Transduction of arginine vasopressin signal in skeletal myogenic cells

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Teti, Anna, Fabio Naro, Mario Molinaro, and Sergio Adamo. Transduction of arginine vasopressin signal in skeletal myogenic cells. *Am. J. Physiol.* 265 (*Cell Physiol.* 34): C113-C121, 1993.—Arginine vasopressin (AVP) induced concentration-dependent (10^{-9} to 10^{-6} M) stimulation of inositol phosphate production and a biphasic increment of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in skeletal myogenic cells in culture. These effects were almost completely abolished when the cells were pretreated with the AVP antagonist [deamino-Pen[1],Val[4],D-Arg[8]]-vasopressin before stimulation with AVP, thus confirming a V_1 receptor-mediated effect. Inositol 1,4,5-trisphosphate production was maximally stimulated within 2–3 s of treatment with AVP, immediately followed by release of Ca^{2+} from intracellular deposits. Both effects were inhibited by treatment with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). Such effect of TPA was reversed by the protein kinase C inhibitor staurosporine. Vasopressin also regulated the intracellular pH of responsive cells with mechanisms involving both Na^+ and anion transport across the plasma membrane. However, unlike in other cell types, AVP stimulated the Na^+-H^+ antiport only simultaneously with a dramatic cell acidification or after treatment with TPA. Response to AVP was observed in L6 and L5 and, to a lesser extent, in chick embryo myogenic cells, regardless of the stage of differentiation (myoblast or myotube). Comparison of different subclones of the L6 cell line demonstrated that the responsiveness to AVP correlated positively with their myogenic potential.

skeletal muscle cells; myogenesis; phosphoinositides; inositol trisphosphate; phorbol esters; cytosolic calcium; cell pH; protein kinase C

ARGININE VASOPRESSIN (AVP) acts as a potent vasoconstrictor, an antidiuretic hormone, and a neurotransmitter. Cellular responses to AVP can be mediated by two classes of receptors (50). The V_1 receptor is coupled to phospholipase C and induces breakdown of phosphoinositides (36), whereas the V_2 receptor is coupled to the adenylate cyclase pathway (41). The two receptors may be simultaneously expressed in the same tissue, accounting for different synchronous functions regulated by the hormone (23, 51, 58, 59).

In cultured smooth muscle and renal glomerular mesangial cells, AVP induces proliferation (22), cell contraction (3), and/or synthesis of prostaglandin I_2 (51), an autocrine-paracrine modulator of AVP action (52). These effects are driven by pathways involving regulation of intracellular ion composition and protein kinase activity. In fact, increases of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (10, 12, 28, 53), protein kinase C (PKC) activity (13), and tyrosine phosphorylation (20) and modulation of intracellular pH (pH_i) (7, 21, 22) have been described in cells or tissues exposed to AVP.

Although the role played by AVP in smooth muscle and mesangial cells is fairly well understood (6, 13, 31), its action on skeletal muscle remains to be elucidated. Previous studies, conducted in vivo on skeletal muscle preparations (29) and in vitro on chick embryo myogenic cell cultures (56), suggest that AVP could influence carbohydrate metabolism. More recently, Wakelam et al. (55) demonstrated the presence of functional AVP V_1 receptors in the L6 myogenic cell line. Furthermore, high levels of immunoreactive AVP have been shown in human fetal muscle, its concentration declining as gestational age increases (32).

To address the question of the functional role played by this neuropeptide in skeletal muscle, we first characterized the response to AVP at the level of signal transduction, including inositol phosphate production, intracellular Ca^{2+} homeostasis, and regulation of pH_i, in several skeletal myogenic cell lines with different origin, stage of differentiation, and myogenic potential. We report here that the AVP-induced intracellular signaling in myogenic cells shows some distinctive differences from those observed in other cell types. Furthermore, the responsiveness to AVP of the cell lines examined has been found to be heterogeneous and independent on the stage of differentiation in culture, but possibly related to the origin and myogenic potential of the different lines or clones.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), reagents, and sterile plasticware for cell culture were from Flow Laboratory (Irvine, UK). D-*Myo*-[^{3}H]inositol (sp act >400 GBq/mmol) was obtained from Du Pont-New England Nuclear (Bad Homburg, Germany). Other tritiated *myo*-inositol mono- and polyphosphates were purchased from Amersham (Amersham, UK) and Du Pont-New England Nuclear. The MF20 anti-skeletal muscle myosin monoclonal antibody was a kind gift of Dr. Donald Fishman (Cornell Medical College, New York, NY). Rhodamine-conjugated goat anti-mouse immunoglobulin was from Cappel Laboratories (Malvern, PA). The acetoxymethyl esters of fura 2 (fura 2-AM) and 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein pentaacetoxymethyl ester (BCECF-AM) were from Molecular Probes (Eugene, OR). Ionomycin and bovine serum albumin (BSA) were from Calbiochem (La Jolla, CA). Acar plastic, cut in 14-mm-square cover slips, was from Allied Engineered Plastics (Pottsville, PA). Synthetic AVP, vasotocin, prostaglandin $F_{2\alpha}$, U-46619 (a stable mimetic of thromboxane A_2), angiotensin II (ANG II), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), type I collagen from calf skin, and all other reagents were from Sigma (St. Louis, MO).

Cell Cultures

Rat myogenic L6 cells (60) were obtained from the American Type Culture Collection (Rockville, MD) and cultured in DMEM containing 4.5 g/l glucose and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS, unless otherwise stated. Clones were obtained by limiting dilution of

cell suspensions in 96-well multiplates. Each clone was expanded and characterized with respect to the ability of undergoing myogenic differentiation and substrate-independent growth. First the cells were seeded in collagen-coated dishes at a density of 50,000/ml and, after 1 day, induced to differentiate by reduction of serum concentration to 2%. After 6 days, the percentage of fusion was measured on Wright-stained dishes (16); parallel dishes were examined by immunofluorescence for the presence of skeletal myosin, using the MF20 anti-skeletal muscle myosin monoclonal antibody, as already described (54). Second each clone was tested for its ability of growing in a substrate-independent manner, seeding 10,000 cells/6-cm dish in a top layer of 0.25% agar over a base layer of 0.5% agar (33). Growth in soft agar was microscopically checked after 7 and 15 days of plating.

