bar) of 7 experiments. ●, carbachol before treatment (A); ●, carbachol as a reference agonist (B); ○, butyltrimethylammonium before treatment; ▲, after a 50-min treatment; △, after a 90-min treatment. [Adapted from Ref. 8 with permission]

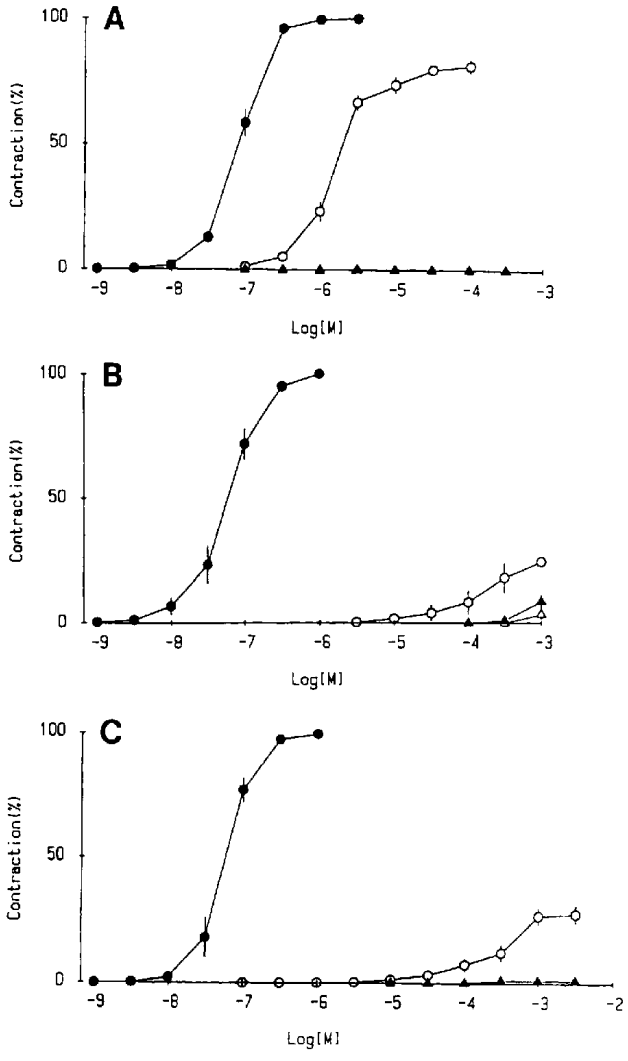

Fig. 3. Irreversible inhibitory effects of 50- and 90-min treatments with PrBCM (3×10^{-6} M) on concentration-response curves of partial agonists, pilocarpine (A), benzoylcholine (B) and heptyltrimethylammonium (C). Ordinate: contraction (%), which is expressed as a percent of the contractile response to carbachol (10^{-6} M). Abscissa: logarithm of drug concentration (M). Each value is expressed as a mean ± S.E. (bar) of 7 experiments. ●, carbachol as a reference agonist; ○, before treatment; ▲, after a 50-min treatment; △, after a 90-min treatment. [Adapted from Ref. 8 with permission]

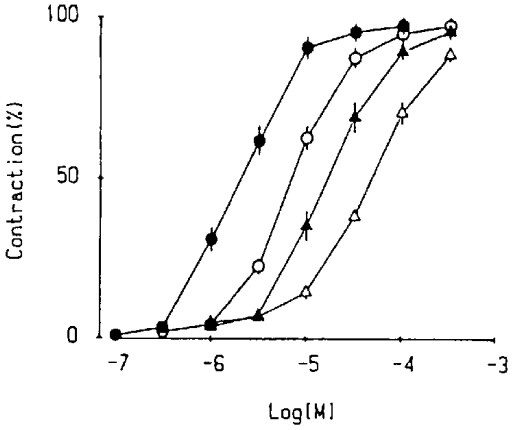

Fig. 4. Inhibitory effect of pilocarpine on the concentration-response curve of carbachol in the longitudinal muscle treated with PrBCM (3×10^{-6} M) for 50 min. Ordinate: contraction (%), which is expressed as a percent of the contractile response of the untreated strip to carbachol (10^{-6} M). Abscissa: logarithm of carbachol concentration (M). Each value is expressed as a mean ± S.E. (bar) of 7 experiments. ●: carbachol alone; ○, ▲ and △: with pilocarpine, 10^{-5}, 3×10^{-5} and 10^{-4} M, respectively. [Adapted from Ref. 8 with permission]

selective) (13); AF-DX116 [11-([2-{(diethylamino)methyl}-1-piperidinyl]acetyl)-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodizepine-6-one] (M_2 [m2]-selective) (14); himbacine (M_2 [m2], M_4 [m4]-selective) (15); and 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) (M_1 [m1], M_3 [m3]-selective) (16).

Concentration-response curves of carbachol after a 50-min treatment with PrBCM and those of pilocarpine without PrBCM-treatment were shifted to the right in a parallel manner by each of the four antagonists. The pA_2 values and slopes of the four antagonists are shown in Table 1. Schild plots of these results yielded a straight line with a slope of 1 (Table 1). The pA_2 values of the four antagonists against pilocarpine did not differ from their values against carbachol after the treatment with PrBCM (Table 1) and were identified with the values for the M_3-subtype. These results suggest that the subtype of PrBCM-sensitive and -resistant muscarinic cholinoceptors in the longitudinal smooth muscle of guinea pig ileum is the M_3-subtype (17). Two different subtypes of M_3-cholinoceptors were identified in the intestinal smooth muscles using PrBCM: one PrBCM-sensitive and the other PrBCM-resistant.

Furthermore, the left atrium of guinea pig, driven

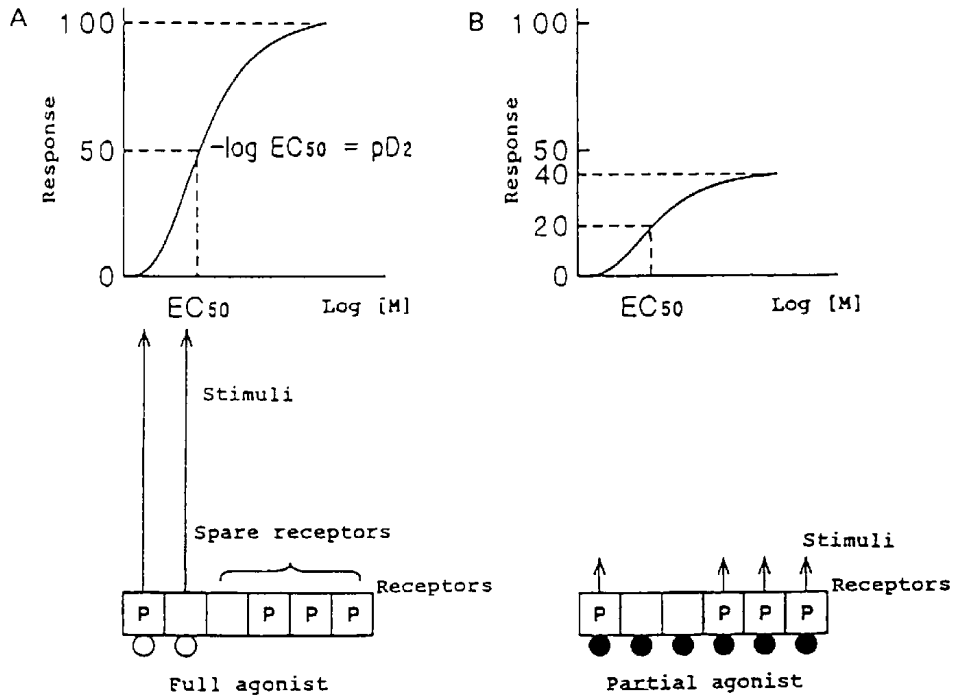

Fig. 5. The difference in full (A) and partial (B) agonist-cholinoceptor mechanisms. Upper figures: concentration-response curves for a full agonist (A) and partial agonist (B), of which the maximum contraction is 40%. Lower figures: interactions of the full agonist (A) and partial agonist (B). Receptors unmarked and marked with P: PrBCM-resistant and PrBCM-sensitive cholinoceptors, respectively. The full agonist interacts with both PrBCM-sensitive and PrBCM-resistant cholinoceptors to induce contraction. However, the partial agonist induces contraction through the interaction with PrBCM-sensitive receptors, but acts as an antagonist of the full agonist on PrBCM-resistant ones.

Table 1. The pA_2 values and slopes for four muscarinic antagonists

Antagonists	PrBCM-sensitive receptors (against pilocarpine)		PrBCM-resistant receptors (against carbachol after treatment with PrBCM)	
	pA_2	slope	pA_2	slope
Pirenzepine	6.56±0.04	1.00±0.12	6.49±0.02	0.94±0.01
AF-DX 116	6.45±0.04	1.03±0.10	6.30±0.06	0.90±0.10
Himbacine	7.02±0.03	0.97±0.01	7.00±0.02	0.96±0.01
4-DAMP	9.05±0.03	0.96±0.01	9.00±0.04	0.95±0.02

The pA_2 values were calculated from parallel shifts of the curves for the agonists by the method of Arunlakshana and Schild as modified by Tallarida et al. slope: slope of the Schild plot. Each value represents the means±S.E. of 6 experiments. [Adapted from Ref. 17 with permission]

electrically, was used as a test organ containing M_2-cholinoceptors. Concentration-response curves of carbachol and butyltrimethylammonium, muscarinic full agonists, were progressively inhibited by 10- and 30-min treatments with PrBCM (10^{-6} M). The 50-min treatment had no further significant inhibitory effect on their curves, while the 30-min treatment completely inhibited the concentration-response curve of pilocarpine, a partial agonist. In the atrium after the 30-min treatment with PrBCM, pilocarpine shifted the concentration-response curves of the full agonists, suggesting a competitive antagonism. These results suggest the existence of two subtypes of M_2-receptors, PrBCM-sensitive and -resistant ones, and also suggest that the full agonists inhibit the twitch through interaction of these two subtypes, while the partial agonist produces inhibition through an activation of the PrBCM-sensitive ones (18). These findings are similar to the difference in full and partial agonist-cholinoceptor mechanisms on M_3-receptor in the intestinal smooth muscle of guinea pig (Fig. 5). PrBCM was reported to be selective for some populations of rat heart muscarinic cholinoceptors (19). It was also reported that PrBCM could not completely inactivate [^{3}H]quinuclidinyl benzilate (QNB) binding sites in the guinea pig heart.

II-1-2. Effects of guanine nucleotide

Muscarinic cholinoceptors are assumed to interact, via a guanine nucleotide binding regulatory protein (G protein)-regulated process, with multiple effector systems leading to inhibition of adenylate cyclase, opening of potassium channels and activation of polyphosphoinositide metabolism (20–28). In this section, the membrane fractions from guinea pig taenia caecum were used (29).

The interaction of cholinoceptors with PrBCM was tested by direct receptor binding techniques with [^{3}H]-QNB and [^{3}H]PrBCM. Pretreatment of the smooth muscle strips with PrBCM (in vivo alkylation) for 10–50 min resulted in progressive decreases in the maximum number of [^{3}H]QNB binding sites. We have confirmed that in vivo alkylation with PrBCM for 50 min reduced the density of [^{3}H]QNB binding sites to about 10% of the control value, but more prolonged alkylation was not accompanied by further reduction in the maximum [^{3}H]QNB binding sites. These results agreed well with those of functional experiments.

Nonlinear regression analyses revealed that carbachol binds to three different affinity sites: the super-high (SH), high (H) and low (L) subsites (Table 2) in membranes obtained from guinea pig taenia caecum, as reported in a variety of tissues (30–34). The pIC_{50} value (the negative logarithm of the 50% inhibitory concentration estimated from its displacement curve) for carbachol was decreased, but that for atropine was not, by in vivo alkylation of the muscle strips with PrBCM for 50 min, a condition under which only the PrBCM-resistant sites remained. The loss in the affinity of carbachol in the PrBCM-pretreated membranes could not be explained by a selective inactivation of the SH and/or H subsites by the in vivo alkylation, because if such higher affinity subsites which accounted for about 90% of the total receptor sites were destroyed by PrBCM, the PrBCM-resistant receptor would essentially be occupied only by the L subsite. Actually, SH, H and L subsites also coexist in the PrBCM-resistant sites; consequently, the apparent decrease in affinity of carbachol to the receptor sites was revealed to be due to both the relative abundance of the H and L subsites and the relative scarcity of the SH subsite in the PrBCM-resistant receptors. The difference in the relative density of each subsite between the PrBCM-sensitive and -resistant sites seems to further support the distinct entities or states of both receptor sites.

