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Different mechanisms of Ca^{2+}-handling following nicotinic acetylcholine receptor stimulation, P_{2U}-purinoceptor stimulation and K^+-induced depolarization in C2C12 myotubes

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1 The increase in intracellular Ca^{2+} on nicotinic acetylcholine receptor (nAChR) stimulation, P_{2U}-purinoceptor stimulation and K^+-induced depolarization was investigated in mouse C2C12 myotubes by use of fura-2 fluorescence to characterize the intracellular organisation of Ca^{2+} releasing stores and Ca^{2+}-entry process.

2 Stimulation of nAChRs with carbachol induced a rapid rise in internal Ca^{2+} ($EC_{50}=0.85\pm0.09$ μM), followed by a sustained phase. The Ca^{2+} response evoked by carbachol (10 μM) was completely blocked by the nAChR antagonist, pancuronium (3 μM), but was not affected by the muscarinic antagonist, atropine (3 μM), or under conditions when Ca^{2+} entry was blocked by La^{3+} (50 μM) or diltiazem (10 μM). Addition of pancuronium (3 μM) during the sustained phase of the carbachol-evoked response did not affect this phase.

3 Stimulation of P_{2U} purinoceptors with ATP (1 mM) induced a somewhat higher biphasic Ca^{2+} response (EC_{50} of the rapid phase: 8.72 ± 0.08 μM) than with carbachol. Pretreatment with La^{3+} abolished the sustained phase of the ATP-induced Ca^{2+} response, while the response was unaffected by diltiazem or pancuronium.

4 Stimulation of the cells with high K^+ (60 mM), producing the same depolarization as with carbachol (10 μM), induced a rapid monophasic Ca^{2+} response, insensitive to diltiazem, pancuronium or La^{3+}.

5 Under Ca^{2+}-free conditions, the sustained phase of the carbachol- and ATP-evoked responses were abolished. Pre-emptying of depolarization-sensitive stores by high K^+ under Ca^{2+}-free conditions did not affect the carbachol- or ATP-evoked Ca^{2+} mobilization and *vice versa*. Preincubation of the cells with ATP in the absence of extracellular Ca^{2+} decreased the amplitude of the subsequent carbachol-induced Ca^{2+} response to 11%, while in the reverse procedure the ATP-induced response was decreased to 65%. Ca^{2+} mobilization evoked by simultaneous addition of optimal concentrations of carbachol and ATP was increased compared to levels obtained with either agonist.

6 Preincubation with high K^+ under normal conditions abolished the sustained phase of the ATP-evoked Ca^{2+} response. The carbachol response consisted only of the sustained phase in the presence of high K^+.

7 The carbachol-induced Ca^{2+} response was completely abolished under low Na^+/Ca^{2+}-free conditions, while under low Na^+ conditions only a sustained Ca^{2+} response was observed. The ATP- and K^+-induced responses were changed compared to Ca^{2+}-free conditions.

8 ATP (300 μM) induced the formation of $Ins(1,4,5)P_3$ under Ca^{2+}-free conditions with a comparable time course to that found for the rise in internal Ca^{2+}. In contrast to ATP, carbachol (10 μM) did not affect $Ins(1,4,5)P_3$ levels under Ca^{2+}-free conditions.

9 It is concluded that the Ca^{2+} release from discrete stores of C2C12 myotubes is induced by stimulation of nAChRs, P_{2U}-purinoceptors and by high K^+. Only the P_{2U}-purinoceptor and nAChR activated stores show considerable overlap in releasable Ca^{2+}. Sustained Ca^{2+}-entry is activated by stimulation of nAChRs and P_{2U}-purinoceptors via separate ion-channels, which are different from the skeletal muscle nAChR-coupled cation-channel.

Keywords: Calcium; P_2-purinoceptor; nucleotide receptor; nicotine acetylcholine receptor; C2C12 myotubes; intracellular calcium store; $Ins(1,4,5)P_3$

Introduction

The increase in intracellular Ca^{2+} of skeletal myotubes has been shown to modulate important functional changes in these cells related to neurotransmission. A short-term increase in cellular Ca^{2+} enhances the rate of desensitization of nicotine acetylcholine receptors (nAChRs; Miledi, 1980), while a long-term increase in Ca^{2+} is involved in down-regulation of nAChRs (Smilowitz *et al.*, 1988; Klarsfeld *et al.*, 1989; Bursztajn *et al.*, 1991) and synthesis of specific acetylcholinesterase isoenzymes (Inestrosa *et al.*, 1983; Fernandez & Hodges-Savola, 1992).

Stimulation of myotubes with the transmitter of motor neurones (acetylcholine), its co-transmitter ATP (Silinsky, 1975) and depolarization of the myotubes have all been reported to increase intracellular Ca^{2+} in myotubes. In myotubes of different origin, the ACh-sensitive receptor which is involved in augmenting Ca^{2+} has invariably been characterized as the muscle type nicotinic receptor (Giovannelli *et al.*, 1991; Grassi *et al.*, 1993). Characterization of ATP-sensitive re-

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ceptors (P_2-purinoceptors) is still largely based on their sensitivity to ATP analogues (Cusack & Hourani, 1990), because of the lack of a specific antagonist at cloned receptors (Webb *et al.*, 1993; Lustig *et al.*, 1993). With the exception of mouse C2C12 myotubes, in which the augmentation of Ca^{2+} is mediated by a P_{2U}-purinoceptor (Henning *et al.*, 1993a), the P_2-purinoceptors involved in other types of myotubes do not fit the proposed subclassification (Cusack & Hourani, 1990).