Rat L5 cells, kindly provided by Dr. S. Scarpa (University La Sapienza, Rome), were cultured in F-14 medium supplemented with either 10% FBS (to sustain proliferation) or 2% FBS (to induce differentiation, 45).

Human rhabdomyosarcoma (RD) cells (2, 35) and C2-C12 (a mouse muscle satellite cell-derived line) were obtained from American Type Culture Collection, cultured and cloned as described above and previously (2). Primary myogenic cell cultures were prepared from 11-day chick embryos as previously described (1). For fluorescence studies, cells were seeded at a density of 10^5 cells/ml onto collagen-coated plastic or glass cover slips.

Phosphoinositide Turnover

Myoblasts (at 2nd-3rd day of culture in 10% FBS-containing medium) and myotubes (at 6th day of culture in 2% FBS-containing medium) were labeled with 5 μCi/ml of D-*myo*-[^{3}H]-inositol in complete medium for 48 h. Cells were then extensively washed with Hanks' balanced salt solution supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.4) and 0.1% BSA; the same medium was used throughout the incubation with the agonists. To measure receptor activation by AVP under various conditions and to identify inositol 1,4,5-trisphosphate [Ins(1,4,5,)P_3] and its metabolites, respectively, two different sets of experiments have been performed (17). *1*) Cells were preincubated with 10 mM LiCl. After 10 min the agonist (or agonist solvent) was added and the incubation was then carried out for further 30 min. These samples were chromatographed on Dowex 1×8–200 as already described (1). 2) Cells were incubated with the agonist but without LiCl. Incubations were arrested at various times, and samples were analyzed by high-performance liquid chromatography (HPLC) as described below. In both sets of experiments, the incubation was stopped by rapidly substituting the medium with ice-cold 10% trichloroacetic acid (TCA). Phytic acid hydrolysate (20 μg P_i/sample; Ref. 57) was added, and extraction of the inositol phosphates was performed as previously described (1), except that TCA was removed with a single extraction with 1 vol of a mixture of trichlorotrifluoroethane and tri-*n*-octylamine (74:26, vol/vol; Ref. 9).

Table 1. *AVP-dependent stimulation of phosphoinositide breakdown in different myogenic cells*

Cell Type Subclone	Fusion, %	Myosin HC Expression, AU	Growth in Soft Agar, AU	Production of AVP-Induced IP, fold stimulation
L6	40±7	++	−	14±3
L6-C5	59±5	+++	−	17±3
L6-A4	32±6	++	±	11±2
L6-C9	16±4	+	−	6±3
L6-H2	−	−	+++	1.2±0.3
L5	70±5	+++	−	19±4
C2-C12	30±8	++	NT	1.1±0.5
RD	20±4	++	+	1.2±0.4
Chick embryo myogenic cells	65±10	+++	NT	1.9±0.4 1.8±0.3*

Values are means ± SD of at least 2 separate experiments, each sample in triplicate. For fusion, cells were cultured under differentiating conditions (see MATERIALS AND METHODS). Production of arginine vasopressin (AVP)-induced inositol phosphate (IP) involved stimulation of inositol monophosphate plus inositol bisphosphate accumulation after 30 min incubation with 3×10^{-7} M AVP in presence of LiCl. Stimulation of phosphoinositide turnover by AVP was measured in both undifferentiated and differentiated L6, L6-C5, L6-A4, L5, C2-C12, RD, and chick embryo myogenic cells (see MATERIALS AND METHODS for respective culture conditions), and no statistical significance was observed. −, Nondetectable; NT, not tested; AU, arbitrary units. * Cells stimulated with 3×10^{-7} M vasotocin.

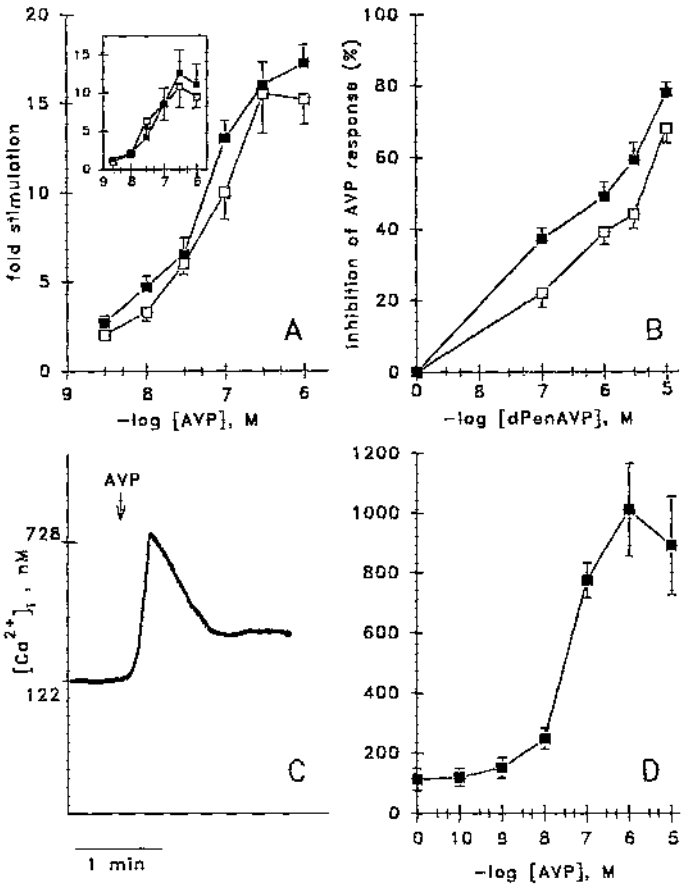

Fig. 1. Effect of arginine vasopressin (AVP) on phosphoinositide breakdown and Ca^{2+} concentration of L6-C5 cells. *A*: stimulation of inositol monophosphate (IP_1) + inositol bisphosphate (IP_2) [inositol trisphosphate (IP_3) in *inset*] generation by different concentrations of AVP after 30 min of incubation (in presence of 10 mM LiCl) in [^{3}H]inositol-prelabeled undifferentiated [mononucleated proliferating cells at 2nd-3rd day of culture in 10% fetal bovine serum (FBS)-containing medium, ■) and differentiated (multinucleated ~60% fusion at 6th day of culture in 2% FBS-containing medium, □) L6-C5 cultures. *B*: inhibition of 10^{-7} M AVP-induced inositol phosphate production elicited by different concentrations of AVP antagonist [deamino-Pen^1,Val^4,D-Arg]vasopressin ([dPen]AVP; ■,myoblasts; □, myotubes). *C*: fluorescence trace of fura 2-loaded L6-C5 myoblasts calibrated for intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) as described in MATERIALS AND METHODS. *D*: dose-dependent increase of $[Ca^{2+}]_i$ in L6-C5 cells treated with AVP. Data represent size of maximal $[Ca^{2+}]_i$ increases induced by doses of AVP indicated on *abscissa*.