It is well-known that the guanine nucleotides lowers the affinity of agonists but not antagonists to many types of receptor systems including muscarinic receptors via interaction of the receptor with G proteins. The decrease in the affinity of carbachol, but not of atropine, by in vivo

Table 2. The pIC_{50} values and the pK_i values for carbachol at super-high (SH)-, high (H)- and low (L)-affinity subsites with their relative densities in the membranes prepared from control and alkylated guinea pig taenia caecum and the effects of 3 mM GTP on these parameters

	n	pIC_{50}	SH		H		L	
			pK_i	%	pK_i	%	pK_i	%
Control	6	5.84±0.08	7.29±0.19	49.2±4.0	5.12±0.21	39.5± 5.3	3.26±0.35	11.4±2.2
Tissue-alkylated no addition	5	5.01±0.06*	7.23±0.19	23.1±4.0*	5.08±0.26	57.3± 3.2*	3.08±0.16	15.9±5.3
Membrane-alkylated no addition	6	5.78±0.05	7.46±0.23	41.3±4.3	5.67±0.16	33.2± 7.5	3.36±0.10	15.6±2.8
+3 mM GTP	3	5.12±0.02*	6.98±0.22	30.1±4.1*	5.09±0.26	56.7±11.5*	3.35±0.24	13.2±3.4

Tissue-alkylation was carried out by incubation of the muscle strips with PrBCM (3×10^{-7} M) for 50 min, and membrane-alkylation was carried out by incubation of the membranes with PrBCM (10^{-8} M) for 30 min. *Significantly different from the corresponding values in the control at $P<0.05$.

alkylation is apparently not due to a mechanism by which PrBCM might act on the receptor systems to convert them to a state similar to that in the presence of the guanine nucleotide, since guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) was able to further decrease the affinity of carbachol in membranes from the PrBCM-pretreated muscles, as in the control membranes. The shift of the pIC_{50} value by GTPγS in the PrBCM-resistant receptor sites suggests that these sites may couple with G proteins to induce a PrBCM-resistant contraction.

To investigate the mode of interaction of PrBCM with the two receptor sites, the binding characteristics were studied in the membrane fractions from muscle strips that had been pretreated with PrBCM (in vitro alkylation) followed by centrifugal washes to remove the alkylating drug. Interestingly, by this in vitro alkylation method, the pIC_{50} values for carbachol were not changed. In view of the results obtained with the in vivo alkylation method, some missing cytoplasmic factor must be responsible for the selective inactivation by PrBCM of the receptor sites for the drug.

Our study showed that GTP may be one of the candidates for such a cytoplasmic factor and that GTPγS makes some populations of the receptor sites resistant to PrBCM. First, the guanine nucleotide reduced the amount of [^{3}H]PrBCM specifically bound to the membranes by about 15%. Although somewhat larger, the value was comparable to the relative density of the PrBCM-resistant sites revealed by Scatchard analyses of [^{3}H]QNB binding sites in the in vivo alkylated membranes. Secondly, simultaneous incubation of membranes with GTP during the in vitro alkylation by PrBCM decreased the pIC_{50} value for carbachol, as observed in in vivo alkylated membranes (GTPγS was not used in this experiment, because the binding of GTP to G proteins is almost irreversible). When membranes were preincubated

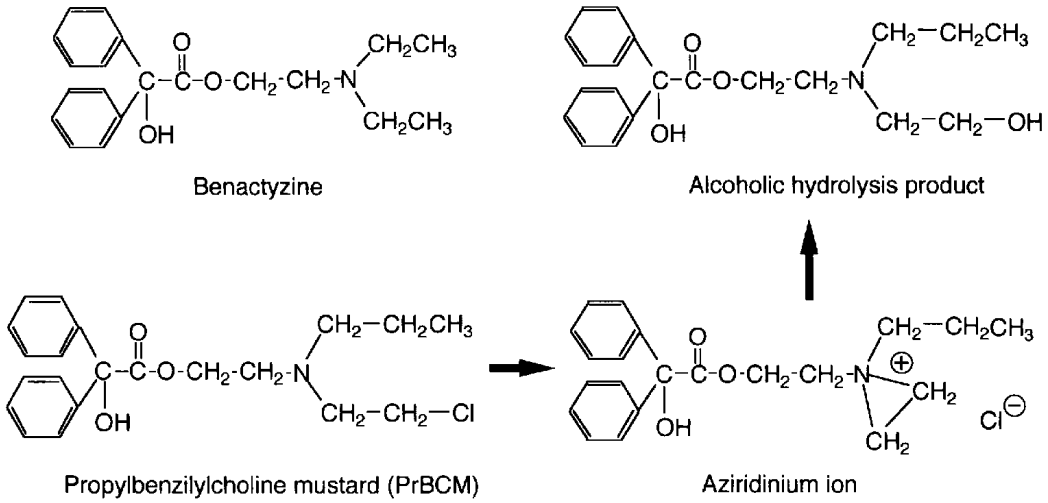

Fig. 6. Structures of propylbenzilylcholine mustard (PrBCM), its aziridinium ion and alcoholic hydrolysis product and benactyzine.

with GTP alone followed by centrifugal washes, the affinity of carbachol was not altered compared with the control value. Thus, the decrease in the affinity of carbachol by in vitro alkylation in the presence of GTP is considered not to be induced via a so-called GTP-shift by the contaminating GTP in the washed membranes due to incomplete removal of the nucleotide (Table 2).

Taken together, the present results suggest that in guinea pig taenia caecum, there coexist two types of muscarinic receptor mechanisms, although it is currently unknown whether the difference in sensitivity of the two sites to PrBCM is due to the distinct entities of the receptor molecules, the same receptor with different modifications or as yet undefined mechanisms. PrBCM can differentiate between the two mechanisms, PrBCM-sensitive and -resistant ones, in the presence of GTP, but in its absence, PrBCM cannot discern the two. In other words, one mechanism is PrBCM-sensitive, irrespective of the presence or absence of GTP, and the other is PrBCM-resistant only when GTP is present (29).

PrBCM discriminates between PrBCM-sensitive and -resistant M_3-cholinoceptors, as described above. One reason for this fact may be that the benzilylcholine moiety in PrBCM discriminates the two subtypes. We tested the effects of benactyzine (Fig. 6) containing the benzilylcholine moiety on both the subtypes. In the untreated ileal muscle of guinea pig, the concentration-response curve of pilocarpine was shifted by benactyzine (3×10^{-8}, 10^{-7} and 3×10^{-7} M) in a parallel fashion. The pA_2 value for benactyzine obtained from the Schild plot of the data was 8.27 ± 0.06. Benactyzine (10^{-8}, 3×10^{-8} and 10^{-7} M) also caused a parallel shift of the curve for carbachol in the PrBCM-treated preparations. The value estimated from the results was 7.82 ± 0.07. The difference between the two pA_2 values (0.45) was significant, suggesting that the affinity of benactyzine for PrBCM-sensitive receptors is about 3 times higher than that for PrBCM-resistant receptors (I. Takayanagi et al., unpublished data).

PrBCM cyclizes spontaneously into quaternary aziridinium ion in aqueous solution at neutral pH, and aziridinium ion alters further into an alcoholic hydrolysis product (Fig. 6). There are PrBCM and its aziridinium and alcoholic hydrolysis product in aqueous solution. Quaternary aziridinium ion is believed to have the highest affinity for cholinoceptor among these three. Aziridinium ion interacts with anionic groups in receptors and binds covalently with a certain portion of the receptors. Thus, PrBCM blocks receptors irreversibly. Young et al. (7) reported that 84.5% of PrBCM exists as aziridinium ion in 8×10^{-4} M PrBCM phosphate buffer (pH 7.5), kept at 32℃ for 20 min. A small amount of this solution was added to bath fluid, a final aziridinium ion concentration of about 10^{-7} M, although the exact concentration in the diluted fluid was unknown. Bath fluid does, however, contain aziridinium ion, alcoholic hydrolysis product and PrBCM; in this paper, the bath fluid containing aziridinium and other components is referred to as aziridinium ion solution. To test the interaction of aziridinium ion with PrBCM-sensitive and -resistant receptors, the preparation was treated with aziridinium ion solution for 3

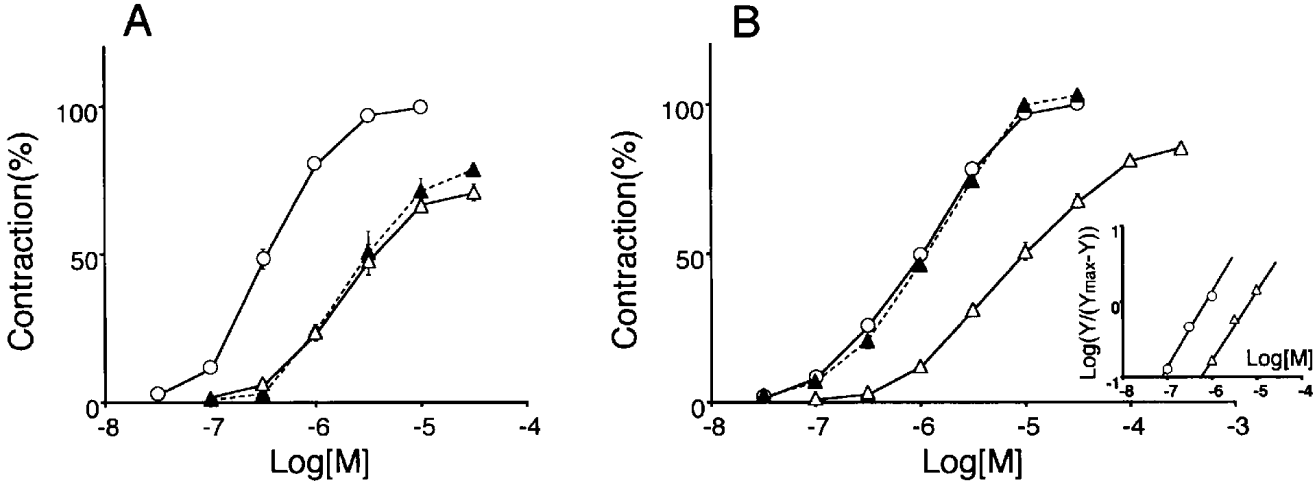

Fig. 7. Effects of propylbenzilylcholine mustard (PrBCM) aziridinium ion solution on the concentration-response curve of pilocarpine (A) and carbachol (B) in the guinea pig ileal muscle untreated (A) and treated with PrBCM (3×10^{-6} M) for 50 min (B). Ordinate: contraction (%), expressed as a percentage of the maximum contraction. Abscissa: log concentration of pilocarpine (A) or carbachol (B). Inset of B: Ordinate: logit, which is the log of $Y/(Y_{max}-Y)$, where Y and Y_{max} are a response to carbachol and the maximum response in the absence or presence of aziridinium ion solution, respectively. Abscissa: log concentration of carbachol. ○: agonist alone, △: with aziridinium ion solution, ▲: after the 60-min washing with normal bath fluid. Each value is expressed as the mean±S.E. (bar) of 4 experiments.

min. In the untreated longitudinal muscle of guinea pig ileum, the concentration-response curve of pilocarpine was depressed by this ion solution. Although the ileal preparation was allowed to equilibrate for 60 min with washing every 10 min, the curve of pilocarpine was not significantly influenced. These results suggest that the 3-min treatment with aziridinium ion solution blocks PrBCM-sensitive receptors (Fig. 7A). In the PrBCM-treated ileal longitudinal muscle, the concentration-response curve of carbachol was reduced by the 3-min treatment with the solution. The response to carbachol returned to one like the control after the preparation was allowed to equilibrate for 60 min with washing every 10 min (Fig. 7B); this indicates that depression of the response to carbachol by aziridinium ion solution is due to an ionic interaction of PrBCM-resistant receptor with aziridinium ion but not to alkylation. As indicated in the relationship between log carbachol concentration and logit, log (Y / (Y_{max} − Y)) (inset of Fig. 7B), where Y is the response to carbachol and Y_{max} is the maximum response in the absence or presence of aziridinium ion solution, the curve of carbachol was shifted by the solution in a parallel fashion, suggesting that aziridinium ion competes with carbachol for PrBCM-resistant cholinoceptors (I. Takayanagi et al., unpublished data). It is generally accepted that a compound with bulky groups acts as a competitive and non-competitive antagonist (35). Aziridinium ion and alcoholic hydrolysis product have bulky groups, whereas benactyzine does not. Benactyzine acts as a pure competitive antagonist, while aziridinium ion and alcoholic hydrolysis product may behave as competitive and non-competitive antagonist, respectively. These results open the following possibilities: PrBCM, which contains benzilylcholine moiety in its molecule, has higher affinity to PrBCM-sensitive receptor than to PrBCM-resistant receptor, and it is able to bind covalently with the former but not with the latter.

It is difficult to explain why PrBCM does not bind covalently with PrBCM-resistant receptors. PrBCM may interact with both possible subtypes of M_3-cholinoceptors in the absence of GTP, that is to say, PrBCM does not discriminate between PrBCM-sensitive and -resistant receptors without GTP (29). However, GTP converts some populations of PrBCM-sensitive receptors to resistant receptors. Thus, PrBCM discriminates between the two subtypes only in the presence of GTP. And thus, the conformation of PrBCM-resistant receptors in the presence of GTP may be suitable to interact with aziridinium ion but not to bind covalently.