C2C12 myotubes possess both a ryanodine-sensitive Ca^{2+} store (sarcoplasmic reticulum) and inositol-(1,4,5) trisphosphate (Ins(1,4,5)P_3)-sensitive Ca^{2+} stores, as judged by functional responses (Grassi *et al.*, 1993; Henning *et al.*, 1993a) and expression of ryanodine and Ins(1,4,5)P_3 receptors (Arai *et al.*, 1992; De Smedt *et al.*, 1994). Stimulation of both nAChR and P_{2U}-purinoceptors in C2C12 myotubes increases intracellular Ca^{2+}, due to mobilizing Ca^{2+} from internal stores as well as initiating Ca^{2+}-entry from the extracellular space. The depolarization caused by extracellular application of high K^+ to the myotubes is thought to cause Ca^{2+} release from the ryanodine-sensitive store via calcium-induced calcium release following the influx of Ca^{2+} via voltage-sensitive L-type Ca^{2+}-channels and through a direct molecular interaction between the L-type Ca^{2+} channel and the ryanodine receptor (Schneider & Chandler, 1973; Meissner, 1994). It has been proposed that both nAChRs (Giovannelli *et al.*, 1991; Grassi *et al.*, 1993) and P_{2U}-purinoceptors (Henning *et al.*, 1993a) activate the phosphoinositide pathway to mobilize Ca^{2+} from internal stores in C2C12 myotubes, which would be remarkable as both types represent receptors from very different families (Noda *et al.*, 1983; Webb *et al.*, 1993; Lustig *et al.*, 1993). The similarities of the signal transduction pathway activated by nicotinic and P_2-purinoceptor agonists or high K^+ were characterized and their interaction was studied by measuring cytoplasmic Ca^{2+} in C2C12 myotubes.

Methods

Cell culture

C2C12 cells, a murine myoblast cell line (Yaffee & Saxel, 1977) were obtained from the American Tissue Type Collection, Rockville, U.S.A. and cultured on glass cover slips at 37°C in Dulbecco's modified essential medium, 7 mM $NaHCO_3$ and 10 mM HEPES (DMEM) supplemented with 10% foetal calf serum. When cells reached confluence, the medium was changed to DMEM supplemented with 5% horse serum. Myotubes were used 5–7 days after initiating myoblast fusion.

Intracellular Ca^{2+} measurement

Cytoplasmic free Ca^{2+} levels were determined by fura-2 fluorescence. Cells plated on glass coverslips were loaded with fura-2-AM (3 μM) for 45 min at 37°C in buffer supplemented with bovine serum albumin (BSA) (0.2%). Before the experiment, cells were washed three times with the buffer of the following composition (mM): NaCl 145, KCl 6, $CaCl_2$ 1.0, $MgCl_2$ 0.5, glucose 10, HEPES 10 (pH 7.4). In the Ca^{2+}-free buffer, $CaCl_2$ was omitted and EGTA (0.4 mM) and $MgCl_2$ (5.2 mM) were added. In the low Na^{2+} buffers, 130 mM NaCl was replaced by glucaminchloride. Recordings were made at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm at 22°C using a fluorescence spectrophotometer (Aminco Bowman 2). Application of the agonists and antagonists did not change the autofluorescence of the myotubes. The actual internal Ca^{2+} concentration of the myotubes could not be determined, because the cells instantly peeled off the cover slips after treatment with permeabilizing agents (e.g. Triton-X or saponin). Therefore, the data are expressed as the ratio between the two wavelengths representing Ca^{2+}-free (380 nm) and Ca^{2+}-bound (340 nm) fluorescent probe.

Ins(1,4,5)P_3 measurement

Ins(1,4,5)P_3 contents of the myotubes was assessed by mass measurement with a radioligand binding assay as described by Henning *et al.* (1993a). Before the experiment, the cells were washed two times in the standard buffer and a final wash was performed with the Ca^{2+}-free buffer. The reaction was stopped by addition of trichloroacetic acid (TCA) after removing the buffer. TCA was extracted 3 times with water-saturated diethylether, the samples were neutralized with 5%, KOH and stored at −40°C. A standard curve for determination of Ins(1,4,5)P_3 mass was constructed with ether-extracted TCA solution.

Data analysis

Data are presented as mean ± s.e.mean and are considered statistically different at $P<0.05$ (paired or unpaired Student's t test). Concentration-response curves were fitted to the averaged data by a least-squares non-linear regression programme (SigmaPlot 4.0, Jandel Scientific).

Drugs

Fura-2-AM were obtained from Boehringer, Manneheim (Germany). Adenosine 5'-triphosphate (ATP) was obtained from Serva, Heidelberg (Germany). Carbachol was obtained from Sigma, St Louis (U.S.A.).