HPLC Separation of Inositol Phosphates

Cell extracts were separated by HPLC using a Gilson dual-pump system (Gilson, Villiers-le-Bel, France) and a Partisil 5 SAX column (11 × 0.47 cm; Whatman, Clifton, NJ). Elution was performed with a 0–1 M ammonium phosphate gradient (pH 3.8; 18, 39) at a flow rate of 1 ml/min. Tritiated *myo*-inositol mono- and polyphosphates were used as standards to optimize the gradient and identify the experimental sample peaks. Adenine and guanine nucleotides were routinely added, as elution markers, to all samples to be separated by HPLC (17). An on-line radioactivity flow detector (Radiomatic, Tampa, FL) was routinely used to monitor the HPLC eluate. Fractions of critical regions of the chromatography were, in some experiments, collected at 0.5-min intervals and statically counted in a beta scintillation spectrometer (Beckman, Fullerton, CA).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ of semiconfluent monolayers of skeletal muscle cell lines was measured by single- or dual-wavelength fluorescence of cells loaded with the Ca^{2+}-sensitive intracellular probe, fura 2 (26, 37). Cells, seeded on plastic or glass cover slips, were loaded with 3 μM fura 2-AM in serum-free medium at 37°C for 60 min. Measurements were performed on the whole cell population, bathed in Krebs-Henseleit-HEPES (KHH) buffer under continuous stirring, with a Perkin-Elmer S5 spectrofluorometer set at 339-nm excitation and 500-nm emission wavelenghts (bandwidth 2.5 nm) and equipped with a temperature-controlled water jacket set at 37°C. Results of some experiments were checked in single cells at dual excitation wavelenghts (340 and 380 nm) using cells seeded on glass cover slips and analyzed with a AR-CM fluorometer (Spex Industries, Edison, NJ) connected with a Diaphot TMD inverted microscope (Nikon, Tokyo, Japan). Calibration of the signal was obtained at the end of each observation and $[Ca^{2+}]_i$ was calculated using previously described formulas (26, 37).

Measurement of pH_i

pH_i was measured by fluorescence excitation of cells loaded with the intracellularly trapped pH indicator, BCECF (43, 48). Semiconfluent monolayers, grown on plastic cover slips, were loaded with 3 μM BCECF-AM in serum-free medium at 37°C for 30 min. In accord with the experimental design, monolayers were then washed in KH solution buffered with either 20 mM HEPES or 24 mM $NaHCO_3$. When HCO_3^--containing media were employed, pH was mantained by holding the solutions in airtight glass containers injected with a 95% air-5% CO_2 mixture until used and performing experiments in stoppered quartz cuvettes. For Na^+-free solutions, choline chloride was used as an isosmotic replacement. Single-wavelength fluorescence was monitored continuously at 37°C with 500-nm excitation and 540-nm emission wavelengths and 2.5-nm bandwidth. Calibration was performed as described by Grinstein et al. (25).

Statistics

Statistical analyses were performed by the *t* test. When required, data were expressed as means ± SE.

RESULTS

Phosphoinositide Turnover

Initial experiments on AVP-dependent induction of phosphoinositide breakdown were performed on L6 cells, clone C5 (L6-C5), a clone that had shown relevant differentiating response when cultured under appropriate conditions (see Table 1). Experiments were performed employing L6-C5 cultures, both as mononucleated undifferentiated cells and as differentiated cells, obtaining superimposable results. Dose-dependent accumulation of inositol phosphates was induced in L6-C5 cells, incubated for 30 min with the agonist in the presence of LiCl (Fig. 1*A*). Phosphoinositide hydrolyisis was effectively stimulated by nanomolar to micromolar AVP. Half-maximal stimulation occurred at ~3×10^{-8} M AVP, judged by the effect on inositol monophosphate plus inositol bisphosphate accumulation. These results confirm those reported by Wakelam et al. (55) in L6 cells. Preincubation of the cells with the AVP antagonist [deamino-Pen^1,Val^4,D-Arg^8]vasopressin (10^{-5} M, 10 min before AVP) inhibited by ~75% the response to 10^{-7} M AVP (Fig. 1*B*), confirming that phosphoinositide hydrolysis was induced by AVP at the level of V_1-type receptors (34).

Stimulation of phosphoinositide turnover by AVP was tested in other L6 clones and in different myogenic cell