II-2. α_1-Adrenoceptor

II-2-1. Effects of treatment with CEC or WB4101

The rabbit thoracic aorta and common iliac artery contain both α_{1A}- and α_{1B}-adrenoceptors. Based on the affinity for WB4101 [2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane] and susceptibility to CEC, the subtypes of α_1-adrenoceptors activated by phenylephrine (a full agonist) and tizanidine (a partial agonist) were evaluated. The α_{1A}-subtype has a high

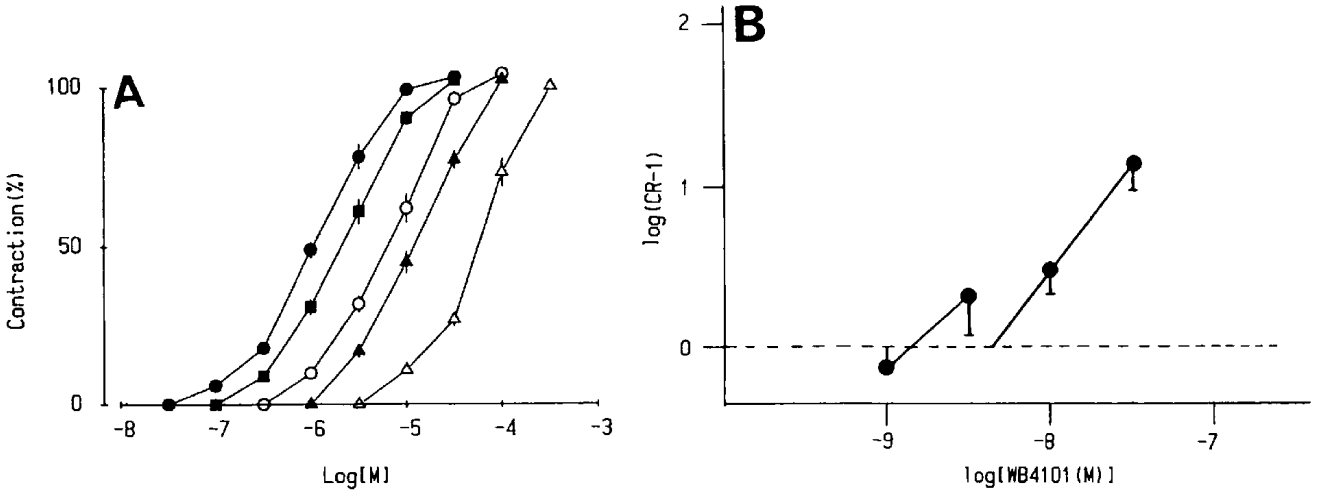

Fig. 8. Effects of WB4101 on the phenylephrine-induced contraction of rabbit thoracic aorta (A) and a Schild plot for the antagonism between phenylephrine and WB4101 (B). A. Ordinate: contraction (%), expressed as a percentage of the maximum contraction induced by norepinephrine (10^{-6} M). Abscissa: log concentration (M) of agonist. ●, agonist alone; ■, with WB4101 (10^{-9} M); ○, with WB4101 (3×10^{-9} M); ▲, with WB4101 (10^{-8} M); and △, with WB4101 (3×10^{-8} M). Each value is expressed as the mean±S.E. (bar) of 5 experiments. B. Ordinate: logarithm of the equieffective concentration ratio (CR) of phenylephrine minus 1. Abscissa: logarithm of the molar concentration of WB4101. [Adapted from Ref. 38 with permission]

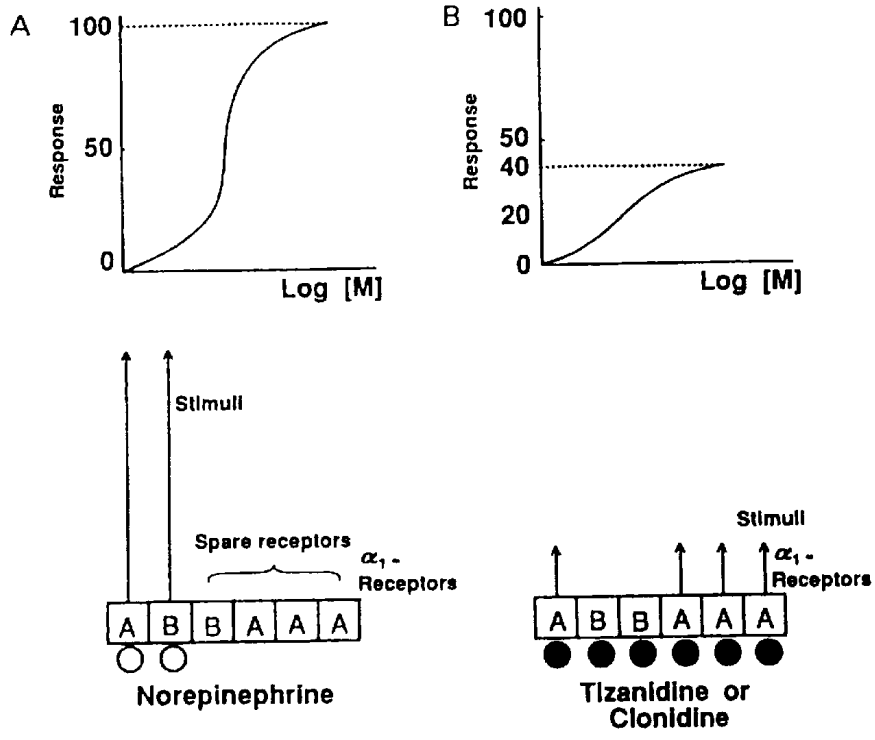

Fig. 9. The difference between the full (for example, norepinephrine: A) and partial agonist (for example, tizanizine or clonidine: B) α_1-adrenoceptor mechanisms in rabbit thoracic aorta. Upper figures: concentration-response curves for the full and partial agonist. Lower figures: interactions of the full and partial agonists with α_1-adrenoceptors. α_1-Adrenoceptors marked with A and B: α_{1A} and α_{1B} subtypes, respectively. The full agonist interacts with both α_{1A} and α_{1B} subtypes to induce contraction. However, the partial agonist induces contraction by interacting with the α_{1A} subtype.

affinity for WB4101, while the α_{1B}-subtype has a low affinity and is selectively susceptible to CEC (an α_{1B}-adrenoceptor-alkylating agent) (36).

CEC partly inhibited the concentration-response curves of phenylephrine, a full agonist in both rabbit common iliac artery and thoracic aorta, suggesting that the contractile response to the full agonist was at least partly mediated through α_{1B}-adrenoceptors. However, the α_{1B}-adrenoceptors are thought to be more prevalent in the common iliac artery than in the thoracic aorta, since CEC more effectively inhibited the responses to the agonist in the common iliac artery than in the thoracic aorta. The curve of tizanidine, a partial agonist, was not significantly influenced by CEC, however. In the thoracic aorta, the slope of the Schild plot obtained from an antagonism between tizanidine and WB4101 was equal to 1, while the slope of the plot from the antagonism between phenylephrine and WB4101 was significantly less than 1. Two pA_2 values for WB4101 were estimated from the antagonism between phenylephrine and WB4101 in rabbit thoracic aorta, suggesting that WB4101 interacts with two distinct receptors (Fig. 8). According to computer-assisted analysis of the nonlinear Schild plot for WB4101 and the α_1-adrenoceptor stimulant in rabbit aorta (37), the high-affinity site for WB4101 obtained herein is the α_{1A}-subtype and the low-affinity site, the α_{1B}-subtype (Fig. 8). These results suggest that the rabbit thoracic aorta contains the α_{1B}-subtype in addition to the α_{1A}-subtype. Phenylephrine, a full agonist, induced contraction through both subtypes and the contractile response to tizanidine, a partial agonist, mainly through the α_{1A}-subtype (38).

Moreover, using norepinephrine, a full agonist, and clonidine, a partial agonist, the similar results were obtained (39). The results confirmed that a full agonist induced contraction through both α_{1A}- and α_{1B}-subtypes, and a partial agonist induced it through only the α_{1A}-subtype (Fig. 9).

II-2-2. Effects of guanine nucleotide

α_1-Adrenoceptors in membrane prepared from rabbit thoracic aorta were selectively labeled with [^{3}H]prazosin, and then the labeled receptors were displaced by the α_1-agonist phenylephrine. The computer analysis of the displacement curve showed the presence of high-affinity and low-affinity sites (Table 3).

Although the low-affinity site was reduced by the pretreatment of tissues with CEC, this site was unaffected

Table 3. Effects of GTPγS (10 μM) on inhibition of the [^{3}H]prazosin binding by chloroethylclonidine in the membrane fractions of rabbit thoracic aorta and effects of chloroethylclonidine on [^{3}H]prazosin binding in single cells prepared from rabbit thoracic aorta

	n	pIC_{50}	nH	High (α_{1A})		Low (α_{1B})		B_{max} Low (%)
				pK_i	B_{max} (fmol/mg protein)	pK_i	B_{max} (fmol/mg protein)	
Tissue								
Untreated	6	5.14±0.15	0.28±0.04*	5.55±0.22	10.07±1.25	4.46±0.18	38.56±1.09	78.38±2.22
CEC	4	5.09±0.10	0.74±0.08*	5.55±0.17	8.26±0.84	4.49±0.26	16.37±0.63†	67.79±3.48$^{\#}$
Membrane								
CEC	3	5.11±0.05	0.29±0.03*	5.83±0.29	8.50±1.40	4.23±0.39	38.77±1.80	80.78±3.74
CEC & GTPγS	3	5.21±0.31	1.02±0.02	5.65±0.31	13.42±1.93	—	—	0
Single cell								
Untreated	3	5.41±0.18	0.39±0.14*	5.82±0.16	0.98±0.43	4.59±0.19	2.43±0.43	71.30±12.6
CEC	3	5.58±0.24	1.04±0.28	5.91±0.24	0.89±1.08	—	—	0

Membrane preparations were incubated with 0.2 nM [^{3}H]prazosin and different concentrations of phenylephrine. Single cells were incubated with 0.2 nM [^{3}H]prazosin and different concentrations of phenylephrine. The IC_{50} value is the molar concentration of unlabeled drug necessary to displace 50% of the specific binding. pIC_{50}, negative logarithm of the IC_{50} value. Negative logarithm of the K_i value (pK_i) and B_{max} for the high- and low-affinity binding sites were calculated from the IC_{50} values. Percentage of low-affinity binding sites (B_{max} Low (%)) was calculated from the equation B_{max} Low (%) = B_{max} Low / (B_{max} High + B_{max} Low) × 100. n, number of experiments; nH, pseudo Hill coefficient; CEC, chloroethylclonidine treatment. The values represent means±S.E. *: significant difference from unity ($P<0.05$). $^{\dagger,\#}$: significant difference from the value in untreated membranes ($P<0.05$).

by the same pretreatment of membrane preparations that did not contain the metabolically stable GTP analog GTPγS. However, in membrane preparations where GTPγS was added and single cell preparations where there was endogeneous GTP, the low-affinity site was completely eliminated by the CEC pretreatment. Displacement studies with the α_1-antagonist WB4101 also revealed high- and low-affinity binding sites labeled by [^{3}H]prazosin. Displacement curves of WB4101 obtained from membrane preparations in the presence of GTPγS did not differ from those in the absence of GTPγS. These results suggest that the low-affinity site of phenylephrine binding labeled with [^{3}H]prazosin was selectively bound by CEC which was used to pretreat the tissues, membrane preparation containing GTPγS and single cells. GTPγS may convert a conformation of α_{1B}-adrenoceptors that is suitable to interact with CEC and then bind covalently with them. Thus, CEC is able to recognize these two distinct subtypes of α_1-adrenoceptors only when GTPγS is present (40). CEC, like PrBCM, cyclizes spontaneously into quarternary aziridinium ion in aqueous solution and then changes into an alcoholic hydrolysis product. Aziridinium ion is thought to interact with anionic groups in α_{1B}-adrenoceptors to bind covalently with them. To test interactions of aziridinium ion with α_{1A} (CEC-resistant)- and α_{1B} (CEC-sensitive)-receptors, the prepa-

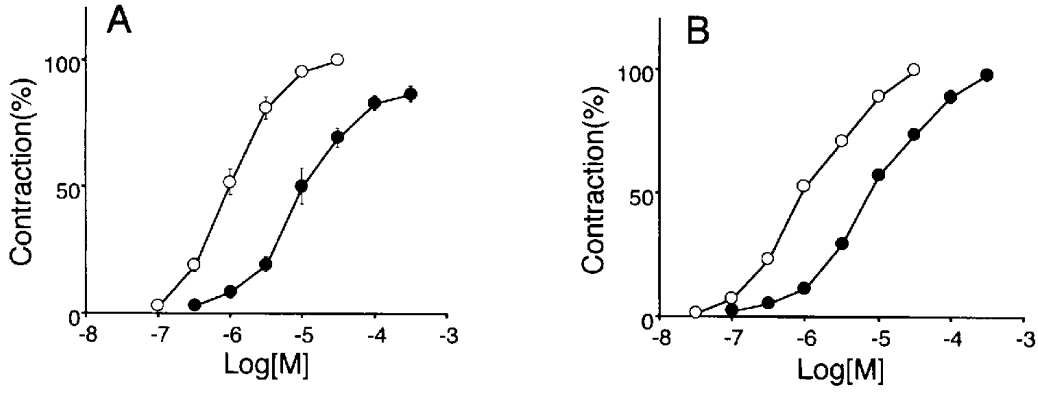

Fig. 10. Effects of chloroethylclonidine (CEC) on norepinephrine-induced contractions of rabbit iris dilator muscle untreated (A) and thoracic aorta treated with CEC (10^{-4} M) for 60 min (B). Ordinate: contraction (%) expressed as a percentage of the maximum contraction. Abscissa: log concentration of norepinephrine. ○: agonist alone, ●: in the presence of CEC (10^{-4} M) for 3 min. Each value is expressed as the mean±S.E. (bar) of 4 experiments.

rations were treated with CEC (10^{-4} M) for 3 min. Rabbit iris dilator muscle was used as an α_{1B}-adrenoceptor predominant preparation and rabbit aorta muscle where α_{1B}-adrenoceptors were almost completely blocked by the 60-min treatment with CEC (10^{-4} M) as an α_{1A}-adrenoceptor-predominant one. Concentration-response curves of norepinephrine obtained in the two preparations were shifted in parallel by the 3-min treatment with CEC (10^{-4} M) (Fig. 10), suggesting that aziridinium ion interacts with both α_{1A}- and α_{1B}-adrenoceptors (I. Takayanagi et al., unpublished data). After these interactions, aziridinium ion binds covalently with α_{1B}-receptors but not with α_{1A}-receptors.

