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MUSCARINIC CHOLINERGIC ENHANCEMENT OF INOSITIDE TURNOVER IN CEREBRAL NERVE ENDINGS IS NOT MEDIATED BY CALCIUM UPTAKE

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Abstract—Muscarinic cholinergic stimulation of rat cerebral nerve endings incubated with $^{32}P_i$ causes an enhancement of the labeling of phosphatidic acid (PA) and phosphatidylinositol (PI). The involvement of Ca^{2+} in the stimulation of PA and PI labeling by carbamylcholine (CCh) was investigated. Enhancement of Ca^{2+}-influx with veratridine and the Ca^{2+}-ionophore A23187 caused a vast decrease of the labeling of the polyphosphoinositides, which was not accompanied by an enhancement of the labeling of PA and PI. The dihydropyridine Ca^{2+}-agonist BAY K 8644 did not affect phospholipid labeling. A23187, veratridine and BAY K 8644 did not enhance stimulation of the labeling of PA and PI by CCh. When Ca^{2+} was omitted from the incubation, A23187 caused an enhancement of basal and CCh-stimulated labeling of PA and PI, possibly indicating a particular feature of A23187 unrelated to its iontophoretic properties. The Ca^{2+}-channel antagonists nimodipine, verapamil and flunarizine were virtually without effect on basal and CCh-stimulated labeling of PI and PA. These data support the notion that the muscarinic cholinergic inositide response is not mediated or controlled by Ca^{2+}-flux.

The enhanced operation of the inositide cycle upon the activation of specific receptors is a well established phenomenon [1–3]. It is generally accepted that the initial event following receptor activation is phosphodiesteratic degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2)* to diacylglycerol and myo-inositol 1,4,5-trisphosphate. The latter is degraded by specific phosphatases. Diacylglycerol is rapidly phosphorylated to phosphatidate (PA), which is converted through a liponucleotide-intermediate to phosphatidylinositol (PI). In two sequential phosphorylation steps, PI is converted to PIP_2, via phosphatidylinositol 4-phosphate (PIP), thus closing the cycle. Receptor-mediated enhancement of the inositide cycle is generally analyzed in two ways. First, the enhanced labeling of PA from ^{32}P reflects the generation of diacylglycerol. Radiolabeled PA is then converted into [^{32}P]-PI. Secondly, under incubation with [^3H]-inositol, radiolabeled inositol phosphates can be measured, especially in the presence of Li^+, which inhibits degradation of myo-inositol 1-phosphate [4]. Both diacylglycerol and myo-inositol 1,4,5-trisphosphate have been implicated in serving a second messenger function. Diacylglycerol can enhance the Ca^{2+}-sensitivity of protein kinase C [5]. Myo-inositol 1,4,5-trisphosphate can stimulate the release of Ca^{2+} from endoplasmic reticular stores [6].

In a number of systems the inositide response to receptor activation is insensitive to depletion of extracellular Ca^{2+} (for refs. 1–3). From the concurrency of receptor mediated enhancement of the inositide cycle and receptor mediated Ca^{2+}-dependent events such as muscle contraction or vesicular secretion, it was suggested that the inositide cycle would trigger the influx of Ca^{2+}, which in turn would initiate the cellular response [1]. Likewise in ileum smooth muscle fragments muscarinic stimulation of PI-labeling was insensitive to various Ca^{2+}-channel antagonists [7]. However, it has been reported that the muscarinic inositide response in iris smooth muscle and nerve endings is abolished by depletion of Ca^{2+} from the medium with the use of chelators, such as EGTA [8–10]. In addition, the presence of the Ca^{2+}-ionophore A23187 has been reported to result in a synergistic enhancement of the muscarinic inositide response in cerebral nerve endings [11]. Since phosphodiesteratic degradation of PIP_2 is a Ca^{2+}-requiring event [12, 13], a Ca^{2+}-influx may here precede and perhaps even mediate the muscarinic inositide response. Alternatively, the inositide effect is dependent on the presence of a minimal level of Ca^{2+}.

In view of the role of Ca^{2+} in neurotransmission, it is of particular interest in the nervous system as to which of these various possibilities most accurately describes the relationship of Ca^{2+}-distribution to receptor mediated enhancement of the inositide turnover. Therefore we have conducted a study to investigate further the role of Ca^{2+} in the enhancement of the inositide turnover in muscarinic cholinergically stimulated nerve endings from rat forebrain.