Results

Stimulation of differentiated C2C12 myotubes with the non-hydrolyzable nAChR agonist, carbachol (10 μM) induced a rapid increase of intracellular Ca^{2+}, followed by a sustained phase (Figure 1a). The increase in intracellular Ca^{2+} evoked by carbachol (10 μM) was blocked by preincubation of the cells with the nicotinic antagonist, pancuronium (3 μM; $n=5$; not shown), but was not affected by the muscarinic antagonist, atropine (3 μM; $n=3$; not shown). Stimulation of the myotubes with ATP (1 mM) evoked a similar biphasic change in intracellular Ca^{2+} to that observed with carbachol (Figure 1b). The amplitude of the rapid phase of the response was used to determine the concentration-response relationship for carbachol and ATP (Figure 1c). Stimulation of the myotubes with ATP produced a larger maximum (Figure 2; Table 1) and a larger EC_{50} was observed compared to carbachol-stimulated cells (ATP: EC_{50}: 8.7 ± 0.1 μM, carbachol: EC_{50} 0.85 ± 0.08 μM).

Responses in the absence of external Ca^{2+}

The basal intracellular Ca^{2+} was decreased slightly under Ca^{2+}-free conditions (10 min; fluorescence 340/380 ratio under normal conditions: 1.04 ± 0.02, under Ca^{2+}-free conditions: 0.91 ± 0.04; $n \geq 12$). In the absence of extracellular Ca^{2+}, the sustained phase of the carbachol (10 μM) and ATP-evoked Ca^{2+} response was abolished and maximal amplitudes were decreased to 67% and 72% compared to values obtained in the presence of external Ca^{2+}, respectively (Figures 2a [left panel], 3a [right panel]; Table 1). It is known that stimulation of C2C12 myotubes with carbachol (10 μM) depolarizes the myotubes by about 40 mV (Henning *et al.*, 1994). To study the contribution of this carbachol-induced depolarization to the Ca^{2+} mobilization, carbachol (10 μM) and high K^+ (60 mM), known to produce similar depolarizations, were compared (Henning *et al.*, 1994). Under Ca^{2+}-free conditions, stimulation of the cells with high K^+ induced a rapid monophasic increase in internal Ca^{2+} (Figure 2a [right panel]). The carbachol (10 μM)-evoked Ca^{2+} response was abolished in the presence of high K^+ (60 mM) and *vice versa* (Figure 2a). It was investigated whether this phenomenon was due to activation of a common Ca^{2+} store. The myotubes were stimulated with the agonist, followed by a brief wash (3 min) with Ca^{2+}-free solution allowing re-

polarization without refilling of the Ca^{2+} stores, and subsequently stimulated for the second time. Following this paradigm, repeated stimulation of the cells with the same agonist, i.e. carbachol (10 μM) or high K^+ (60 mM), did not evoke a second rise in internal Ca^{2+} (Figure 2b). In contrast, stimulation with high K^+ followed by the brief wash and subsequent stimulation with carbachol (10 μM) produced a Ca^{2+} response with the same amplitude as that observed without the high K^+ response in advance (Figure 2c [right panel]). In the reverse experiment, when cells were stimulated with carbachol (10 μM) followed by the wash, stimulation with the high K^+ (60 mM) produced a normal Ca^{2+} response (Figure 2c [left panel]). After cessation of the first carbachol-induced depolarization by blocking its nAChRs with pancuronium instead of washing, the subsequent Ca^{2+} response on stimulation with high K^+ was also restored (not shown). Involvement of the depolarization in Ca^{2+} mobilization was further investigated by preventing the carbachol-induced depolarization under low Na^+/Ca^{2+}-free conditions (Henning *et al.*, 1994). Under these conditions, the carbachol-evoked Ca^{2+} mobilization was abolished (Figure 2d [left panel]), whereas the response induced by high K^+ was unaffected (Figure 2d [right panel]).

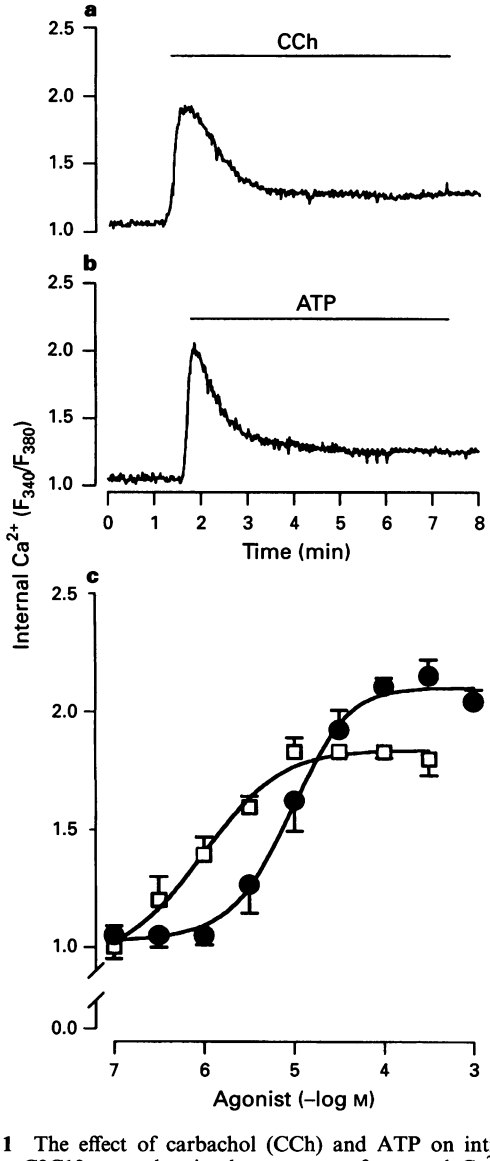

Figure 1 The effect of carbachol (CCh) and ATP on intracellular Ca^{2+} in C2C12 myotubes in the presence of external Ca^{2+}, using fura-2 fluorescence. (a) Typical responses caused by carbachol (10 μM); (b) typical responses to ATP (1 mM); (c) concentration-dependent changes in the amplitude of the peak of the increase in fluorescence (mean ± s.e.mean) caused by carbachol (□, $n \geq 12$) and ATP (●, $n \geq 9$). Data are expressed as fluorescence ratio ($R = F_{340}/F_{380}$).