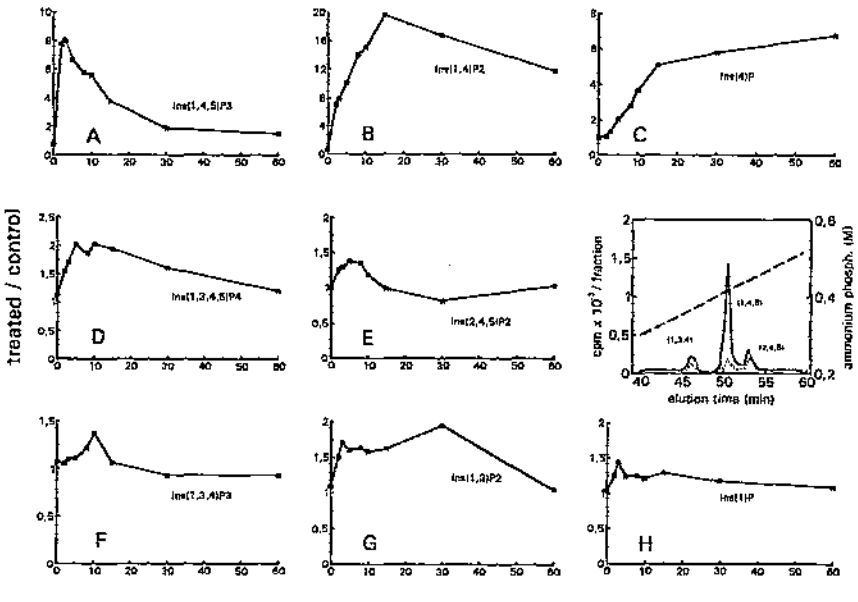

Fig. 2. Time course of stimulation of specific [^{3}H]-inositol phosphates in L6-C5 cells treated with AVP. *A–H*: extracts of control and 3 × 10^{-7} M AVP-treated cells were subjected to anion-exchange high-performance liquid chromatography as detailed in MATERIALS AND METHODS. Radioactivity corresponding to each identified peak is plotted vs. time of incubation with AVP (without addition of lithium). *Inset*: detail of elution pattern of IP_3 region of AVP-stimulated (solid line) or control (dotted line) L6-C5 cells at 5 s of stimulation.

lines, such as L5, C2, and RD, as well as in chick embryo primary myogenic cell cultures. The results (shown in Table 1) indicate that L6 clones A4 and C9 (which show a lower myogenic potential than clone C5) exhibited a lower AVP-induced production of inositol phosphates than L6-C5. The L6-H2 clone (characterized by a transformed, possibly tumorigenic phenotype and exhibiting no myogenic differentiation) did not show an increase of inositol phosphates in response to AVP. The myogenic cell line L5 exhibited AVP-induced stimulation of phosphoinositide turnover comparable to that observed in L6-C5 cells at both stages of differentiation tested (myoblasts and myotubes). C2-C12 mouse satellite cells, and human RD cells did not respond to AVP (although phosphoinositide hydrolysis was induced in these 2 cell lines by 20 μM acetylcholine; not shown). Finally, a modest stimulation of phosphoinositide turnover was induced by AVP in primary cultures of chick embryo myogenic cells (Table 1). A similar stimulation of phosphoinositide breakdown was obtained in these cells on treatment with equimolar concentrations of the avian AVP analogue, vasotocin.

In another set of experiments, aimed to study Ins(1,4,5)P_3 production and metabolism, [^{3}H]inositol-labeled L6-C5 and L5 cells were stimulated with 3×10^{-7} M AVP for short times in the absence of LiCl and the extracts were separated by ion-exchange HPLC (Fig. 2). Ins(1,4,5)P_3 production was rapidly and effectively stimulated by AVP. Figure 2*A* shows that the level of this second messenger increased about eightfold over the control level after 2–3 s of treatment with AVP, and then slowly declined to about twice the untreated control value after 1 min of stimulation. The increase of inositol 1,4-bisphosphate [Ins(1,4)P_2; Fig. 2*B*] reached a maximum at ~15–20 s, whereas the stimulation of inositol 4-monophosphate [Ins(4)P_1; Fig. 2*C*] continued throughout the 60-s time period shown. Although with a lower level of stimulation than Ins(1,4,5)P_3 (Fig. 2*D*), inositol tetrakisphosphate (not further resolved with our elution conditions) increased over the control level, reaching a maximum at ~8–10 s of stimulation with AVP. Another [^{3}H]inositol-labeled metabolite (tentatively identified as inositol 2,4,5-trisphosphate [Ins(2,4,5)P_3] on the basis of its elution, since no authentic standard was available) was rapidly and transiently (although moderately) stimulated by AVP in L6 cells (Fig. 2*E*). A slight increase of inositol 1,3,4-trisphosphate [Ins(1,3,4)P_3], inositol 1,3-bisphosphate [Ins(1,3)P_2], and inositol 1-monophosphate [Ins-(1)P_1] was also observed (Fig. 2, *F–H*).

The tumor promoter TPA, a powerful activator of PKC (11), rapidly, although partially, inhibited the response of L6-C5 cells to AVP. Pretreatment of L6-C5 cells with

Table 2. *Effect of 10^{-7} M AVP on $[Ca^{2+}]_i$ on L6-C5 myoblasts*

Conditions	n	$[Ca^{2+}]_i$, nM		
		Basal	AVP peak	AVP plateau
Control	6	114±7	655±93*	174±17†
EGTA (3 mM)	4	95±13	500±35	93±15‡
EGTA (3 mM) + ionomycin (0.5 μM)	4	50±10	50±10	50±10
Nifedipine (10^{-5} M)	3	141±5	580±41	243±44
Nicardipine (10^{-5} M)	3	147±13	631±84	296±32

Values are means ± SE. $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration. * $P < 0.002$ vs. basal. † $P < 0.005$ vs. basal. ‡ $P < 0.05$ vs. AVP plateau in control.

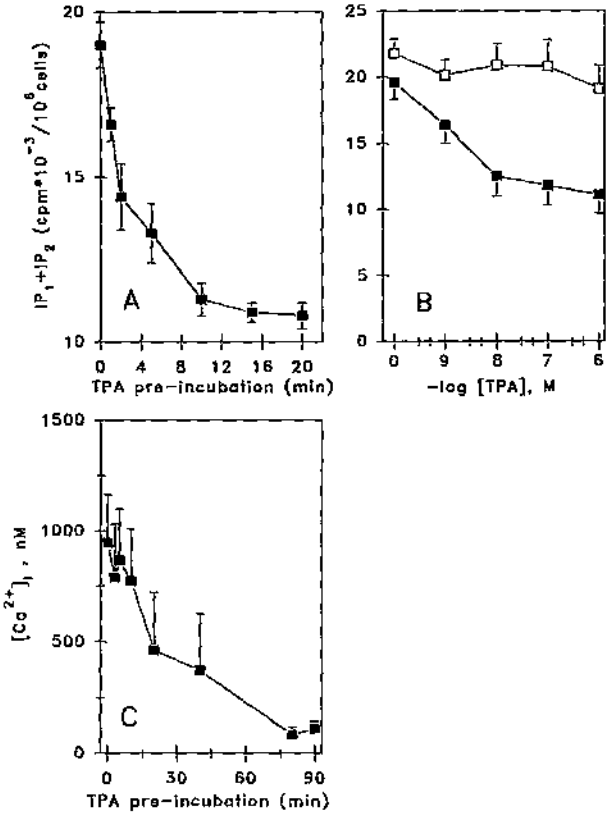

Fig. 3. Modification by 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) of response to AVP of L6-C5 cells. *A*: [^{3}H]inositol-labeled cells were preincubated with 10^{-7} M TPA for times indicated before 30-min incubation with 3×10^{-7} M AVP. *B*: cells were incubated with (□) or without (■) 10^{-6} M staurosporine for 10 min before 20-min incubation with 10 mM LiCl and TPA at concentrations indicated. At end of this period, 3×10^{-7} M AVP was added and incubation proceeded for further 30 min. *C*: L6-C5 myoblasts were pretreated with 10^{-7} M TPA for times indicated on *abscissa* before addition of 10^{-7} M AVP. Data are means ± SE of $[Ca^{2+}]_i$ rapid phase peak.