MATERIALS AND METHODS

Nerve endings were prepared by differential and sucrose gradient centrifugation [10, 11] with minor modification [14]. Briefly, male Wister rats (Win-

* Abbreviations used: CCh, carbamylcholine; EGTA, ethyleneglycolbis(β-aminoethylether)-*N'N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

kelmann, F.R.G.) were decapitated and the forebrain was rapidly excised and placed in 9 vol. of 0.32 M sucrose. Following homogenization (10 strokes, 1200 rpm, Potter-Elvehjem) the material was centrifuged for 10 min at 1000 *g* (Sorvall, SS-34 rotor). The supernatant was collected and the pellet was centrifuged again after rehomogenization (4 strokes) in 5 vol. of 0.32 M sucrose. The combined supernatants were centrifuged for 20 min at 10,000 *g* (Sorvall, SS-34 rotor). The supernatant was discarded and the pellet, resuspended in 0.32 M sucrose was layered on top of a discontinuous gradient consisting of 15 ml of 1.2 M and 15 ml of 0.8 M sucrose. Following centrifugation for 2 hr at 23,500 rpm (Beckman SW 28 rotor) the 0.8:1.2 M interface was collected. After careful dilution of the interface with 1 vol. of 0.16 M sucrose, nerve endings were pelleted by centrifugation for 45 min at 27,000 *g* (Sorvall SS-34) and resuspended in incubation medium (30 mM HEPES, 142 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl_2$, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 5.6 mM glucose; pH 7.4). In experiments without Ca^{2+}, $CaCl_2$ was omitted from the medium. All procedures were carried out at 0–4°.

Nerve endings (about 0.7 mg of protein) were incubated in a final volume of 0.5 ml incubation medium with various drugs and 10–20 μCi of $^{32}P_i$ (carrier free, Amersham, Braunschweig, F.R.G.) for 30 min at 37° in a water bath with shaking [14, 15]. The incubation was terminated by the addition of 1.5 ml of chloroform:methanol (1:2, by vol.), and phospholipids were extracted under acidic conditions [13] and separated by thin layer chromatography of K-oxalate impregnated TLC plates (Merck, Darmstadt, F.R.G., silica gel 60, 20 × 20 × 0.025 cm), using the solvent chloroform:acetone:methanol:acetic acid:H_2O (40:15:13:12:7, by vol.) [13, 16]. Following localization by iodine staining and autoradiography, labeled bands were scraped off, and radiotracer was quantitated by scintillation counting.

Protein was determined spectrophotometrically using bovine serum albumin standard [17]. All chemicals were reagent grade or better and obtained through commercial sources. Verapamil was from Knoll AG (Ludwigshafen, F.R.G.), veratridine from Sigma Chemie GmbH (Taufkirchen, F.R.G.), A23187 from Calbiochem (San Diego, CA, U.S.A.), flunarizine from Janssen Pharmaceutica (Beerse, Belgium). BAY K 8644 and nimodipine were locally synthesized (Bayer AG, Wuppertal-Elberfeld, F.R.G.). Statistical analysis was performed using Student's *t*-test (two-tailed).

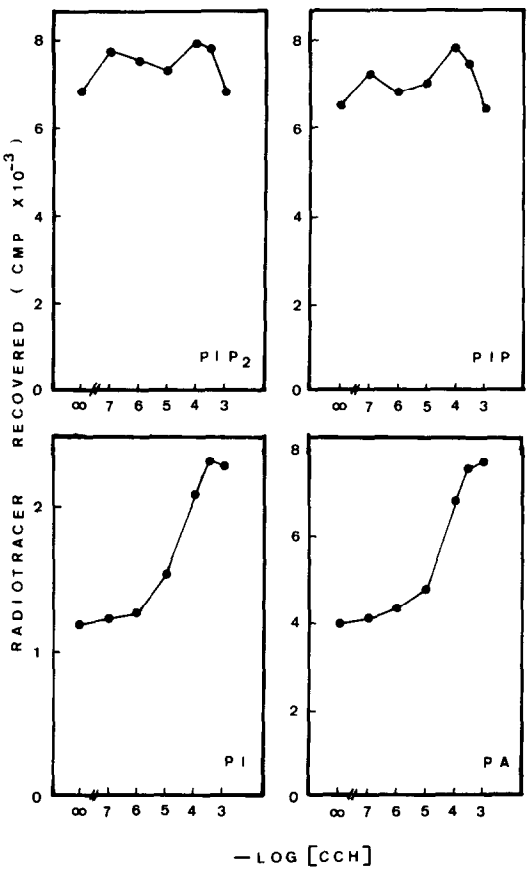

Fig. 1. Effect of carbamylcholine on the phospholipid labeling in nerve endings. Nerve endings were incubated as described in Materials and Methods with various concentrations (M) of CCh. Data are from an experiment in duplicate (range < 10%), representative of ten.