III. The relationship between changes in cytosolic Ca^{2+} concentration and tension development

A reliable method of measuring smooth muscle contraction simultaneously with Ca^{2+} signals in vascular smooth muscle using dual wave length excitation of a fluorescent dye was introduced by Ozaki et al. (41). Sato et al. (42) reported that in rat vascular smooth muscle, greater tension development was induced at the same level of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the presence of norepinephrine, a specific receptor stimulant, than in the presence of a high concentration of K^+, a depolarizing agent, and suggested that norepinephrine increased the Ca^{2+}-sensitivity of the contractile elements. Huang et al. (43) studied the modes by which endothelin-1 (ET) induces Ca^{2+}-influx and the relative functional importance of the different sources of Ca^{2+} for ET-induced contraction using fura 2-loaded and -unloaded rat aortic strips. ET caused an increase in $[Ca^{2+}]_i$ followed by a tonic contraction in Ca^{2+}-containing solution, and it produced a transient elevation of $[Ca^{2+}]_i$ followed by a small sustained contraction in Ca^{2+}-free medium. ET also significantly stimulated ^{45}Ca influx into the La^{3+}-inaccessible fraction. With the same change of $[Ca^{2+}]_i$, ET caused a larger tension than that induced by high K^+. ET-induced contraction and $[Ca^{2+}]_i$ elevation were not significantly inhibited by $10^{-7}-3\times10^{-7}$ M nicardipine which nearly abolished the contraction and $[Ca^{2+}]_i$ elevation produced by high K^+. During treatment of the strips with high K^+, addition of ET induced further increases in $[Ca^{2+}]_i$ and muscle tension, and vice versa. In Ca^{2+}-free medium, ET-induced contraction was influenced neither by ryanodine-treatment nor by high K^+-treatment, although the former attenuated and the latter potentiated the $[Ca^{2+}]_i$ transient induced by ET. Furthermore, the sustained contraction induced by ET under Ca^{2+}-free conditions began to develop after the $[Ca^{2+}]_i$ level returned to the baseline. Thus, it seems that the Ca^{2+} released from the ryanodine-sensitive and -insensitive Ca^{2+} stores by ET

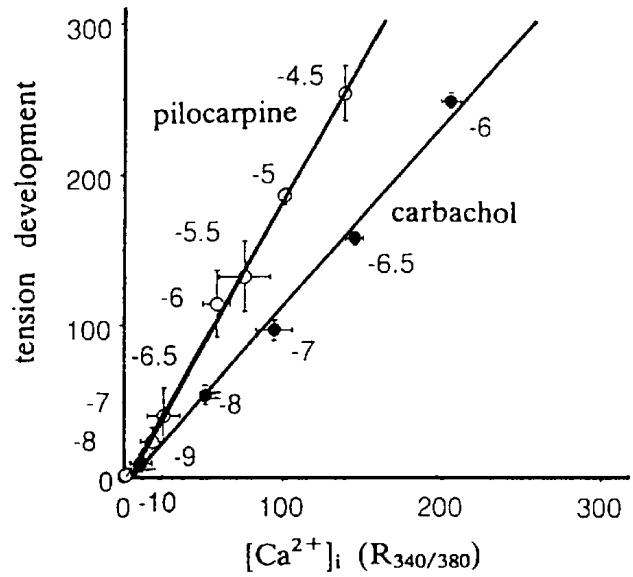

Fig. 11. Relationship between $[Ca^{2+}]_i$ ($R_{340/380}$) and tension development at various concentrations of carbachol and pilocarpine. Ordinate and abscissa: tension (%) and $R_{340/380}$ (%), respectively. The 70-mM K^+-induced increase is designated as 100%. Each point is expressed as a mean ± S.E. (bar) of 3 experiments. Each number shown is the negative logarithm of the concentration used. ○, pilocarpine; ●, carbachol. [Adapted from Ref. 49 with permission]

may provide only a minor or indirect contribution, if any, to the tension development. Huang et al. (43) suggested that ET might cause a contraction mainly by stimulating Ca^{2+}-influx through a Ca^{2+} channel(s) other than the voltage-dependent type of Ca^{2+} channel and by increasing the sensitivity of the contractile filaments to Ca^{2+} or activating them Ca^{2+}-independently. Further studies are necessary to understand the mechanisms presented herein. These data indicate that specific receptor stimulants increase the Ca^{2+}-sensitivity of contractile elements.

First, the relationship between changes in $[Ca^{2+}]_i$ and muscle tension development induced by carbachol and pilocarpine in longitudinal smooth muscles either treated or untreated with PrBCM was investigated in order to clarify the different nature of PrBCM-sensitive and -resistant receptors.

Carbachol and pilocarpine caused a rapid increase in $[Ca^{2+}]_i$ that was accompanied by an increase in muscle tension. The peak amplitude of both $[Ca^{2+}]_i$ and muscle tension induced by the test agonist were compared with those by 70 mM K^+. Both $[Ca^{2+}]_i$ and muscle tension, expressed as a percentage of those induced by 70 mM K^+, were increased by the agonists in a concentration-dependent manner. Figure 11 shows the existence of a positive correlation between $[Ca^{2+}]_i$ and tension development due to both carbachol in preparations treated with PrBCM and pilocarpine in untreated ones ($r=0.9967$ for

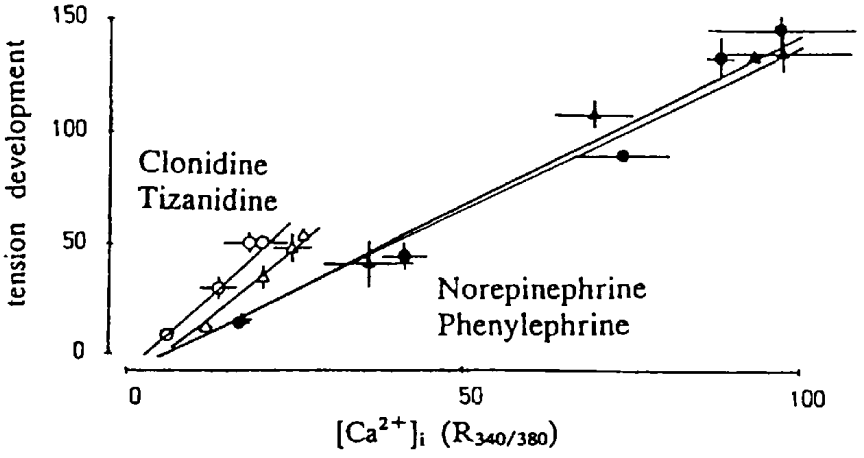

Fig. 12. Relationship between $[Ca^{2+}]_i$ ($R_{340/380}$) and tension development at various concentrations of four agonists. ●, norepinephrine; ▲, phenylephrine; ○, clonidine; and △, tizanidine. Ordinate and abscissa, tension (%) and $[Ca^{2+}]_i$ ($R_{340/380}$), respectively, of which 100% represents the 70 mM K^+-induced increase. Each point represents the mean±S.E. (bar) of four experiments. [Adapted from Ref. 51 with permission]

carbachol and r=0.9978 for pilocarpine). Slopes of the regression lines were 1.23±0.058 for carbachol that mainly activates PrBCM-resistant cholinoceptors and 1.85±0.071 for pilocarpine that only interacts with PrBCM-sensitive ones. The slope for pilocarpine was significantly steeper than that for carbachol. These results suggest that greater tension is induced for a given increase in $[Ca^{2+}]_i$ in the presence of pilocarpine, which activates PrBCM-sensitive receptors, than in the presence of carbachol, which activates PrBCM-resistant ones. It is well-known that contraction of smooth muscles is regulated not only by $[Ca^{2+}]_i$ but also by the Ca^{2+}-sensitivity of the contractile elements (42–45). In the guinea pig intestinal smooth muscle, pilocarpine was a partial agonist with high intrinsic activity in a normal physiological solution, while it behaved as a partial agonist with lower intrinsic activity in the solution from which Ca^{2+} was removed. Also, carbachol has a higher inositol-triphosphate formation capacity than pilocarpine (46). Konno and Takayanagi (46) concluded that carbachol, a full agonist, was apparently able to mobilize stored Ca^{2+} better than pilocarpine. Consequently, the contractile response to pilocarpine is more dependent on Ca^{2+}-influx than is the response to carbachol. They (46) also reported that pilocarpine was a partial agonist with lower intrinsic activity in phosphatidylinositol turnover. Taking the aforementioned observations into account (46), the present results suggest that the activation of PrBCM-sensitive receptors might enhance the Ca^{2+}-sensitivity of the contractile elements (47–49).

Secondly, fura 2-loaded thoracic aorta strips from rabbits were used. Norepinephrine, phenylephrine, clonidine and tizanidine induced an increase in $[Ca^{2+}]$ and muscle tension in a concentration-dependent manner. A positive correlation between $[Ca^{2+}]_i$ and tension development owing to the agonists was noted (Fig. 12). The slope of regression lines between $[Ca^{2+}]_i$ and tension development for clonidine and tizanidine, α_1-adrenergic partial agonists, were significantly steeper than those for norepinephrine and phenylephrine, α_1-adrenergic full agonists. The intrinsic activities of the partial agonists obtained from tension development were greater than those from changes in $[Ca^{2+}]_i$. Furthermore, the slopes of the regression lines between $[Ca^{2+}]_i$ and tension development for both the full agonists in the CEC-treated preparations, which did not contain α_{1B}-adrenoceptors, were significantly steeper than those in the untreated ones, which contained both α_{1A}- and α_{1B}-adrenoceptors. These results suggest that the partial agonists, α_{1A}-adrenoceptor activators, cause a greater muscle tension than the full agonists, α_{1A}- and α_{1B}-adrenoceptor activators, at the same level of $[Ca^{2+}]_i$ (50, 51).

IV. Intracellular signal transduction pathways

It is generally accepted that the primary trigger for smooth muscle contraction is the elevation of $[Ca^{2+}]_i$ surrounding the myofilaments and subsequent phosphorylation of the 20 kDa myosin light chain (MLC) by Ca^{2+}/calmodulin-related myosin light chain kinase (MLCK), which activates actomyosin ATPase (52). Receptor stimulations evoked by agonists produce various signal transducing substances in smooth muscle cells and thereby activate the contractile apparatus. As we have mentioned in Section III, the observed Ca^{2+}-tension relationship produced by full and partial agonist suggests

the presence of an additional regulatory mechanism that modulates the Ca^{2+}-sensitivity of contractile elements: MLC-phosphorylation-dependent and -independent pathways, which are regulated by protein kinase Cs (PKCs) stimulated by phorbol esters (53) or low molecular weight GTP binding protein families (54–56).

Adrenoceptors and cholinoceptors belong to the family of seven transmembrane segment receptors, which are heterotrimeric ($\alpha\beta\gamma$) G protein-coupled to phosphoinositide-phospholipase C, and cause a rapid hydrolysis of inositol phospholipids, resulting in the generation of two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (57). The former releases Ca^{2-} from the cellular storage sites and activate Ca^{2+} /calmodulin-dependent kinases including PKC, with the subsequent phosphorylation of MLC and the initiation of contraction. DAG is the endogenous activator of PKC, a serine/threonine kinase that plays an important role in signal transduction during contractile responses (58, 59). In vascular (60–62) and tracheal (63) smooth muscle, there is substantial evidence indicating that PKC activation is involved in increased myofilamental Ca^{2+}-sensitivity. Pharmacological procedures have provided data showing that PKC induced contraction requires less Ca^{2+} than those elicited by membrane depolarization, enabling us to see an important role for PKC in the regulation of smooth muscle contraction. On the other hand, in addition to the increase in intracellular Ca^{2+} concentration released from Ca^{2+} storages, receptor stimulation evoked Ca^{2+}-influx from extracellular fluid through voltage-dependent and/or receptor-operated Ca^{2+} channels. The existence of a link between the phospholipase C pathway and Ca^{2+} channels (64) and the existence of two pharmacologically distinct receptor subtypes that couple with a different Ca^{2+} signalling mechanism and having different physiological roles were propounded (37). We already described that the partial agonists cause greater muscle tension than the full agonists at the same level of $[Ca^{2-}]_i$ (Figs. 11 and 12). Therefore, in rabbit aorta and guinea pig ileum, which contain two receptor subtypes, the final physiological contractile responses is considered to be caused by the intracellular communication between at least two pathways.