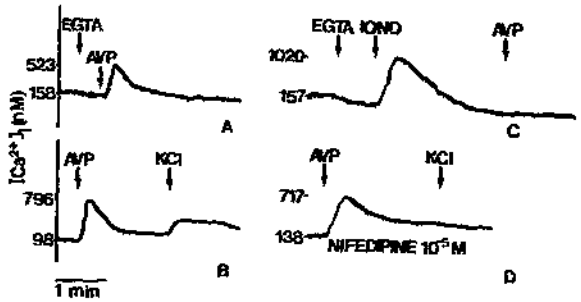

Fig. 4. AVP-dependent modulation of $[Ca^{2+}]_i$ in L6-C5 cells. *A*: 10^{-7} M AVP-induced $[Ca^{2+}]_i$ transient in Ca^{2+}-free buffer obtained by addition of 3 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). *B*: inhibition of response to 10^{-7} M AVP in cells pretreated with 0.5 μM ionomycin (Iono) to discharge intracellular Ca^{2+} deposits. *C*: $[Ca^{2+}]_i$ increase in cells treated with 10^{-7} AVP and with 75 mM KCl. *D*: effect of nifedipine on $[Ca^{2+}]_i$ elevations induced by 10^{-7} M AVP or by 75 mM KCl.

10^{-7} M TPA decreased by ~40% the production of inositol phosphates induced by treatment with 10^{-9} to 10^{-6} M AVP for 30 min (Fig. 3*B*). The effect was measurable within 2-3 min of pretreatment with TPA and reached a maximum after 10 min (Fig. 3*A*). No effect of pretreatment with TPA was observed in the absence of stimulation with AVP.

To test whether the inhibitory effect of TPA was dependent on stimulation of PKC activity, L6-C5 cells were incubated with 10^{-6} M staurosporine (an inhibitor of PKC; Ref. 47) for 10 min before the addition of 10 mM LiCl and TPA at different concentrations. Preincubation was continued for 20 min, 10^{-7} M AVP was then added, and the cells were incubated for further 30 min. Staurosporine virtually abolished the inhibitory effect of TPA on AVP-induced accumulation of inositol phosphates (Fig. 3*B*).

Regulation of $[Ca^{2+}]_i$

A prompt transient increase in $[Ca^{2+}]_i$ was induced by 10^{-7} M AVP in L6-C5 cells (Fig. 1*C*). Treatment with AVP triggered a rapid (16 ± 3 s) peak of $[Ca^{2+}]_i$ followed by a stable lower level plateau (Table 2). Responses were dose dependent (Fig. 1*D*), with half-maximal dose at 3×10^{-8} M. Other vasoconstrictors, such as prostaglandin $F_{2\alpha}$, U-46619, and ANG II, at doses of 10^{-6} M failed to induce significant changes of $[Ca^{2+}]_i$ (not shown).

In cells equilibrated in the presence of 3 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; to chelate Ca^{2+} and obtain a virtually Ca^{2+}-free solution), 10^{-7} M AVP still rapidly increased $[Ca^{2+}]_i$, but such an increase was not followed by a stable plateau, since $[Ca^{2+}]_i$ promptly returned to near basal levels (Fig. 4*A*, Table 2). When intracellular Ca^{2+} stores were discharged by pretreating the cells with 0.5 μM of the Ca^{2+} ionophore ionomycin in 3 mM EGTA-containing buffer, no change of $[Ca^{2+}]_i$ was observed upon addition of 10^{-7} M AVP (Fig. 4*B*, Table 2). Furthermore, 10^{-5} M nifedipine or nicardipine, two dihydropyridine antagonists of L-type voltage sensitive Ca^{2+} channels (VSCCs), failed to prevent AVP-induced $[Ca^{2+}]_i$ transients (Fig. 4*D*, Table 2), although they inhibited Ca^{2+} influx upon addition of 75 mM of the membrane-depolarizing agent KCl (Fig. 4, *C* and *D*). Finally, pretreatment with 10^{-7} M TPA induced a time-related inhibition of AVP-dependent increase of $[Ca^{2+}]_i$ (Fig. 3*C*).

As shown for the stimulation of phosphoinositide hydrolysis, AVP-dependent $[Ca^{2+}]_i$ transients similar to that recorded in L6-C5 undifferentiated cells were observed in L6, L6-A4, L6-C9, and L5 myoblasts and in L6-C5 and L5 myotubes (Table 3). Interestingly, the nonmyogenic L6-H2 clone displayed a response to 10^{-7} M AVP much lower than that of other clones and C2-C12 satellite cells failed to respond to AVP (Table 3). All cell types, however, responded to membrane depolarization by 75 mM KCl (Table 2), indicating that all of them expressed functional VSCCs.

Regulation of pH_i

We investigated the pattern of regulation of pH_i by AVP in L6-C5 cells, adopting two different experimental designs to provide evidence for the regulation of myogenic cell pH both in the presence and absence of HCO_3^-.