RESULTS

Incubation of nerve endings with the muscarinic cholinergic agonist carbamylcholine (CCh) caused a concentration-dependent enhancement of the label-

Table 1. Involvement of Ca^{2+} in effects of A23187 and CCh on phospholipid labeling in nerve endings

Additions		PIP_2		PIP		PI		PA	
Ca	A23187	Control	CCh	Control	CCh	Control	CCh	Control	CCh
−	−	100 ± 6	88 ± 5	100 ± 3	95 ± 5	100 ± 4	128 ± 4^{b}	100 ± 2	167 ± 8^{b}
−	+	98 ± 2	68 ± 4^{b,d}	148 ± 10^{d}	77 ± 8^{b}	126 ± 4^{e}	219 ± 26^{a,d}	163 ± 12^{d}	321 ± 30^{a,d}
+	−	61 ± 6	58 ± 5	71 ± 2	59 ± 5	81 ± 8	148 ± 9^{b}	101 ± 2	172 ± 10^{b}
+	+	12 ± 2^{e}	10 ± 1^{e}	12 ± 3^{e}	12 ± 1^{e}	74 ± 4	103 ± 8^{a,d}	81 ± 8^{c}	159 ± 4^{b}

Nerve endings were incubated as described in Materials and Methods. The additions were 10^{-5} M A23187 and 10^{-3} M CCh as indicated. The presence (+) or omission (−) of Ca^{2+} from the medium is indicated. Data (mean ± SEM, N = 6) are from two independent experiments and expressed as percent of the values obtained in an incubation without Ca, A23187 and CCh. The values were significantly different from those in the absence of CCh when [a] $P \leq 0.02$ or [b] $P \leq 0.001$ and from those in the absence of A23187 when [c] $P \leq 0.05$, [d] $P \leq 0.02$ and [e] $P \leq 0.001$.