To study the interaction between the ATP-, carbachol- and high K^+-evoked Ca^{2+} release processes, the myotubes were stimulated with one of the agonists followed by subsequent addition of the other agonist under Ca^{2+}-free conditions. Prestimulation of the cells with carbachol (10 μM) reduced the amplitude of the Ca^{2+} response evoked by ATP (1 mM) to 65% of its control value (Figure 3a [left panel]; Table 1). In the reverse procedure, the Ca^{2+} response evoked by carbachol (10 μM) was strongly decreased to 11% after prestimulation with ATP (1 mM; Figure 3a [right panel]; Table 1). Simultaneous stimulation of the cells with ATP (1 mM) and carbachol (10 μM) resulted in a significant increase in the amplitude of the Ca^{2+} response compared to that evoked by stimulation with either agonist (Table 1). The amplitude of the response evoked by simultaneous addition of ATP and carbachol amounted to 76% of their summed value (Table 1). The Ca^{2+} response induced by ATP (1 mM) was unaffected in the presence of high K^+ (60 mM) and *vice versa* (Figure 3b).

Responses in the presence of external Ca^{2+}

In contrast to the biphasic Ca^{2+} response obtained with ATP and carbachol, high K^+ induced a fast monophasic increase in cellular Ca^{2+} under normal conditions (Figure 4a [right panel]). Interaction between carbachol-, ATP- and high K^+-evoked responses was further investigated by stimulating the myotubes with one of the agonists followed by subsequent addition of another agonist. In the presence of carbachol (10 μM), the monophasic response to high K^+ was completely abolished (Figure 4a [left panel]). In the reverse experiment, stimulation of the cells with carbachol (10 μM) induced a sustained rise in internal Ca^{2+} only in the presence of high K^+ (60 mM; Figure 4a [right panel]). In the presence of high K^+ (60 mM), the rapid phase of the ATP (1 mM)-induced Ca^{2+}

Table 1 The action of nAChR and P_2-purinoceptor agonist on cytoplasmic Ca^{2+} in C2C12 myotubes under normal and Ca^{2+}-free conditions

	Ca^{2+} conditions		*Ca^{2+}-free conditions*	
Agonist	*peak*	*plateau*	*peak*	*plateau*
ATP (1 mM)	1.11 ± 0.04	0.31 ± 0.05	0.80 ± 0.05	–
CCh (10 μM)	0.83 ± 0.07	0.21 ± 0.02	0.56 ± 0.05	–
ATP after CCh	1.02 ± 0.07	–	0.52 ± 0.06	–
CCh after ATP	0.52 ± 0.05	–	0.06 ± 0.02	–
CCh + ATP	1.42 ± 0.10	0.23 ± 0.05	1.04 ± 0.10	–
La^{3+} + ATP	1.10 ± 0.06	–	ND	ND
La^{3+} CCh	1.00 ± 0.15	0.28 ± 0.04	ND	ND

Values are expressed as the increase in fluorescence ratio (F340/380) over basal levels ($n \geq 12$, except La^{3+}: $n = 5$). Basal fluorescence ratio under normal conditions measured 1.04 ± 0.02 and under Ca^{2+}-free conditions 0.91 ± 0.04. The plateau was measured 4 min after addition of the agonist. CCh = carbachol; ND = no data obtained; – = no increase over basal levels was detected ($P > 0.05$). La^{3+} (50 μM) was added 8 min in advance.