In β-escin skinned vascular smooth muscle of rabbit thoracic aorta, Ca^{2+}-sensitivity of the contraction induced by constant cytoplasmic Ca^{2-} of 3×10^{-7} M (pCa 6.5) was increased through both α_{1A}- and α_{1B}-subtypes activated by norepinephrine (10^{-5} M), but through only the α_{1A}-subtype activated by methoxamine (10^{-4} M) or clonidine (10^{-4} M) (38). With α_{1B}-adrenoceptor-alkylating agent CEC-pretreated tissue, the Ca^{2+}-sensitization produced by norepinephrine was similar to that produced by methoxamine and clonidine, which was not affected by the CEC-pretreatment. In the presence of GTP or GTPγS, the Ca^{2-}-sensitization of Ca^{2-}-evoked contraction produced by α_1-adrenoceptor agonists and that through the α_{1A}-subtype is inhibited by the PKC inhibitor, H-7 [1-(5-isoquinolinylsulfonyl)-methyl-piperazine]. The activators of PKC, phorbol esters, showed effects similar to those of agonists in that they increased the Ca^{2+}-sensitivity of MLC-phosphorylation (60, 65) and activated a mechanism that is independent on MLC-

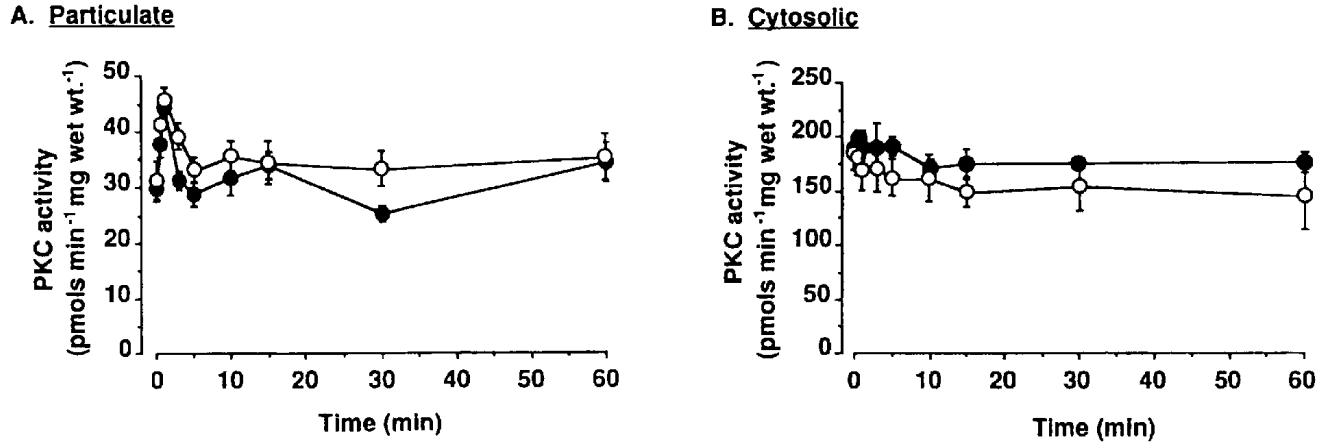

Fig. 13. Time course of protein kinase C (PKC) distribution between the particulate (A) and cytosolic (B) fractions in response to clonidine alone (●) or clonidine with *Clostridium botulinum* C_3 pretreatment (○) in β-escin-permeabilized thoracic aorta. Ordinate: PKC activity (pmol/min·mg wet weight) produced by clonidine (10^{-4} M). Abscissa: time (min) from application of clonidine. All PKC distributions were performed in the presence of 50 μM guanosine 5′-triphosphate (GTP) plus 0.3 μM Ca^{2-}. Each value is presented as the mean±S.E. (bar) for 5–6 determinations. [Reprinted from Ref. 67 (Eur. J. Pharmacol., Vol. 290, pp. 19–27, 1995) with kind permission of Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands]

Table 4. Effects of guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) or *Clostridium botulinum* C_3 (C_3) on norepinephrine (NE), clonidine (Clon) and phorbol 12-myristate 13-acetate (TPA) enhanced Ca^{2+}-sensitization in the chloroethylclonidine-pretreated tissues

	n	pCa 6.5		Agonists & pCa 6.5		pCa 5.0	Agonists & pCa 6.5 /pCa 6.5
		Tension (mg)	%	Tension (mg)	%	Tension (mg)	
Untreated							
NE	3	8.73±0.78	28.7±2.63	14.2±1.85	44.4±4.47	31.8±1.92	1.54±0.06[a]
Clon	3	7.57±0.30	27.2±0.99	11.1±1.45	39.3±5.59	28.4±0.38	1.53±0.12[b]
TPA	3	7.68±1.32	23.3±3.79	19.5±0.92	59.6±2.54	32.8±0.33	2.75±0.40[c]
GDPβS-treated							
NE	3	7.63±0.32	27.1±1.01	7.23±0.13	25.7±0.58	28.1±0.29	0.96±0.03[d]
Clon	3	7.85±1.39	26.5±4.62	7.96±1.34	26.9±4.44	29.6±0.31	1.02±0.01[e]
TPA	3	7.09±0.51	23.5±2.06	10.8±0.88	36.9±3.18	30.3±0.94	1.60±0.20[f]
C_3-treated							
NE	3	8.83±1.87	30.7±6.29	9.12±2.02	31.7±6.77	28.7±0.50	1.03±0.03[g]
Clon	3	8.73±0.95	29.5±4.24	8.84±1.21	30.0±5.00	29.8±1.22	1.00±0.05[h]
TPA	3	7.29±1.11	23.6±2.83	11.0±0.94	36.0±1.88	30.5±1.01	1.56±0.11[i]

The table shows the amplitude of the contraction at a given concentration of either stimulant and the maximum response evoked by 10 μM Ca^{2+} expressed as a relative of 100%. Tissues are pretreated with chloroethylclonidine (10^{-4} M). GDPβS: 1 mM, C_3: treatment with 5 μg/ml, NE: 10^{-5} M, Clon: 10^{-4} M, TPA: 10^{-6} M. Values are given as means±S.E. a–d, b–e, c–f, a–g, b–h, c–i: significantly different from each other ($P<0.05$).

phosphorylation (66), being supported by the observation that the potent MLCK inhibitor KT5926 [(8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-(14-*n*-propoxy-2,3,9,10-8,11-epoxy,1*H*,8*H*,11*H*,-2,7b,11a-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one] decreased a part of the contraction potentiated by phorbol esters (67). After the exposure of muscle strips to clonidine, there is a rapid increase in the activity of PKC in the particulate fraction (Fig. 13A, closed circles), while the activity of PKC in cytosolic protein tended to concomitantly decrease. In the preparations with down-regulated PKC activity by the treatment with phorbol ester, the Ca^{2+}-sensitizing effects of phorbol ester were completely abolished (68, 69). Because agonists activate PKC through the formation of DAG, we can be fairly certain that PKCs, including in atypical isoforms, participate in the Ca^{2+}-sensitizing pathways activated by agonists in smooth muscle. Furthermore, the Ca^{2+}-sensitization induced by agonist is inhibited by the pretreatment of the muscle with *Clostridium botulinum* C_3 (C_3). Earlier, Morii et al. (70) reported the existence and tissue distribution of G_b (Rho) in aorta and suggested that this protein has a contractile function as shown by its wide distribution in the body. Low molecular weight GTP-binding proteins (Ras superfamily) are mainly proteins of the Ras family, Rho family and Rab family. These three families are involved in controlling a diverse set of essential cellular functions including proliferation, cytoskeletal organization and intracellular vesicle transport (71). As shown in Table 4, agonist-enhanced Ca^{2+}-sensitivity of the skinned smooth muscle contraction is almost eliminated by C_3, and this is supported by similar observations that the GTPγS-enhanced Ca^{2+}-sensitivity was abolished by C_3 (56). By using a biochemical technique, purified C_3 from the culture medium of a type C strain has been shown to ADP-ribosylate Rho on asparagine 41 (72–75); and it is concluded that Rho A p21, which belongs to the Ras p21-like low molecular weight GTP-binding protein superfamily, is involved in the Ca^{2+}-sensitization of smooth muscle contraction (56). The Ca^{2+}-sensitization through the activation produced by the α_1-adrenoceptor agonists clonidine and norepinephrine in the CEC-treated preparation, which shows the KT5926-resistant response, may have been mediated through GTP-binding protein-coupled inhibition of MLC phosphatase in phosphorylation-dependent pathways. Though the protein phosphatases modulated by GTP-binding protein(s) remain to be identified, C_3-sensitive low molecular weight GTP-binding protein(s) may be involved in the inhibitory effect of the negative modulation of phosphatase activity (76).

The activation of PKC produced by the α_{1A}-subtype stimulant clonidine was not affected by C_3-treatment as shown in Fig. 13. However, the PKC inhibitor H-7 inhibited the Ca^{2+}-sensitization produced by norepinephrine (62), and phorbol ester-induced Ca^{2+}-sensitization is also partly inhibited by C_3 (67). These findings suggest that PKC is important in the regulation of Ca^{2+}-sensitiv-

ity to the agonist-induced contractions. Moreover, in the skinned smooth muscle treated with C_3, PKC activated by clonidine had the same level of activity as PKC activated by clonidine without C_3, showing no regulatory effect of PKC on C_3-sensitive protein (Fig. 13); this indicates that PKC, which produces Ca^{2+}-sensitization in smooth muscle, is in a position unaffected by C_3-sensitive GTP-binding proteins: a step above, upstream from C_3-sensitive GTP-binding proteins in the Ca^{2+}-sensitizing cascade or independent of C_3-sensitive GTP-binding proteins. With α_{1B}-adrenoceptor-alkylation agent CEC-treated tissue, where only the α_{1A}-subtype remained, the enhancement of Ca^{2+} contraction produced by norepinephrine was not affected by the potent MLCK inhibitor KT5926.

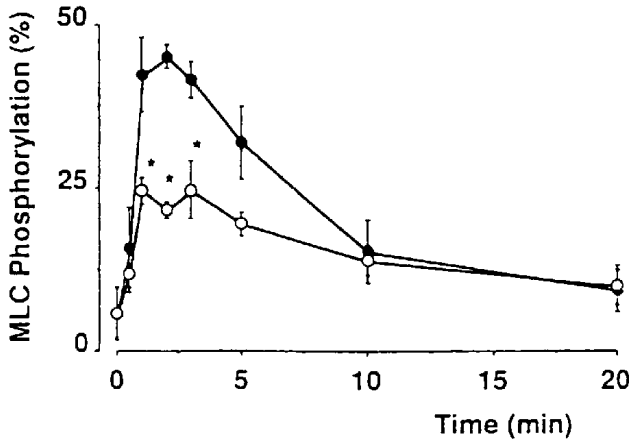

Fig. 14. Effects of norepinephrine and clonidine on myosin light chain phosphorylation in rabbit thoracic aorta. Ordinate: myosin light chain phosphorylation (%) produced by norepinephrine (10^{-5} M) (●) and clonidine (10^{-4} M) (○). Abscissa: time (min) from application of each agonist. Each value is presented as the mean ± S.E. (bar) of 4 separate experiments. *Significant difference from the corresponding value ($P < 0.05$). [Reprinted from Ref. 62 (Eur. J. Pharmacol., Vol. 265, pp. 133–139, 1994) with kind permission of Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands]

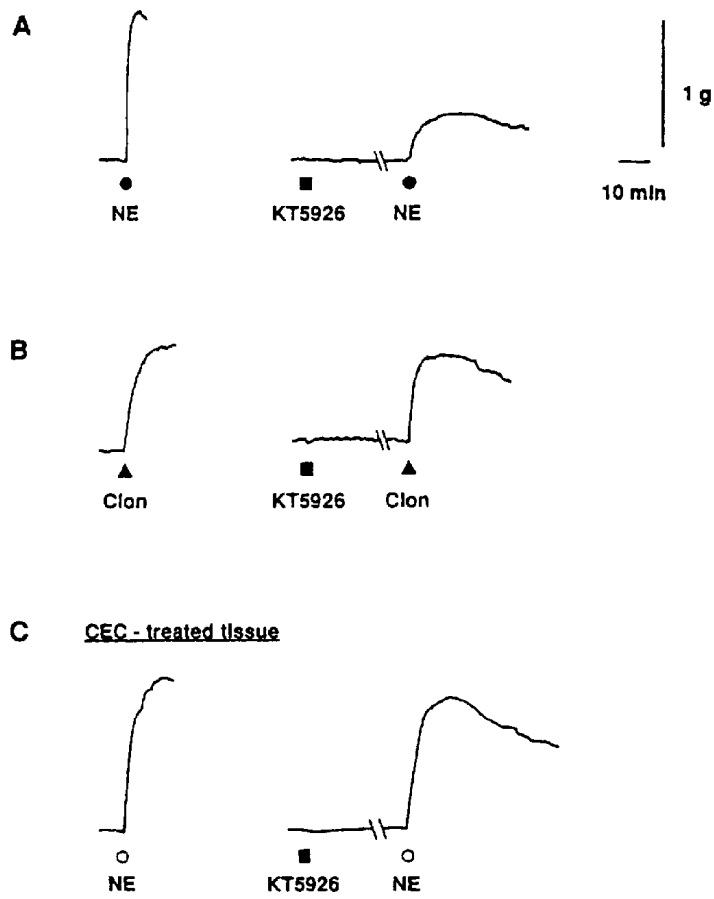

Fig. 15. Typical tracings illustrating the effect of KT5926 on the contractile response of the rabbit aorta to norepinephrine (A), clonidine (B) and norepinephrine in chloroethylclonidine-treated tissues (C). ■, 3×10^{-6} M KT5926. In A: ●, 3×10^{-7} M norepinephrine. In B: ▲, 3×10^{-6} M clonidine. In C: ○, 3×10^{-6} M norepinephrine after a 60-min treatment with 10^{-4} M chloroethylclonidine (CEC). [Reprinted from Ref. 77 (Gen. Pharmacol., Vol. 26, pp. 357–362, 1995) with kind permission of Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands]

