previously was predominantly located in the plasma membrane. The binding of the isotope-labelled thiamine to yeast plasma membrane increased in proportion to protein concentration in the range 0.25 to 2 mg/ml (data not shown). The optimal pH of yeast plasma membrane was between 5.0 and 5.3. This value is comparable to the optimal pH of the thiamine uptake by yeat cells. The binding of [^{14}C]thiamine to the plasma membrane was saturable. The figure shows that the binding activity increased linearly with increasing concentration of thiamine up to 0.1 μM and then began to saturate, attaining a plateau at the concentration of 1 μM. Analysis of the data by the method of Scatchard showed that there was a single class of binding sites which had a dissociation constant (K_d) for thiamine of 0.11 μM and a maximum binding (B_{max}) of 97.9 pmol of [^{14}C]thiamine/mg of protein. The K_d value of yeast plasma membrane to thiamine was close to the apparent K_m (0.18 μM) of thiamine transport in *S. cerevisiae*. As shown in table 2, the binding activity of yeast plasma membrane to [^{14}C]thiamine (1 μM) was inhibited by thiamine analogs, such as chloroethylthiamine (a gift from Sankyo Co. Ltd (Tokyo)) which have been known to be specific inhibitors of yeast thiamine transport. In particular, O-benzoylthiamine disulfide (a gift from Tanabe Chemical Industries Ltd (Osaka)) which is very lipophilic and without effect on the soluble thiamine-binding protein as described previously[10], inhibited thiamine binding by yeast plasma membrane markedly.

In a previous paper[12] we reported that 4-azido-2-nitrobenzoylthiamine, a photoreactive thiamine derivative, irreversibly and specifically inactivated the yeast thiamine transport system under the irradiation of visible light. It was therefore suggested that this inactivation was due to the binding of 4-azido-2-nitrobenzoylthiamine to some components in the thiamine transport system, and it led us to study the inactivation of the binding activity of yeast plasma membrane to thiamine with 4-azido-2-nitrobenzoylthiamine. 2 ml of membrane fraction (1 mg of protein/ml) in 0.05 M acetate buffer, pH 5.0, containing 0.02% NaN_3, was incubated with 4-azido-2-nitrobenzoylthiamine (5 μM) for 10 min in the dark at 4 °C. The reaction mixture was then photolyzed with a Toshiba black light (40 W) at a distance of 25 cm for 15 min, followed by dilution with the chilled buffer described above (30 ml). The diluted solution was then centrifuged at 123,000 × g for 1 h and the plasma membranes were washed further with 30 ml of the same buffer. The resuspended plasma membranes were used immediately for the binding assay by centrifugation. 0.5–1 mg of membranes were incubated with 1 μM [^{14}C]thiamine in 2.5 ml of binding buffer (0.05 M acetate buffer, pH 5.0, containing 0.02% NaN_3) with or without 1 mM unlabeled thiamine for 60 min at 4 °C, then centrifuged for 10 min at 100,000 × g. This was sufficient to pellet the membrane while giving a minimal redistribution of the free [^{14}C]thiamine. Aliquots before and after centrifugation were taken with the difference in radioactivity equated to thiamine bound to membranes. As shown in table 3, the thiamine-binding activity of yeast plasma membranes was decreased to 47.8% of that in the dark by the treatment with 4-azido-2-nitrobenzoylthiamine under visible light. These results suggested that there is a plasma membrane-bound protein which specifically binds thiamine, and might possibly be a functional component involved in the yeast thiamine transport system.

Furthermore, it was found that the plasma membrane fraction from *Saccharomyces cerevisiae* PT-R_2, a thiamine transport mutant, has a thiamine-binding activity of only 1.1% of that from the parent strain (table 4). Evidence which has been presented above shows that the thiamine-binding protein present in the plasma membrane may participate directly in thiamine transport in *Saccharomyces cerevisiae*.