Table 3. *Effect of 10^{-7} M AVP and 75 mM KCl on $[Ca^{2+}]_i$ of L6, L5, and C2 cell lines*

Clones	Stage	*n*	$[Ca^{2+}]_i$, nM		
			Basal	AVP	KCl
L6	MB	3	153±45	680±267	367±65
L6-A4	MB	3	190±13	769±23	492±109
L6-C5	MB	6	114±7	655±93	657±114
L6-C5	MT	3	150±32	925±150	637±139
L6-H2	MB	3	190±14	308±10	392±10
L5	MB	4	133±5	609±19	447±208
L5	MT	4	115±25	643±50	424±38
C2-C12	MB	3	120±4	120±4	245±128

Values are means ± SE. MB, myoblast; MT, myotubes.

pH_i regulation in the absence of HCO_3^-. Under these conditions, L6-C5 cells demonstrated a pH_i of 7.26 ± 0.06, which was indefinitely stable. In this circumstance 10^{-7} M AVP induced a decrease of pH_i (Fig. 5*A*), reaching a plateau within 215 ± 36 s. The fall of pH_i was sustained, and the cells maintained their low pH_i for several minutes in the continuous presence of AVP (Fig. 5*A*).

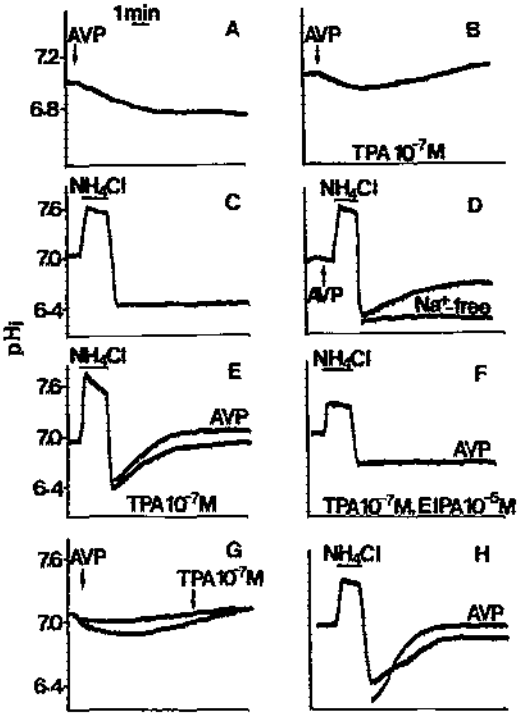

Fig. 5. AVP-dependent regulation of pH_i in L6-C5 cells. *A-F*: fluorescence traces of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein-loaded L6-C5 myoblasts bathed in *N*2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered solution, calibrated for pH_i as described in MATERIALS AND METHODS. *A*: effect of 10^{-7} M AVP. *B*: effect of 10^{-7} M AVP in cells treated with TPA. *C*: lack of pH_i recovery in cells acid-loaded by addition and subsequent withdrawal of 20 mM NH_4Cl. *D*: realkalinization in cells acid-loaded by NH_4Cl prepulse in presence of 10^{-7} M AVP. Realkalinization was abolished in Na^+-free buffer. *E*: pH_i recovery from acid loading by NH_4Cl prepulse in cells treated with 10^{-7} M TPA alone or together with 10^{-7} M AVP. *F*: lack of pH_i recovery in cells treated as shown in *F* but in presence of 10^{-6} M EIPA. *G-H*: pH_i in L6-C5 myoblasts bathed in HCO_3^--buffered solution. *G*: effect of 10^{-7} M AVP in presence or absence of TPA. *H*: NH_4Cl prepulse in untreated cells and cells exposed to 10^{-7} M AVP.

Pretreatment of the cells for 3 min with 10^{-7} M TPA before the addition of 10^{-7} M AVP failed to affect basal pH_i but induced an AVP-dependent slight and transient (87 ± 15 s) drop of pH_i (Fig. 5*B*), which was followed by a net recovery to near basal levels within 237 ± 63 s from the stimulus (Table 4).

When cells were acid loaded by applying and then withdrawing a KH solution containing 20 mM NH_4Cl (8), pH_i promptly dropped to 6.48 ± 0.05 (−0.52 ± 0.1 pH units) but failed to recover, keeping stable for several minutes (Fig. 5*C*). By contrast, addition of 10^{-7} M AVP before the NH_4Cl prepulse induced a substantial pH_i recovery (Fig. 5*D*, Table 5), which reached a plateau within 95 ± 6 s (recovery rate, normalized at 30 s from the acid loading, was 0.010 pH units/s). Pretreatment of the monolayers with 10^{-5} M ethylisopropyl amiloride (EIPA) or removal of extracellular Na^+ by isotonic substitution with choline (Fig. 5*D*), reduced basal pH_i and completely abolished the AVP-induced pH_i recovery (Table 5).

When cells were treated with 10^{-7} M TPA and then acid loaded by NH_4^+ prepulse, a pH_i recovery (Fig. 5*E*, Table 5) within 107 ± 9 s (recovery rate at 30 s: 0.009 pH units/s) was stimulated, with a mechanism blocked by either treatment with 10^{-5} M EIPA or removal of external Na^+ (Table 5). Both EIPA and Na^+ removal reduced basal pH_i by −0.19 ± 0.04 and −0.47 ± 0.04, respectively. Furthermore, in the presence of TPA, 10^{-7} M AVP had an additive effect (Fig. 5*E*), leading to a higher recovery achieved within 77 ± 2 s (Table 5; recovery rate at 30 s: 0.014 pH units/s).

pH_i regulation in presence of HCO_3^-. In this circumstance, basal pH_i was 7.02 ± 0.06 (Table 4). In contrast to what observed in the absence of HCO_3^-, addition of 10^{-7} M AVP induced a transient drop of pH_i followed by an alkalinization that was completed within 92 ± 3 s (Fig. 5*G*, Table 4). Pretreatment with 10^{-7} M TPA inhibited AVP-induced cell acidification and subsequent realkalinization (Fig. 5*G*, Table 4).

When cells were acid loaded by NH_4Cl prepulse, a dramatic acidification was observed, rapidly followed by a recovery (Fig. 5*H*, Table 5) within 85 ± 10 s (recovery rate at 30 s: 0.005 pH units/s). Addition of 10^{-7} M AVP before the NH_4Cl Prepulse increased the pH_i recovery (Fig. 5*H*) which reached the plateau within 106 ± 7 s (Table 5; recovery rate at 30 s: 0.011 pH units/s). Pretreating cells with EIPA (10^{-5} M, 5 min), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS, 5 × 10^{-4} M, 30 min), or 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS, 5 × 10^{-4} M, 30 min) before the addition of 10^{-7} M AVP inhibited the AVP-dependent realkalinization (Table 4).