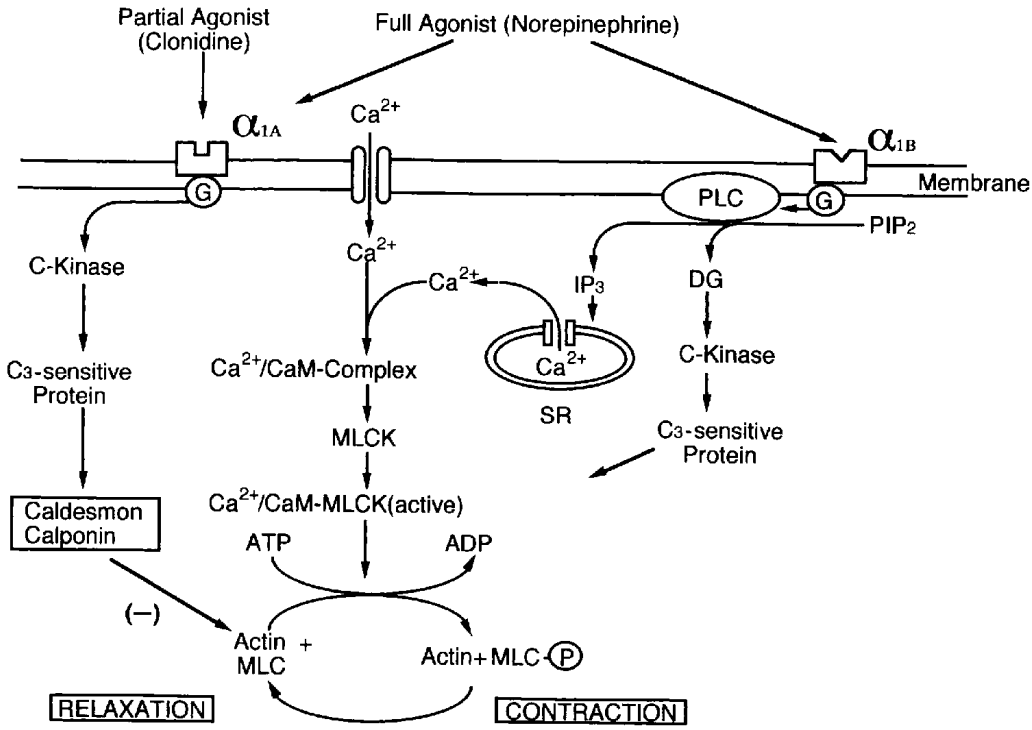

Fig. 16. Possible mechanisms for tension development mediated through α_{1A}- and α_{1B}-adenoceptors in β-escin permeabilized thoracic aorta strip from rabbit. PLC: phospholipase C, PIP_2: phosphatidilinositol biphosphate, DG: diacylglycerol, IP_3: inositol trisphosphate, SR: sarcoplasmic reticulum, CaM: calmodulin, MLC(K): myosin light chain (kinase), (−): inhibition, C_3: *Clostridium botulinum* C_3. [Reprinted from Ref. 81 (Yakugaku Zasshi, Vol. 116, No. 6, pp. 417–440, 1996) with kind permission of Pharmaceutical Society of Japan]

Moreover, the amount of MLC-phosphorylation induced by 10^{-5} M norepinephrine was greater than that by 10^{-4} M clonidine (Fig. 14), indicating that the different Ca^{2+}-sensitizing effect of these two agonists is not related to the amount of phosphorylated MLC. Using intact aortic strips, the MLCK inhibitor KT5926 markedly inhibited the contractile response to norepinephrine (the residual response: 47.6±7.40%, n=4), but only slightly inhibited the contractile response to clonidine (the residual response: 80.6±6.25%, n=4) and that to norepinephrine in CEC-treated tissue (Fig. 15). These results mean that norepinephrine predominantly produces the contractile response through phosphorylation of MLC by the activation of MLCK, whereas clonidine induces the contraction through the phosphorylation-independent pathway of MLC; this shows that the α_{1A}-adrenoceptor subtype activates the MLC-phosphorylation-independent pathway in rabbit vascular smooth muscle (77). In addition, K-252a [(8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one], a potent inhibitor of protein kinases, also caused similar inhibitory effects to KT5926 on the contractile response to norepinephrine and clonidine with

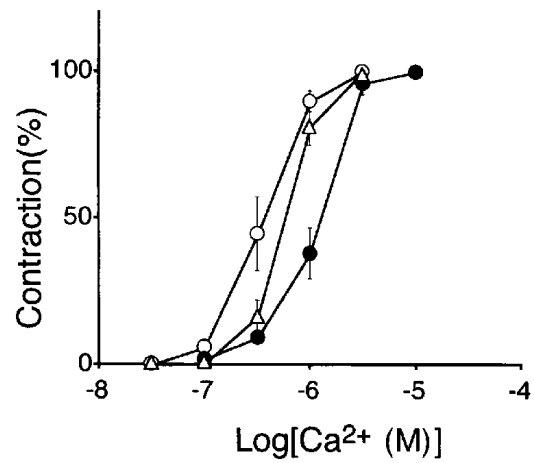

Fig. 17. Augmentation by pilocarpine and carbachol of Ca^{2+} concentration-response curves in α-toxin skinned guinea pig ileum longitudinal muscle. Ordinate: percentage of maximum response to 10 μM Ca^{2+}. Abscissa: logarithm of molar concentration of Ca^{2+}. ●, Ca^{2+} alone; ○, with 50 μM GTP and pilocarpine (3×10^{-4} M); △, with 50 μM GTP and carbachol (10^{-5} M). Each value is expressed as the mean±S.E. (bar) of 4–5 experiments. Note that the contractions by carbachol were obtained in the PrBCM-treated preparation and those by pilocarpine in the untreated one.

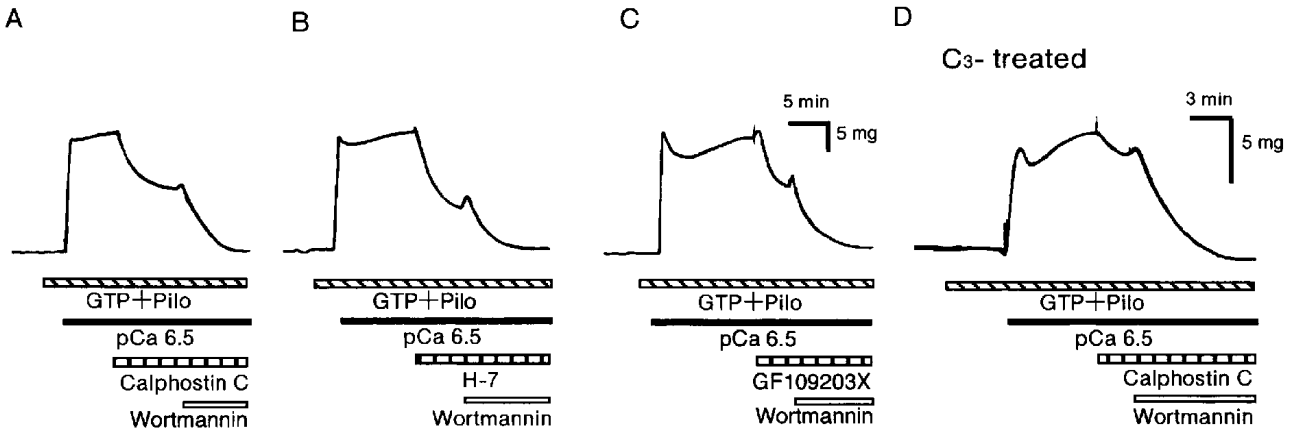

Fig. 18. Typical traces of the inhibitory effects of protein kinase C inhibitors, calphostin C (A), H-7 (B) and GF109203X (C), on Ca^{2+}-induced contraction in the presence of GTP and pilocarpine. Skinned strips were contracted by 3×10^{-7} M Ca^{2+} (pCa 6.5) in the presence of 5×10^{-5} M GTP and 3×10^{-4} M pilocarpine (Pilo) (hatched bars). Calphostin C (10^{-6} M), H-7 (10^{-5} M) and GF109203X (10^{-6} M) and a myosin light chain kinase inhibitor wortmannin (10^{-5} M) were applied as indicated by the striped bar and open bar, respectively. In panel D, the skinned smooth muscle was pretreated with 5 μg/ml *Clostridium botulinum* C_3 (C_3) for 20 min at 25℃ in the relaxing solution containing 10 μM α-nicotinamide adenosine dinucleotide and 50 μM GTP. Note that the traces were selected as typical ones from 5 experiments.

CEC-untreated or -treated tissues (77). These findings suggest that the contraction induced through α_1-adrenoceptors is initiated by a direct mechanism, possibly phosphorylation of MLC, which is mainly activated through the α_{1B}-adrenoceptor subtype, and that the agonists may increase the Ca^{2+}-sensitivity of contractile elements by increasing the Ca^{2+}-sensitivity of MLC-phosphorylation. On the other hand, although actin-binding proteins, calponin and caldesmon bind to actin and inhibit the actin-myosin interactions, this activity is abolished by Ca^{2+}/calmodulin (78–80). The dissociation of these proteins from actin-filaments results in the Ca^{2+}-sensitization of muscle contraction. α_{1A}-Adrenoceptors stimulated by clonidine activate the actin-myosin interactions through an actin regulating protein such as calponin or caldesmon on the grounds that the amount of MLC-phosphorylation and inhibitory effects of MLCK inhibitor on clonidine-induced contraction is smaller than those on the norepinephrine-induced one. Both α_1-adrenoceptor subtypes (α_{1A} and α_{1B}) increase the Ca^{2+}-sensitivity of contractile elements. The Ca^{2+}-sensitization produced by α_{1A}-subtype receptors is mainly caused by the MLC-phosphorylation-independent pathway mediated through PKC and low molecular weight GTP-binding protein (Rho A p21), and that produced by α_{1B}-subtype receptors is mainly caused by the MLC-phosphorylation dependent on Ca^{2+} (Fig. 16) (81).

We have observed the similar findings on M_3-cholinoceptors. As mentioned in Section II-1-1, in intestinal smooth muscles of the guinea pig, M_3-cholinoceptors are differentiated by PrBCM into two subtypes: PrBCM-sensitive and -resistant subtypes. The partial agonist pilocarpine activates only the PrBCM-sensitive one, but the full agonist carbachol activates both the PrBCM-sensitive and -resistant ones (5, 6, 8). As shown in Fig. 17, the Ca^{2+}-sensitivity of the contractile elements is increased by pilocarpine more effectively than by carbachol in guinea pig intestinal muscle, suggesting that in the Ca^{2+}-sensitivity of contractile elements, the post receptor processes activated by pilocarpine are partly different from those activated by carbachol. The contraction induced by pCa 6.5 in the presence of pilocarpine and GTP was reduced by PKC inhibitors, calphostin C, H-7 and GF 109203X [2-(1-(3-dimethylaminopropyl)-indo-(3-yl)-3-(indol-3-yl)-maleimide], and an MLCK inhibitor, 10^{-5} M wortmannin (Fig. 18: A, B and C). As shown in Fig. 11, Ca^{2+} contraction was increased more effectively in the presence of pilocarpine than in the presence of carbachol. These results confirmed the findings that in the fura 2-loaded intestinal smooth muscle of guinea pig, pilocarpine induces greater contraction than carbachol at the same level of $[Ca^{2+}]_i$. As the Ca^{2+}-sensitization in various smooth muscles is accompanied by an activation of PKC (62, 67, 82–84) in the α-toxin-skinned ileal smooth muscle of guinea pig, all the PKC inhibitors used partly inhibited contraction induced by Ca^{2+} in the presence of pilocarpine, and the following application of the MLCK inhibitor wortmannin completely inhibited the residual Ca^{2+}-contraction to the resting level (Fig. 18: A, B and C). The Ca^{2+}-sensitization enhanced by pilocarpine is partly related to PKC and also to MLC-phosphorylation. In addition, the inhibitory

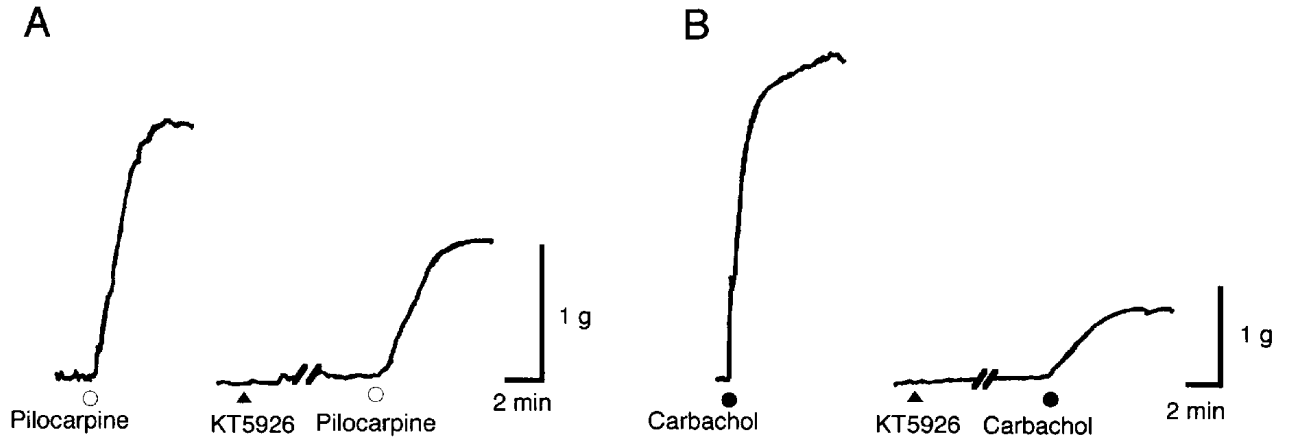

Fig. 19. Typical tracings illustrating the effect of KT5926 on the contraction of ileum induced by pilocarpine (A) and carbachol (B). ▲, KT5926 (3×10^{-6} M). A: in the untreated preparation, ○, pilocarpine (10^{-6} M). B: in the preparation treated with PrBCM (3×10^{-6} M) for 50 min, ●, carbachol (10^{-7} M).

effect of calphostin C, a PKC inhibitor, on the Ca^{2+}-induced contraction of the botulinum ADP-ribosyltransferase C_3-treated preparation (Fig. 18D) was significantly smaller than that on the untreated one (Fig. 18A), indicating that the low molecular weight GTP-binding protein (Rho) is also involved in the Ca^{2+}-sensitization enhanced by pilocarpine (I. Takayanagi et al., unpublished data). Itagaki et al. (85) also suggested the involvement of a Rho p21-like low molecular weight GTP-binding protein in the regulation of Ca^{2+}-sensitivity using β-escin-skinned smooth muscle of guinea pig ileum. It is possible that Rho p21 is involved in the Ca^{2+}-sensitization of smooth muscle contraction mediated by PrBCM-sensitive M_3-cholinoceptor.