Table 3. Photoinactivation with 4-azido-2-nitrobenzoylthiamine of thiamine-binding activity in yeast plasma membrane

Addition	Irradiation	Thiamine-binding (%)
4-Azido-2-nitrobenzoyl thiamine (5 μM)	(−)	100
	(+)	47.8

Table 4. Thiamine-binding activity of plasma membrane from a parent strain and a thiamine transport mutant of *S. cerevisiae*

Strain	Thiamine-binding specific activity (pmol/mg of protein)	%
S. cerevisiae (parent strain)	145.4	100
Thiamine transport mutant (PT-R_2)	1.6	1.1

1 Kawasaki, T., Miyata, I., Esaki, K., and Nose, Y., Archs Biochem. Biophys. *131* (1969) 223.
2 Neujahr, H.Y., Acta chem. scand. *17* (1963) 1902.
3 Henderson, G.B., Zevery, E.M., Kadner, R.J., and Huennekens, F.M., J. supramol. Struct. *6* (1977) 239.
4 Iwashima, A., Nishino, H., and Nose, Y., Biochim. biophys. Acta *330* (1973) 222.
5 Iwashima, A., Wakabayashi, Y., and Nose, Y., Biochim. biophys. Acta *413* (1975) 413.
6 Griffith, T.W., and Leach, F.R., Archs Biochem. biophys. *159* (1973) 658.
7 Matsuura, A., Iwashima, A., and Nose, Y., Biochem. biophys. Res. Commun. *51* (1973) 241.
8 Nishimune, T., and Hayashi, R., Biochim. biophys. Acta *328* (1973) 124.
9 Iwashima, A., and Nishimura, H., Biochim. biophys. Acta *577* (1979) 217.
10 Iwashima, A., Nishimura, H., and Nose, Y., Biochim. biophys. Acta *557* (1979) 460.
11 Serrano, R., Molec. cell. Biochem, *22* (1978) 51.
12 Sempuku, K., Nishimura, H., and Iwashima, A., Biochim. biophys. Acta *645* (1981) 226.

0014-4754/86/060607-02$1.50 + 0.20/0

Use of ^{3}H-QNB in the isolation of plasma membrane from smooth muscle of the urinary bladder: Effect of oxalate on calcium uptake by the membrane fractions

S. Batra

Department of Obstetrics and Gynecology, University of Lund and AB Leo Research Laboratories, Box 941, S-25109 Helsingborg (Sweden), 5 March 1985

Summary. Specific binding of tritiated quinuclidinyl benzilate (^{3}H-QNB) to surface membrane muscarinic receptors was utilized to identify plasma membrane (PM) fractions from smooth muscle of the rabbit urinary bladder. Accumulation of ^{3}H-QNB in the PM fraction was 4–5-fold higher than that in fractions of endoplasmic reticulum (EM) or mitochondria (M). A similar pattern of

distribution was found for 5'-nucleotidase. ^{3}H-QNB binding therefore appears to be a suitable marker for plasma membrane of the urinary bladder. Data on ATP-dependent calcium uptake by PM and ER fractions showed that oxalate highly potentiated calcium uptake by both fractions and consequently this feature cannot be used to identify ER fractions specifically.
Key words. ^{3}H-QNB; plasma membrane; smooth muscle; muscarinic receptors; calcium-uptake; urinary bladder.

Several techniques are available for the preparation of plasma membrane, but they have usually been tailored for the preparation of membranes from particular cell types or for the study of specific membrane functions. Preparation of plasma membrane from smooth muscle has generally been more difficult than from other tissues such as the liver or the heart[1].

Plasma membrane preparations can be characterized b a variety of methods and in general the methods used have been tedious and inaccurate. A majority of workers have relied heavily on the analysis of specific marker enzymes. However, this involves the assumption that the specificity pattern of these enzymes worked out for the liver is applicable also to the tissue in question. More recently, markers for specific surface membrane receptors have been used for the characterization of plasma membranes[2–4]. This method does not suffer from the above disadvantage, particularly when the right choice of marker-ligand is made. Since the urinary bladder is highly cholinergic in its innervation, and since a highly selective radioligand for muscarinic receptors, namely quinuclidinyl benzilate (QNB), is available, in the present study QNB binding was used to specifically identify plasma membrane from smooth muscle of the urinary bladder. In view of the recent debate on whether potentiation by oxalate of ATP-dependent calcium uptake is a unique feature of endoplasmic reticulum of smooth muscle[5–8], calcium uptake in membrane fractions isolated by the present procedure was also examined.