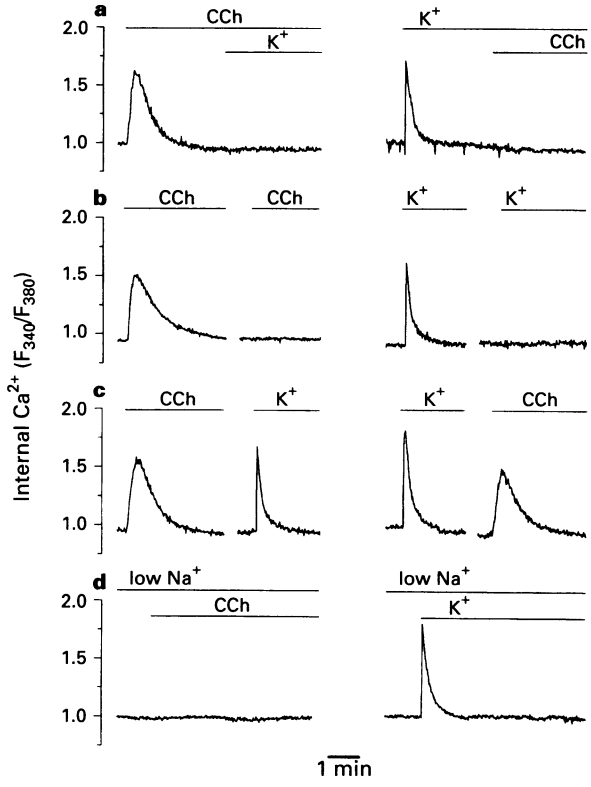

Figure 2 Changes in intracellular Ca^{2+} evoked by carbachol and high K^+ in the absence of external Ca^{2+}. (a) Abolition of the response to high K^+ (60 mM) in the presence of carbachol (10 μM; left panel), and *vice versa* (right panel); (b) a second stimulation by carbachol (10 μM) did not produce a Ca^{2+} response after termination of the preceding carbachol-evoked response by washing under Ca^{2+}-free conditions (left panel). Similarly, a second stimulation with high K^+ was not effective, after a preceding K^+ response followed by a wash under Ca^{2+}-free conditions (right panel); (c) The second response to carbachol was unaffected if the preceding high K^+-induced response was terminated by a brief wash with Ca^{2+}-free medium (left panel). Similarly, the high K^+-evoked response was unaffected after termination of the preceding carbachol response by washing under Ca^{2+}-free conditions; (d) The response to carbachol (10 μM) was completely abolished under low Na^{2+} conditions (15 mM; left panel), whereas the response evoked by high K^+ (60 mM) was unaffected under these conditions (right panel). Data are expressed as fluorescence ratio ($R = F_{340}/F_{380}$); tracings are representative of 5 or more experiments.

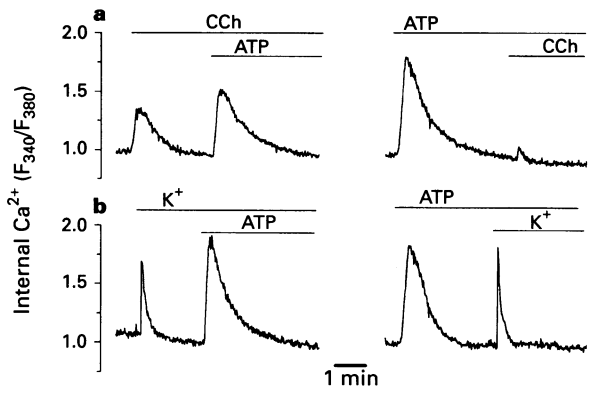

Figure 3 Changes in intracellular Ca^{2+} evoked by carbachol, ATP and high K^+ in the absence of external Ca^{2+}. (a) The response to carbachol (10 μM) showing abolition of the sustained phase, followed by the response to ATP (1 mM; left panel). The response evoked by ATP (1 mM) also showing abolition of the sustained phase, followed by the response to carbachol (10 μM; right panel); (b) the response to high K^+ (60 mM), followed by the response evoked by ATP (1 mM; left panel). The ATP-induced response, followed by the response to high K^+ (60 mM; right panel). Data are expressed as fluorescence ratio ($R = F_{340}/F_{380}$); tracings are representative of 6 or more experiments.

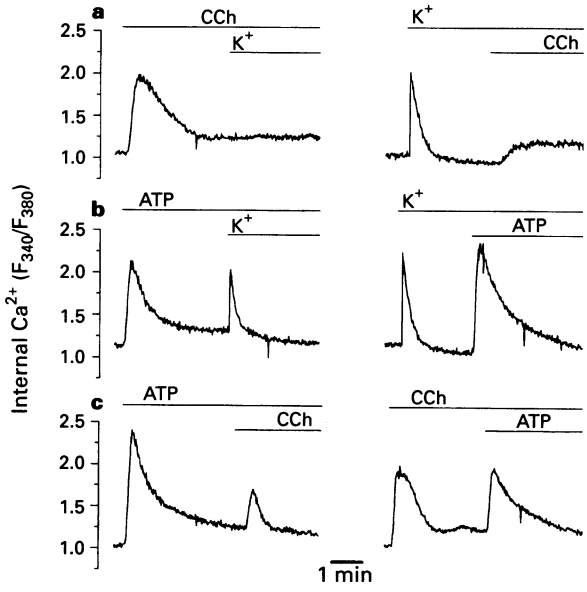

Figure 4 Changes in intracellular Ca^{2+} evoked by carbachol, ATP and high K^+ under normal conditions. (a) Abolition of the response evoked by high K^+ (60 mM) in the presence of carbachol (10 μM; left panel). In the presence of high K^+ (60 mM), carbachol (10 μM) induced a sustained response (left panel); (b) high K^+ evoked response was unaffected in the presence of ATP (1 mM; left panel). The sustained phase of the ATP-induced response was reduced in the presence of high K^+ (right panel); (c) decrease in amplitude and abolition of the sustained phase of the carbachol (10 μM)-evoked response in the presence of ATP (1 mM; left panel). Similarly, the response of ATP (1 mM) was reduced in amplitude and lacked the sustained phase in the presence of carbachol (10 μM; right panel). Data are expressed as fluorescence ratio ($R = F_{340}/F_{380}$); tracings are representative of 7 or more experiments.

response was unaffected, but the sustained phase was abolished (Figure 4b [right panel]). The high K^+ (60 mM) response was unaffected in the presence of ATP (1 mM; Figure 4b [left panel]). Prestimulation with carbachol reduced the amplitude of the second Ca^{2+} response evoked by ATP (1 mM) to 92% and abolished its sustained phase (Figure 4c [right panel]; Table 1). In the reverse experiment, prestimulation with ATP (1 mM) reduced the amplitude of the carbachol (10 μM)-evoked response to 63% and also abolished the second sustained phase (Figure 4c [left panel]; Table 1).