The concentration-response curve of pilocarpine in the untreated preparation or of carbachol in the PrBCM-treated preparation, either of which contains only PrBCM-resistant cholinoceptor, was clearly reduced by the Ca^{2+}-antagonist nicardipine (3×10^{-9} and 10^{-8} M) in a non-competitive fashion. The negative logarithm of the nicardipine concentration reducing the maximum response to agonist to one half the maximum response was obtained. The value obtained from the antagonism against pilocarpine was 8.25 ± 0.03 and that against carbachol was 8.22 ± 0.03. These two values are not significantly different, suggesting that Ca^{2+}-influxes through activation of PrBCM-sensitive and -resistant receptors were equally inhibited by nicardipine and that the contractions through both subtypes are mainly through Ca^{2+}-influxes (I. Takayanagi et al., unpublished data). Konno and Takayanagi (46) reported that carbachol was apparently able to mobilize intracellularly stored Ca^{2+} more greatly than pilocarpine. Therefore, an MLC kinase inhibitor KT5926 inhibited the contractile response of the PrBCM-treated preparation to carbachol more than that of the untreated preparation to pilocarpine (Fig. 19), although the phosphorylation of MLC produced by carbachol was not significantly different from that by pilocarpine (I. Takayanagi et al., unpublished data). Concentrations of carbachol (10^{-7} M) and pilocarpine (10^{-6} M) used herein were equipotent.

Wortmannin, another MLCK inhibitor, induced similar effects. In PrBCM-treated strips, it inhibited the contractile response to carbachol (the residual response: $55.6\pm6.7\%$, $n=4$), but only slightly inhibited the contractile response to pilocarpine (the residual response: $71.4\pm1.01\%$, $n=4$). The above-mentioned results suggest that both M_3-cholinoceptor subtypes (PrBCM-sensitive and PrBCM-resistant) also increase the Ca^{2+}-sensitivity of contractile elements and suggest that the Ca^{2+}-sensitization produced by PrBCM-sensitive subtype receptors is mainly caused by the MLC-phosphorylation-independent pathway mediated through PKC and low molecular weight GTP-binding protein, whereas the Ca^{2+}-sensitization produced by the PrBCM-resistant subtype is mainly caused by Ca^{2+} through the MLC-phosphorylation-dependent pathway.

Based on these results, we would propose the following hypothetical system of second messengers, intracellular transducing mechanisms and pathways in smooth muscle contraction: Stimulation by agonists induces the activation of phospholipase C mediated through the heterotrimeric G protein and produces inositol phosphates that release Ca^{2+} from intracellular Ca^{2+} stores and DAG that activates PKC. The PKC activates low molecular weight GTP-binding protein(s) linked to actin-binding proteins, calponin and/or caldesmon. The receptor stimulations through α_{1A}-adrenoceptor and PrBCM-sensitive M_3-

cholinoceptor subtypes mainly activate the MLC-phosphorylation-independent pathway mediated through PKC and low molecular weight GTP-binding protein at low concentration of intracellular Ca^{2+} and produce efficiently sustained smooth muscle contraction, whereas the stimulations through α_{1B}-adrenoceptor and PrBCM-resistant M_3-cholinoceptor subtypes activate the MLC-phosphorylation-dependent pathway directly related to Ca^{2+}/calmodulin (Fig. 16) (81).

V. Conclusion

The mechanisms of the two subtypes that were discriminated by β-chloroethylamines, PrBCM or CEC were investigated. There are two subtypes of M_3-cholinoceptors, PrBCM-sensitive and -resistant. The full agonists contract the intestinal smooth muscle through the interaction of these two subtypes, while the partial agonists produce the contraction through the activation of the PrBCM-sensitive ones. PrBCM can differentiate between the two mechanisms in the presence of GTP, but in its absence, PrBCM cannot distinguish the two. In other words, one mechanism is PrBCM-sensitive, being operative irrespective of the presence or absence of GTP, and the other is PrBCM-resistant, being operative only when GTP is present. One reason for this may be that the benzilylcholine moiety in PrBCM discriminates the two subtypes. The affinity of benactyzine for the PrBCM-sensitive receptor is about 3 times higher than that for the PrBCM-resistant one. These results lead to the following possibilities: PrBCM, which contains a benzilylcholine moiety in its structure has higher affinity to PrBCM-sensitive receptors than to PrBCM-resistant receptors and is able to bind covalently with the former but not with the latter. The conformation of PrBCM-resistant receptors in the presence of GTP may be suitable for interaction with aziridinium ion but not for binding it covalently.

On the other hand, the rabbit thoracic aorta contains the α_{1B}-subtype in addition to the α_{1A}-subtype. The full agonist induces contraction through both α_{1A}- and α_{1B}-subtypes, and the partial agonist induces contraction through only the α_{1A}-subtype. GTP may convert α_{1B}-adrenoceptors to a conformation suitable for interaction with CEC and then bind covalently with them. Thus, CEC is able to recognize these two distinct subtypes of α_1-adrenoceptors only when GTP is present. Aziridinium ion is believed to interact with anionic groups in α_{1B}-adrenoceptors to bind covalently. After interactions of aziridinium ion with α_{1A}- and α_{1B}-adrenoceptors, the ion binds covalently with α_{1B}-receptors but not with α_{1A}-receptors. Although β-adrenoceptor mechanisms in guinea pig taenia caecum were not discussed in this review, β-adrenoceptors also have two different types of binding sites, high- and low-affinity sites, which could be discriminated by some partial agonists. The competitive antagonistic effect of the partial agonist is due to its ability to compete with the full agonist for the high-affinity site, while the partial agonist interacts with the low-affinity site to induce the β-adrenergic effect (86–89).

β-Chloroethylamine-sensitive and -resistant receptors may be coupled to different signal transduction mechanisms. The receptor stimulations through α_{1A}-adrenoceptor and PrBCM-sensitive M_3-cholinoceptor subtypes mainly activate the MLC-phosphorylation-independent pathway mediated through PKC and low molecular weight GTP-binding protein at low concentration of intracellular Ca^{2+} and produce efficiently sustained smooth muscle contraction, whereas the stimulations through α_{1B}-adrenoceptor and PrBCM-resistant M_3-cholinoceptor subtypes activate the MLC-phosphorylation-dependent pathway directly related to Ca^{2+}/calmodulin (Fig. 16).

REFERENCES

1 Stephenson RP: A modification of receptor theory. Br J Pharmacol **11**, 379–393 (1956)

2 Nickerson M: Receptor occupancy and tissue receptor. Nature **178**, 697–698 (1956)

3 Van Rossum JM and Ariëns EJ: Receptor reserve and threshold-phenomena. II. Arch Int Pharmacodyn Ther **136**, 385–413 (1962)

4 Hulme EC, Birdsall NJM and Buckley NJ: Muscarinic receptor subtype. Annu Rev Pharmacol Toxicol **30**, 633–673 (1990)

5 Hisayama T, Kumagai N and Takayanagi I: Two apparently distinct muscarinic cholinoceptor mechanisms in guinea-pig taenia caecum. Jpn J Pharmacol **46**, 414–417 (1988)

6 Takayanagi I, Hisayama T, Kiuchi Y and Sudo H: Propylbenzilylcholine mustard discriminates between two subtypes of muscarinic cholinoceptors in guinea-pig taenia caecum. Arch Int Pharmacodyn Ther **298**, 210–219 (1989)

7 Young JM, Hiley R and Burgen ASV: Homologues of benzilylcholine mustard. J Pharm Pharmacol **24**, 950–954 (1972)

8 Takayanagi I, Harada M and Koike K: A difference in receptor mechanisms for muscarinic full and partial agonist. Jpn J Pharmacol **56**, 23–31 (1991)

9 Dörje F, Wess J, Lambrecht G, Tacke R, Mutschler E and Brann MR: Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J Pharmacol Exp Ther **256**, 727–733 (1991)

10 Caulfield MP: Muscarinic receptors: characterization, coupling and function. Pharmacol Ther **58**, 319–379 (1993)

11 Friebe TP, Mutschler E and Lambrecht G: Muscarinic receptor subtype: pharmacological characterization, molecular structure, location, function and receptor-effector coupling. Pharm Ztg Wiss **138**, 3–11 (1993)

12 Wess J: Molecular basis of muscarinic acetylcholine receptor function. Trends Pharmacol Sci **14**, 308–313 (1993)

13 Hammer R, Berrie CP, Birdsall NJM, Burgen ASV and Hulme EC: Pirenzepine distinguishes between different subclasses of muscarinic receptors. Nature **283**, 90–92 (1980)