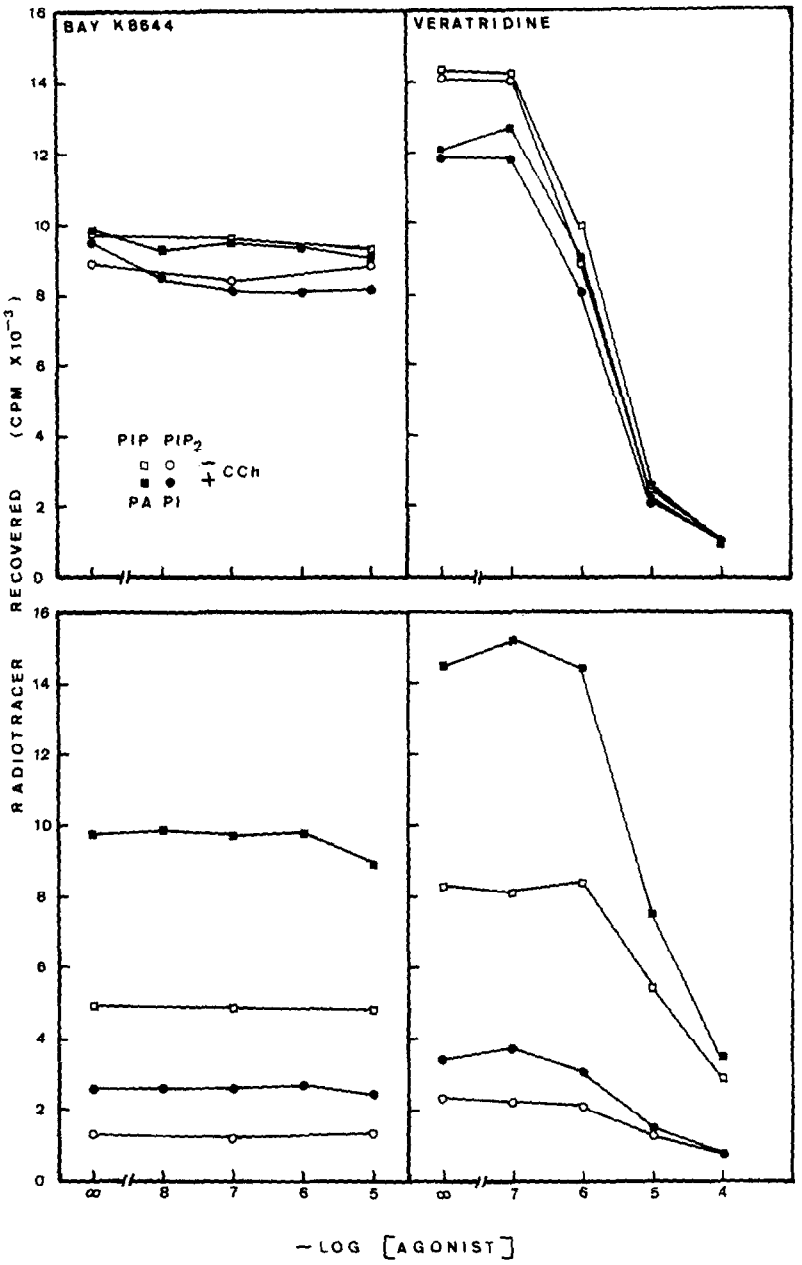

Fig. 2. Effects of BAY K 8644 and veratridine on basal and CCh-stimulated phospholipid labeling in nerve endings. Nerve endings were incubated as described in Materials and Methods in the presence of various concentrations (M) of BAY K 8644 (left) or veratridine (right) without (○, □) or with (●, ■) 1 mM CCh. Radiotracer recovered as different phospholipids is shown in the top part for PIP_2 (○, ●) and PIP (□, ■) and in the bottom part for PI (○, ●) and PA (□, ■). Data are from experiments in duplicate (range < 5%). In an independent experiment 10^{-5} M BAY K 8644 also appeared without an effect on basal and CCh-stimulated labeling. Veratridine data are representative of three independent experiments.

ing of PA and PI from $^{32}P_i$ (Fig. 1), in agreement with previous reports [9–11, 14, 15, 18]. Optimal concentrations of CCh were found at 3×10^{-4} M and above. Labeling of PIP_2 and PIP was not affected by the addition of CCh.

The effects of the Ca^{2+}-ionophore A23187 on the CCh-induced enhancement of the labeling of PA and the inositides was studied in the absence and presence of Ca^{2+} in the incubation medium (Table 1). When Ca^{2+} was omitted from the medium, 1 mM CCh was somewhat less effective in stimulating labeling of PA and PI than in the presence of Ca^{2+}. A23187 caused a stimulation of PA and PI labeling in the absence of Ca^{2+}, which was comparable to that seen with CCh. Concurrent addition of A23187 and CCh resulted in a much larger stimulation of the labeling of PA and PI than seen with either substance alone. In the presence of Ca^{2+}, however, A23187