Table 4. *Effect of AVP on pH_i of L6-C5 myoblasts in HEPES- or HCO_3^--buffered solutions*

Conditions	*n*	AVP (10^{-7} M)	
		Acidification	Alkalinization
Control-HEPES	5	−0.33±0.09*	ND
TPA (10^{-7} M)-HEPES	3	−0.12±0.02	+0.12±0.02
Control/HCO_3^-	3	−0.07±0.02	+0.26±0.04†
TPA (10^{-7} M)-HCO_3^-	3	−0.02±0.002	+0.07±0.005
EIPA (10^{-5} M)-HCO_3^-	3	−0.07±0.02	+0.06±0.02
DIDS (5 × 10^{-4} M)-HCO_3^-	3	−0.43±0.07	ND
SITS (5 × 10^{-4} M)-HCO_3^-	3	−0.14±0.06	+0.07±0.10

Values are means ± SE in intracellular pH (pH_i) units; ND, not detectable; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; EIPA, ethylisopropyl amiloride; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocyanostilbene 2,2′-disulfonic acid. * $P < 0.001$ vs. basal. † $P < 0.01$ vs. basal.

Table 5. *Effect of AVP on pH_i recovery of L6-C5 myoblasts acid loaded with 20 mM NH_4Cl prepulse*

Conditions	*n*	pH_i Recovery after NH_4Cl Prepulse	
		Without AVP	10^{-7} M AVP
Control-HEPES	3	+0.05±0.02	+0.65±0.07*
EIPA-HEPES	3		ND
Na^+-free-HEPES	3		ND
TPA-HEPES	3	+0.47±0.03†	+0.69±0.02‡
TPA-HEPES-EIPA	3	ND	ND
TPA-HEPES-Na^+-free	3	ND	ND
Control-HCO_3^-	3	+0.31±0.02	+0.67±0.03*

Values are means ± SE in pH_i units. * $P < 0.01$ vs. untreated. † $P < 0.05$ vs. untreated. ‡ $P < 0.01$ vs. cells treated with TPA alone.

DISCUSSSION

The results described in this report represent the first comprehensive characterization of the intracellular signaling systems activated by AVP in skeletal myogenic cells.

We have shown that Ins(1,4,5)P_3 is produced within seconds of treatment with AVP and is followed by a prompt transient increase of $[Ca^{2+}]_i$. Increase of $[Ca^{2+}]_i$ is consistent with the IP_3 production. In fact, the peak of $[Ca^{2+}]_i$ was preceded by the increase of Ins(1,4,5)P_3 and depended on mobilization of Ca^{2+} from cellular deposits, since it was abolished after discharge of the stores by treatment with the Ca^{2+} ionophore ionomycin. Furthermore, a good correlation exists between the inositol phosphate and the Ca^{2+} response to AVP of the various lines and clones tested.

AVP-dependent inositol phosphate production in the responsive myogenic cell clones shows characteristics similar to those observed in other canonical models of AVP sensitive cells (36, 49, 50), confirming the physiological nature of the action of AVP in skeletal myogenic cells. Analysis of inositol phosphates produced after short treatment with AVP indicates that the time course of Ins(1,4,5)P_3 stimulation is compatible with the hypothesis of direct hydrolysis of phosphatidylinositol bisphosphate and with the time course observed for the release of Ca^{2+} from Ins(1,4,5)P_3-sensitive intracellular stores. Furthermore, comparison of the time courses of production of the different inositol phosphates identified suggests that most of the Ins(1,4,5)P_3 is directly dephosphorylated to Ins(1,4)P_2 and Ins(4)P. Only a small amount of Ins(1,4,5)P_3 undergoes phosphorylation to inositol tetrakisphosphate, which is then dephosphorylated to Ins(1,3,4)P_3, and Ins(1,3)P_2. The metabolism of inositol phosphates observed in myogenic cells is similar to that described in AVP-stimulated WRK1 cells (40). However, it differs from that observed in AVP-stimulated rat glomerulosa cells [where stimulation of Ins(1,4,5)P_3 is much

lower than in our conditions; Ref. 27] and in the same cells challenged with ANG II, where the stimulation of $Ins(1,3,4)P_3$ is severalfold higher than that measured in our experiments (5).

The increase of $[Ca^{2+}]_i$ observed in myogenic responsive cells was due to release of Ca^{2+} from intracellular deposits (transient peak) and to Ca^{2+} influx from the extracellular fluid through membrane channels (sustained phase). The channels were insensitive to membrane potential, since treatment with dihydropyridine derivatives, known as L-type VSCC antagonists, at dose a adequate to prevent KCl-induced $[Ca^{2+}]_i$ elevation failed to substantially modify the AVP-dependent $[Ca^{2+}]_i$ increase. This suggests that other types of Ca^{2+} entry devices are activated by the treatment with AVP, the identity of which remains, at present, unknown.

The response to AVP appears to be regulated by PKC, possibly at the level of the receptor, as shown for other agonist-target systems (19, 30). In fact, pretreatment of the cells with TPA partially inhibited the AVP-induced phosphoinositide breakdown, an effect reversed by staurosporine. The AVP-dependent increase of $[Ca^{2+}]_i$ was also inhibited by pretreatment with TPA, but the temporal pattern of such effect seems to be different from that of inositol phosphates production. This discrepancy is probably due to the different technical approaches employed for measuring the two parameters, since the measurement of $[Ca^{2+}]_i$ followed in time the events upon addition of AVP, whereas inositol phosphates were measured at the end of a 30 min incubation with AVP applied after the preincubation with TPA. However, it should be pointed out that only a partial (40%) inhibition of inositol phosphate production was induced by TPA, suggesting that the residual AVP-induced inositol phosphate levels could still sustain the release of Ca^{2+} from intracellular stores. This finding is different from that observed in other cell types responsive to AVP (13) in which a pretreatment of 3 min with TPA is adequate to generate rapid and complete inhibition of the AVP-dependent $[Ca^{2+}]_i$ increase.