Materials and methods. Chemicals. Tritiated 1-quinuclidinyl benzilate (^{3}H-QNB) with a specific activity of 33 Ci/mmol was purchased from New England Nuclear Corporation. The radiochemical purity of ^{3}H-QNB was checked by thin-layer chromatography (Merck Silica Gel 60 plates) in a solvent system of chloroform, methanol and ammonia solution (80:20:1). Over 98% of the radioactivity migrated as a single peak as revealed by Berthold Radiochromatogram scanner model LB 2723. Atropine and 5'-AMP (sodium salt) were purchased from Sigma Chemical Co.

Preparation of subcellular fractions and ^{3}H-QNB distribution. White female rabbits weighing between 2.7 and 3.2 kg were killed by a blow on the head and exsanguinated. The bladder was excised and placed in ice-cold sucrose (0.25 M), Hepes (10 mM) solution, pH 7.2. Generally bladders from 4–5 rabbits were collected. After trimming excess fat and connective tissue the bladder was cut open and mucosa was removed by thorough scraping. The tissue, after weighing, was homogenized in about 4 vols of the above sucrose-Hepes buffer with a Polytron homogenizer (PGA 10–35) for 2 s at one half speed. The homogenate was centrifuged at 200 × g for 3 min. A supernatant fluid was removed with a Pasteur pipette and saved. The residue was resuspended in the original volume of buffer and homogenized and centrifuged again as above. This process of residue homogenization and centrifugation was repeatead six times; the supernatant fluid was saved after each centrifugation. The pooled supernatant was filtered through two layers of gauze, and the filtrate centrifuged at 175000 g for 20 min in Beckman L-5-65 centrifuge. The pellet (HI) left after discarding the supernatant was suspended in 20 ml of buffer and used for loading with ^{3}H-QNB prior to centrifugation on a discontinuous sucrose gradient. Aliquots (2.5 ml) of HI suspension were incubated with 0.2 nM ^{3}H-QNB with or without 0.2 μM atropine. One set of tubes was incubated at 37 °C for 30 min, and the other at 2 °C for 20 h.

Incubation at low temperature was carried out to avoid changes in sedimentation behavior that could occur by exposure to 37 °C. After incubation the suspension was subjected to sucrose-gradient centrifugation.

Three sucrose solutions, 6 ml (1.5 M), 4 ml (1.22 M) and 2.5 ml (1.1 M) were layered successively in 6 (20 ml) Beckman cellulose nitrate tubes. The QNB loaded HI suspension was layered on top of the last sucrose layer and centrifuged at 106,000 × g for 1.5 h in a Beckman L2-65 centrifuge using a SW 25 rotor. The top band separating at the interface of the gradient and subcellular suspension was enriched in plasma membrane (PM). Fragmented endoplasmic reticulum (ER) and a mitochondrial (M) fraction were removed in the following bands, respectively. The residue at the bottom containing nuclei, myofibrills and cell debris, was discarded. The PM, ER and M bands from each of the six gradient tubes were carefully aspirated with a Pasteur pipette and suspended in sucrose Hepes buffer. They were then centrifuged at 175,000 × g for 30 min. The final pellet from each