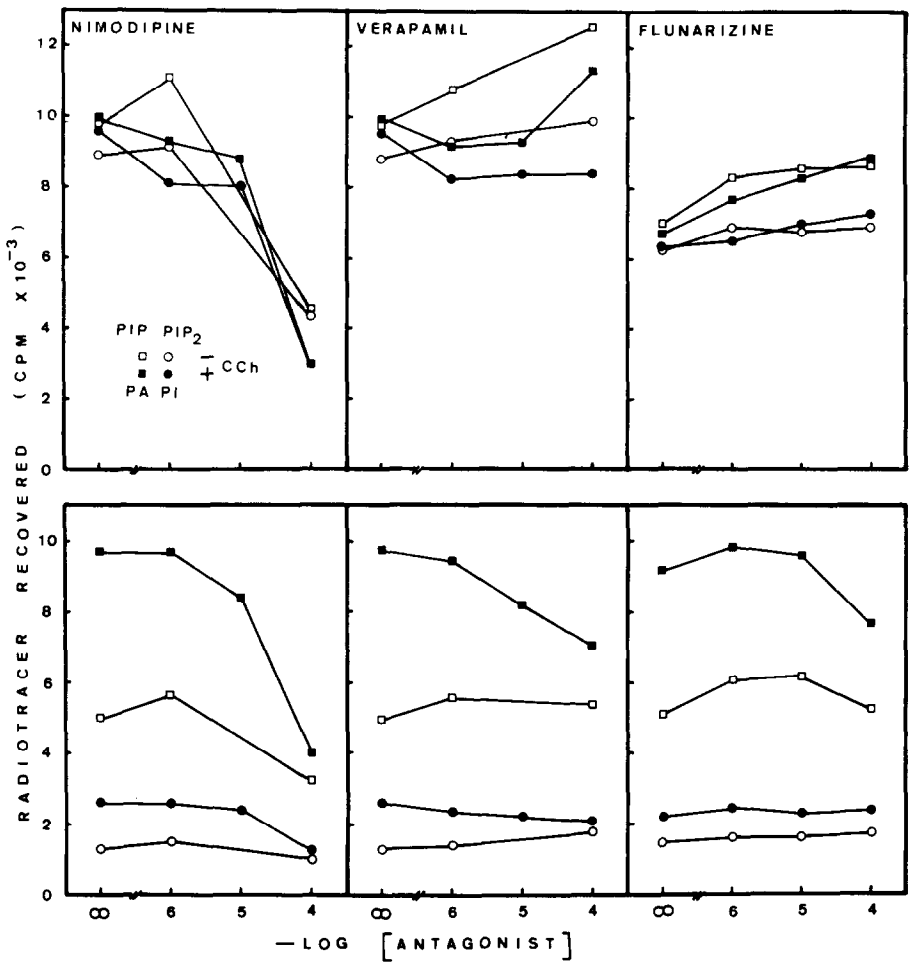

Fig. 3. Effects of nimodipine, verapamil and flunarizine on basal and CCh-stimulated phospholipid labeling in nerve endings. Nerve endings were incubated as described in Materials and Methods in the presence of various concentrations (M) of nimodipine (left), verapamil (middle) and flunarizine (right), without (○, □) or with (●, ■) 1 mM CCh. Radiotracer recovered as different phospholipids is shown in the top part for PIP_2 (○, ●) and PIP (□, ■) and in the bottom part for PI (○, ●) and PA (□, ■). Data are from experiments performed in duplicate (range < 10%). The nimodipine and verapamil data are representative of two independent experiments. In an independent experiment 10^{-4} M flunarizine also appeared without an effect on basal and CCh-stimulated labeling.

did not enhance basal and CCh-stimulated labeling of PA and PI. In fact, a slight decrease of the basal and CCh-stimulated labeling could be seen. The effect of A23187 on the labeling of the polyphosphoinositides varied with the presence or absence of Ca^{2+} in the medium (Table 1). In the absence of the cation, A23187 stimulated basal labeling of PIP while that of PIP_2 was not affected. In the presence of Ca^{2+}, A23187 caused a reduction of the labeling of both polyphosphoinositides. Only when A23187 was present and Ca^{2+} omitted, CCh caused a decrease of the labeling of both PIP_2 and PIP.

The effect of veratridine and BAY K 8644 on basal and CCh-stimulated phospholipid labeling is shown in Fig. 2. Veratridine caused a dose-dependent reduction of the labeling of all four phospholipids. At high concentrations of veratridine, the labeling of PIP_2 and PIP was almost abolished. At no concentration of veratridine tested did we find an enhancement of the basal and CCh stimulated labeling of PA and PI. The Ca^{2+}-channel activating dihydropyridine BAY K 8644, at the various concentrations tested, did not affect either basal or CCh-stimulated labeling. Omitting Ca^{2+} from the medium did not alter the effects of veratridine and BAY K 8644 (data not shown).

The effect of three different Ca^{2+}-channel antagonists on basal and CCh-stimulated phospholipid labeling is shown in Fig. 3. Nimodipine caused an inhibition of the CCh-stimulated labeling of PA and PI, but only at the high concentration of 10^{-4} M. Labeling of PIP_2 and PIP was also diminished in the presence of nimodipine. Likewise only high concentrations of verapamil caused a small inhibition of the CCh stimulated labeling of PA and PI. A minimal inhibition of the CCh-stimulated labeling of PA and PI was obtained with flunarizine. Both verapamil and flunarizine produced a small enhancement of the labeling of PIP.