Intracellular signaling depending on phospholipase C activation has been shown to involve pH_i modifications that are thought to be relevant to the biological response (22, 38, 42). In conditions in which all ion transport mechanisms involving HCO_3^- are inactivated by incubation in a HCO_3^--free buffer, AVP induced intracellular acidification in L6-C5 skeletal myogenic cells similar to that described in other cell types (8). However, although in renal glomerular mesangial cells, this early acidification is regularly followed by substantial net realkalinization (possibly related to mitogenic effects of AVP; Refs. 8, 22), this alkalinization was lacking in L6-C5 cells. This is, to our knowledge, a novel condition requiring further characterization. The capability of L6-C5 cells of regulating their pH_i upon addition of AVP is influenced by TPA. In fact, in the absence of HCO_3^- AVP is capable of acidifying the cell but fails to induce realkalinization. Conversely, a mechanism for realkalinization is activated by AVP in TPA-pretreated cells, possibly through a PKC-dependent phosphorylation.

Even though induction of realkalinization was lacking L6-C5 cells were capable of regulating pH_i by the Na^+-H^+ antiport. Cells exposed to NH_4Cl prepulse (7) to induce an acute acidification still failed to recover basal pH_i. However, these cells did not lack a HCO_3^--independent mechanism of pH_i control, but this was stimulated only in particular conditions. In fact, pretreatment with AVP followed by acute acidification by NH_4Cl prepulse induced a substantial alkalinization by a Na^+-dependent EIPA-sensitive mechanisms. We hypothesize, according to previous reports (7), that such mechanism is represented by a Na^+-H^+ antiport, which displays a distinctive behavior in L6-C5 cells. In fact, in these cells acute acidification or treatment with AVP are not capable of triggering stimulation of the antiport, which may be achieved only by preactivation of PKC by TPA, or by a combination of both acute acidification and treatment with AVP. The mechanisms leading to such individual regulation are still unknown. Preliminary results from this laboratory indicate that in L6-C5 cells 10^{-7} M AVP fails to activate the PKC, a Na^+-H^+ antiporter-regulating agent, within 15 min, whereas TPA activates the PKC α-isoform (Adamo and Nervi, unpublished results). This would explain why a realkalinizing effect is observed in TPA plus AVP-treated but not in AVP-treated cells. The sensitivity of basal pH_i on removal of Na^+ or to treatment with EIPA, both leading to a net cell acidification, confirms the presence of the Na^+/H^+ antiporter, which seems to actively alkalinize but is balanced by an opposing acidification mechanism, the nature of which remains to be elucidated.

In the presence of HCO_3^-, AVP-induced cell acidification was promptly followed by realkalinization, slightly overshooting the basal pH_i. Furthermore, an NH_4Cl prepulse induced a pH_i recovery, which was additionally stimulated by AVP treatment. AVP-induced cell alkalinization was sensitive to both DIDS and SITS, suggesting a role for an anion transport, such as Na^+-dependent and Na^+-independent Cl^--HCO_3^- countertransport (8, 21), or Na^+-HCO_3^- cotransport (24) in the regulation of skeletal muscle pH_i. Nevertheless, from these data a conclusion on the nature of the mechanism cannot be drawn. Moreover, under these experimental conditions, EIPA was equally active in inhibiting alkalinization, indicating that, in the presence of HCO_3^-, the Na^+-H^+ countertransport also accounts for the final effects induced by AVP. Interestingly, in this condition, TPA reduced the effect of AVP, probably stimulating an alkalinizing mechanism that contrasts the AVP-induced acidification. As a consequence, AVP-dependent realkalinization is equally reduced. Taken together these data indicate that regulation of pH_i in L6-C5 cells is complex and a comprehensive model still difficult to hypothesize. Further work is in progress to elucidate this issue.

Response to AVP was not equally induced in every cell population tested. Comparison of different myogenic cell clones or lines indicates that although no major differences exist between the stage of differentiations of mononucleated myoblast and multinucleated myotube, some myogenic cell clones or lines did not appear to possess functional V_1 receptors. In fact, the satellite cell-derived

line C2-C12 as well as cells exhibiting a transformed phenotype, such as RD and the L6-H2 subclone, failed to respond to AVP. Conversely, other cellular models, probably more representative of the embryonic myogenic cells (L5 and L6) exhibited the ability to respond to stimulation with AVP. Chick embryo primary myogenic cells showed a response to AVP lower compared with that of L6 and L5 responsive clones. However, it should be pointed out that we observed a similar difference when we measured phosphoinositide breakdown induced by a different agonist, acetylcholine, even though ^{125}I-labeled bungarotoxin-specific binding indicated a higher complement of acetylcholine receptors in chick embryo myotubes than in L6 and L5 myotubes (unpublished data). The reason of this discrepancy is at present unclear. We can hypothesize that differences may reside at several levels of the signal-transduction pathways (e.g., phosphoinositol lipid pools, G proteins, and 1-phosphatidylinositol and 1-phosphatidylinositol-4-phosphate kinases). Further work is necessary to address this interesting question.

The heterogeneity of the response to AVP of skeletal myogenic cells of different origin could be explained on the basis of the recent observation that immunoreactive AVP is abundant in human skeletal muscle at the beginning of the fetal period of development, declines throughout gestation, and reaches very low levels at birth (32). Furthermore, embryonic, fetal, and satellite myogenic cells are known to display differential lineage- and stage-specific gene expression throughout their life span (15, 46). They therefore mantain substantial differences in their behavior and regulation, even though all of them eventually differentiate into skeletal muscle fibers. AVP has recently been shown to increase adenocorticotropic hormone (ACTH) levels in sheep fetuses (4). Because ACTH and other proopiomelanocortin-derived peptides are mitogenic for mammalian embryonic skeletal myogenic cells (14), it is tempting to speculate that AVP and ACTH may synergistically act as growth factors for embryonic skeletal muscle. This hypothesis is not in contrast to the fact that postmitotic differentiated myotubes also respond to AVP. It may be speculated that the same agonist and signal-transduction systems regulate different functions (mitotic activity in myoblasts, carbohydrate metabolism, contraction in muscle fibers; Refs. 29, 56) at different stages of differentiation of the same cell type.

A comprehensive description of the mechanisms of AVP intracellular signal transduction in skeletal myogenic cells has been provided in this paper. Further work is in progress to elucidate the mechanisms underlying the heterogeneity of the response to AVP of myogenic cells and the overall physiological role of AVP during myogenesis.

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