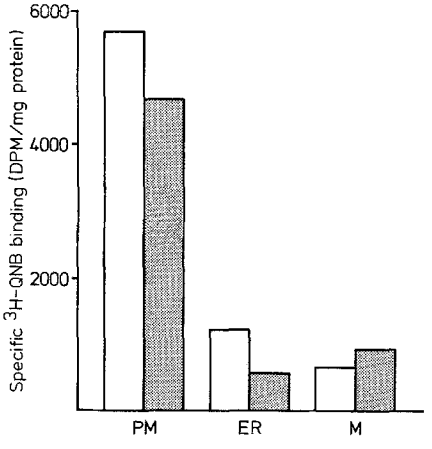

Figure 1. Distribution of ^{3}H-QNB in plasma membrane (PM) endoplasmic reticulum (ER) and mitochondrial (M) fractions prepared by density-gradient centrifugation of the parent HI fraction, which prior to centrifugation was incubated with ^{3}H-QNB at 37 °C for 30 min (open bars) or at 2 °C for 20 h (stippled bars). Values are means of duplicate determinations from two individual preparations.

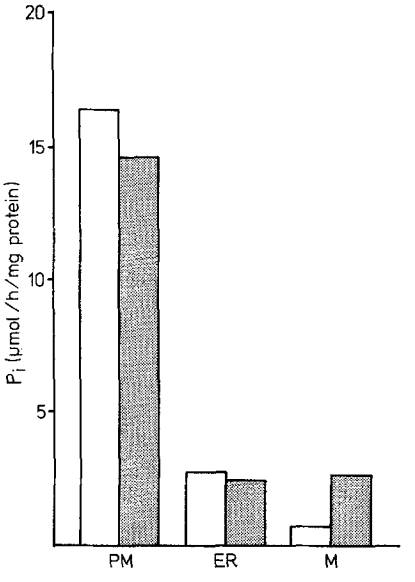

Figure 2. Distribution of 5'-nucleotidase activity in subcellular fractions prepared as in figure 1.

was suspended in sucrose Hepes buffer and appropriate aliquots used for the determination of radioactivity, protein concentration and 5'-nucleotidase activity. Protein concentration was determined by the method of Lowry et al.[9].

Determination of 5'-nucleotidase. The activity of 5'-nucleotidase was determined by incubation of each fraction in a mixture containing 10 mM each of adenosine 5'-monophosphate and $MgCl_2$ in Tris-HCl buffer (100 mM pH 7.5) and about 100 µg of membrane protein in a total volume of 0.2 ml. After incubation at 37 °C for 20 min, the reaction was terminated by the addition of 3.8 ml ice-cold trichloroacetic acid. The liberated phosphate was measured after extraction with butyl acetate according to the method of Sanui[10].

Measurement of calcium uptake. Calcium uptake was energized by ATP and measured by a Millipore filtration technique as described previously[11,12]. Aliquots of plasma membrane or endoplasmic reticulum suspensions (approximately 100 µg protein) were incubated at 37 °C in 1 ml of a medium containing 5 mM each of $MgCl_2$, ATP and NaN_3, 0.1 M KCl, 2.5 µM free Ca^{2+} buffered with Ca-EGTA, trace amounts of $^{45}CaCl_2$, 30 mM Hepes buffer at pH 7.0. The concentration of oxalate when used was 10 mM.

Results and discussion. The yield of PM, ER and M fractions by the present method was approximately 2, 0.4 and 6 mg/g tissue respectively. Although a number of reports in recent years have appeared on the preparation of membrane fractions from different smooth muscles, to my knowledge this is the first attempt to prepare plasma membrane fractions from the smooth muscle of the urinary bladder.

The subcellular distribution of radioactivity after loading of the parent fraction (HI) with ^{3}H-QNB is shown in figure 1. There was no noteworthy difference in the pattern of distribution whether the fractions during QNB-loading were incubated at 37 °C for 30 min. or at 2 °C for a longer time (20 h). The binding of QNB depicted in figure 1 is specific binding since it is the difference between that measured in the absence of atropine (total binding) and in the presence of 1000-fold excess atropine (nonspecific binding). The nonspecific binding was approximately 20% of the total. This confirms recent findings that QNB binds with high specificity to tissues that are richly innervated by cholinergic nerves[13–15]. In fact our recent data (unpublished) showed that binding of QNB to PM fractions isolated by the present procedure was totally specific.