The carbachol and ATP-induced Ca^{2+} responses were studied in the presence of diltiazem, pancuronium and La^{3+} to determine common features. Diltiazem (10 μM), a blocker of voltage-operated Ca^{2+} channels, did not affect the carbachol (10 μM), ATP (1 mM) or K^+-induced Ca^{2+} response with respect to their rapid and sustained phases (not shown; $n \geq 4$). Preincubation or addition of La^{3+} (50 μM), assumed to block receptor-activated Ca^{2+}-entry, did not affect the carbachol-induced response (Figure 5a [left panel]; Table 1). In contrast, the sustained phase of the ATP response was abolished by addition of La^{3+} (Figure 5a [right panel]; Table 1). The Ca^{2+}-entry through the nAChR coupled cation-channels was investigated by adding pancuronium during the sustained phase of the carbachol response. Pancuronium by itself did not affect intracellular Ca^{2+} (not shown) and addition of pancuronium during the sustained phase of carbachol-evoked Ca^{2+} response did not affect this phase either (Figure 5b [left panel]). In contrast, preincubation with pancuronium (3 μM) completely

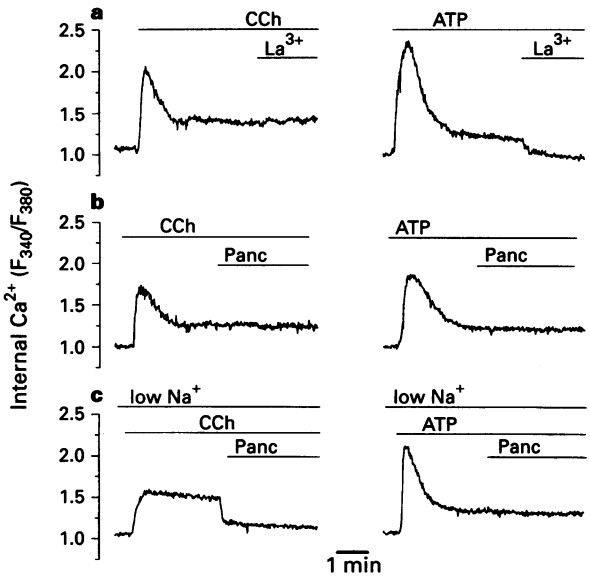

Figure 5 Changes in intracellular Ca^{2+} evoked by carbachol and ATP in the presence of extracellular Ca^{2+}. (a) The sustained phase of the response induced by carbachol (10 μM) was unchanged by addition of La^{3+} (50 μM; left panel), while the sustained phase following ATP (1 mM) was inhibited under these conditions (right panel); (b) administration of the nAChR antagonist, pancuronium (3 μM) during the sustained phase did not affect this phase of responses evoked by carbachol (10 μM, left panel) or ATP (1 mM; right panel). (c) Augmentation of the carbachol-evoked response under low Na^+ conditions (15 mM; left panel) is partly blocked by administration of pancuronium during this phase (3 μM; left panel). Low Na^+ conditions or pancuronium (3 μM) did not affect the ATP-induced response (right panel). Data are expressed as fluorescence ratio ($R = F_{340}/F_{380}$); tracings are representative of 6 or more experiments.

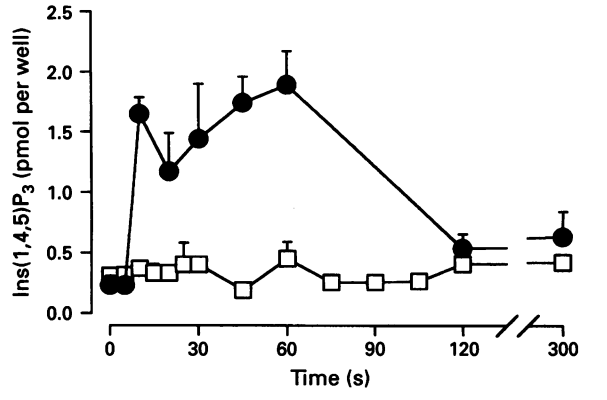

Figure 6 The levels of $Ins(1,4,5)P_3$ following stimulation of the myotubes with carbachol (□, 10 μM) or ATP (●, 1 mM) in the absence of external Ca^{2+}. Data are expressed as mean ± s.e.mean ($n = 5$; determined in duplicate).

abolished the biphasic Ca^{2+} response induced by carbachol (10 μM; $n = 5$; not shown). The ATP-induced response (Figure 5b [right panel]) or the high K^+ evoked responses were not affected by pancuronium (3 μM; not shown).

The carbachol and ATP responses were also investigated under low Na^+ conditions (15 mM). When carbachol (10 μM) induced a slow and sustained increase in intracellular Ca^{2+} (Figure 5c [left panel]). The amplitude of the sustained phase was increased compared to this phase under normal Na^+ conditions (Figure 5b [left panel]). This sustained phase observed under low Na^+ conditions was partially sensitive to pancuronium (3 μM; Figure 5c [left panel]), in contrast with that under normal conditions (Figure 5b [left panel]). The ATP-induced biphasic Ca^{2+} response was not affected under low Na^+ conditions (Figure 5c [right panel]).