14 Hammer R, Giraldo E, Schiavi GB, Monferini E and Ladinsky H: Binding profile of a novel cardioselective muscarinic receptor antagonist, AF-DX116, to membranes of peripheral tissues and brain in the rat. Life Sci **38**, 1653–1662 (1986)
15 Anwar-ul S, Gilani H and Cobbin LB: The cardioselectivity of himbacine: a muscarine receptor antagonist. Naunyn Schmiedebergs Arch Pharmacol **332**, 16–20 (1986)
16 Barlow RB, Berry KJ, Glenton PAM, Nikolaou NM and Soh KS: A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea pig atrial pacemaker cells at 29℃ and in ileum at 29℃ and 37℃. Br J Pharmacol **58**, 613–620 (1976)
17 Harada M, Koike K and Takayanagi I: Characterization of subtype of propylbenzilylcholine mustard (PrBCM)-sensitive and -resistant muscarinic cholinoceptors in guinea pig ileal muscle. Jpn J Pharmacol **59**, 485–487 (1992)
18 Takayanagi I, Koike K and Saito K: Propylbenzilylcholine mustard-sensitive and -resistant muscarinic receptors in cardiac muscle. Gen Pharmacol **22**, 691–694 (1991)
19 Christophe J, De Neef P, Robberecht P and Waelbroeck M: Propylbenzilylcholine mustard is selective for rat heart muscarinic receptors having a low affinity for agonists. Br J Pharmacol **88**, 63–70 (1986)
20 Fukuda K, Kubo T, Maeda A, Akiba I, Bujo H, Nakai J, Mishina M, Higashida H, Neher E, Marty A and Numa S: Selective effector coupling of muscarinic acetylcholine receptor subtypes. Trends Pharmacol Sci **10**, Supp, 4–10 (1989)
21 Jakobs KH, Aktories K and Schultz G: GTP-dependent inhibition of cardiac adenylate cyclase by muscarinic cholinergic agonists. Naunyn Schmiedebergs Arch Pharmacol **310**, 113–119 (1979)
22 Lichtshtein D, Boone G and Blume A: Muscarinic receptor regulation of NG-108-15 adenylate cyclase: requirement for Na^+ and GTP. J Cyclic Nucleotide Res **5**, 367–375 (1979)
23 Brown SL and Brown JH: Muscarinic stimulation of phosphatidylinositol metabolism in atria. Mol Pharmacol **24**, 351–356 (1983)
24 Brown JH and Masters SB: Muscarinic regulation of phosphatidylinositol turnover and cyclic nucleotide metabolism in the heart. Fed Proc **43**, 2613–2617 (1984)
25 Masters SB, Harden TK and Brown JH: Relationship between phosphoinositide and calcium responses to muscarinic agonists in astrocytoma cells. Mol Pharmacol **26**, 149–155 (1984)
26 Pfaffinger PJ, Martin JM, Hunter D, Nathans NM and Hille B: GTP-binding proteins couple cardiac muscarinic receptors to a K channel. Nature **317**, 536–538 (1985)
27 Breitwieser GE and Szabo G: Uncoupling of cardiac muscarinic and α-adrenergic receptors from ion channels by a guanine nucleotide analogue. Nature **317**, 538–540 (1985)
28 Peralta EG, Ashkenazi A, Winslow JW, Ramachandran J and Capon DJ: Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. Nature **334**, 434–437 (1988)
29 Kiuchi Y, Kumagai N, Hisayama T and Takayanagi I: Guanosine 5′-triphosphate converts some populations of propylbenzilylcholine mustard-sensitive muscarinic cholinoceptor sites to sites resistant to the drug in intestinal smooth muscle. Jpn J Pharmacol **55**, 329–338 (1991)
30 Birdsall NJM, Burgen ASV and Hulme EC: The binding of agonists to brain muscarinic receptors. Mol Pharmacol **14**, 723–736 (1978)
31 Ehlert FJ, Roeske WL and Yamamura HI: Muscarinic receptor: Regulation by guanine nucleotides, ion and *N*-ethylmaleimide. Fed Proc **40**, 153–159 (1981)
32 Harden TK, Meeker RB and Martin MW: Interaction of a radiolabeled agonist with cardiac muscarinic cholinergic receptors. J Pharmacol Exp Ther **227**, 570–577 (1983)
33 Uchida S, Matsumoto K, Mizushima A, Osugi T, Higuchi H and Yoshida H: Effects of guanine nucleotide and sulfhydryl reagent of subpopulations of muscarinic acetylcholine receptors in mammalian hearts: possible evidence for interconversion of super-high and low affinity agonist binding sites. Eur J Pharmacol **100**, 291–298 (1984)
34 Mizushima A, Uchida S, Zhou XM, Kagiya T and Yoshida H: Cardiac M_2 receptors consist of two different types, both regulated by GTP. Eur J Pharmacol **135**, 403–409 (1987)
35 Adachi S, Koike K and Takayanagi I: Pharmacological characteristics of indoline derivatives in muscarinic receptor subtypes. Pharmacology **53**, 250–258 (1996)
36 Han C, Abel PW and Minneman KP: Heterogeneity of α_1-adrenergic receptors revealed by chloroethylclonidine. Mol Pharmacol **32**, 505–510 (1987)
37 Suzuki E, Tsujimoto G, Tamura K and Hashimoto K: Two pharmacologically distinct α_1-adrenoceptor subtypes in the contraction of rabbit aorta: each subtype couples with a different Ca^{2-} signalling mechanism and plays a different physiological role. Mol Pharmacol **38**, 725–736 (1990)
38 Takayanagi I, Harada M, Koike K and Satoh M: Differences in α_1-adrenoceptor mechanisms for phenylephrine and tizanidine in rabbit thoracic aorta and common iliac artery. Can J Physiol Pharmacol **69**, 1819–1824 (1991)
39 Satoh M, Kojima C and Takayanagi I: Pharmacological characterization of contractile responses induced by α_1-agonists, norepinephrine and clonidine, by selective antagonists of their subtypes in rabbit thoracic aorta. Jpn J Pharmacol **60**, 169–177 (1992)
40 Satoh M, Kokubu N and Takayanagi I: Chloroethylclonidine discriminates between α_{1A}- and α_{1B}-adrenoceptors in the presence of guanosine 5′-triphosphate in rabbit thoracic aorta. Jpn J Pharmacol **63**, 1–8 (1993)
41 Ozaki H, Sato K, Satoh T and Karaki H: Simultaneous recordings of calcium signals and mechanical activity using fluorescent dye fura 2 in isolated strips of vascular smooth muscle. Jpn J Pharmacol **45**, 429–433 (1987)
42 Sato K, Ozaki H and Karaki H: Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura 2. J Pharmacol Exp Ther **246**, 294–300 (1988)
43 Huang XN, Hisayama T and Takayanagi I: Endothelin-1 induced contraction of rat aorta: contributions made by Ca^{2+} influx and activation of contractile apparatus associated with no change in cytoplasmic Ca^{2+} level. Naunyn Schmiedebergs Arch Pharmacol **341**, 80–87 (1990)
44 Karaki H: Ca^{2+} localization and sensitivity in vascular smooth muscle. Trends Pharmacol Sci **10**, 320–325 (1989)
45 Nishimura J, Khalil RA and van Breemen C: Agonist-induced vascular tone. Hypertension **13**, 835–844 (1989)
46 Konno F and Takayanagi I: Relationship between the contractile responses and their coupling second messenger systems for muscarinic drugs in the guinea pig ileal longitudinal muscle.

Arch Int Pharmacodyn Ther **301**, 15–29 (1989)
47 Takayanagi I and Ohtsuki H: Greater contraction is induced by pilocarpine than by carbachol at the same levels of cytosolic Ca^{2+} concentration in isolated longitudinal muscle of guinea pig ileum. Jpn J Pharmacol **53**, 525–528 (1990)
48 Takayanagi I, Kiuchi Y, Ohtsuki H and Harada M: Activation of propylbenzilylcholine mustard-sensitive muscarinic cholinoceptors more effectively utilizes cytosolic Ca^{2+} for contraction in guinea pig intestinal smooth muscle. Eur J Pharmacol **187**, 139–142 (1990)
49 Takayanagi I, Ohtsuki H, Saito K, Koike K and Satoh M: Propylbenzilylcholine mustard (PrBCM)-sensitive cholinoceptors and contractile response to partial agonist in guinea pig ileal muscle. Jpn J Pharmacol **56**, 151–158 (1991)
50 Takayanagi I and Onozuka S: Greater tension is developed at the same level of cytosolic Ca^{2+} concentration in the presence of clonidine, an adrenergic partial agonist, than in the presence of norepinephrine. J Pharmacobiodyn **12**, 781–786 (1989)
51 Takayanagi I and Onozuka S: α_1-Adrenergic partial agonists utilize cytosolic Ca^{2+} more effectively for contraction in aortic smooth muscle. Can J Physiol Pharmacol **68**, 1329–1333 (1990)
52 Adelstein RS and Eisenberg E: Regulation and kinetics of the actin-myosin-ATP interaction. Annu Rev Biochem **49**, 921–956 (1980)
53 Chatterjee M and Tejada M: Phorbol ester-induced contraction in chemically skinned vascular smooth muscle. Am J Physiol **251**, C356–C361 (1986)
54 Rubin EJ, Gill MD, Bouquet P and Popoff MR: Functional modification of a 21 kilodalton G protein when ADP-ribosylated by exoenzyme C_3 of *Clostridium botulinum*. Mol Cell Biol **8**, 418–426 (1988)
55 Kitazawa T, Kobayashi S, Horiuchi K, Somlyo AV and Somlyo AP: Receptor-coupled, permeabilized smooth muscle. J Biol Chem **264**, 5339–5342 (1989)
56 Hirata K, Kikuchi A, Sasaki T, Kuroda S, Kaibuchi K, Matsuura Y, Seki H, Saida K and Takai Y: Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. J Biol Chem **267**, 8719–8722 (1992)
57 Lee MW and Severson DL: Signal transduction in vascular smooth muscle: diacylglycerol second messengers and PKC action. Am J Physiol **267**, C659–C678 (1994)
58 Nishizuka Y: Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase. Science **258**, 607–614 (1992)
59 Pears CJ and Parker PJ: Domain interactions of protein kinase C. J Cell Sci **100**, 683–686 (1991)
60 Collins EM, Walsh MP and Morgan KG: Contraction of single vascular smooth muscle cells by phenylephrine at constant $[Ca^{2+}]_i$. Am J Physiol **262**, H754–H762 (1992)
61 Nishimura J, Morland S, Ahn HY, Kawase T, Moreland RS and van Breemen C: Endothelin increases myofilament Ca^{2+}-sensitivity in α-toxin-permeabilized rabbit mesenteric artery. Circ Res **71**, 951–959 (1992)
62 Satoh M, Kojima C, Kokubu N and Takayanagi I: α_1-Adrenoceptor subtypes mediating the regulation and modulation of Ca^{2+}-sensitization in rabbit thoracic aorta. Eur J Pharmacol **265**, 133–139 (1994)
63 Park S and Rasmussen H: Carbachol-induced protein phosphorylation changes in bovine tracheal smooth muscle. J Biol Chem **261**, 15734–15739 (1986)
64 Blakeley AG, Brown DA, Cunnane TC, French AM, McGrath JC and Scott NC: Effects of nifedipine on electrical and mechanical responses of rat and guinea pig vas deferens. Nature **294**, 759–761 (1981)
65 Colburn JC, Michnoff CH, Hsu LC, Slaughter CA, Kamm KE and Stull JT: Sites phosphorylated in myosin light chain in contracting smooth muscle. J Biol Chem **263**, 19166–19173 (1988)
66 Nishiye E, Somlyo AV, Török K and Somlyo AP: The effects of MgADP on cross-bridge kinetics: A laser flash photolysis study of guinea-pig smooth muscle. J Physiol (Lond) **460**, 247–271 (1993)
67 Kokubu N, Satoh M and Takayanagi I: Involvement of botulinum C_3-sensitive GTP-binding proteins in α_1-adrenoceptor subtypes mediating Ca^{2+}-sensitization. Eur J Pharmacol **290**, 19–27 (1995)
68 Hori M, Sato K, Sakata K, Ozaki H, Takano-Ohmuro H, Tsuchiya T, Sugi H, Kato I and Karaki H: Receptor agonists induce myosin phosphorylation-dependent and phosphorylation-independent contractions in vascular smooth muscle. J Pharmacol Exp Ther **261**, 506–512 (1992)
69 Ohanian V, Ohanian J, Shaw L, Scarth S, Parker PJ and Heagerty AM: Identification of protein kinase C isoforms in rat mesenteric small arteries and their possible role in agonist-induced contraction. Circ Res **78**, 806–812 (1996)
70 Morii N, Sekine A, Ohashi Y, Nakao K, Imura H, Fujiwara M and Narumiya S: Purification and properties of the cytosolic substrate for botulinum ADP-ribosyltransferase. J Biol Chem **263**, 12420–12426 (1988)
71 Hall A: The cellular functions of small GTP-binding protein. Science **249**, 635–640 (1990)
72 Narumiya S, Sekine A and Fujiwara M: Substrate for botulinum ADP-ribosyltransferase, Gb, has an amino acid sequence homologous to a putative rho gene product. J Biol Chem **263**, 17255–17257 (1988)
73 Aktories K, Braun U, Rösener S, Just I and Hall A: The rho gene product expressed in *E. coli* is a substrate of botulinum ADP-ribosyltransferase C_3. Biochem Biophys Res Commun **158**, 209–213 (1989)
74 Braun U, Habermann B, Just I, Aktories K and Vandekerckhove J: Purification of the 22 kDa protein substrate of botulinum ADP-ribosyltransferase C_3 from porcine brain cytosol and its characterization as a GTP-binding protein highly homologous to the rho gene product. FEBS Lett **243**, 70–76 (1989)
75 Sekine A, Fujiwara M and Narumiya S: Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. J Biol Chem **264**, 8602–8605 (1989)
76 Kitazawa T, Masuo M and Somlyo AP: G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. Proc Natl Acad Sci USA **88**, 9307–9310 (1991)
77 Satoh M, Kokubu N, Matsuo K and Takayanagi I: α_{1A}-Adrenoceptor subtype effectively increases Ca^{2+}-sensitivity for contraction in rabbit thoracic aorta. Gen Pharmacol **26**, 357–362 (1995)
78 Clark T, Ngai PK, Sutherland C, Gröschel-Stewart U and Walsh MP: Vascular smooth muscle caldesmon. J Biol Chem **261**, 8028–8035 (1986)
79 Abe M, Takahashi T and Hiwada K: Simplified co-purification of vascular smooth muscle calponin and caldesmon. J Biochem

107, 507–509 (1990)

80 Abe M, Takahashi T and Hiwada K: Effect of calponin on actin-activated myosin ATPase activity. J Biochem **108**, 835–838 (1990)

81 Takayanagi I: Pharmacological studies on drug receptor mechanisms. Yakugaku Zasshi **116**, 417–440 (1996) (Abstract, figures and tables in English)

82 Nishimura J and van Breemen C: Direct regulation of smooth muscle contractile elements by second messengers. Biochem Biophys Res Commun **163**, 929–935 (1989)

83 Anabuki J, Hori M, Ozaki H, Kato I and Karaki H: Mechanisms of pinacidil-induced vasodilation. Eur J Pharmacol **190**, 373–379 (1990)

84 Singer HA: Phorbol ester-induced stress and myosin light chain phosphorylation in swine carotid medial smooth muscle. J Pharmacol Exp Ther **252**, 1068–1074 (1990)

85 Itagaki M, Komori S, Unno T, Syuto B and Ohashi H: Possible involvement of a small G-protein sensitive to exoenzyme C3 of *Clostridium botulinum* in the regulation of myofilament Ca^{2+} sensitivity in β-escin skinned smooth muscle. Jpn J Pharmacol **67**, 1–7 (1995)

86 Koike K and Takayanagi I: A possible mechanism in interaction of a partial agonist with β-adrenoceptor in guinea-pig taenia caecum: Effects of Gpp(NH)p on its two different binding sites. Jpn J Pharmacol **38**, 169–176 (1985)

87 Koike K and Takayanagi I: A beta-adrenergic partial agonist (befunolol) discriminates two different affinity sites. Jpn J Pharmacol **42**, 325–328 (1986)

88 Takayanagi I, Koike K and Nakagoshi A: Beta-adrenergic partial agonists and two different affinity sites in beta-adrenoceptor. *In* Recent Research on Neurotransmitter Receptors, Edited by Yoshida H, pp 41–52, Excerpta Medica, Amsterdam (1986)

89 Takayanagi I, Koike K and Nakagoshi A: Interactions of some partial agonists with high and low affinity binding sites in β-adrenoceptors. Can J Physiol Pharmacol **65**, 18–22 (1987)