Binding of ^{3}H-QNB in fractions containing ER and M was about 10–20% of that obtained in the PM fractions, attesting to the suitability of this marker for identification of the plasma membrane from the urinary bladder. This claim is substantiated by the data shown in figure 2 on the activity of 5'-nucleotidase in these fractions. The activity of 5'-nucleotidase in the M and ER fractions was approximately 10–15% of that measured in the PM fraction. Here again there was no difference in the pattern of distribution of the enzyme activity whether the HI fraction was exposed to 37 °C for 30 min or kept at 2 °C for 20 h during the QNB-incubations. Thus the data on the marker enzyme for plasma membrane and the ligand binding to surface membrane receptors are in good agreement. QNB binding therefore appears to be a suitable marker for plasma membrane of the urinary bladder and could similarly be used for preparations from other tissues known to have a high population of muscarinic receptors such as salivary glands or gastrointestinal tissue.

ATP-dependent calcium uptake has been studied in membranes prepared from several smooth muscles[16–19], but not that of the urinary bladder. Some data on calcium uptake by strips of intact urinary bladder tissue have recently been reported by Anderson[20].

The data on calcium uptake by PM and ER fractions is shown in figure 3. The calcium uptake medium contained NaN_3, but separate experiments showed that more than 90% of the total uptake in both fractions was azide insensitive. Since azide is a potent inhibitor of calcium uptake by mitochondria, contamination by mitochondria in these fractions could be considered to be negligible.

In controls without oxalate, calcium uptake by both fractions was relatively low but comparable to that reported for similar fractions isolated from other smooth muscles[8,11,18].

Oxalate (10 mM) potentiated calcium uptake by as much as 50-fold and 110-fold in PM and ER fractions respectively. This is an unusually high stimulation by oxalate anions of calcium uptake in membrane fractions isolated from smooth muscle; however, considerable variation in this respect was found by Kwan et al.[8], who compared preparations from three different smooth muscles. In the data presented by these authors membrane preparations from rat vas deferens showed by far the greatest potentiation (approximately 50-fold) compared to those from rat fundus (8-fold) and dog aorta (27-fold). Batra[11] reported that under certain conditions the presence of oxalate caused a 6-fold increase in calcium uptake by the microsomal fraction of the rabbit myometrium. Since oxalate considerably potentiated calcium uptake by both PM and ER in the present study, it is concluded that this property cannot be used with impunity to identify ER fractions of smooth muscle.

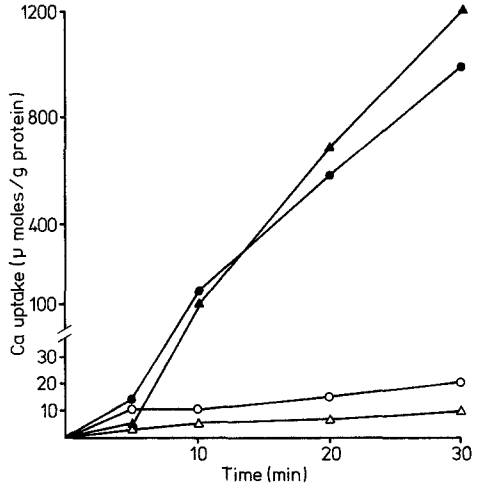

Figure 3. Time course of ATP-dependent calcium uptake by ER (triangles) and PM (circles) in the absence (open symbols) and presence (closed symbols) of 10 mM oxalate. Each point is the mean of 4–5 determinations.

Acknowledgments. I thank Bodil Nilsson and Bengt Nordgren for technical assistance. This study was supported by the Swedish Medical Research Council (Project No. 04X-07150-01A).