Ins(1,4,5)P_3 formation

Involvement of the phospholipase C pathway in the release of internal Ca^{2+} was determined by measuring the $Ins(1,4,5)P_3$ formation after stimulation of the cells with carbachol or ATP under Ca^{2+}-free conditions. Incubation of the myotubes for 5 min with ATP (300 μM) induced a transient 4 fold increase in $Ins(1,4,5)P_3$, showing a peak after about 45 s (Figure 6). In contrast, stimulation of the cells with carbachol (10 μM) did not affect basal $Ins(1,4,5)P_3$ levels (Figure 6).

Discussion

These results show that nAChR stimulation, P_2-purinoceptor stimulation and high external K^+ increase the internal Ca^{2+} concentration in C2C12 myotubes. Both carbachol and ATP evoked a biphasic response characterized by a rapid rise in intracellular Ca^{2+} followed by a sustained phase, whereas high K^+ induced a fast transient increase in Ca^{2+}.

High K^+-induced Ca^{2+} response

The high K^+-induced transient rise in internal Ca^{2+} is commonly attributed to depolarization of the myotubes activating voltage-operated L-type Ca^{2+}-channels. In turn, ryanodine receptors on the sarcoplasmic reticulum are activated by Ca^{2+} entering the cell and via direct molecular interaction of the Ca^{2+}-channel with the ryanodine receptor (Schneider & Chandler, 1973; Meissner, 1994). In accord, the high K^+ induced Ca^{2+} response was abolished if the cells were depolarized in advance. When Ca^{2+}-entry is prevented under Ca^{2+}-free conditions or with diltiazem, the high K^+-induced Ca^{2+} response is due to the remaining direct molecular interaction between the Ca^{2+} channel and the ryanodine receptor (Schneider & Chandler, 1973; Meissner, 1994) under these conditions. It is noted that the contribution of Ca^{2+}-entry to the high K^+-induced response is difficult to assess, in view of the limited time resolution of the Ca^{2+} measurement.

nAChR-mediated Ca^{2+} response

The Ca^{2+} response observed with carbachol was evoked via nicotinic AChRs, for it was blocked by pretreatment with the receptor antagonist, pancuronium, while the muscarinic antagonist, atropine, was not effective. It is known that stimulation of C2C12 myotubes with carbachol (10 μM) induces a sustained depolarization, due to Na^+-influx through the nAChR coupled cation-channel. This depolarization is comparable to that caused by high K^+ (60 mM; Henning *et al.*, 1994). Nevertheless, the nAChR-mediated Ca^{2+} response is quite different from that evoked by high K^+. The Ca^{2+} response caused by second addition of carbachol was completely blocked by pre-stimulation of the cells with the same agonist, if refilling of the Ca^{2+} stores was prevented in the absence of external Ca^{2+}. The same was observed when high K^+ was used instead of carbachol. However, a second stimulation with carbachol or high K^+ was unaffected by prestimulation with the other agonist. Therefore, carbachol and high K^+ mobilize Ca^{2+} from different internal stores. As it is generally assumed that high K^+ causes Ca^{2+} release from the ryanodine-sensitive store, our observations imply that the nAChR-activated Ca^{2+} stores are different from the ryanodine store, in agreement with a previous report (Grassi *et al.*, 1993). The nAChR mediated Ca^{2+} mobilization was abolished by preventing the Na^+-influx via the receptor gated channels by reducing the Na^+ gradient or by decreasing the electrical gradient by high K^+ conditions. It is tempting to speculate that the Na^+-influx triggers emp-

tying of the internal store (Grassi *et al.*, 1993). Recently, increased cytosolic Na^+ has been shown to augment thapsigargin-induced Ca^{2+} release in smooth muscle cells (Borin *et al.*, 1994). Whether a similar mechanism is involved in the nAChR-mediated mobilization of Ca^{2+} from internal stores remains to be investigated. Involvement of inositol phosphates in nAChR-mediated Ca^{2+} mobilization, as suggested previously (Giovannelli *et al.*, 1991; Grassi *et al.*, 1993), seems unlikely in view of the absence of $Ins(1,4,5)P_3$ formation. Another implication of our observations is that nAChR stimulation and the concomitant decrease in membrane potential does not empty the depolarization sensitive Ca^{2+} store. This might be due to the relatively slow development of the depolarization compared to that evoked by high K^+, causing inactivation of L-type Ca^{2+} channels.