DISCUSSION

Stimulation of Ca^{2+}-uptake with veratridine, BAY K 8644 or the ionophore A23187 did not mimic or enhance the CCh-induced stimulation of PA and PI labeling. Furthermore, the Ca^{2+}-channel blockers nimodipine, verapamil and flunarizine could not inhibit the inositide response to CCh. These data indicate that the muscarinic cholinergic enhancement of labeling of PA and PI in nerve endings is not mediated or controlled by a Ca^{2+}-influx.

In the absence of Ca^{2+}, A23187 and CCh synergistically enhanced labeling of PA and PI. This observation is in accordance with a previous report [11]. However, we found that the addition of Ca^{2+} to the medium abolished the stimulatory effects of A23187. In addition, neither veratridine nor BAY K 8644 could mimic or enhance the CCh-stimulation of PA and PI labeling. It seems unlikely that A23187 acts by overcoming an intracellular limitation of Ca^{2+} since the stimulation by CCh in the presence of A23187 without Ca^{2+} in the medium was much larger than that obtained in the presence of Ca^{2+}. It is suggested that the potentiating effect of A23187 on the CCh-induced labeling of PA and PI, which is observed only when Ca^{2+} is omitted from the medium, is a particular feature of A23187 and is possibly not related to its iontophoretic properties.

In the presence of A23187, CCh caused a decreased labeling of PIP_2 and PIP. Under control conditions, no such decrease of the labeling of the polyphosphoinositides by CCh is seen (see also [11]). Since the polyphosphoinositides are labeled with ^{32}P to equilibrium, the absence of a decrease may be explained by a rapid replenishment of the PIP_2 through PIP from PI, as suggested by Downes and Wüsteman [19]. A control thereof occurs by a possible product-feedback inhibition of the PI- and PIP-kinases [20], or an indirect "turn off" mechanism involving diacylglycerol activation of the protein kinase C resulting in an inhibition of PIP kinase [21]. The efficiency of the controlling system for the levels of PIP_2 and PIP may differ between various systems. For instance, muscarinic cholinergic stimulation of parotid acinar cells caused a decreased labeling of PIP_2 [22]. Therefore it is possible that A23187 interferes with the replenishment-control of the polyphosphoinositides.

Based on structural features and lipophilicity, Ca^{2+}-channel antagonists can be divided into three different groups: dihydropyridines, diphenylalkylamines and other [23]. We have found that an example from each group, being nimodipine, flunarizine and verapamil respectively, was virtually unable to inhibit the inositide response to CCh. In accord with these findings is the report that several Ca^{2+}-antagonists were unable to inhibit the CCh-induced inositide effect in guinea pig ileum [7]. Several reports have appeared recently to indicate that the Ca^{2+}-channel antagonists can inhibit Ca^{2+}-uptake into nerve endings with similar potency as that found in peripheral preparations [24–33]. If in the present study these channels were activated in the course of the muscarinic acetylcholine receptor-mediated enhancement of PA and PI labeling, an inhibition by the Ca^{2+}-channel antagonists most likely would have been observed. At concentrations at which the different antagonists are known to be capable of inhibiting synaptosomal Ca^{2+}-uptake, no effects on CCh-stimulated PA and PI labeling were seen. It is possible that at the high concentrations of Ca^{2+}-channel antagonists required to cause an inhibition of the CCh-stimulated PA and PI labeling, a less specific and general deterioration of the nerve endings underlies the effects observed. Therefore, inhibition of Ca^{2+}-uptake seems not to inhibit the inositide response to muscarinic acetylcholine receptor activation in nerve endings.

EGTA has, however, been found to inhibit the receptor mediated inositide response [8–10]. Since Ca^{2+}-flux across the plasma membrane should be considered as a very dynamic process, chelation of extracellular Ca^{2+} by EGTA most likely will have its effect on the intracellular availability of the cation as well [34]. An indirect effect of EGTA on Ca^{2+}-dependent events, such as the inositide phosphodiesterase, is hence to be expected. The data from studies with EGTA should therefore not be taken as evidence for a Ca^{2+}-flux to mediate the stimulated inositide turnover to receptor-activation, but may only indicate that the cation is involved in the response. It is concluded that muscarinic acetylcholine stimulation of inositide turnover in nerve endings is not mediated by an influx of Ca^{2+}. This conclusion consents to the notion that receptor-mediated enhanced operation of the inositide cycle triggers the intracellular mobilization of Ca^{2+}, rather than that it is a consequence thereof.

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