1 Batra, S., in: Vascular Neuroeffector Mechanisms, p. 3. Eds J.A. Bevan, T. Godfraind, R.A., Maxell and P.M. Vanhoulte. Raven Press, New York 1980.
2 Matlib, M.A., Crankshaw, J., Garfield, R.E., Crankshaw, D.J., Kwan, C.Y., Branda, L.A., and Daniel, E.E., J. biol. Chem. *254* (1979) 1834.
3 Vallieres, J., Fortier, M., Somlyo, A.V., and Somlyo, A.P., Am. J. Biochem. *9* (1978) 487.
4 Sachs, G., Jackson, R.J., and Rabon, E.C., Am. J. Physiol. *238* (1980) G 151.
5 Wibo, M., Morel, N., and Godfraind, T., Archs int. Pharmacodyn. *250* (1981) 333.
6 Ford, G.D., and Hess, M.L., Am. J. Physiol. *242* (1982) 242.
7 Kwan, C.Y., Am. J. Physiol. *244* (1983) 115.
8 Kwan, C.Y., Grover, A.K., Triggle, C.R., and Daniel, E.E., Biochem. Int. *6* (1983) 713.
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1981) 265.
10 Sanui, H., Analyt. Biochem. *60* (1974) 489.

11 Batra, S., Acta physiol. scand. *104* (1978) 68.
12 Batra, S., Acta physiol. scand. *114* (1983) 91.
13 Andersson, G.F., and Marks, B.H., J. Pharmac. exp. Ther. *221* (1982) 598.
14 Nilvebrant, L., and Sparf, B., Acta pharmac. toxic. *52* (1983) 30.
15 Taft Jr, W.C., Abdel-Latif, A.A., and Akhtar, R.A., Biochem. Pharmac. *29* (1980) 2713.
16 Batra, S.C., and Daniel, E.E., Comp. Biochem. Physiol. *38* (1971) 369.
17 Batra, S., in: Excitation-contraction Coupling in Smooth Muscle, p.225. Eds R. Casteels, T. Godfraind and J.C. Ruegg, Elsevier/North-Holland, Amsterdam 1977.
18 Daniel, E.E., Grover, A.K., and Kwan, C.Y., Fedn Proc. *41* (1982) 2898.
19 Raeymaekers, L., Wuytack, F., Eggermont, J., Schutter, G., and Casteels, R., Biochem. J. *210* (1983) 315.
20 Anderson, G.F., J. Pharmac. exp. Ther. *220* (1982) 347.

0014-4754/86/060608-04$1.50 + 0.20/0

Methyllycaconitine, a naturally occurring insecticide with a high affinity for the insect cholinergic receptor

K.R. Jennings*, D.G. Brown and D.P. Wright Jr[1]

Agricultural Research Division, American Cyanamid Co., P.O. Box 400, Princeton (New Jersey 08540, USA), 8 May 1985

Summary. Studies of extracts of *Delphinium* seeds, long known to be insecticidal, revealed that a principal insecticidal toxin was methyllycaconitine, which is shown to be a potent inhibitor of α-bungarotoxin binding to housefly heads ($K_{inh} = 2.5 \times 10^{-10} \pm 0.5 \times 10^{-10}$M).
Key words. Insecticide; nicotinic receptor; methyllycaconitine; *Delphinium* alkaloids.

The extract of seeds from *Delphinium* has a long history of being used as an insecticide. Pliny the Elder described the use of this seed extract as a topical treatment for 'vermin in the head and other parts of the body'[2].

Recently, work on toxic components of *Delphinium brownii*, a cattle-stock poison in Western Canada, has led to the identification of a principal toxic component, methyllycaconitine (MLA, fig. 1) which displays neuromuscular blocking activity at nicotinic receptors in a rat phrenic nerve-diaphragm preparation[3]. We report here the results of a study on the insecticidal activity of the seeds of *Delphinium* hybrid, cv. 'Pacific Giant, King Arthur' and the identification of a possible site of action at the insect nicotinic receptor.