In the presence of external Ca^{2+}, stimulation of nAChRs also induced Ca^{2+}-entry, as represented by the sustained phase of the Ca^{2+} response. The nAChR-mediated Ca^{2+}-entry is not due to Ca^{2+}-gating through the nAChR-channel, for the sustained phase was unaffected by administration of pancuronium during this phase. However, the sustained phase is activated through stimulation of nAChRs because pretreatment with pancuronium completely prevented Ca^{2+}-entry. Further, the nAChR-mediated Ca^{2+}-entry is independent of the membrane depolarization or Na^+-influx, for the sustained Ca^{2+} response was still observed under low Na^+ conditions and high K^+ depolarization. Moreover, the sustained phase of the carbachol-induced response under low Na^+ conditions was increased in amplitude and partially sensitive to pancuronium. Both phenomena can be explained, assuming a substantial additional Ca^{2+}-entry through nAChR gated channels becoming prominent under low Na^+ conditions. Further, voltage-operated Ca^{2+}-channels are apparently not involved in the activation of nAChR-mediated Ca^{2+}-entry for the sustained phase of the Ca^{2+} response was unaffected by diltiazem or by depolarization of the cells. It is also unlikely that the nAChR-evoked Ca^{2+}-entry is dependent on the filling state of the Ca^{2+} stores (Putney & Bird, 1993; Randriamampita & Tsien, 1993), because Ca^{2+}-entry was still observed under conditions blocking Ca^{2+} mobilization such as low external Na^+ or high K^+. Finally, the nAChR-mediated Ca^{2+}-entry in C2C12 cells was not affected by La^{3+}, which supports the idea that a process is involved which differs from Ca^{2+}-entry induced by stimulation of G-protein coupled receptors activating phospholipase C (Den Hertog *et al.*, 1992; Henning *et al.*, 1993a). An intriguing possibility explaining nAChR mediated Ca^{2+}-entry is expression of a nAChR α-subunit different from the skeletal muscle type α_1-subunit giving rise to the formation of nAChR channels which have a high Ca^{2+} permeability and which are sensitive to pancuronium, as observed in neuronal nAChRs (Sargent, 1993). Recently, rat skeletal muscle myotubes have beeen reported to express neuronal α_4-, α_5- and α_7-nAChR subunits (Corriveau *et al.*, 1995), some of which are involved in formation of neuronal nAChR channels which have a high Ca^{2+} permeability and are insensitive to pancuronium (Sargent, 1993).

P_2-purinoceptor-mediated Ca^{2+} response

At least three different P_2-purinoceptors are present in mouse C2C12 myotubes: a P_{2U}-purinoceptor coupled to phospholipase C (Hemming *et al.*, 1992; 1993a) and two unclassified P_2-purinoceptors activating Na^+-influx (Henning *et al.*, 1992) and the formation of cyclic AMP (Henning *et al.*, 1993b), respectively. Experiments using different purinoceptor agonists have shown that the ATP-evoked increase in cytoplasmic Ca^{2+} in these cells is exclusively mediated by the nucleotide type P_{2U}-purinoceptor (Henning *et al.*, 1993a). The possibility was excluded that the small ATP-induced membrane depolarization caused by Na^+-influx contributes to the internal rise in Ca^{2+} (Henning *et al.*, 1992), which is in agreement with the unaffected ATP-induced Ca^{2+} response observed under low Na^+ conditions. Thus, stimulation of the P_{2U}-purinoceptor under Ca^{2+}-free conditions resulted in a transient formation of $Ins(1,4,5)P_3$ associated with a rapid increase in internal Ca^{2+}.

The maximum amplitude of the ATP-evoked rapid phase of the Ca^{2+} response as observed in the presence of external Ca^{2+} was decreased under Ca^{2+}-free conditions. This need not imply that Ca^{2+}-entry contributes to the rapid phase, as its reduction may be related to the decreased basal intracellular Ca^{2+} as observed under Ca^{2+}-free conditions, affecting various steps in the Ca^{2+} mobilization process such as PLC activation (Uhing *et al.*, 1986) or $Ins(1,4,5)P_3$ receptor function (Bezprozvanny *et al.*, 1992).

Stimulation of the P_{2U}-purinoceptor also activated Ca^{2+}-entry from the extracellular space, represented by the sustained Ca^{2+} response. The absence of the sustained phase in the presence of high K^+ or carbachol suggests that the mechanism responsible for P_{2U}-purinoceptor-induced Ca^{2+}-entry is dependent on the electro-chemical gradient and therefore voltage-dependent. Capacitive Ca^{2+}-entry, following emptying of $Ins(1,4,5)P_3$ sensitive stores (Putney & Bird, 1993; Randriamampita & Tsien, 1993), has not been observed in C2C12 myotubes (Henning, unpublished). Thus the mechanism leading to Ca^{2+}-entry on P_{2U}-purinoceptor is unknown. Possibly, arachidonic acid formed from diacylglycerol may act as a messenger to promote Ca^{2+}-entry, as found on stimulation of PLC coupled receptors in DDT_1 MF-2 cells (van der Zee *et al.*, 1995).

Interaction between nAChR and P_2-purinoceptor

So far, the results show that activation of Ca^{2+} mobilization and entry are different following activation of nAChRs and P_{2U}-purinoceptors in mouse C2C12 myotubes. Nevertheless, interaction between these receptor-induced Ca^{2+} responses was observed. In particular, nAChR-evoked Ca^{2+} mobilization was significantly inhibited by prestimulation of P_{2U}-purinoceptors. This interaction is not necessarily caused by 'cross-talk' on the level of Ca^{2+} stores, but might also be due to nAChR desensitization following P_{2U}-mediated activation of protein kinase C (Huganir *et al.*, 1986). Further, the Ca^{2+}-entry activated by stimulation of either receptor type was abolished by prestimulation of the other receptor. *In vivo*, the P_2-purinoceptor agonist ATP is released as a co-transmitter with acetylcholine (Silinsky, 1975). Therefore, the cross-talk between the two systems may serve to modulate skeletal muscle function.

In summary, it was shown that Ca^{2+} release from discrete stores of C2C12 myotubes is induced by stimulation of nAChRs, P_{2U}-purinoceptors and by high K^+. Only the P_{2U}-purinoceptor and nAChR activated stores show considerable overlap in releasable Ca^{2+}. Sustained Ca^{2+}-entry is activated by stimulation of nAChRs and P_{2U}-purinoceptors via separate ion-channels, which are different from the skeletal muscle nAChR-coupled cation-channel.

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