Chloroform extracts of crushed seeds of the *Delphinium* plant were tested for insecticidal activity against a number of species of insects and mites (table) and found to produce mortality and protect leaves from feeding damage. This extract was also tested for nicotinic activity against insect cholinergic receptors in an assay measuring the inhibition of [^{3}H]-propionyl-α-bungarotoxin (^{3}H α-BGTx) binding to *Musca domestica* head homogenate[4]. α-Bungarotoxin is an antagonist of nicotinic acetylcholine receptors in insects[5], having a $K_D = 1.1 \pm 0.1$ nM. The *Delphinium* seed extract displayed a very potent inhibition of binding of the radioligand to the receptor, being much more potent than the standard, nicotine. In an effort to identify this cholinergic agent, extracts of *Delphinium* seeds were separated into alkaloidal and non-alkaloidal fractions. Both the cholinergic and insecticidal activities were found to reside in the alkaloidal fraction. The alkaloidal fraction was further separated by preparative silica gel thin layer chromatography (TLC) yielding 6 clearly defined alkaloidal fractions. One of these, the major alkaloid, with an R_f of 0.43 on silica gel (Merck, Silica Gel 60, 0.25 mm) when eluted with cyclohexane:chloroform:diethylamine (5:4:1) was found to have very potent cholinergic activity, having a K_{inh} value for displacing ^{3}H α-BGTx of less than 0.5 nM. This fraction was chemically characterized by mass spectrometry, proton and C^{13} NMR spectroscopy and identified as methyllycaconitine. This MLA fraction was also tested and found to display good insecticidal activity against *Spodoptera eridania* and *Musca domestica* (data not shown). A sample of MLA.citrate was obtained and tested for activity in the insect nicotinic cholinergic receptor assay. The MLA.citrate displayed identical cholinergic activity to that observed for the MLA alkaloid fraction purified from *Delphinium* seeds by TLC (fig. 2). The K_{inh} value calculated for MLA.citrate was $2.5 \times 10^{-10} \pm 0.5 \times 10^{-10}$ M. The cholinergic activity of this alkaloid at the insect nicotinic receptor is much more potent than that reported for the rat muscle receptor, where the ED_{50} was determined to be 2.3×10^{-6} M[3]. Aconitine, another aconite alkaloid reported to be more potent than MLA at the rat muscle nicotinic receptor, was found to be considerably less active than MLA at the insect receptor, having a K_{inh} of $2.7 \times 10^{-4} \pm 0.8 \times 10^{-4}$ M. Lycoctonine, which lacks the aromatic ester function of MLA (fig. 1), also inhibited 3H α-BGTx binding to the insect cholinergic receptor, but with a K_{inh} of $3.8 \times 10^{-7} \pm 0.6 \times 10^{-7}$ M. In addition to its lower potency at inhibiting ^{3}H α-BGTx binding, it was also ineffective as an insecticide when tested against *Spodoptera eridania*.

The rank order of potency for inhibition of α-bungarotoxin binding in the insect preparation is MLA > lycoctonine > aconitine; in the rat phrenic nerve-diaphragm preparation[3]

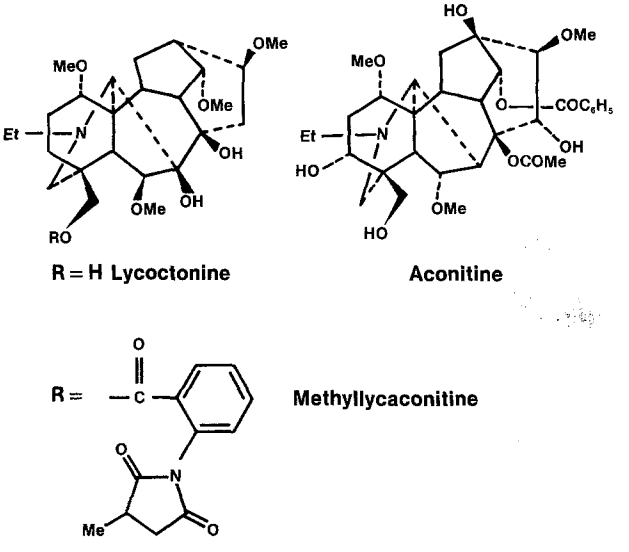

Figure 1. Structures of aconite alkaloids investigated.